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Plant regeneration from axillary bud, callus and somatic embryo in carnation (*Dianthus caryophyllus*) and assessment of genetic fidelity using RAPD-PCR analysis

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ABSTRACT

Plant regeneration was achieved from axillary bud, callus and somatic embryos of *Dianthus caryophyllus* cv.Yellow Dot Com from node and leaf explants cultured on Murashige and Skoog (MS) medium supplemented with plant growth regulators. The explant nodal cutting responded well for direct regeneration while leaf explant was selected for callus induction. The highest number of direct adventitious shoots were achieved with 9.08 µM TDZ and 5.37 µM NAA. The maximum callus induction was achieved with 2.06 µM 2, 4-D and 2.68 µM NAA. The calli derived from MS medium supplemented with 5.37 µM NAA and 2.27 µM TDZ 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. The calli derived from leaf explants cultured on medium containing 2.06 µM 2, 4-D and 2.68 µM NAA were highly friable, had poor regeneration potential and were selected for cell suspension studies. The cultures were allowed to grow into micro-colonies in liquid medium and subsequently into embryogenic calli on semi-solid and solid MS medium which later differentiated into somatic embryos without growth regulators. Plantlets were obtained from *in vitro* derived shoots/somatic embryos with 60-80% survival after 30 day of transfer to pots. The plants regenerated from axillary buds, callus and somatic embryos were compared with mother plant to assess genetic fidelity using RAPD and 2.94%, 26.47% and 20.58% variation was observed, respectively.

Key words: Callus, Carnation, *Dianthus caryophyllus*, Growth regulators, Plantlets, Polymerase chain reaction, Somatic embryogenesis

The genetic variability within carnation (*Dianthus caryophyllus* L.) is relatively limited, therefore, breeding potential for new flower colours and patterns, as well as resistance to biotic and abiotic stresses is also limited (Bhatt *et al.* 1989). Carnation is vegetatively propagated which further limits its available genetic pool. That is why biotechnological tools are being used for improvement of this crop. However, availability of regeneration protocol is the first step towards the use of biotechnological approaches. Numerous studies have been made to regenerate carnation *in vitro* using different explants (Thakur *et al.* 2002, Wankhede *et al.* 2006 and Kanwar *et al.* 2009). Shoot regeneration in carnation is influenced by genotype, explant and the balance of growth regulators (Kallak *et al.* 1997). Efficient regeneration protocols for *in vitro* propagation of carnation *via* somatic embryogenesis have also been reported (Karami *et al.* 2007 and Ali *et al.* 2008). Clonal propagation through cell culturing provides the ability to regenerate plants from single cell or clumps of 2-5 cells. It makes possible the propagation of plants of any age, the induction, isolation and selection of mutants, the production of somatic hybrids and modification of cells by genetic engineering for crop improvement. However, the major problem associated with micropropagation is occurrence of somaclonal variations among the sub-clones of parental line arising as a direct consequence of *in vitro* culture of plant cell, tissue and organ. The plant growth regulators especially synthetic ones at sub- and super-optimal levels have been shown to be associated with somaclonal variations (Martin 2006). The frequency of these variations varies with the source of explant and their pattern of regeneration, media composition and cultural conditions (Bhatia *et al.* 2009). The occurrence of cryptic genetic defects arising *via* somaclonal variation in the regenerates can seriously limit the utility of the micropropagation system (Salvi *et al.* 2001). The genetic integrity of the micropropagated plants can be evaluated by molecular analysis. RAPD has become a widely employed technique used for detection of clonal fidelity since it has the advantage of being technically simple, quick to perform and requiring only small amounts of DNA, no prior sequence information required and do not use radioactive

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probes (Lakshmanan *et al.* 2007). RAPD analysis has been applied for the detection of somaclonal variations in number of crops (Ozden-Tokatli *et al.* 2006, Bhatia *et al.* 2011 and Pandy *et al*. 2012). The present work was carried out in carnation cv. Yellow dot Com with the objectives to regenerate plants from different regeneration pathways and to compare the regenerants with parent plants for the assessment of genetic fidelity using RAPD technique.

MATERIALS AND METHODS

Stem cuttings (8 -10 cm) from the apical portion of carnation cv. Yellow dot Com were procured from the Department of Floriculture and Landscaping, University of Horticulture and Forestry, Solan (Himachal Pradesh), India. Nodal (0.8-1 cm) and leaf (0.5 cm^2) segments were cut and surface sterilized with 0.1% $HgCl_2$ (mercuric chloride) for 2-3 min followed by 3-4 washings with sterilized distilled water and used as explants for establishment of *in vitro* shoot and callus cultures.

