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*Full Length Research Paper*

# *In vitro* selection for resistance to *Fusarium oxysporum* f. sp. dianthi and detection of genetic polymorphism via RAPD analysis in carnation

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**Embryogenic calli derived from leaf base explants of two carnation cultivars, Lia and White Liberty, were cultured *in vitro* for resistance to toxic metabolites produced by *Fusarium oxysporum* f.sp.dianthi, using two selection methods, the double – layer culture and culture filtrate techniques. Results indicated that the reduction of callus growth rate was higher with the *in vitro* selection method using double layer than with the *in vitro* selection method using culture filtrate. Results also revealed that embryogenic callus percentage, shoot formation and root induction percentages were affected by *in vitro* selection methods. Regenerated plants from the two tested cultivars were grown to maturity in the greenhouse. Five primers were used to amplify DNA of the two selected carnation cultivars and their sixteen somaclones. A total of 62 amplification products were obtained, out of which 96.15% showed polymorphism. Genetic similarity among the eighteen genotypes ranged from 0.32 to 0.91. Using RAPD technique, the regenerated somaclonal variant lines and their parents were classified into two clusters: The cultivar Lia and its 8 somaclones were grouped in one cluster while the cultivar White Liberty and its somaclones were included in another cluster. The present study indicated that the use of RAPD technique was sensitive and powerful to detect genetic variation at the level of DNA among carnation variants. This might be of particular importance in the future, dealing with *in vitro* selection for *Fusarium* – resistant lines.**

**Key words:** Carnation, *Dianthus caryophyllus* L., *Fusarium oxysporum* f.sp.dianthi, *in vitro* selection.

## INTRODUCTION

Carnation (*Dianthus caryoplyllus* L.), is one of the most important cut flowers around the world. Carnation flowers are, also, an aromatic, stimulant herb that has been used in tonic cordials in the past to treat fevers, though this use is now obsolete. It is traditionally prescribed in European herbal medicine to treat coronary and nervous disorders. The flowers are considered to be alexiteric, antispasmodic, cardiotonic, diaphoretic and nervine. Carnations are attacked by a number of pathogens, including fungi, bacteria and viruses. Vascular wilt, caused by *Fusarium oxysporum* f.sp.*dianthi*, is considered to be the most serious and sever disease affecting carnation (Lahdenpera, 1987). Chemical control is not effective; biological systems (suppressive soils) are being studied currently but not yet routinely applied (McCain et al., 1980; Garibaldi and Gullino, 1989). Steam sterilization of the soil is an expensive method of cultivation. There are genetic sources of resistance in the carnation germplasm (Schiva et al., 1982). Resistance is a qualitative character and consequently, through selection, it is possible to obtain more resistant varieties (Schiva et al., 1985). *In vitro* selection for disease resistance presents an excellent opportunity to assess the direct application of tissue culture to crop improvement. Genetic variation can be enhanced by cell and tissue culture (somaclonal variation technology) and may possibly lead to new source of resistance, and in *vitro*

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selection procedures may increase the efficiency of selection. Induced mutants exhibiting partial or complete disease resistance can be isolated (Line et al., 1974). The principal limitation to the widespread use of induced mutagenesis lays in the low recovery frequencies ($10^{-4}$ to $10^{-6}$) in $M_2$ populations (Brock, 1979) of specific single gene mutants. This necessitates the screening of very large populations to identify disease resistant individuals. However, somaclonal variation often occurs at higher frequencies (up to 10% per cycle of regeneration) than chemical or radiation induced mutation, making it a viable alternative to mutagenesis and a valuable tool for the plant geneticist to introduce variation into breeding programme (Skirvin et al., 2000). They, also, reported that somaclonal variation has been implicated in many phenotypical changes in plants, and named cultivars have been released from somaclonal variation studies. The objectives of this study were to investigate the effect of Fusarium toxins on the *in vitro* traits and to obtain well defined phenotypes, resistant to culture filtrate of *F. oxysporum* f. sp. dianthi and to detect the polymorphism among somaclones and their parents using RAPD markers.

## MATERIALS AND METHODS

### Isolation and identification of pathogenic fungi

Root-rotted and damped-off carnation plants were collected from Beissar farm-EL-Montazah-Alexandria, Egypt, during the growing seasons of 2005 and 2006. The diseased plants were cut into small pieces of 0.5 cm, washed thoroughly with tap water, surface sterilized by dipping in a 1% sodium hypochlorite solution for 2 min. then rinsed several times in sterile water and dried between sterilized filter paper. The surface sterilized pieces were placed on potato dextrose agar medium (PDA) supplemented with streptomycin sulphate at the rate of (50 µg $ml^{-1}$) in Petri-dishes and kept at room temperature, 28°C, for 5 days. The isolated fungi were purified by using single-spore isolation and hyphal-tip methods. Pure cultures were grown on PDA medium. The fungal isolates were identified as *F. oxysporum* f. sp. dianthi. The obtained isolates were identified in the Plant Pathology Department, Faculty of Agriculture, Alexandria University, according to Barnett and Hunter (1972).

