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MOLECULAR ANALYSES OF GLADIOLUS LINES WITH IMPROVED RESISTANCE AGAINST FUSARIUM WILT

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Abstract

The genetic changes/variations taken place during the process of *in-vitro* selection for improvement of Fusarium resistance in the susceptible Gladiolus cultivar were assessed. The selected cell lines showing resistance against *Fusarium oxysporum* were analyzed with total of 29 amplified reproducible RAPD fragments. The number of fragments per primer ranged from 4 in S13 and R8 to 10 fragments in S19. The total size of the amplified products varied from 200bp to 1800bp. All the primers were found to be polymorphic and produced different percentages of polymorphism. The average number of fragments per primer was 6 from which 62% were found to be polymorphic fragments. Similarity indices were generated from RAPD data. Since all morphological characteristics of the resistant lines were non significant when compared with control therefore, it can be concluded that the phylogenetic differences among various groups are because of the activation of tolerance level in the highly susceptible Gladiolus cultivar against Fusarium wilt.

Abbreviations: CAMB (Centre for Advanced Molecular Biology), CV (Cultivar), FOG (*Fusarium oxysporum* f. sp. gladioli) and UPGMA (Unweighted Pair Group Method Arithmetic Average)

Introduction

Gladiolus is one of the most widely cultivated, economically important flowering plants. The luxuriance unique colorful spikes of some high demanding Gladiolus cultivars have attained immense importance in the community of flower lovers. During the last two decades, biotechnological approaches have been adopted to improve agricultural practices that have opened vistas for plant improvement and utilization (Memon *et al*., 2010). It is possible to generate Fusarium resistance through breeding or transformation of resistant gene (s), but all these techniques will also change other important characteristics like color, shape and size of flower which can deprive the market demand of a well adapted variety. According to Wenzel & Foroughi-Wehr (1990), it is most likely that the progenies of the selected somaclones may not differ significantly from the starting material. The direct use of *in-vitro* selection at cellular level is a powerful and valuable approach especially when the improvements of one or two easily identifiable characters are desired in an important variety. The main advantage of *in-vitro* selection is that the basic genotype of the candidate variety is usually altered slightly for the targeted trait only as compared with hybridization of two distinct varieties. Desired character (s) like disease resistant can be activated without altering unique flower colour of a variety, thus reducing the time required to breed and improve variety with the same desired character through conventional hybridization method. As reported by Dallavalle *et al*., (2002), the soil born fungus, *Fusarium oxysporum* Schlecht: f. sp. gladioli (Massey) Snyd & Hans (FOG) (Massey 1926; Nelson *et al*., 1981) which causes yellowing and corm rot, is the main pathogen of Gladiolus. Remotti & Löffler (1996) investigated the role of Fusaric Acid in the development of Fusarium corm rot of Gladiolus. According to their findings, Fusarium infected gladioli symptoms are stunted growth related partial or complete corm rot. As reported by Nasir & Riazuddin (2009) that plant cells maintain character of totipotency for long period, but their regenerative ability may be lost under certain conditions e.g., when higher concentrations of purified fungal toxin are applied during *in-vitro* selection for disease resistance. According to their findings, modified selection protocol has enhanced the plantlets regeneration from the selected cells. To determine whether the resistance is stable; the progenies of somaclones or vegetatively propagated somaclones have to be retested for resistance to the pathogen. Nasir & Riazuddin (2008) demonstrated that the *In-vitro* selected cell lines of Gladiolus through activation of Fusarium resistance trait showed the same response whether or not inoculated with conidia of *Fusarium oxysporum* for *In-vivo* plant bioassays. Plantlets of all the selected cell lines exhibited significant growth as compared with control after two applications of conidia of *Fusarium oxysporum*.

Several cultivars have shown that traits such as short straw, early maturity and especially resistance to certain diseases can be introduced in otherwise well-adapted varieties without significantly altering their original attributes. In vegetatively propagated crops like Gladiolus, mutants are usually used directly as improved variety. It has been reported that Gamma irradiation is found useful for the induction of *Fusarium* mildew resistance in the ornamental plant species, for example, two carnation mutants, selected from Gamma-irradiated cuttings were designated as more resistant to *Fusarium oxysporum*. Another Gamma-irradiated carnation mutant (Loncerda) proved to be highly resistant, to Fusarium (Mitteau & Silvy, 1983).