The sterilized nodal segments (0.8-1 cm) were cultured on MS (Murashige and Skoog 1962) medium supplemented with growth regulators, viz. 4.44 and 8.88 µM benzyladenine (BA), 5.37 µM naphthaleneacetic acid (NAA), 4.54 and 9.08 µM thidiazuron (TDZ) and 5.07 µM indoleacetic acid (IAA) (Table 1) for direct shoot regeneration. The cultures without growth regulators served as control. Data were recorded on explants producing shoots and the number of shoots per explant after four weeks of culture.

The sterilized leaf segments (0.5 cm^2) were inoculated on MS medium supplemented with 2.06 and 4.12 µM 2, 4-dichlorophenoxyacetic acid (2, 4-D), 2.68, 5.37 and 10.74 µM NAA, 2.22 and 4.44 µM BA and 2.27, 4.54 and 6.81 µM TDZ alone or in combination for callus initiation (Table 2).

Table 1 Effect of growth regulators on direct adventitious shoot formation from nodal cutting

Treatment (µM)				Percent explants	Number of
BA	TDZ	NAA	IAA	producing shoots	shoots/explants
0	0	0	0	0(00.00)	0
4.44	0	0	0	0(00.00)	0
8.88	0	0	0	68.33(55.75)	2.26
0	4.54	0	0	0(00.00)	0
0	9.08	0	0	76.67(61.11)	3.41
0	4.45	0	5.07	0(00.00)	0
0	9.08	0	5.07	83.33(65.90)	2.92
0	4.45	5.37	0	0(00.00)	0
0	9.08	5.37	0	88.33(70.02)	4.66
0	0	0	5.07	0(00.00)	0
0	0	5.37	0	0(00.00)	0
4.44	0	5.37	0	0(00.00)	0
4.44	4.45	0	0	0(00.00)	0
8.88	0	0	5.07	0(00.00)	0
8.88	0	5.37	0	63.33(5273)	1.24
$LSD_{0.05}$				2.80	0.12

Figures in the parenthesis are the arc sine transformed values

One-month-old calli (0.8-1 cm^2) were transferred to shoot induction medium comprising of MS basal salts supplemented with cytokinins alone or in combination with auxins (Table 3). Data were recorded on explants producing calli, calli producing shoots and number of shoots per callus after four weeks of culture.

Suspension cultures were obtained using the most friable callus derived from leaf explant. Twenty Erlenmeyer flasks (100 ml) containing 1.25-1.50 g callus per 20 ml liquid MS medium supplemented with 2.06 µM 2,4-D and 2.68 µM NAA were maintained . The cultures were placed on an orbital platform shaker at 100-120 rpm under dark conditions at 25±2ºC. The cultures were subcultured at weekly intervals to obtain maximum cell density.

The content was filtered through autoclaved double layered muslin cloth to separate debris from cells and cells from micro calli. The cultures were further maintained by regular sub-culturing in fresh liquid medium by transferring approximately 5 ml of suspension in 20 ml liquid medium for maintenance of stock solution. The viability of the isolated cells was examined using Evans's blue dye. The density of the isolated cells ($1\text{-}3 \times 10^4$ cells/ml) was adjusted with the help a haemocytometer by diluting the cell suspension with culture medium. The behaviour of freely suspended cells was examined using a microscope (45 x) for observation and photographic records. 2 ml of the suspension was added to Petri plates (4 cm dia) containing 10 ml of semisolid and solid MS medium supplemented with 2.06 µM 2,4-D and 2.68 µM NAA. The Petri plates were swirled to ensure proper mixing of cells in the agar medium. The Petri plates were sealed with parafilm and incubated in dark for cell growth and differentiation. Ten pieces (0.5 g) of embryogenic callus derived from cell suspension (in three replications) were subsequently transferred onto MS medium without growth regulators for somatic embryo initiation. The percent calli producing somatic embryos were recorded at 6 weeks of culture. The somatic embryos were transferred to growth regulator free MS medium for regeneration of plantlets. The plantlets were removed from the culture vessels after 4 weeks and transferred to glasshouse for acclimatization.