### Pathogenecity test

The pathogenic potentialities of the isolated fungi were tested under greenhouse conditions. Plastic pots of 16 cm in diameter, were filled with autoclaved peat moss and sandy soil (1:1 v/v), and aerated for one week. Fungal inoculate of the tested fungi isolate was prepared by culturing on sterilized sorghum grains which kept in 500 ml conical flasks, then incubated at room temperature (28°C) for 2 weeks. The soil was infected with the fungal inoculate, (10 g $kg^{-1}$ soil) and left for one week for the establishment of the inoculate. Pots were irrigated daily for one week before planting. The non inoculated treatment was treated similarly but with the addition of sterilized sorghum grains. Ten surface sterilized carnation offsets were sown in each pot, and five replicates were used for each treatment. Pots were irrigated every other day after planting. During the three weeks after planting the percentages of pre-and post-emergence damping-off, of each treatment, were recorded.

### The double - layer culture technique

The double–layer culture technique classically utilized in microbiology (Lepoivre et al., 1986) was modified as follows: vials containing 40 ml of potato-dextrose agar (PDA) medium (consisting of extract of 200 g potato tubers. 20 g glucose and 7 g agar per liter), were inoculated with mycelium of *F. oxysporum* f. sp. dianthi. These cultures were grown at 26°C for 4 days in a 16/8 h light – dark cycle, then at 5°C for 3 days in continuous light. Subsequently, the vials were autoclaved at 121°C for 15 min to kill the fungal cells (Joffe, 1974). After 2 to 3 h, the cooled agar medium containing the thermostable toxic compounds (Patey and Gilbert, 1989) was over layered with 40 ml of MS callus-growing medium supplemented with 1.0 mg/l of BAP and 0.01 mg/l of NAA. After diffusion of the toxic materials from the fungal culture into the upper nutrient layer (7 days), 5-weeks-old carnation calli were placed on the upper medium (5 calli/vial) for 5 weeks at 25 ± 2°C under 16 h photoperiods, and toxin-sensitivity was assessed on the basis of callus weight. The surviving calli were transferred to MS regeneration medium containing 1.0 mg /l of BAP + 0. 01 mg / l of NAA. The regenerated plants were transplanted into pots and grown to maturity in the greenhouse.

### Preparation of culture filtrates

The culture filtrates of the fungi were prepared according to Tuite (1969). The fungi were cultured in liquid PD medium in conical flasks of 100 ml volume, and were maintained at 25°C in the dark for 50 days. The cultures were then put in a large flask of 1 liter capacity to be homogeneous and the fungal mycelia were eliminated. The remaining liquid was then centrifuged (5000 r.p.m.) for 20 min, then the remaining mate were eliminated. The filtrates were passed through a double layer of filter paper. Hyphae were eliminated from the culture fluid by filtration with 8 µ mesh membrane filter, and the pH of the fluid was adjusted to 5.75 with 1 N of NaOH and/or 1N of HCl. The culture filtrates were then sterilized by 0.2 µmesh membrane filter. The sterilized culture filtrates were mixed with concentrations of (10, 20, 30, 40 and 50%), respectively with melted MS subculture medium containing 1. 2% (w/v) agar and solidified in jars each containing 40 ml. Six week-old carnation calli were placed (3 calli/jar) for 4 weeks at 25 ± 2°C in 16 h photoperiod. The regenerated shoots were transferred to rooting medium (MS without growth regulators). The resulted plantlets, 4–6 cm in length, were transferred to small pots of peat moss, then were put in the greenhouse and grown to mature plants.

### Molecular characterization

### Plant material

PCR analysis was carried out using the genomic DNA from the carnation cultivars, Lia and White liberty, and their somaclones which were obtained by *in vitro* selection. The carnation cultivars as well as their somaclones lines were grown in pots.

### DNA extraction

Frozen young leaves (500 mg) of Carnation genotypes were ground to a powder in a mortar with liquid nitrogen. The DNA extraction was done using CTAB method (Sagahi-Maroof et al., 1984).

