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In vitro Organogenesis from Nodal Derived Callus in Carnation (*Dianthus caryophyllus* L.) cv. 'Master' and Assessment of Genetic Fidelity of Acclimatized Plantlets

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A B S T R A C T

An efficient protocol for organogenesis and plant regeneration has been developed for *Dianthus caryophyllus* L. cv. 'Master' using nodal explant. Optimal callus formation (79.17%) from nodal explants was obtained on MS medium supplemented with 1.5 mg/l 2,4-D and 1.0 mg/l NAA. The highest shoot induction response (65.28%) was recorded on MS medium supplemented with 1.5 mg/l TDZ, 0.25 mg/l Kinetin and 0.25 mg/l NAA leading to maximum average no. of shoots per callus clump (5.14) and shoot length (1.43 cm). The highest percentage of root regeneration (84.44%) was observed on MS medium supplemented with 1.0 mg/l IBA and 100 mg/l activated charcoal. The *in vitro* raised plantlets were successfully acclimatized under *ex vitro* conditions and grew well in the glasshouse. All the regenerated plantlets appeared normal with respect to morphological characteristics with 100% survival rate. Somaclonal variation in tissue culture is a common phenomenon which makes it mandatory to check for genetic stability of plants. The genetic variation in the *in vitro* raised plants was confirmed using Inter Simple Sequence Repeats(ISSR) markers. Comparison of the bands with the mother plant revealed that the genetic variation frequency reached 6.54 % using fifteen ISSR markers.

Introduction

Dianthus caryophyllus L., commonly known as Carnation, belongs to the family Caryophyllaceae, is an important floricultural crop all over the world and ranks just next to Rose in popularity (Roychowdhury *et al.*, 2012)owing to their diverse flower color, ability to withstand long distance transportation, and remarkable ability to rehydrate after continuous shipping (Thu *et al.*, 2020). It is grown in several parts of the world and is believed to be the native of Mediterranean region. The generic name *Dianthus* comes from the writings of Theophratus who lived about 300 B.C. Carnation has great commercial value as a cut flower due to its excellent keeping quality, wide array of colour and forms. Carnation, apart from producing cut flowers can also become useful in gardening for bedding, edging, borders, pots and rock gardens.

Carnations are grown commercially in India in places having mild climate in Solan, Shimla, Kalimpong, Kodaikanal, Mandi, Kullu, Srinagar, Ooty andYercaud. Carnation plants are half hardy herbaceous perennial.

The flowers are solitary, terminally formed; the petals are broad with frilled margins and the calyx cylindrical with bracts at the base. The demand for carnation cut flower is gaining momentum with increasing aesthetic sense and higher socio-economic standard of the people.

In recent years, the plant tissue culture technique has gained greater momentum on commercial application in the field of plant biotechnology and floriculture. The most successful and most widely used discipline of plant tissue culture technique is micrpopropagation which refers to the propagation of plants by using meristem tip culture which is the transfer of apical buds and surrounding leaf primordia to sterile culture conditions(Ali *et al.,* 2004;Naz *et al.,* 2012).

Carnation is considered a difficult plant for *in vitro* shoot induction. There is difficulty in organogenesis in most *Dianthus* species, especially carnation (Kanwar and Kumar, 2009). Establishment of callus cultures and use of plants regenerated from calli via shoot organogenesis hold a potential for the production of useful somaclonal variants. In the past, callus cultures in carnation have been established from a range of vegetative explants such as leaf (Casanova *et al.,* 2004; Kumar *et al.,* 2006; Ranade and Kanwar, 2014; Thakur and Kanwar, 2018) andnodal segments (Nontaswatsari *et al.,* 2002; Bora *et al.,* 2007).

However, tissue culture raised plants involving a prolonged callus phase is considered to be unreliable due to the genomic changes of *in vitro* raised plantlets. Molecular techniques are valuable tools used in the analysis of genetic fidelity of *in vitro* raised plants. Detection and analysis of genetic variation can help in understand the molecular basis of various biological phenomena in plants (Kumar *et al.,* 2011). Thus the genetic fidelity of the *in vitro* derived plantlets should be tested as early as possible.

This paper reports an efficient, high frequency plant regeneration protocol via indirect organogenesis in *Dianthus caryophyllus* L. cv. 'Master', red colour cultivar of carnation as well as to apply molecular marker analysis using ISSR markers for the detection of genetic polymorphism.