In all the cultures, MS medium supplemented with 3% (w/v) sucrose and the pH of the medium was adjusted to 5.6-5.8 with 1 N NaOH and/or 1 N HCl before autoclaving at 1 kg cm^2 for 15 min. Agar (0.8%, w/v, Difcobacto was used for gelling. All the cultures were incubated at 24±2ºC under a 16/8-h (day/night) photoperiod with light intensity of 50-60 µmol/m^2/s. All the cultures were transferred to fresh medium at 30 days interval.

In all the experiments three replications was taken with ten explants per treatment. The experiment was repeated three times with similar results. Data recorded for different parameters were subjected to completely randomized design (Gomez and Gomez 1984). The statistical analysis based on the mean values per treatment was made using analysis of variance technique for CRD.

Genomic DNA was extracted from young leaves of

Table 2 Effect of growth regulators on percent callus induction, growth and type of callus

Treatment (µM)				Callus induction*(%)	Growth of callus	Type of callus
2,4-D	NAA	BA	TDZ			
0	0	0	0	0(0.0)	-	-
2.06	0	0	0	20.02(26.56)	+	F
4.12	0	0	0	26.66(30.78)	+	F
0	2.68	0	0	33.33(35.01)	++	N
0	5.37	0	0	60.00(56.15)	+++	N
0	10.74	0	0	80.00(63.43)	+++	N
0	0	2.22	0	0(0.0)	-	-
0	0	4.44	0	0(0.0)	-	-
0	0	0	2.27	40.00(38.85)	++	N
0	0	0	4.54	26.66(30.78)	++	N
0	0	0	6.81	20.00(26.56)	++	N
2.06	2.68	0	0	93.33(81.14)	++++	F
2.06	5.37	0	0	66.66(55.36)	++++	F
4.12	2.68	0	0	53.33(47.30)	+++	F
4.12	5.37	0	0	73.33(59.28)	++++	F
0	2.68	4.44	0	33.33(35.01)	++	N
0	5.37	2.22	0	53.33(46.92)	++	N
0	5.37	4.44	0	40.00(39.23)	++	N
0	5.37	0	2.27	86.66(72.29)	+++	N
0	5.37	0	4.54	66.66(54.99)	+++	N
$LSD_{0.05}$				(20.88)		

*Mean of 30 explants; -: No callus formation; Growth of callus: +; Fair callus growth; ++: Good callus growth; +++: Very good callus growth; ++++: Excellent callus growth; Type of callus: F: Friable; N: Nodular; Figures in the parentheses are arc sine transformed values

Table 3 Effect of growth regulators on callus producing shoots and mean number of shoots per callus

Treatment (µM)					Callus producing shoots (%)*	Mean number of shoots/callus
BA	TDZ	Zeatin	NAA	IAA		
4.44	0	0	0	0	0(0.0)	0
8.88	0	0	0	0	0(0.0)	0
0	4.54	0	0	0	0(0.0)	0
0	9.08	0	0	0	0(0.0)	0
0	0	4.56	0	0	0(0.0)	0
0	0	9.12	0	0	0(0.0)	0
4.44	0	0	5.37	0	0(0.0)	0
8.88	0	0	5.37	0	0(0.0)	0
4.44	0	0	0	5.07	0(0.0)	0
8.88	0	0	0	5.07	0(0.0)	0
0	4.54	0	5.37	0	0(0.0)	0
0	9.08	0	5.37	0	0(0.0)	0
0	4.54	0	0	5.07	0(0.0)	0
0	9.08	0	0	10.14	16.67(15.00)	1.66
0	0	4.56	5.37	0	0(0.0)	0
0	0	9.12	5.37	0	0(0.0)	0
0	0	4.56	0	5.07	33.33(30.00)	3.66
0	0	9.12	0	5.07	66.67(60.00)	4.00
$LSD_{0.05}$					(18.96)	0.30

*Mean of 30 explants. Figures in parentheses are arc sine transformed values

donor plant and ten randomly selected *in vitro*-derived hardened plants regenerated each from axillary buds, callus and cell suspension following the method of Virscek Marn *et al*. (1999) with some modifications. Twelve random decamer primers were used for initial screening. The amplification reaction volume was 25µl for RAPD, containing 1U of Taq DNA polymerase, TaqDNA polymerase buffer (1 X) containing 1.5 mM $MgCl_2$,10 pmol of primer, 2.5 mM $MgCl_2$; 10 pmol of decamer primer, 2.5 mM of deoxynucleotide triphosphate (dNTP) and 30-40 ng of template DNA. Reaction mixtures were exposed to the following conditions: preliminary denaturation of DNA at 94ºC for 4 min followed by 40 cycles of denaturation at 94ºC for 30 sec. Primer annealing at temperature specific to primer for 1 min and amplification at 72ºC for 2 min. After 40 cycles, there was a final extension step of 8 min at 72ºC followed by 4ºC soak until recovery. 1 kbp DNA ladder was used as the size marker. The amplification products of RAPD were analysed on 1.4 % agarose gel prepared in 1X TAE buffer and visualised in transilluminator under UV light.