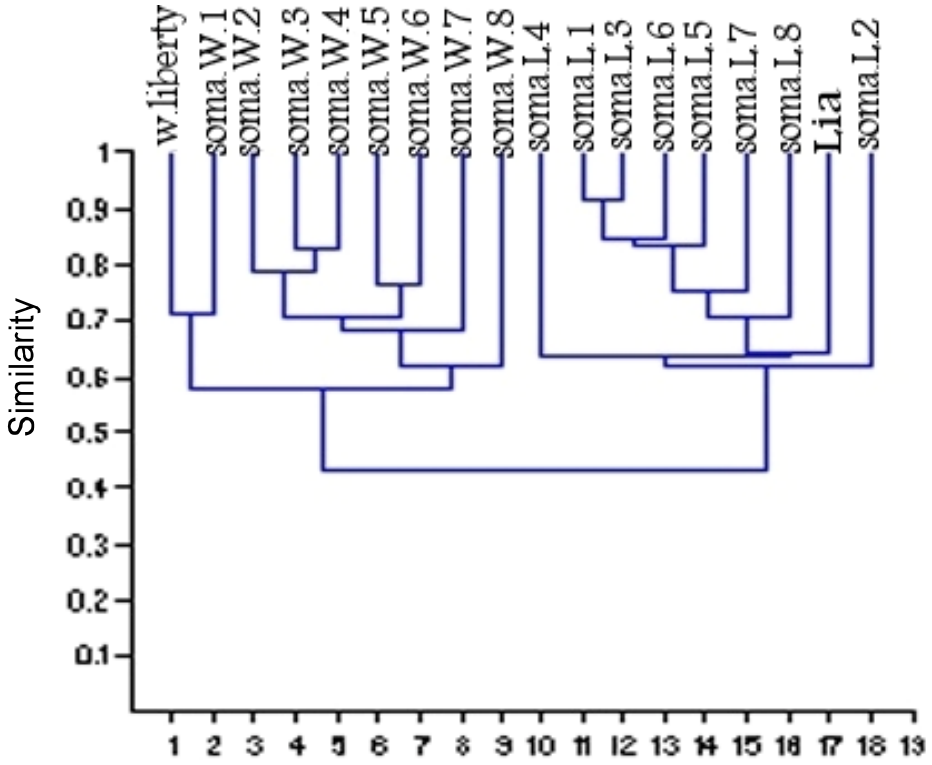


**Figure 1.** Dendogram of genetic distances, constructed using RAPD data and the UPGMA method of clustering, shows DNA similarity between somaclonal variant lines and their parents.

***PCR amplification***

Five primers (Table 3), obtained from Pharmacia Biotech. (Amersham Pharmacia Biotech., UK Limited, HP79NA, England), were tested in this experiment, to amplify the templated DNA. Amplification reaction volumes were 25 µl, each containing 1X PCR buffer with $MgCl_2$ [50 mM KCl, 10 mM tris –HCl (pH =9.0), 2 mM $MgCl_2$ and 1% Trition X – 100], 200 µM of each of dATP, dGTP, dCTP and dTTP, 50 PM primer, 50 ng template DNA and 1.5 µl of Taq polymerase. Reaction mixtures were exposed to the following conditions: 94°C for 3 min., followed by 45 cycles of 1 min. at 94°C, 1 min. at 36°C, 2 min. at 72°C, and a final 7 min. extension at 72°C. Amplification products were visualized with DNA marker on 1.6% agarose gel with 1X TBE buffer and were detected by staining with an Ethidium Bromide solution for 30 min. Gels were, then distained in deionized water for 10 min. and photographed on Polaroid films under U. V. light.

**Data handling and cluster analysis**

Data were, statistically, analyzed as a 2 – factor experiment (cultivars and selection methods) in a completely randomized design with six replicates. Shoot number was subjected to square root transformation prior to statistical analysis (Steel and Torrie, 1980). Comparisons among means were made using the Least Significant Differences test (LSD) The data were analyzed, using SAS program, version 6 (1985).

Data were scored for computer analysis on the basis of the presence of the amplified products for each primer. If a product was present in a cultivar, it was designated as “1”, if absent, it was designated as “0”, after excluding the unreproducible bands. Pair-wise comparisons of cultivars, based on the presence or absence of unique and shared polymorphic products, were used to determine similarity coefficients, according to Jaccard (1908). The similarity coefficients were then used to construct dendograms, using the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) employing the SAHN (Sequential, Agglomerative, Hierarchical, and Nested clustering) from the NTSYS–PC (Numerical Taxonomy and Multivariate Analysis System), version 1.80 (Applied Biostatistics) program (Rohlf, 1993) (Figure 1).