This is the first report on the application of RAPD-PCR analyses to assess whether selected cell lines those are showing varying levels of tolerance against *Fusarium* wilt have also been altered genetically or not.

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Materials and Methods

Plant materials and DNA extractions: DNA from leaves of *In-vitro* selected, Gamma irradiated cell lines and control of Gladiolus cultivar Friendship (CV-1) was extracted using the CTAB method, described by Rogers & Bendich, (1985), with modifications. 0.1 g of fresh tissue from each individual plantlet was grounded to a fine powder in liquid nitrogen using pre-cooled mortar and pestle. Added 0.5ml of extraction buffer (2% w/v CTAB, 100mM Tris HCl, pH 8.0, 20mM EDTA, pH 8.0 1.4M NaCl) and 0.1ml of 20% SDS. The mixture was thoroughly stirred and incubated at 65^{o}C for 10 minutes, 0.5 ml of 5M potassium acetate was added and kept at 0^{o}C for 20 minutes. The DNA was precipitated by adding 10ml of isopropanol. The pellet was air dried and dissolved in 700 µl of deionized water. Single stranded RNA was digested with RNase-A (10mg/ml) for 30 minutes at 37°C, extracted once with chloroform isoamylalcohol (24:1) and precipitated by adding 1/10th volume of 3M Na acetate and equal volume of isopropanol. The pellet was washed with 70% ethanol. The pellet was air dried completely and was resuspended in autoclaved distilled water.

Quantification of DNA: The DNA was quantified by measuring its absorption on spectrophotometer in buffer solution (TE Buffer, pH 7.5) at 260nm. Absorption of 1 optical density corresponds to 50µg/ml dsDNA.

DNA was also quantified by electrophoresis (3V/Am) in 1% agarose gels (w/v) by comparison to known concentration (100µg) of lamda phage DNA. The DNA was visualized by ethidium bromide staining and DNA solutions were then diluted to 100µg/µl for PCR reaction.

Amplification conditions and analyzing: PCR amplifications were performed in a DNA Engine (MJ Research) Thermocycler, as described by Vieria *et al.,* (1997), with modifications. The reaction mixture contained 10mM tris-HCl, pH 8.3, 50mM KCl, 2.5mM MgCl2, 1mM dNTPs, 2.5µM primer, 250-300µg template DNA, 2 units of Taq polymerase, and an ultra pure autoclaved water in a final volume of 25µl. The reactions were subjected to 45 cycles, after an initial denaturation at 94^{o}C for 2 minutes. Each cycle consisted of 1 minute at 94^{o}C 1 minute at 36 - 42^{o}C and 1minute at 72^{o}C with a final 5min extension at 72^{o}C.

Amplicons were analyzed by electrophoresis in 1% Agarose gel with 1X TBE buffer (0.09M tris-borate, 1M EDTA (pH 8.0). A DNA ladder (100bp, Pharmacia) was used as molecular weight marker. The gels were photographed under UV light, using a Gel-Documentation 2000 system (Bio-Rad).

Data evaluation: The amplification of DNA samples was repeated thrice and only reproducible bands on several runs were considered for analyses. Each amplified product was scored across all the 10 resistant lines and control of CV-1 for 5 out of 20 random 10-mer primers. Whereas, Gamma irradiated D1 line could only be amplified by S-13 and S19 primers. Bands were scored as present (+) or absent (-) for each cell line, weak and spurious bands were not included in the analyses. A pair wise similarity matrix as reported by Dice, (1945) was constructed using the DICE similarity index SD=2a / (2a + b + c), where 'a' is number of shared bands between a pair of plants 'X' and 'Y'; 'b' is the number of bands present in plant 'X' and absent in plant 'Y' and 'c' is the number of bands present in 'Y' and absent in plant 'X'. Similarity estimates were analyzed using Unweighted Pair Group Method Arithmetic Averages (UPGMA) provided by Mega 2.1 software and the resulting cluster were represented as dendrogram.