Data were scored for computer analysis on the basis of the presence of the amplified products for each primer. If a product was present, it was designated as '1', if absent, it was designated as '0', after excluding the irreproducible bands. Pair wise comparisons of control and variants, based on the presence or absence of unique and shared polymorphic products, were used to determine similarity coefficients (Jaccard 1908).

RESULTS AND DISCUSSION

Direct shoot regeneration: The results revealed that the regenerative pathway influenced the ability of the explant to regenerate plants. The explants responded very specifically to the growth regulators. Direct adventitious shoots regenerated from nodal explant when 8.88 µM BA and 9.08 µM TDZ were used alone or in combination with 5.07 µM IAA or 5.37 µM NAA (Table 1). About 88.35% of the nodal explants produced 4.66 shoots with 9.08 µM TDZ and 5.37 µM NAA (Fig 1a). No regeneration was recorded on growth regulator free medium or with 4.4 µM BA, 4.54 µM TDZ, 5.37 µM NAA and 5.07 µM IAA alone or in combination.

After trying several combinations of growth regulators,

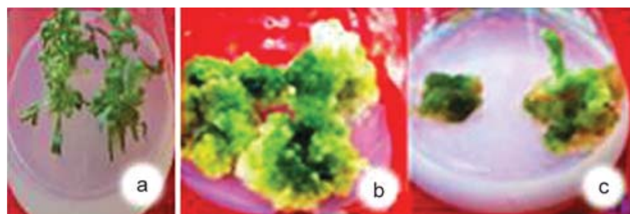

Fig 1 (a) Shoot regeneration from axillary buds on MS medium supplemented with 9.08 µM TDZ and 5.37 µM NAA, (b) callus formation on MS medium supplemented with 5.37 µM NAA and 2.27 µM TDZ, 30 day after culture, (c) shoot regeneration from callus on MS medium supplemented with 9.12 µM zeatin and 5.07 µM IAA.

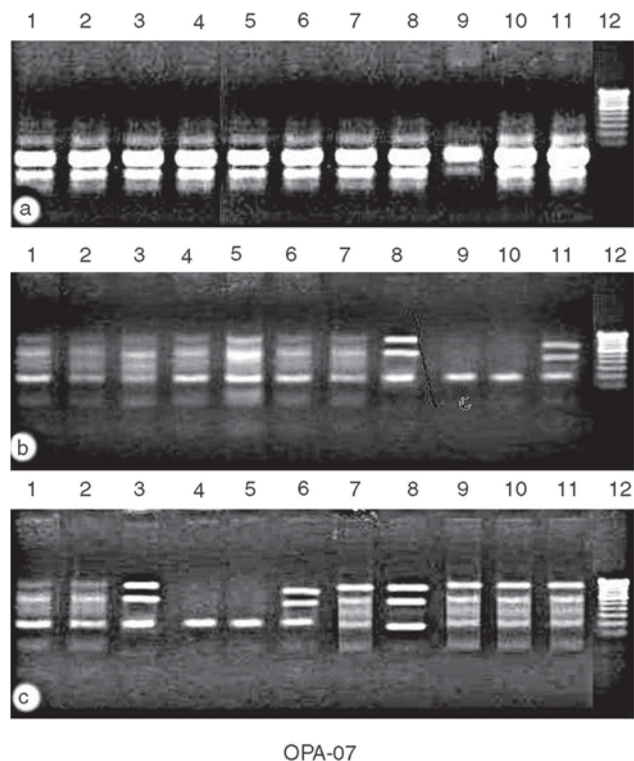

Fig 2 RAPD amplification profile of carnation cv. 'Yellow dot Com' revealed by primer OPA-07. (a) 1: parent plants, 2 -11: axillary bud raised plants 12: DNA marker (1 kb) (b) 1: parent plants, 2 -11: somatic embryo raised plants 12: DNA marker (1 kb) (c)1: parent plants, 2 -11: callus raised plants 12: DNA marker (1 kb).