## RESULTS AND DISCUSSION

### Effect of toxin on *in vitro* traits

Two *in vitro* selection methods were used. The first method was the *in vitro* selection of carnation cells for in sensitivity to toxic metabolites of *F. oxysporum* f. sp. dianthi via a double layer culture technique. The second method was the *in vitro* selection of carnation cells resistant to culture filtrate of *F. oxysporum* f. sp. dianthi. In a preliminary experiment, calli of carnation were cultured on medium with different concentrations of pathogen filtrate (10, 20, 30, 40, and 50%). The culture filtrate at 10% was found inhibitory to the growth of cell culture since no growth occurred above the 10% (v/v) level of the filtrate. Therefore, the treatment 10% culture filtrate was used for *in vitro* selection of carnation cells resistant to culture filtrate of *F. oxysporum* f. sp. dianthi

**Table 1.** Means of callus growth rate (%) and embryogenic callus (%) as influenced by selection methods and cultivars x selection methods interaction.

| Cultivar | Callus growth rate (%) | | | Embryogenic callus (%) | | |
|---|---|---|---|---|---|---|
| | Selection method | | | Selection method | | |
| | Control | 10% culture filtrate | Double layer | Control | 10% culture filtrate | Double layer |
| Lia | 139.67 | 32.49 | -18.75 | 77.16 | 36.95 | 19.02 |
| White liberty | 198.75 | 22.23 | -24.24 | 80.37 | 13.62 | 23.72 |
| Selection method mean | 169.21 | 27.36 | -21.49 | 78.76 | 25.28 | 21.37 |
| L.S.D(0.05) Cultivar × selection method | | 28.13 | | | 9.74 | |

**Table 2.** Means of shoot formation (%) and root induction (%) as influenced by cultivars x selection methods interaction.

| Selection method | Trait | |
|---|---|---|
| | Shoot formation | Root induction |
| Control | 5.81 | 5.54 |
| 10% culture filtrate | 0.47 | 0.20 |
| Double layer | 0.24 | 0.17 |

L.S.D. $_{(0.05)}$ for selection method means of shoot formation = 0. 50; L.S.D. $_{(0.05)}$ for selection method means of root formation = 0. 47.

## Callus growth rate

The analysis of variance indicated that the percentage of callus growth rate was, highly, significantly influenced by *in vitro* selection methods and the interaction between cultivars and *in vitro* selection methods. The cultivars had no significant effect on callus growth rate. The effects of selection method on the growth rate of callus for the two carnation cultivars are shown in Table 1. It was clear from the table that the reduction of the growth rate of callus was higher with the *in vitro* selection method using double layer than with the *in vitro* selection method using culture filtrate. Moreover, results in Table 1 indicated that the interaction was significant between carnation cultivars and *in vitro* selection method for callus growth rate. The interaction resulted to the greater reduction in "White Liberty" cultivar compared to "Lia" cultivar.

## Embryogenic callus

Data of the analysis of variance revealed that embryogenic callus percentage was significantly affected by *in vitro* methods and the cultivar x *in vitro* method interaction, whereas the variations between cultivars were statistically insignificant. The control gave the significantly highest embryogenic callus percentage (78.76%) compared to 10% culture filtrate and the double layer methods (25.28 and 21.37%, respectively) as shown in Table 1. Means of the two *in vitro* selection methods was statistically equal. The cultivar x selection method interaction (Table 1) arose with the differential response of the two cultivars to *in vitro* selection method. Whereas Lia cultivar showed higher embryogenic callus percentage for the 10% culture filtrate method (36.95%) compared to double layer method (19.02%). White liberty cultivar showed a completely opposite trend where the double layer method gave significantly higher embryogenic callus percentage (23.72%) compared to the culture filtrate method (13.62%).

## Shoot formation

Data of the analysis of variance revealed that shoot formation percentage was affected by *in vitro* selection methods, whereas, the variation between cultivars and the variations due to the *in vitro* selection and cultivars interaction were statistically insignificant. The control gave the significantly highest shoot formation percentage (5.81%) compared to 10% culture filtrate method (0.47%) and the double layer method (0.24%) as shown in Table 2. Means of the two *in vitro* selection methods were statistically similar. Whole plant regeneration at flowering stage had been obtained under greenhouse (Figure 2).

## Root induction

The analysis of variance indicated that the percentage of root induction was highly significantly influenced by *in vitro* selection methods. The cultivars and the *in vitro* selection x cultivars interaction did not significantly affect