Materials and Methods

Plant material

Fresh leaves were obtained from greenhouse grown superior mother plants of carnation *Dianthus caryophyllus* L. cv. 'Master'. The surface sterilization of explants was done in the laminar air flow cabinet using 0.2 per cent Bavistin solution for 5-10 minutes and 0.5 per cent sodium hypochlorite solution (4% chlorine available) for 10-20 minutes followed by washing 3 times with sterile distilled water.

Callus induction

Nodal segment (1.0-1.2 cm) explants were excised and cultured on solid MS medium (Murashige and Skoog, 1962) supplemented with different concentrations of 2,4-D ranging from 0.5 mg/l to 2.5 mg/l alone and in combination with 0.25 mg/l to 1.0 mg/l NAA for callus induction. The cultures were kept in darkness for two weeks for the induction of callus followed by transfer to 16 hours photoperiod for another two weeks.

In vitro shoot bud induction

After the establishment of callus cultures, the calli were subjected to solid MS medium supplemented with 1.5 mg/l TDZ, 0.25 mg/l Kinetin and 0.25 mg/l NAA from our previous report (Thakur and Kanwar, 2018). The cultures were incubated at 25±2ºC temperature and 16 hr photoperiod for the induction of adventitious shoot buds.

Effect of subculturing on shoot bud induction

Keeping in view the above observations, experiment was conducted to study the effect of subculturing on regeneration potential of nodal derived callus. Calli were subcultured on solid MS medium supplemented with 1.5 mg/l TDZ, 0.25 mg/l Kinetin and 0.25 mg/l NAA and incubated at 25±2ºC under 16 hr photoperiod for four weeks. The callus was subcultured at an interval of every four weeks for five times. Data was recorded for average number of shoot bud induction and average shoot length for every subculture passage.

Shoot multiplication

For multiplication of microshoots, the established microshoots were cut aseptically into 1.5 to 2.0 cm long segments and were inoculated on the medium found best for shoot proliferation (Solid MS medium supplemented with 2.0 mg/l Kinetin and 0.25 mg/l NAA) from our previous report by (Thakur and Kanwar, 2018). In order to multiply microshoots *in vitro*, they were subcultured at the interval of four weeks for five times on same multiplication medium.

Root induction in microshoots

In vitro raised microshoots of 3.0-4.0 cm length were cut with sterile blade and were transferred to the rooting medium. The microshoots were inoculated on the half strength MS medium supplemented with different concentrations and combinations of IBA (0.5 to 2.5 mg/l) and activated charcoal (50-100 mg/l) designed for the purpose of root induction. The data was recorded as average number of roots per shoot and average root length.

Hardening and acclimatization

The plantlets were taken out of culture tubes and hardening was done in plastic cups filled with sterile cocopeat and perlite (2:1 ratio). The plants were watered with half strength liquid MS medium at regular intervals for 30 days. Thereafter successfully acclimatized plantlets were transferred to the bigger pots and exposed to sunlight and watered twice a week.

Statistical analysis

All the experiments were conducted in a completely randomized design (CRD). The data recorded on different parameters was subjected to analysis of variance using CRD(Gomez and Gomez, 1984) while Arc sine transformation was applied for the data expressed in percentages. All the experiments were studied through statistical analysis using SPSS software for windows version 16.0.

Molecular analysis

The genomic DNA was isolated from new emerging green leaves using CTAB method with certain modifications to check the genetic uniformity of *in vitro* raised plants (Doyle and Doyle, 1987). DNA amplification of mother plant and fourteen *in vitro* raised plants was conducted using ISSR markers. Amplified DNA of mother plant and fourteen *in vitro* raised plants were then separated on agarose gel.

Results and Discussion

Sterilization of explants

For surface sterilization of nodal explants, the treatment comprising of 0.2 percent Bavistin for 5 minutes and 0.5 per cent sodium hypochlorite for 15 minutes resulted in highest percentage of uncontaminated cultures (87.50%) after four weeks of incubation. Decreasing the time of treatment resulted in less percentage of uncontaminated cultures while longer duration of treatment resulted in death of explants.

Callus induction

The results presented in Table 1 reveals that out of all combinations tested, MS medium supplemented with 1.5 mg/l 2,4-D and 1.0 mg/l NAA showed maximum per cent callus induction (79.17%) followed by treatment comprising of solid MS medium supplemented with 2.0 mg/l 2,4-D and 0.5 mg/l NAA which resulted in 65.28% callus induction. The callus obtained was compact in texture and green in colour (Fig. 1a-d).