Results

RAPD analyses: This is first report of RAPD application to understand whether *in-vitro* generated Fusarium resistant Gladiolus lines have also been genetically improved during the process of *in-vitro* selection using various selection pressure of the Fusaric acid or not. RAPD analyses of 10 *Fusarium* resistant, one Gamma irradiated and control were performed with total of 29 amplified reproducible fragments produced from 5 out of 20 random decamer primers. The number of fragments per primer ranged from 4 in S13 and R8 to 10 fragments in S19. The total size of the amplified products varied from 200bp to 1800bp. All the primers were found to be polymorphic and produced different percentages of polymorphism. The average number of fragments per primer was 6 from which 62% were found to be polymorphic fragments. RAPD amplification profiles obtained using five decamer primers in 10 FOG resistant cell lines, one Gamma irradiated and control of CV-1 are shown (Table 1). Gamma irradiated cell line could only be amplified by S-13 and S-19 primers. R8 primer produces 7 fragments (Fig. 1); R15 primer produces 6 fragments (Fig. 2). S13 primer produces 4 fragments (Fig. 3). S18 produces 5 fragments (Fig. 4). S19 primer produces 10 fragments (Fig. 5). It was observed that 75% fragments of R8 primer, 60% fragments of R15, 50% fragments of S13, 60% fragments of 13 and 66% fragments of S19 were found to be polymorphic.

Cluster analyses: Cluster analyses were done on the basis of similarity co-efficient generated from RAPD data of 29 fragments. Similarity indices ranged from 0.40 to 0.95 among 10 resistant lines, one Gamma irradiated and control were noted. All the ten resistant lines were grouped into one major cluster, which gave similarity co-efficient range from 0.40 to 0.95 (Fig. 6). The dendrogram shown in Fig. 6 representing the similarity indices data generated through RAPD-PCR analyses were grouped into one cluster. The dendrogram has categorized RAPD data of all the lines and control in the form of 8 different groups. The control and CAMB-G05 fall in group-1; CAMB-G10 and CAMB-G02 in group-2; CAMB-G07 and CAMB-G08 in group-3; CAMB-G04 and CAMB-G09 in group-4; CAMB-G03 in group-5; CAMB-G06 in group-6 and CAMB-G01 in group-7 whereas, the Gamma irradiated CAMB-G11 showed entirely different genetic identity when compared with control or any other group.

Table 1. Description of five decamer primers used for RAPD-PCR analyses of *In-vitro* selected, Gamma irradiated and control plants of CV-1.

	Primer	Sequence 5'-3'	Resolved bands	Scored bands	Monomorphic bands	Polymorphic bands	Fragment size of scored bands
1.	R-8	-CCCGTTGCCT-	4	4	1	3	500-1800
2.	R-15	-GGACAACGAG-	6	6	2	4	250-500
3.	S-13	-TTCAGGGTGG-	4	4	2	2	250-600
4.	S-18	-CTGGCGAACT-	5	5	2	3	250-800
5.	S-19	-GAGTCAGCAG-	10	10	4	6	200-700
				29	**11**	**18**	

Monomorphic Bands → Same Bands (similar Bands)
Polymorphic Bands → Different Bands (present in few but absent in others /not present in all)

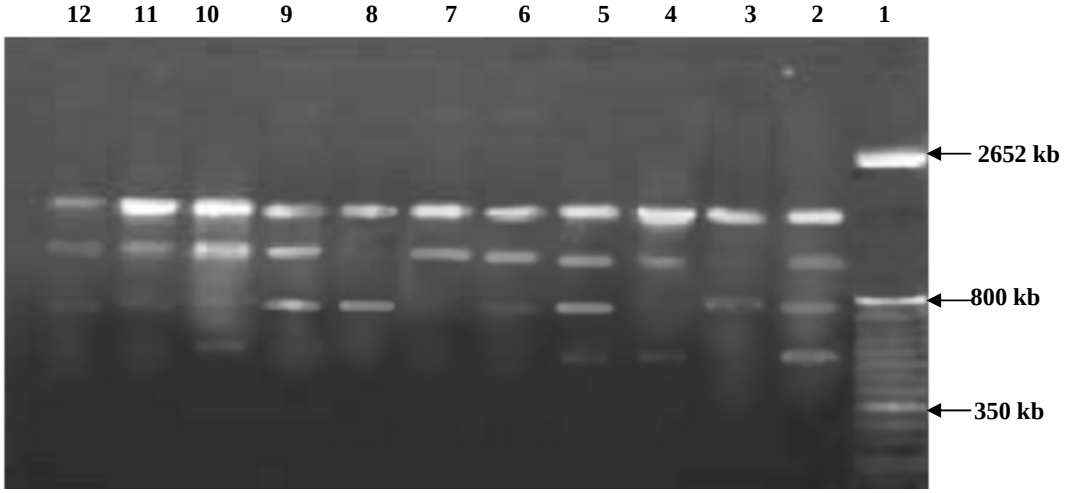

Fig. 1. RAPD profile generated from Gladiolus lines using R-8 Primer.