MS medium supplemented with 5.37 µM NAA and 2.27 µM TDZ were identified as the best for maximum shoot regeneration potential.

About 67% of the calli derived from leaf explant produced shoots on MS medium supplemented with 4.56 µM zeatin and 5.07 µM IAA and 9.12 µM zeatin and 5.07 µM IAA, respectively (Fig 1b).

Callus mediated regeneration

The callus was initiated from the leaf explant when the explants was transferred to medium supplemented with 2,4-D, NAA, TDZ alone or in combination. The maximum callus (93.3%) was observed on the medium containing 2.06 µM2, 4-D and 2.68 µM NAA followed by 86% with 5.37 µM NAA and 2.27 µM TDZ (Fig 1c).The best callus growth was recorded when 2.06 and 4.12 µM 2,4-D was used in combination with 2.68 or 5.37 µM NAA (Table 2). However, callus cultured on medium devoid of growth regulators did not show any response regarding regeneration. Difficulty in regenerating plants from callus due to their highly dedifferentiation nature has been observed previously (Shiba and Mii 2005).

The average number of 3-4 shoots per callus was observed with these treatments. Shoots were not observed on medium with 4.44 and 8.88 µM BA, zeatin or TDZ when used alone (Table 3). Thakur *et al.* (2002) reported best

callus growth from internode segments of two carnation cultivars Cabret and Feyenoord with 0.5 mg/L NAA and 0.5 mg/L 2, 4-D. High frequency callus induction was achieved with 1 mg/L 2, 4-D, 2.2 mg/L TDZ and 0.12 mg/L zeatin from leaf segments of carnation cultivar Candy (Kumar *et al.* 2006). Kanwar and Kumar (2009) reported highest average number of shoots (6.50 shoots) per leaf callus with 2 mg/L TDZ and 1 mg/L IAA in cultivar Indios.

The variation in the number of shoots may be due to the difference in the cultivar, type of the explant and culture conditions used. Shoot regeneration in carnation is reported to be influenced by genotype, explant and the balance of growth regulators (Kallak *et al.* 1997).

The best callus mass and growth was observed on the medium with 2.06 μM 2, 4-D and 2.68 μM NAA but the callus was friable and did not regenerate shoots. This callus was used for cell suspension studies. The optimum concentration of 2.06 μM 2, 4-D and 2.68 μM NAA was found to be best for isolation and separation of cells in liquid MS medium when kept on the shaker at a shaking speed of 100-120 rpm in darkness. The cell density was increased by sub-culturing suspension into fresh liquid medium at weekly intervals and the maximum cell density was found after two months of inoculation. The first divisions in cells occurred within a week of suspension culture, the dividing cells changed to elongate or kidney shaped and showed bulge at one position and septum in the culture before the formation of two cells. Two dividing cells also showed papillae indicating cell growth and their preparedness for cell division to form four celled stage. 8-celled colonies emerged within one week of first cell division. Cells were allowed to divide in the liquid medium till the formation of micro-colonies of more than 32 cells followed by their culturing on the same but semi-solid (0.6% agar) or solid medium (0.8% agar). Brown spherical micro-calli were observed within 4 weeks of culturing on solid medium, which were allowed to increase in size to form macro-callus. The macro-calli were allowed to increase subsequently in size for 3-4 weeks to form embryogenic callus on MS medium containing 2.06 μM 2, 4-D and 2.68 μM NAA. About 50-60% of the calli produced globular somatic embryos (Fig 2h) within 2-3 weeks on growth regulator free medium.

After 3 weeks globular shaped embryos developed further to form torpedo and cotyledonary shaped embryos. Earlier, Shiba and Mii (2005) reported highly regenerable cell suspension cultures in *Dianthus acicularis* with 1 mg/L 2,4-D while Choudhary and Chin (1995) reported initiation of cell suspension cultures in carnation from friable callus with 5, 10, 15 or 20 μM 2, 4-D.