Radojevic *et al*., (2010) reported plant regeneration in different carnation species using nodal segments as explants on MS medium supplemented with 1.0 mg/l NAA, 0.5 mg/l IBA and 1.0 mg/l BAP for shoot induction. Kumar *et al*., (2016) achieved maximum callus induction with 2.06 µM 2, 4-D and 2.68 µM NAA in *Dianthus caryophyllus*cv. Yellow Dot Com from leaf explants.

Callus differentiation and shoot bud induction

The callus induced from nodal explant was cut into small pieces (1.0-1.5 cm) and cultured on MS medium supplemented with 1.5 mg/l TDZ, 0.25 mg/l Kinetin and 0.25 mg/l NAA which resulted in maximum 65.28 per cent shoot regeneration with average 5.14 number of shoots per callus clump and shoot length of 1.43 cm. Shoot bud induction initiated from the callus after 2 weeks and data was recorded after 4 weeks of incubation (Fig. 1e).

Earlier, we have reported that out of all the treatments maximum per cent shoot regeneration was observed on treatment comprising of solid MS medium supplemented with 1.5 mg/l TDZ, 0.25 mg/l Kinetin and 0.25 mg/l NAA which resulted in maximum 80.56 per cent shoot regeneration with average 6.01 number of shoots per callus clump and maximum shoot length of 1.93 cm when leaf segment was used as explant in *Dianthus caryophyllus* L. cv. 'Master' (Thakur and Kanwar, 2018).

Radojevic *et al.,* (2010)found 1.0 mg/l NAA, 0.5 mg/l IBA and 1.0 mg/l BAP best for *in vitro* establishment of nodal segments in *Dianthus caryophyllus* L. Hassan *et al.,* (2011) reported the best shoot formation response on MS medium supplemented with higher concentration of BA (4.0 mg/l) in carnation. *In vitro* shoot regeneration in carnation from nodal segment was obtained on medium containing 2.0 mg/l BA. Kumar *et al.,* (2016)reported that the calli derived from MS medium supplemented with 5.37 µM NAA and 2.27 µM TDZ in carnationcv. Yellow Dot Com were found to have maximum shoot regeneration potential. The highest number of calli producing shoots and average number of shoots per callus was recorded on MS medium supplemented with 9.12 µM zeatin and 5.07 µM IAA.

Maximum 73.61 per cent callus inducing shoot buds were observed after third subculture which is significantly higher than rest of subculturings. Similarly, average number of shoots also statistically increased

from5.14 to 8.51 with the advancement of subculture from first to third thereafter started decreasing. However, average shoot length remains statistically apart from first subculture to third subculture, thereafter, started decreasing from1.49 to 1.40 cm (Table 2). It was noted that although the per cent shoot bud induction, number of shoot buds and shoot length increased slightly up to third subculture thereafter declined. The gradual decline in the morphogenic potential of callus may be due to accumulation of inhibitory substances (Halperin, 1986; Thakur and Kanwar, 2018), decline in metabolism, transport and interaction between growth regulators.

Table.1 Effect of different concentrations of 2,4-D alone and in combination with NAA supplemented in solid MS medium on per cent callus induction

Growth regulators (mg/l)2,4-D	NAA	Per cent callus induction[1,2]	Type	Colour	Growth
0.0	**0.00**	0.00 (0.00)^{r}	-	-	-
0.5	**0.00**	2.78 (9.60)^{q}	**C**[3]	**LG**[6]	+[7]
1.0	**0.00**	5.56 (13.64)^{o}	**C**	**LG**	+
1.5	**0.00**	8.33 (16.77)^{n}	**C**	**LG**	+
2.0	**0.00**	13.89 (21.87)^{l}	**C**	**LG**	+
2.5	**0.00**	16.67 (24.09)^{k}	**C**	**LG**	+
0.5	**0.25**	4.17 (11.78)^{p}	**C**	**G**[5]	+
1.0	**0.25**	11.11 (19.46)^{m}	**C**	**G**	+
1.5	**0.25**	20.83 (27.15)^{j}	**C**	**G**	+
2.0	**0.25**	40.28 (39.39)^{f}	**C**	**G**	++
2.5	**0.25**	30.56 (33.56)^{h}	**C**	**G**	++
0.5	**0.50**	19.44 (26.15)^{j}	**C**	**G**	++
1.0	**0.50**	27.78 (31.81)^{i}	**C**	**G**	++
1.5	**0.50**	43.05 (41.00)^{e}	**C**	**G**	+++
2.0	**0.50**	65.28 (53.90)^{b}	**C**	**G**	+++
2.5	**0.50**	47.22 (43.41)^{d}	**C**	**G**	+++
0.5	**1.00**	30.56 (33.56)^{h}	**C**	**G**	++
1.0	**1.00**	56.94 (48.99)^{c}	**C**	**G**	+++
1.5	**1.00**	**79.17 (62.85)^{a}**	**C**	**G**	+++
2.0	**1.00**	55.56 (48.20)^{c}	**C**	**G**	+++
2.5	**1.00**	33.33 (35.26)^{g}	**C**	**G**	++
CD0.05		**1.27**			
SE+		**0.61**			