Lane 1 50 bp ladder (Invitrogen)
Lane 2 Control CV-1
Lane 3 CAMB-G01
Lane 4 CAMB-G02
Lane 5 CAMB-G03
Lane 6 CAMB-G04
Lane 7 CAMB- G05
Lane 9 CAMB- G06
Lane 9 CAMB- G07
Lane 10 CAMB- G08
Lane 11 CAMB- G09
Lane 12 CAMB- G10

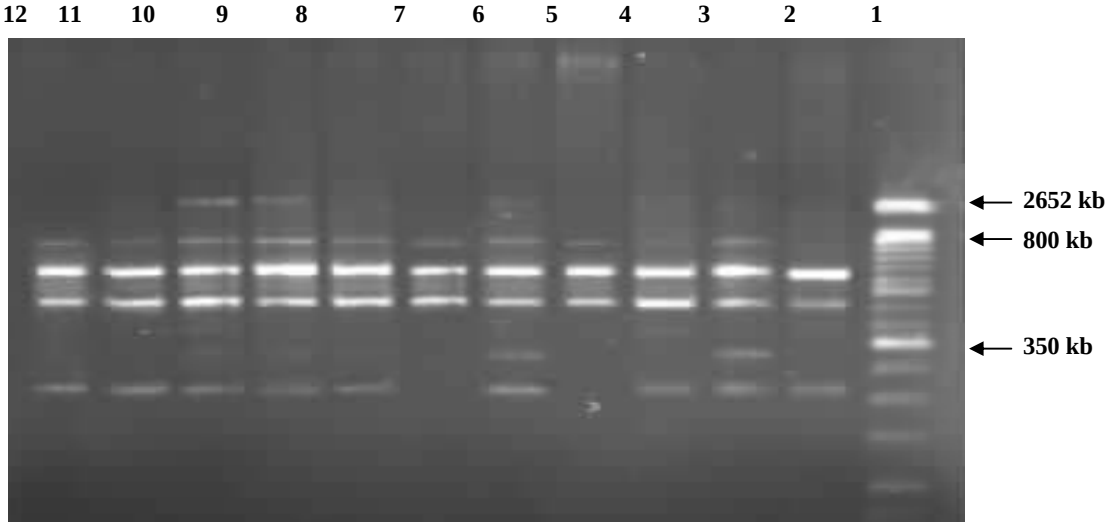

Fig. 2. RAPD profile generated from Gladiolus lines using R-15 Primer.

Lane 1 50 bp ladder (Invitrogen)
Lane 2 Control CV-1
Lane 3 CAMB-G01
Lane 4 CAMB-G02
Lane 5 CAMB-G03
Lane 6 CAMB-G04
Lane 7 CAMB-G05
Lane 8 CAMB-G06
Lane 9 CAMB-G07
Lane 10 CAMB-G08
Lane 11 CAMB-G09
Lane 12 CAMB-G10