In the present investigation, the optimum quantity of callus for suspension culture was found to be 1.25 – 1.50 g per 20 ml of liquid MS medium containing 2.06 μM 2,4-D and 2.68 μM NAA. These results are in agreement with Kumar and Kanwar (2007), who inoculated 0.8-1 g callus per 20 ml in *Gerbera jamesonii* and Shiba and Mii (2005) who inoculated 0.5 g callus per 30 ml of liquid medium in *Dianthus acicularis*. In the present investigation, optimum plating cell density was adjusted to 1-3 × 10^4 cells per ml. It was also observed that single cell showed poor division, whereas cell aggregates of 2-5 showed enhanced divisions leading to micro-callus formation. Pradhan *et al.* (1998) also derived callus from cell suspension culture of *Dalbergia latifolia* by plating cell clumps of 20-25 cells on solid MS medium with 10.18 μM NAA and 2.2 μM BA and 10% coconut water. Scott *et al.* (1988) reported that single cell of *Shorea roxbhurgii* failed to form colonies, whereas small cluster of 5-6 cells did divide to form callus. This may be attributed due to the reason that the single cell may be damaged during isolation or were unable to adapt to the phase change from liquid to solid medium where the material was less readily available and inhibitory metabolites builds up around cells (Torrey and Reinert 1961).

The somatic embryos were transferred to the same medium for regeneration of plantlets and about 80% of the somatic embryos germinated to form complete plantlets. Similar results were reported earlier by Seo *et al.* (2007) and Karami *et al.* (2007). After four weeks, the plantlets

Table 4 Nucleotide sequences and RAPD amplification results of the primer used in the PCRAmplification

Primer	Sequence	Scorable bands	MotherPlant	Monomophic bands			Polymorphic bands			Fragment size (bp)
				A	B	C	A	B	C	
OPA-07	5’-GAAACGGGTG-3’	4	4	3	2	1	1	2	3	1000-500
OPA-11	5’-CAATCGCCGT-3’	3	3	3	3	3	0	0	0*	1000-700
OPA-17	5’-GACCGCTTGT-3’	3	3	3	3	3	0	0	0	600-200
OPB-12	5’-CCTTGACGCA-3’	3	3	3	3	3	0	0	0	1000-600
OPC-12	5’-TGTCATCCCC-3’	4	4	4	4	4	0	0	0	900-500
OPC-18	5’- TGAGTGGGTG-3’	2	2	2	2	2	0	0	0	1000-700
OPE-03	5’-CCAGATGCAC-3’	5	5	5	1	5	0	4	0	1000-500
OPE-06	5’-AAGACCCCTC-3’	2	2	2	2	1	0	0	1	800-400
OPE-07	5’-AGATGCAGCC-3’	3	3	3	3	3	0	0	0	700-300
OPG-09	5’-CTTCACCCGA-3’	3	3	2	2	2	0	1	1	900-600
OPK-09	5’-CCCTACGGAC-3’	2	2	2	0	0	0	2	2	900-700
	Total	34	34	33	25	27	1	9	7	

A = Plants raised from axillary bud; B = Plants raised from somatic embryo; C – Plants raised from callus.

obtained from axillary bud, callus and somatic embryos were removed from culture vessels and transferred to pots in glasshouse with 60-80% survival 30 days after transfer to pots.

Somaclonal variation generally occurs during tissue culture when indirect regeneration is stimulated resulting in plants that can be genetically altered (Kaeppler and Phillips 1993, Kaeppler *et al*. 2000). Due to their uncontrollable and unpredictable nature, their occurrence is a serious drawback in propagation of elite species. Detection and analysis of genetic variation can help in understana the molecular basis of various biological phenomenons in plants (Kumar *et al.* 2010 and Kumar *et al*. 2011). Variation induced in tissue cultured plants are most likely reflected in the banding profiles developed by different marker system (Phillips *et al*. 1994). The RAPD profile showed 2.94%, 26.47% and 20.58% polymorphism in plants raised via different pathways. The lowest polymorphism was detected in plants raised through axillary buds as only one primer (OPA-7) showed variation (Table 4). Highest polymorphism was observed in the plants raised from somatic embryos. These findings support the fact that a meristem-based micropropagation system is much more stable genetically than those in which regeneration occurs via callus or cell suspension phase. Joshi and Dhawan (2007) also reported that plants regenerated from adventitious buds around axillary buds or from other well-developed meristematic tissue showed the lowest tendency for genetic variation. Ozden-Tokatli *et al.* (2006) showed the presence of somaclonal variations in microshoots of pistachio after many cycles of axillary bud proliferation. The genetic alteration may be due to the activation of plant retrotransposons in response to stress caused by tissue culture. In the present study, a total of 20 decamer primers were used for initial screening with the mother plant of carnation but only 11 RAPD primers gave clear and reproducible bands. The number of scorable bands for each primer varied from 2 (OPE-06, OPK-09) to 5 (OPE-03) (Table 4). The 11 primers produced 34 distinct and scorable bands with an average 3.33 bands per primer. Each primer generated a unique set of amplification products ranging from 200 (OPA-17) to 1000 bp (OPA-07, OPA-11, OPB-12, OPC-18, OPE-03). Of the eleven RAPD primers, ten primers exhibited identical RAPD profile (Fig 2a). One primer OPA-07 showed difference between micropropagated and mother plants. Primers OPA-07, OPE-03, OPG-09 and OPK-09 showed variations in somatic embryo-raised plants (Fig 2b), whereas primers OPA-07, OPE-06, OPG-09 and OPK-09 exhibited polymorphism in callus-raised plants (Fig 2c).