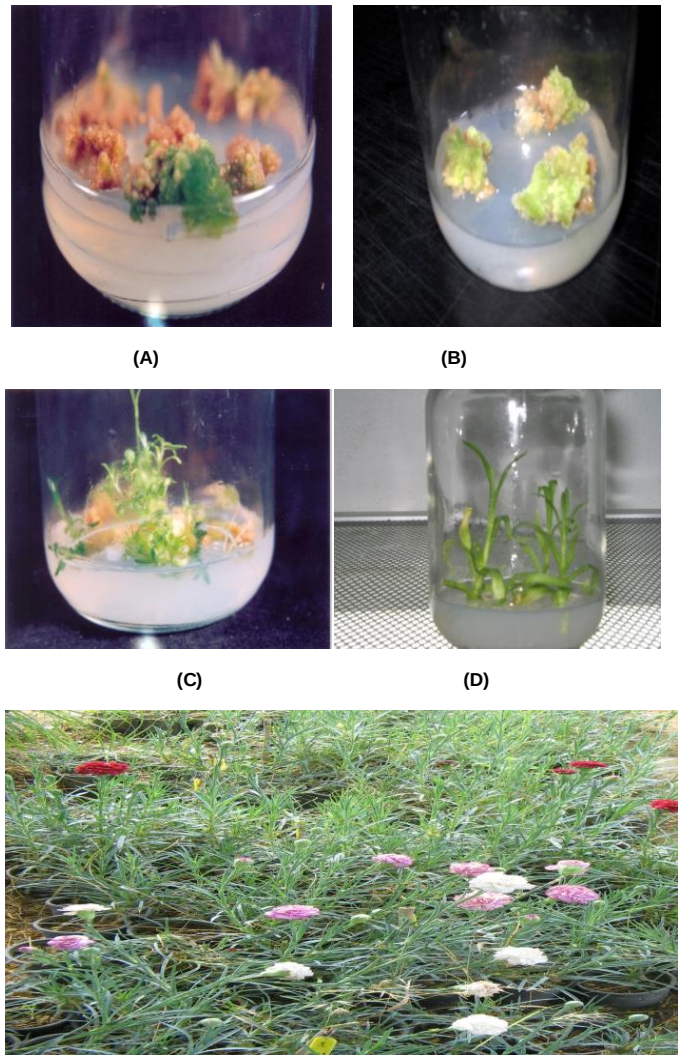


**Figure 2.** *In vitro* selection to *Fusarium oxysporum* in carnation: (A) Callus formation on 10% Fusarium culture filtrate, (B) Embryogenic callus on the double layer media, (C) Shoot formation on 10% *Fusarium* culture filtrate media, (D) Shoot formation on rooting media for root induction, (E) Whole plant regeneration at flowering stage.

the root induction. The control gave the significantly highest root induction percentage (5.54%) compared to 10% culture filtrate and the double layer methods (0.20 and 0.17%, respectively) as shown in Table 2. Means of the two *in vitro* selection methods was statistically equal.

Although conventional breeding practices have been broadly successful in developing disease resistant cultivars, inefficient screening procedures and the lack of appropriate genetic variation can limit progress in particular cases. *In vitro* selection for disease resistance presents an excellent opportunity to assess the direct application of tissue culture to crop improvement (Barakat and Abdel – Latif, 1996). Genetic variation can be enhanced by somaclonal variation technology and may possibly lead to new sources of resistance, and the *in vitro* selection procedures may increase the efficiency of selection.

Results in this study demonstrated that the culture containing 10% Fusarium culture filtrate was suitable for selecting carnation calli resistant to toxic metabolites of the fungus. In agreement with Arcioni et al. (1987), it was found that inhibition of callus growth was due to the *Fusarium* metabolites present in the selective media. A number of previous studies have also found that the toxin present in the culture filtrate was able to inhibit cell growth and the cells of the host species were more sensitive to the toxin than those of non – host plants (Malepszy and El Kazzaz, 1990). Binding et al. (1970)

**Table 3.** Number of amplification and polymorphic products, using five primers in carnation cultivars and their somaclones.

| Primer number | Nucleotide sequence 5`to 3` | Number of amplification[a] | Number of polymorphic[b] | Polymorphism b/a (%) |
|---|---|---|---|---|
| 1 | GACCGCTTGT | 13 | 10 | 76.9 |
| 2 | GGTGCGGGAA | 12 | 11 | 91.7 |
| 3 | GGTGACGCAG | 13 | 11 | 84.6 |
| 4 | AGCCAGCGAA | 10 | 8 | 80.0 |
| 5 | CAATCGCCGT | 14 | 12 | 85.7 |

was the first to demonstrate the possibility of *in vitro* selection of callus cultures for desirable traits. Many attempts have been made since then to apply such selection schemes for crop improvement, including resistance to *Fusarium* wilt (Arai and Takeuchi, 1993; Scala et al., 1998; Mosquera et al., 1999).