[1]Figures in parentheses are arc sine transformed values

[2]Means followed by different letters are significantly different at *P*=0.05 according to Duncan's multiple range test

[3]C:Compact [5]G Green [7]+ : Slow

[4]F:Friable [6]LG Lightgreen ++ : Moderate

+++: Fast

- : Noresponse

Table.2 Effect of subculturing of callus on shoot proliferation on MS medium supplemented with 1.5 mg/l TDZ, 0.25 mg/l Kinetin and 0.25 mg/l NAAat an interval of four weeks for five times

Callus subculture passage	[1,2]Shoot bud induction (per cent)	[2]Average number of shoots per callus piece	[2]Average shoot length (cm)
I	65.28 (53.90)^{c}	5.14^{e}	**1.43^{a,b,c}**
II	70.38 (57.03)^{b}	6.16^{d}	**1.47 ^{a,b}**
III	**73.61 (59.09)^{a}**	**8.51^{a}**	**1.49 ^{a}**
IV	69.44 (56.45)^{b}	7.52^{b}	**1.39 ^{c}**
V	59.72 (50.61)^{d}	6.62^{c}	**1.40 ^{b,c}**
CD0.05	**2.13**	**0.51**	**0.08**
SE+	**0.83**	**0.20**	**0.03**

[1]Figures in parentheses are arc sine transformedvalues

[2]Means followed by different letters are significantly different at *P*=0.05 according to Duncan's multiple range test

Table.3 Effect of different concentration of IBA and activated charcoal (AC) on *in vitro* rooting

IBA (mg/l)	AC (mg/l)	[1,2]Per cent Rooting	[2]Average no. of roots	[2]Average root length (cm)
-	-	0.00 (0.00)^{p}	0.00^{k}	**0.00^{k}**
0.5	50.0	8.89 (17.35)^{o}	1.33^{j}	**1.51^{j}**
1.0	50.0	13.33 (21.41)^{j}	1.63^{j}	**1.62^{j}**
1.5	50.0	24.44 (29.62)^{f}	2.50^{h}	**1.88^{i}**
2.0	50.0	17.78 (24.93)^{i}	3.37^{f}	**1.99^{h,i}**
2.5	50.0	11.11 (19.44)^{l}	3.77^{e}	**2.18^{g,h}**
0.5	100.0	77.78 (61.89)^{b}	6.10^{b}	**3.37^{b,c}**
1.0	100.0	**84.44 (66.78)^{a}**	**7.67^{a}**	**3.53^{a}**
1.5	100.0	68.89 (56.10)^{c}	4.70^{d}	**3.26^{c}**
2.0	100.0	57.08 (49.07)^{d}	5.47^{c}	**2.44^{e,f}**
2.5	100.0	35.56 (36.61)^{e}	4.63^{d}	**2.34^{f,g}**
0.5	200.0	13.33 (21.39)^{k}	3.07^{g}	**2.94^{d}**
1.0	200.0	17.78 (24.94)^{h}	2.53^{h}	**2.59^{e}**
1.5	200.0	22.22 (28.12)^{g}	2.33^{h}	**2.42^{e,f,g}**
2.0	200.0	8.89 (17.33)^{m}	1.97^{i}	**2.20^{f,g,h}**
2.5	200.0	2.22 (8.56)^{o}	1.53^{j}	**1.91^{i}**
CD0.05		**1.51**	**0.30**	**0.23**
SE+		**0.71**	**0.14**	**0.11**

[1]Figures in parentheses are arc sine transformed values

[2]Means followed by different letters are significantly different at *P*=0.05 according to Duncan's multiple range test