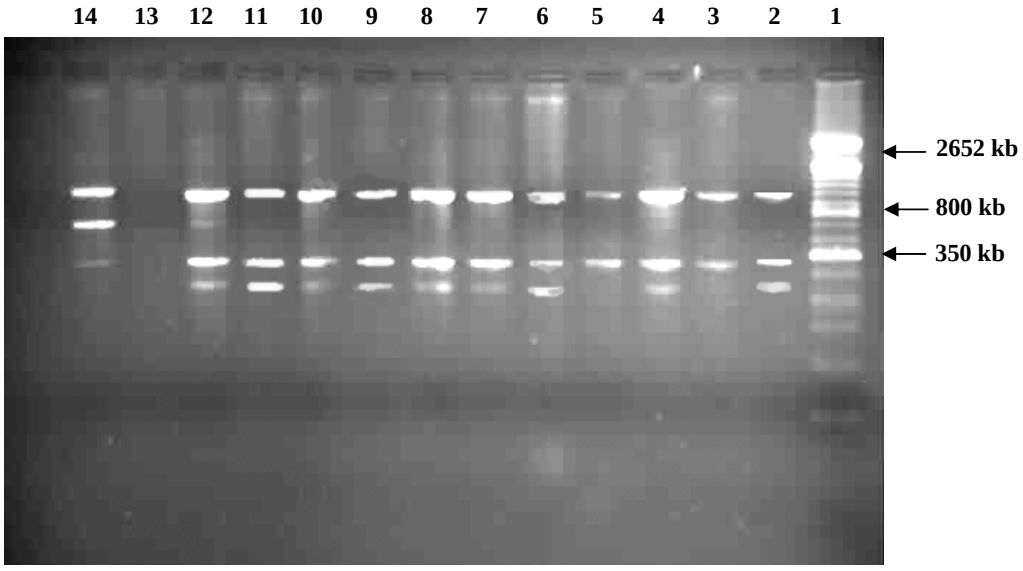

Fig. 3. RAPD profile generated from Gladiolus lines using S-13 Primer.

Lane 1 50 bp ladder (Invitrogen)
Lane 2 Control CV-1
Lane 3 CAMB-G01
Lane 4 CAMB-G02
Lane 5 CAMB-G03
Lane 6 CAMB-G04
Lane 7 CAMB-G05
Lane 8 CAMB-G06
Lane 9 CAMB-G07
Lane 10 CAMB-G08
Lane 11 CAMB-G09
Lane 12 CAMB-G10
Lane 13 Negative Control
Lane 14 Gamma Irradiated

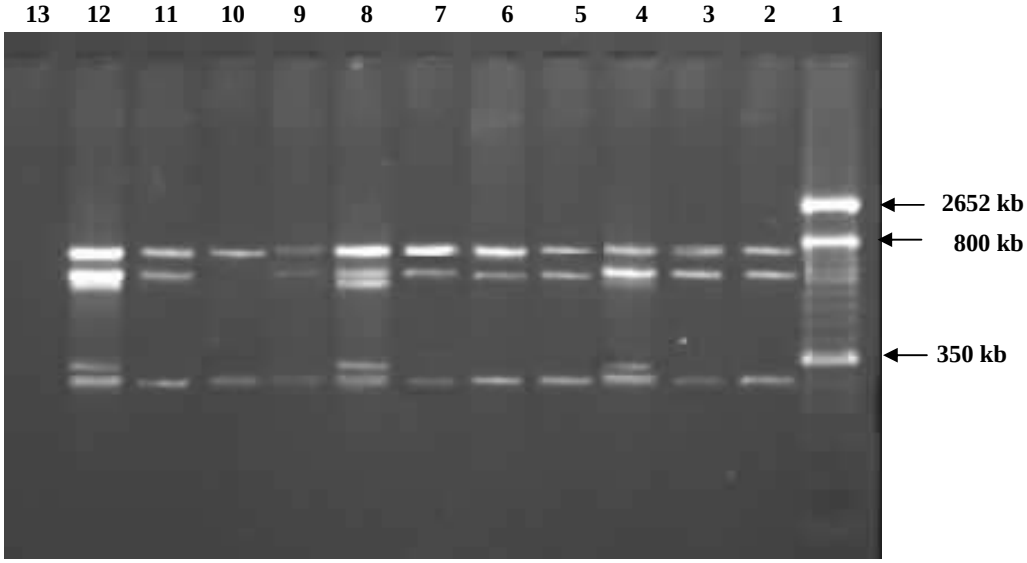

Fig. 4. RAPD profile generated from Gladiolus lines using S-18 Primer.

Lane 1 50 bp ladder (Invitrogen)
Lane 2 Control CV-1
Lane 3 CAMB-G01
Lane 4 CAMB-G02
Lane 5 CAMB-G03
Lane 6 CAMB-G04
Lane 7 CAMB-G05
Lane 8 CAMB-G06
Lane 9 CAMB-G07
Lane 10 CAMB-G08
Lane 11 CAMB-G09
Lane 12 CAMB-G10
Lane 13 -VE CONTROL

Data regarding phylogenetic analyses and average Fresh Weight (FW) of Fusarium treated cormels confirm that only one trait i.e. Fusarium resistance has been activated in at least four selected cell lines while other important characteristics like flower color, flower shape, flower size, plant height and maturity were non significant as compared to control of CV-1. During plant bioassays under field condition, the Cell lines CAMB-G1, CAMB-G4 CAMB-G6 and CAMB-G9 produced healthier and uniform size corms and the morphological characteristics were also non significant as compared with control. The differences among all selected cell-lines and control of CV-1 in the formation of cormlets under normal *in-vitro* condition were non-significant as assessed

by fresh weight of the cormlets (Table 2). While fresh weight of the cormels produced after infestation with fungal conidia under open field condition were highly significant which when correlated with phylogenetic data confirmed that the *in-vitro* selection protocol has activated Fusarium resistance in the four cell lines and also discriminated various groups on the basis of resistant, tolerant or susceptible gladiolus lines (Table 2 & Fig. 6). The dendrogram has separated high fresh weight cell lines from the low weight cell lines which confirm our findings that only single dominant trait of Fusarium resistance has been activated *In-vitro.*