An alternative of the *in vitro* selection method using culture filtrate, the double – layer culture technique is based upon the *in vitro* selection of somaclonal variants insensitive to toxic metabolites of *F. oxysporum* f. sp. dianthi. The callus growth rate of carnation on the upper medium layer was strongly inhibited. Majority of calli ceased growth, turned brown and died within 4 weeks after being transferred on the upper layer. The regeneration of shoots from some calli had succeeded after transferring the calli on subculture media. The obtained number of plants was 11 and 8, for the cultivars Lia and White liberty, respectively. These results were similar to those of Ahmed et al. (1991) and Abdalla et al. (2002). Thus, the double layer technique seems to be a useful method of selecting *Fusarium* – resistant callus of carnation and is definitely cheaper than the *in vitro* selection methods using either culture filtrate or commercially available *Fusarium* toxins. Recently, Svabova and Lebeda (2005) reported that the basic advantages of using *in vitro* cultures when compared with natural conditions are: (1) unfavourable weather and climate conditions are avoided, which enables to assess quantitative differences in polygenic traits more easily and precisely; (2) a large number of individuals can be tested in a small space; (3) it is easier to manipulate mutants, haploids, somaclones with higher variability in the genome; and (4) mass screening of mutants for resistance is facilitated.

## RAPD analysis

### *Screening for polymorphic primers in carnation cultivars and their somaclones*

Five primers were screened for their ability to amplify the genomic DNA of the two carnation cultivars, Lia and White liberty, and their somaclones. The number of DNA fragments amplified ranged from 10 to 14 depending on the primer and the DNA sample with a mean value of 12.4 bands per primer (Table 3). These values are rather high for RAPD amplification, compared to the average numbers of amplified bands recorded in other crops; namely three fragments in *Triticum turgidium* L. (Joshi and Nguyen, 1993), 4.3 fragments in *Solanum tuberosum* L. (Masuelli et al*.,* 1995) but much lower than that reported in Chrysanthemum varieties and their somaclones (Barakat et al*.,* 2002). The size of fragments ranged from 200 to 1600 bp. A total of 62 fragments were produced by the five primers. Of these 62 amplified fragments, 16.7% were not polymorphic; whereas, the remaining bands (83.3%) were polymorphic in one or other of the eighteen genotypes (two cultivars and their somaclones). However, Figure 3 shows the amplification profiles, generated by primer 4 across the carnation cultivars and their somaclones. The 8 scorable bands were polymorphic across the carnation genotypes. Previously, RAPD markers tightly linked to the locus controlling carnation, *D. caryophyllus*, flower type were identified by Scovel et al. (1998). Genotype identification of ornamental species by RAPD had been reported by Benedetti et al. (2001). Likewise, RAPD analysis had been used also to identify DNA markers correlated to *F. oxysporum* resistance in the greenhouse carnation (Scovel et al., 2001). Williams et al. (1990) reported that polymorphism among individuals could arise through nucleotide change that prevented amplification by introducing either a mismatch at one priming site, deletion of a priming site, insertions that rendered priming sites too distant to support amplification and insertions or deletions that change the size of the amplified product. Recently, RAPD analysis was used for the identification of molecular markers associated with flower longevity that could be used for the early screening of $F_1$ progenies with a long vase life (Benedetti et al., 2005).

## Cluster analysis

One of the goals of the present study was to investigate the efficiency of RAPD markers in determining, accurately, the genetic relationship between somaclones and their parents. The RAPD markers, produced by five primers, were used to construct a similarity matrix. Simple matching coefficient, ranging from 0.32 to 0.91, suggested a broad genetic base for carnation genotypes.