Fig.1 *In vitro* plant regeneration in *Dianthus caryophyllus* L. cv. 'Master' from nodal explant a) Nodal explant cultured on callus induction medium b) Callus initiation from cut ends of the explant c) Callus induction after two weeks d) Callus proliferation after four weeks e) *In vitro* shoot bud induction from callus f) *In vitro* shoot multiplication g) Root regeneration in *in vitro* developed shoots

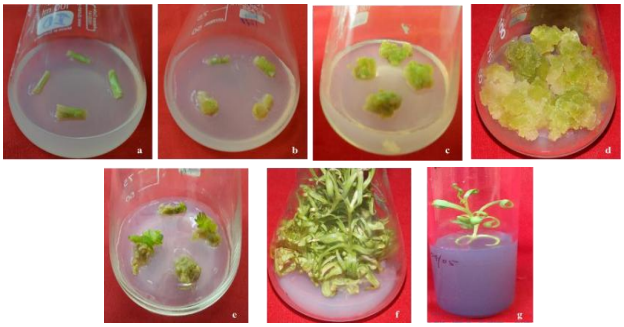
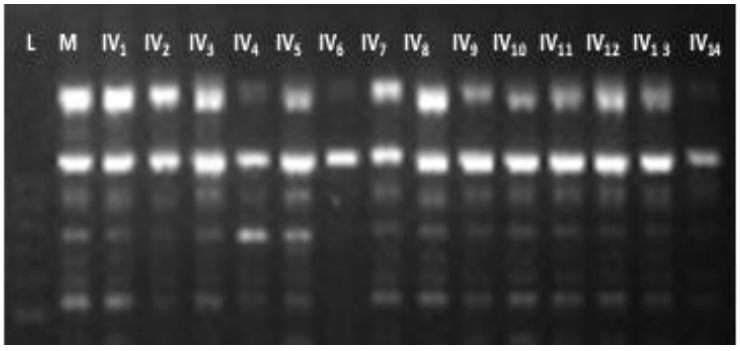


Fig.2 Hardening of *Dianthus caryophyllus* L. cv. 'Master' a) Hardened plantlets in plastic cups b) Hardened plantsin pots after one month of hardening c-d) Flowering in carnation cv. 'Master'

Fig.3 ISSR pattern of mother plant and *in vitro* raised plants of *Dianthus caryophyllus* L. cv. 'Master' by ISSR primer (hb-19) L: denotes 1kbp DNA ladder, M: denotes mother plant, IV1-IV14:*in vitro* raised plants



Sahoo *et al.*, (1997) reported gradual decline in the regeneration potential of the callus derived from juvenile as well as mature explants that did not show regeneration after five subculture passages. It was observed that concentration as well as combination of plant growth hormones is a key factor in induction of shoot buds.

Shoot multiplication

For shoot multiplication, the individual shoots from the shoot clumps were separated and cultured on the MS medium supplemented with 2.0 mg/l Kinetin and 0.25 mg/l NAA till a sufficient rate of shoot multiplication was achieved. This resulted in 11.83 average number of shoots with 3.44 average shoot length (Fig. 1f).

Bora *et al.,* (2007) reported high rate of multiplication of nodal segment of on MS medium supplemented with 0.5 mg/l NAA, 0.5 mg/l Kn and 0.5 mg/l GA3 in carnation. Mizory *et al.,* (2014) found 5.7 average number of shoots per explant on medium supplemented with 3.0 mg/l BA and 0.1 mg/l NAA. Whereas, Hassan *et al,* .(2011) reported very high multiplication rate with average of 38.00 shoots per explants on medium supplemented with 0.5mg/lBAP and 0.1 mg/l NAA. *In vitro* multiplication of shoots (10.22 per explant) was highest on medium supplemented with 1.0 mg/l TDZand0.1 mg/l TIBA in carnation (Ranade and Kanwar, 2014).

In vitro root induction

There was a great deal of variation among cultivars of carnation in their requirements for plant growth regulators *in vitro* and rooting of carnation cultivars and was highly genotype dependent. Auxin plays a major role in root induction through its effect on the first cell division which forms root initials. The microshoots measuring 3.0-4.0 cm in length were isolated and excised for transfer to root induction medium. It was observed that treatment which comprised of half strength MS medium supplemented with 1.0 mg/l IBA and 100 mg/l activated charcoal showed maximum rooting (84.44 per cent) with 7.67 average number of roots having 3.53 cm root length (Table 3). Root initiation started generally after 15-20 days of culturing (Fig.1g).