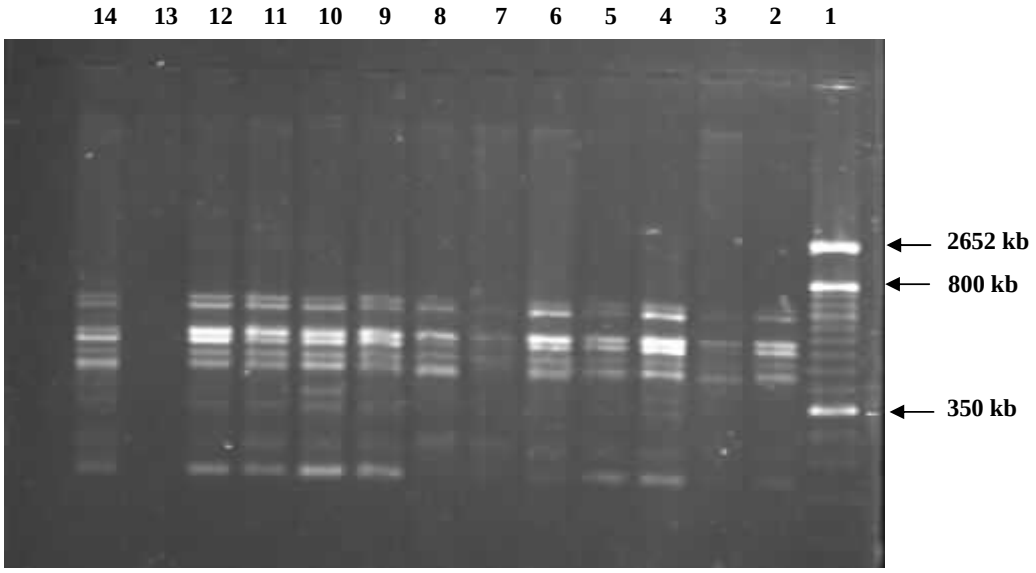

Fig. 5. RAPD profile generated from Gladiolus lines using S-19 Primer.

Lane 1 50 bp ladder (Invitrogen)
Lane 2 Control CV-1
Lane 3 CAMB-G01
Lane 4 CAMB-G02
Lane 5 CAMB-G03
Lane 6 CAMB-G04
Lane 7 CAMB-G05
Lane 8 CAMB-G06
Lane 9 CAMB-G07
Lane 10 CAMB-G08
Lane 11 CAMB-G09
Lane 12 CAMB-G10
Lane 13 Negative Control
Lane 14 GAMMA IRRADIATED

Table 2. Field Performance of toxin free VS_1 *In vitro* produced cormlets, grown *In vivo* and inoculated with fungal conidia. Average Fresh Weight (FW) of VS_1 and VS_2 was evaluated. Mean ± standard error shown.

Line Nos.	*In vitro* (VS_1) Avg. FW	*In vivo* (VS_2) Avg. FW
Control	1.46 ± 0.046	0.09 ± 0.011
CAMB-G01	1.51 ± 0.141	2.88 ± 0.427
CAMB-G02	1.29 ± 0.064	1.08 ± 0.054
CAMB-G03	1.52 ± 0.092	1.29 ± 0.103
CAMB-G04	1.526 ± 0.032	3.566 ± 0.057
CAMB-G05	1.706 ± 0.063	1.023 ± 0.02
CAMB-G06	1.60 ± 0.018	2.83 ± 0.093
CAMB-G07	1.51 ± 0.091	1.503 ± 0.242
CAMB-G08	1.31 ± 0.065	1.47 ± 0.177
CAMB-G09	1.45 ± 0.17	3.193 ± 0.150
CAMB-G10	1.63 ± 0.013	1.583 ± 0.083