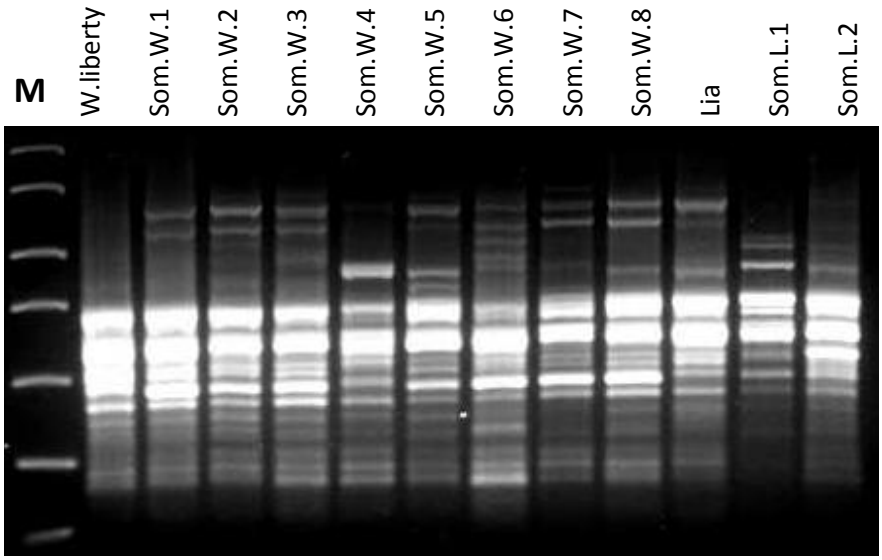


**Figure 3.** RAPD polymorphism in carnation cultivars and their somaclones.

Figure 1 represents the clustering of carnation cultivars generated by UPGMA analysis of the parents; namely, “Lia” and “White liberty”, and their somaclones. The results of characterization analysis revealed a high diversity between the two cultivars. Carnation genotypes appeared clustered into two well-defined groups; the first group comprised the “Lia” cultivar and its somaclones. The second group included the “White liberty” cultivar and its somaclones. Each group containing four or five clusters and, more or less, at the same rate of similarity. The highest level of similarity in Lia group was found for Lia-somaclone 1 “soma L. 1” and “soma L. 3”, with almost 0.91 similarity and the lowest level of similarity in “Lia” group was found for “soma L. 2”, with almost 0.62. Moreover, in the “White liberty” group, the highest and the lowest level of similarity were found for “soma W. 2” and “soma W. 4” (83%) and for “soma W. 1” and “soma W. 5” (54%), respectively.

These results indicated that RAPD technique could be successfully applied to ornamental crops. DNA markers are currently used to identify varieties and to analyze inter – and intra – specific genetic relatedness (Rajapakse and Ballard, 1997). Genetic diversity of *Dianthus chinensis* and *D. caryophyllus* with RAPD analysis was reported by Wen et al. (2003). (You Bo et al. (2004) ) used RAPD technique to amplify the genomic DNA fragments of 87 carnation cultivars. Their results revealed that the 87 cultivars were divided into 10 groups at the DNA level. These results have significance for the genetic breeding and identification of cultivars of carnation. The present study indicated that the use of RAPD technique was sensitive and powerful to detect genetic variation at the level of DNA among carnation cultivars and their somaclones. RAPD analysis also allows the detection of changes due to *in vitro* selection methods to *F. oxysporum*.

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## REFERENCES

Abdalla MY, Mutate MI, Barakat MN, El–Rokaeba AA (2002). *In vitro* selection for resistance to *Fusarium graminearum* in wheat by tissue culture and RAPD techniques. Alex. J. Agric. Res., 47 (1):67-75.

Ahmed KZ, Mesterhazy A, Sagi F (1991). *In vitro* techniques for selecting wheat (*Triticum aestivum* L.) for *Fusarium* resistance. I: Double – layer technique. Euphytica, 57:251-257.

Arai M, Takeuchi M (1993). Influence of Fusarium wilt toxin (s) on carnation cells. Pl. Cell Tiss. Org. Cult., 34:278-293.

Arcioni S, Pizzotti M, Damiani F (1987). *In vitro* selection of alfalfa plants resistant to *Fusarium oxysporum* f. sp. medicaginis. Theor. Appl. Genet., 74: 700-705.

Barakat MN, Abdel-Latif TH (1996). *In vitro* selection of wheat callus tolerant to high levels of salt and plant regeneration. Euphytica, 91: 127-140.

Barakat MN, Abdel Fattah RS, Badr M, El-Torky MG (2010). *In vitro* culture and plant regeneration derived from ray florets of Chrysanthemum *morifolium*. Afr. J. Biotechnol., 9(8): 1151-1158.Barnett HL, Hunter BB (1972). Illustrated genera of imperfect fungi. Burgess Pub. Co. Minneapolis, USA, p. 241.

Benedetti LD, Mercuri A, Bruna S, Burchi G, Schiva T (2001). Genotype identification of ornamental species by RAPD analysis. Acta Hort., 546: 391-394.

Benedetti L, Braglia L, Bruna S, Burchi G, Mercuri A, Schiva T (2005). PCR–based markers and cut flower longevity in carnation. Acta Hort., 683: 437-442.

Binding H., Binding K, Starub J (1970). Selection in Gewebekulturen mit haploiden Zellen. Naturwissensch, 3: 138-139.