Pairwise similarity coefficient among parent plants and axillary bud, somatic embryo and callus-raised plants ranged from 0.97 -1.00, 0.65-1.00 and 0.75 to 1.00, respectively (Tables 5, 6 and 7). In this way, more than 97% similarity was obtained from samples raised from axillary buds indicating higher similarity and fidelity. On the other hand, 75 to 80% similarity was obtained in somatic embryo and callus derived plants indicating that some variations have occurred during these pathways.

Table 5 Jaccard's similarity matrix of parent (P) and 10 axillary bud-derived plants

	P	1	2	3	4	5	6	7	8	9	10
P	1.00										
1	1.00	1.00									
2	1.00	1.00	1.00								
3	0.97	0.97	0.97	1.00							
4	1.00	1.00	1.00	0.97	1.00						
5	1.00	1.00	1.00	0.97	1.00	1.00					
6	1.00	1.00	1.00	0.97	1.00	1.00	1.00				
7	1.00	1.00	1.00	0.97	1.00	1.00	1.00	1.00			
8	1.00	1.00	1.00	0.97	1.00	1.00	1.00	1.00	1.00		
9	1.00	1.00	1.00	0.97	1.00	1.00	1.00	1.00	1.00	1.00	
10	1.00	1.00	1.00	0.97	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table 6 Jaccard's similarity matrix of parent (P) and 10 somatic embryo-derived plants

	P	1	2	3	4	5	6	7	8	9	10
P	1.00										
1	0.91	1.00									
2	0.91	1.00	1.00								
3	0.81	0.84	0.84	1.00							
4	0.81	0.84	0.84	1.00	1.00						
5	0.93	0.96	0.96	0.87	0.87	1.00					
6	1.00	0.91	0.91	0.81	0.81	0.93	1.00				
7	0.93	0.85	0.85	0.75	0.65	0.87	0.93	1.00			
8	1.00	0.91	0.91	0.81	0.81	0.93	1.00	0.93	1.00		
9	1.00	0.91	0.91	0.81	0.81	0.93	1.00	0.93	1.00	1.00	
10	0.93	0.85	0.85	0.65	0.70	0.87	0.93	1.00	0.93	0.93	1.00

Table 7 Jaccard's similarity matrix of parent and callus culture-derived plants

	P	1	2	3	4	5	6	7	8	9	10
P	1.00										
1	0.93	1.00									
2	0.87	0.93	1.00								
3	0.90	0.96	0.90	1.00							
4	0.81	0.87	0.93	0.90	1.00						
5	0.90	0.96	0.96	0.93	0.90	1.00					
6	1.00	0.93	0.87	0.90	0.81	0.90	1.00				
7	1.00	0.93	0.87	0.90	0.81	0.90	1.00	1.00			
8	0.93	0.87	0.81	0.84	0.75	0.84	0.93	0.93	1.00		
9	0.90	0.84	0.84	0.81	0.78	0.81	0.90	0.90	0.96	1.00	
10	1.00	0.93	0.87	0.90	0.81	0.90	1.00	1.00	0.93	0.90	1.00

The plants regenerated from callus, cell suspension or protoplast culture of certain species differed genetically despite the fact that the cultures originated from a highly homogenous background (Xu 2010). In conclusion a good degree of clonal fidelity and low frequency of genetic variations confirm the utility of micropropagation technique for exploitation at commercial level. However, callus and

somatic embryo mediated regeneration may be used to improve genetic variability of those species which have otherwise limited germplasm resources to be utilized in improvement program.

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