Discussion

RAPD analyses: RAPD-PCR analyses of *in-vitro* selected cell-lines, gamma irradiated cell line and control of Gladiolus CV-1 were performed with a total of 29 amplified reproducible DNA fragments produced from 5 out of 20 random decamer primes. The number of DNA fragments per primer ranged from 4 by S13 and R8 to 10 DNA fragments in S19. The sizes of all amplified fragments varied from 200 bp to 1800 bp. All the primers were found to be polymorphic and produced different percentages of polymorphism. The average number of amplified DNA fragment per primer was 6 out of which 62% were found to be polymorphic. On similar findings Bindiya & Kanwar, (2003) reported a total of 286 amplification products from 19 random decamer primers producing 32% product polymorphic in micro propagated plants of *Robina pseudoacacia* L. On another findings De Laia, *et al.,* (2000) reported a total of 62 amplification products from 15 random decamer primers out of which 39 products were polymorphic in sub cultured clone A of Eucalyptus. Similarly, Goto, *et al.,* (1998) scored a total of 134 bands using 30 decamer primers in micropropagated shoots of *Pinus thunburghii* and the number of fragments for each primer varied form 2 to 7.

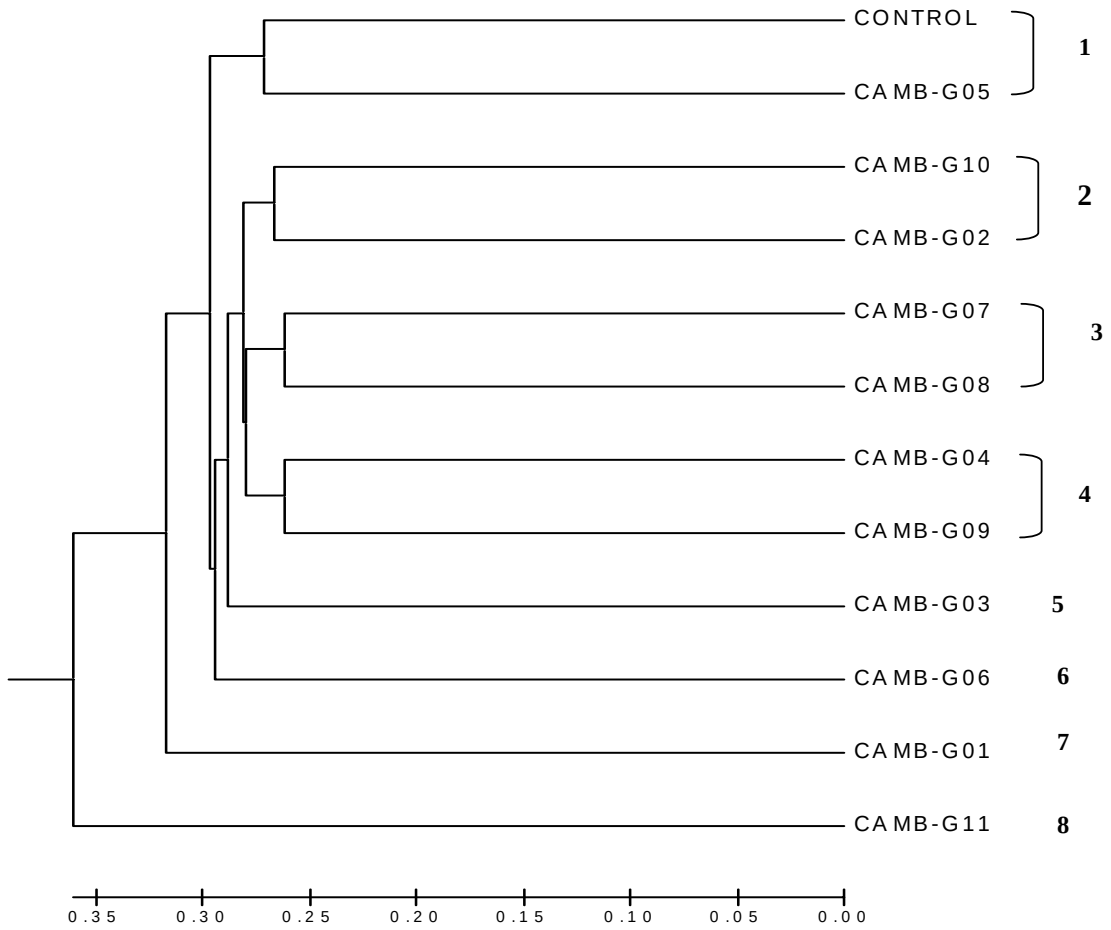

Fig. 6. Dendrogram generated from the 29 RAPD fragments among ten *in-vitro* selected one Gamma irradiated and one control of Gladiolus CV-1, using five primers. RAPD fragments obtained were visually scored as either present (+) or absent (-). Genetic distances were calculated by sample matching method and clustering was performed by UPGMA, using a programme provided by the Mega 2.1.