Brock RD (1979). Seed protein improvement in cereals and grain

legumes. IAEA (ED.) Vienna IAEA, 1: 43-55.
Garibaldi A, Gullino ML (1989) Progress towards Fusarium resistance in carnation. Pro Eucarpia Meeting on Carnation and Gerbera. Alassio, pp. 111-114.
Jaccard P (1908). Nouvelles recherches sur la distribution lorale. Bull. Soc. Vaud. Sci. Nat., 44: 223-270.
Joffe AZ (1974). Toxicity of *Fusarium poae* and *F. sporotrichoides* and its relation to alimentary toxic aleukia. I. F. H. purchase (Ed), Mycotoxins. Elsevier, Amsterdam, pp. 229-262.
Joshi CP, Nguyen HT (1995). RAPD analysis based intervarietal genetic relationships among hexaploid wheats. Plant Sci. Lim., 93 (1-2): 95-103.
Lahdenpera ML (1987). The control of *Fusarium* wilt on carnation with a streptomyces–preparation. Acta Hort., 216: 85-92.
Lepoivre P, Viseur J, Duhem K, Carles N (1986) Double-layer culture technique as a tool for selection of calluses resistant to toxic material from plant pathogenic fungi. In. J. Semal. (Ed): Somaclonal variations and crop improvement. Nijhoff, Dordrecht, pp. 45-52
Line RF, Konzak CF, Allan RE (1974). Induced mutations for disease resistance in crop pants. IAEA (ed) Vienna, pp. 125-132.
Malepszy S, El Kazzaz A (1990). *In vitro* culture of Cucumis sativus. XI. Selection of resistance to *Fusarium oxysporum*. Acta Hortic., 280: 455-458.
Masuelli RW, Tanimoto EY, Brown CR, Comai L (1995). Irregular meiosis in a somatic hybrid between *Solanum bulbocastanum* and *S. tuberosum* detected by species-specific PCR markers and cytological analysis Theor. Appl. Genet., 91: 401-408.
McCain A, Pyeatt LE, Byrne TG, Farnham DS (1980). Suppressive soil reduces carnation disease. California Agric., 34: 5-9.
Mosquera T, Rodriguez LE, Bernal C, Lopez F (1999). *In vitro* selection for resistance to *Fusarium oxysporum* f. sp. dianthi in carnation. Acta Hortic., 482: 309-311.
Patey AL, Gilbert J (1989). Fate of Fusarium mycotoxins in cereals during food processing and methods for their detoxification. In. J. Chetkowski (Ed.): *Fusarium* mycotoxins, Taxonomy and Pathogenecity. Elsevier, Amesterdam, pp. 399-420.
Rajapakse S, Ballard RE (1997) Cultivar identification using molecular methods. In: Geneve RL. Preece JE. Merkle SA (Ed) Biotechnology of ornamental plants. CAB International. Wallinford. UK (Biotechnology in agriculture series), 16: 153-164.
Rohlf FG (1993). Numerical Taxonomy and Multivirate Analysis System. Exeter Software, New York.
Sagahi-Maroof M, Jorgensens K, Allard R (1984). Ribosomal DNA spacer length polymorphisms in barely: Mendelian inheritance, chromosome location and population dynamics. Proc. Natl. Acad. Sci., 81: 8014-8018.
SAS Institute Inc (1985). SAS/STAT. Guide for personal computers. Version 6, 4th ed. Vol. 2 Cary NC, USA.
Scala A, Tegli S, Goggioli V, Lagazio C (1998). Host genotype and temperature-dependent colonization of *in vitro* carnation cultures by *Fusarium oxysporum* f. sp. dianthi race2. Phytoparasitica, 26: 213-222.
Schiva T, Guda CD, D´Aquila F, Bianchini C, Garibaldi A (1982). Selection for resistance oat *Fusarium oxysporum* f.sp.dianthi. Annali Istituo sperimental floricoltura XIII 1: 115-132.
Schiva T, Guda CD, Mercuri A (1985).Analysis genetic for resistance to *Fusarium oxysporumf*. sp. dianthi.Annali Institution Sperimentale Floricoltura XVI, 1: 23-38.
Scovel G, Ben-Yephet Y, Ovadis M, Reuven M, Vainstein M (2001). Markers assisted selection for resistance to *Fusarium oxysporum* in the greenhouse carnation. Acta Hort., 552: 151-156.
Skirvin RM, Coyner M, Norton MA, Motoike S, Gorvin D (2000) Somaclonal variation: do we know what cause it? Pl. Tiss. Cult., 2: 46-48.
Steel RGD, Torrie JH (1980). Principles and Procedures of Statistics. A Biometrical Approach. (2 nd edition). McGraw Hill Book.
Svabova L, Lebeda A (2005). *In vitro* selection for improved plant resistance to toxic-producing pathogens. J. Phytopath., 153: 52-64.
Tuite J (1969). Plant pathological methods, fungi and bacteria. Library of Congress. Cat. Card no.69-17858. Burgess publishing Co. 426, South Sixth st. Minneapolis, Minn., p. 55415.
Wen W, YouMing C, HuiYu Z, MinRen H (2003). Genetic diversity of *Dianthus chinensis* L. and *D. caryophyllus* L. with RAPD. J. Nanj. For. Univ., 27 (4): 72-74.
Williams GK, Kubelik AR, Livak KL, Rafalski JA, Tingey SV (1990). DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., 18: 6531-6535.
You Bo S, Chun L, Jing M, Xijun M, Ming Y (2004). RAPD identification of different carnation cultivars. Acta Hort. Sin., 31(1): 109-111.