Thakur and Kanwar(2018) observed that the half strength MS basal medium supplemented with 1.5 mg/l IBA and 0.02% activated charcoal showed maximum rooting (98.19 per cent) with 9.60 average number of roots per microshoot having 4.24 cm root length. Ali *et al.,* (2008) found that among the various concentrations of NAA and IBA, the best rooting response were obtained on MS medium containing 1.0 mg/l NAA. Rahman *et al.,* (2014) obtained highest percentage of rooting on MS medium supplemented with 0.3 mg/l IBA inred gerbera. In contrary to our results, Daod *et al.,* (2019) found NAA to be more efficient in inducing rooting as compared to IBA.

Hardening and acclimatization

Acclimation is the final but frequently most critical step in a successful micropropagation system. After complete development of roots, plantlets were taken out of the culture tubes, taking all the precautions to avoid any damage to its delicate root system. Each plantlet was given a dip in Bavistin solution to avoid fungal contamination. Plastic cups were first filled with sterile cocopeat and perlite, and the plantlets were carefully placed in the cups (Fig. 2a). After acclimation to *ex vitro* conditions, the well acclimatized plants were transferred to big pots (Fig. 2b). Hundred percent survival rate was observed in transferred plantlets during acclimatization process.

Thakur and Kanwar (2018) observed that per cent survival of hardened plants was 97.68% in the carnation cv. 'Master' using leaf as explant. In contrast, Kumar *et al.*(2016) obtained 60-80% survival in *in vitro* derived shoots/somatic embryos of *Dianthus caryophyllus* cv. Yellow Dot Com after 30 day of transfer to pots.

Molecular analysis

Even though the *in vitro* raised plants were phenotypically identical with the mother plant at flowering stage (Fig. 2c-d) which partly suggest the minimal or absence of somaclonal variations. But genetic variation has been frequently observed in plants regenerated from tissue cultures. The degree and type of variation is affected by several factors, including genotype and culture conditions (Lee and Phillips, 1988). Jain (2001) proposed that cultures which are exposed to 2,4-D for prolonged periods can accumulate mutations. So, the assessment of the genetic stability of *in vitro* derived clones is an essential step in the application of biotechnology for micropropagation of true-to-type clones(Kumar *et al.,* 2016). ISSR markers are considered suitable to detect variations among micropropagated plants since a simple sequence repeat targets the fastevolving hypervariable sequences (Rahman and Rajora, 2001; Joshi and Dhawan, 2007).

In the present study, fifteen ISSR markers were used to assess the genetic fidelity among fourteen *in vitro* raised plants and the mother plant which produced clear, reproducible and scorable bands (Fig. 3). Molecular marker analysis demonstrated 93.46% genetic similarity between mother plants and *in vitro* raised plants. A low level of variation (6.54%) was detected in the *in vitro* raised plants, this could be explained by the somaclonal variation that could appear as a result of indirect regeneration through callus (Fig. 3). Thus, the molecular profiling by using ISSR markers proved to be a reliable method for assessing genetic stability of *in vitro* raised plants.

Similarly, Kumar *et al.,* (2016) reported 2.94%, 26.47% and 20.58% variation in plants regenerated from axillary buds, callus and somatic embryos in *Dianthus caryophyllus* cv. Yellow Dot Com. In contrast to our results, Kumar *et al.*(2011) observed 100% genetic similarities between the micropropagated plants and mother plants using RAPD and ISSR markers. Bhatia *et al.,* (2011) evaluated genetic fidelity with 100% similarity of *in vitro* propagated plants and mother plant of gerbera using RAPD and ISSR markers.

Hence, it becomes imperative to regularly check the genetic integrity of the *in vitro* raised plants in order to produce clonally uniform progeny. However, for better analysis of genetic homogeneity and for discarding the possibility of somaclonal variants, it was always recommended to use more than one marker while carrying out the genetic stability studies (Lakshmanan *et al.,* 2007). Findings of the present study elucidate that an efficient indirect regeneration protocol from nodal explants of carnation cv. 'Master' was established. Further, the genetic homogeneity of *in vitro* regenerated plantlets was confirmed by ISSR markers which showed a low level of genetic variation. Therefore, the present protocol may be applied commercially as alternative means for rapid propagation of this cultivar.

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