Cluster analyses: Cluster analyses were performed on the basis of similarity co-efficient generated from the RAPD data of 29 fragments. Significant variations from the similarity indices were detected. The data of similarity co-efficient generated from five RAPD primers used were grouped into one major cluster and found in the range of 0.4 to 0.95 of similarity level.

We observed that variation in DNA sequence is present in the *In vitro* selected FOG resistant and Gamma irradiated plants as compared with control. Williams *et al.,* (1993) reported that polymorphism in amplified fragments might resulted from changes which alter the size of target DNA (e.g. insertion, deletions, inversions). The number of copies of particular sequence in the genome may vary after cell culture resulting in de-amplification and amplification of genes (Larkin *et al.,* 1989).

Using RAPD techniques, various authors have reported the absence of genetic variation in micropropagated plants like *Picea mariana* (Isabel, 1993), *Festuca pratensis* (Valles *et al.,* 1993), *Pinus thumburghii* (Goto *et al.,* 1998), *Picea abies* (Heinze, & Schmidt, 1995). Whereas, some investigators reported presence of genetic variations in micropagated *Popalus deltoids* (Rani, *et al.,* 1995), *Beta vulgarus* (Munthali**,** *et al.,* 1996), Peach (Hashmi, *et al.,* 1997), *Robinia pseudoacacia* (Major, *et al.,* 1998; Bindiya & Kanwar**,** 2003), *Popules remutoides* (Rahman & Rajora, 2001*).*

Our findings clearly demonstrate that variations have taken place in the *In-vitro* selected and Gamma irradiated plants as compared with control. Rani, *et al.*, (1995) reported variation to the extent of 26% in micro-propagated plants of *Populus deltoids*. Major, *et al*., (1998) reported similarity co-efficient ranging from 0.51 to 0.95 between 12 tissue cultured samples of Robinia and suggested that this variability was due to accumulating mutations during long term cultures. Watanable *et al*., (1998) reported genetic similarity co-efficient in range of 84-97% in regenerated plants of *Angelica acutiloba* transplanted to the field. Similarly, Bindiya, & Kanwar, (2003) reported similarity indices ranged from 0.86 to 0.96 among 18 micropropagated plants of *Robinia pseudoacacia*.

Findings associated with vegetative cycle of the plant such as plant growth and resistance to FOG have been studied and tabulated. Data regarding phylogenetic analyses confirms that only one trait i.e. Fusarium

resistance has been activated in at least four selected cell lines while other important morphological characteristics like flower color, flower shape, flower size, plant height, maturity were non significant as compared to control. During plant bioassays the Cell lines CAMB-G1, CAMB-G4 CAMB-G6 and CAMB-G9 produced healthier and uniform size corms and the morphological characteristics were also non significant as compared with control. The differences among all selected cell-lines and control of CV-1 in the formation of cormlets under normal *in-vitro* condition were non-significant as assessed by fresh weight (FW) of the cormlets. While fresh weight of the cormels produced after infestation with fungal conidia were highly significant which when correlated with phylogenetic data confirmed that the *in-vitro* selection protocol has activated Fusarium resistance in the four cell lines and also discriminated various groups on the basis of resistant, tolerant or susceptible gladiolus lines. Since the morphological characteristics of the selected lines were non significant and the dendrogram has separated high fresh weight cell lines from the low weight cell lines that confirms our findings that only single dominant trait of Fusarium resistance has been activated *in-vitro*. Wenzel & Foroughi-Wehr (1990) confirmed our findings that it is most likely that the progenies of the selected somaclones may not differ significantly from the starting material. Further, Nasir & Riazuddin (2008) demonstrated that the plants regenerated from the *in-vitro* selected cell-lines of Gladiolus, express Fusarium resistant trait, which also confirms the findings that polymorphism in the phylogenetic data is due to activation of Fusarium resistant trait.

Our results suggest that RAPD techniques can be successfully used to assess genetic variations, which usually arise during the *In-vitro* selection process via cell suspension or callus cultures.

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