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In vitro cell selection and multiplication of *Fusarium oxysporum* f. sp. *dianthi* insensitive calli in carnation cultivars Tempo and Raggio-de-Sole

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Abstract
In present investigation, for the development of Fusarium tolerant varieties in carnation, highly susceptible cultivars *viz*. Tempo and Raggio de Sole were used. Calli of caraation cultivars were irradiated to different doses of gamma irradiation. And Callus surviving at highest dose of gamma rays was mass multiplied on callus multiplication medium (MS medium +10 µM NAA + 10 µM kin). Irridiated calli after multiplication, was treated with different concentrations of culture filtrate of *Fusarium oxysporum* f.sp. *dianthi*. 20% Culture resulted *Fusarium* wilt insensitive mutants at the end of 3rd selection cycle.

Keywords: *In vitro* cell selection, culture filtrate, Fusarium, disease resistance

Introduction
Carnation (*Dianthus caryophyllus* L.) is a member of family Caryophyllaceae is one of the world's most popular and economic cut flowers due to its keeping quality, wide range of forms, colors and ability to withstand long distance transportation. Carnation ranks among top ten cut flowers in the world and is commercially grown in India for its cut flower production. In carnation cultivation, there are several fungal diseases which poses problems and are the major contributing factor to low production and economic losse. Among these diseases, *Fusarium* wilt has become the most serious disease (Holley and Baker, 1991) [13]. The organism can colonize the vascular system without symptom expressions. Because of the soil borne nature of the pathogen, the control of this disease has been difficult. Therefore, breeding for resistance in carnation is at high priority.
In vitro multiplication of elite plant genotypes is the widely used commercial application of plant biotechnology. Today, tissue culture technique has been used extensively for micropropagation of almost all the commercially grown genotypes of carnation for quick multiplication of disease free planting material. Through tissue culture techniques, the progenies could be developed at a higher pace. These cell and tissue culture techniques aid in developing the genotypes with altered plant morphology, with a series of flower colour variations and resistance to biotic and abiotic stress as desired by the carnation breeders. Keeping this in view, the present experiment was conducted to develop disease tolerant varieties in carnation through cell selection against *Fusarium oxysporum.*

Materials and Methods
An experiment was conducted on *in vitro* cell selection of irradiated calli of carnation against *Fusarium oxysporum* f. sp. *Dianthi* in the tissue culture laboratory of Department of Floriculture and Landscaping, UHF, Nauni. Diseased samles were collected from the field and surface sterilized. After surface sterilization, the samples were cut to 4-5 mm size under laminar flow cabinet and treated with Mercuric Chloride (0.1%) following quick dip method and then washed with distilled water. Before inoculation of sterilized samples on slants of PDA (Potato Dextrose Agar medium), these were dried on sterilized filter paper. The inoculated slants were incubated in BOD at a temperature of 25°C for 7-10 days. The fungal growth appeared was identified under microscope on the basis of morphological characters after identification, isolated pure culture of pathogen was maintained on Potato Dextrose Agar.

Culture was kept at 25 ºC in BOD incubator for 7-10 days till uniform fluffy mycelial growth was obtained. The culture was stored at low temperature (4 ºC) and subcultured regularly for further use.

For Pathogenicity test the mycelial mats homogenized in a sterilized blender for 1 minute with some quantity of sterilized distilled water. The mixture was obtained through muslin cloth to get clump free suspension. For pathogenicity test, freshly prepared mycelial suspension of 10 ml was poured around ten plants of carnation raised in pots containing sterilized media. The plants were observed for the disease appearance.

For the preparation of culture filtrate of pathogen**, t**he pure culture of pathogen was cultured in liquid Richard's medium. Small bits (2 mm in dia) of fungal mycelium were taken out with the help of sterilized cork borer and one bit was inoculated in each 500 ml flask containing 250 ml medium. The inoculated flasks were kept in dark at 25±2 °C temperature. After for 2 weeks culture was obtained. The culture filtrate was separated under laminar flow conditions.

For testing the toxicity of the culture filtrate, calli were first macerated using 0.5 per cent macerozyme to separate single cells and then put in 100 per cent culture filtrate for 48 hours. After 48 hours, samples treated were stained with 0.1% erythrocin B, which stains only dead cells and these were seen under light microscope.

For *in vitro* cell selection, toxic medium was prepared by adding the different concentrations of CF of *Fusarium oxysporum* f. sp. *dianthi.* in callus multiplication medium was thoroughly mixed with culture filtrate and poured in pre-sterilized petri-plates. Callus derived after giving different gamma rays treatment was cut into small pieces of about 20 mg each and inoculated onto toxic medium in petri-plates under laminar flow cabinet. Inoculated petri-plates were subsequently sealed with parafilm and incubated in culture room. The trial was conducted in three Factorial CRD with three replications. Data on per cent survival of calli was recorded after 20 days of inoculation.

Result

The growing calli pieces after irradiation with different doses of gamma rays were grown on callus multiplication medium containing different concentrations of culture filtrate (CF) of *Fusarium oxysporum* f. sp. *dianthi*.

Per cent survival of calli

The perusal of data presented in Table 1. reveals that among different doses of gamma rays, minimum per cent survival was observed at highest gamma irradiation dose i.e. 50 Gy (48.29%). In contrast, maximum per cent survival among irradiation treatments excluding control was observed at lowest irradiation dose of 10 Gy (60.15%). In comparison to this, survival of calli pieces was 100.00 per cent in control.

Among different concentrations of CF of *Fusarium oxysporum* f. sp. *dianthi*, calli did not survive at 25 per cent concentration of CF. Minimum survival of calli was observed at 20 per cent CF (12.66%). In contrast, maximum survival was observed at 5 per cent concentration of CF (93.00%), survival of calli grown without CF was 100 per cent. In general, survival of calli was more in cultivar 'Raggio-de-Sole' (56.73%) than 'Tempo' (55.81%). Differences observed between different doses of gamma rays and cultivars were found to be non-significant.

Interaction between concentrations of CF and cultivars (Table 2.) revealed that at highest concentration (25%) the calli did not survive in any of the cultivar. Survival of calli decreased with the increase in concentration of CF and minimum survival of calli (11.03%) was recorded in case of 'Raggio-de-Sole' when 20 per cent CF was added in the medium. Cultivar 'Tempo' also showed minimum survival of calli (14.29%) at same concentration. On contrary, maximum survival of calli was observed with lowest concentration of CF (5%) in both cultivars 'Tempo' (93.64%) and 'Raggio-de-Sole' (92.36%).

Interaction between doses of gamma rays and concentrations of cultivar filtrate revealed minimum survival of 50 Gy gamma rays irradiated calli grown in MS medium having 20% CF (3.87%). In contrast, maximum (98.41%) survival was observed of unirradiated calli at 5 per cent CF which was found to be at par with 10 Gy irradiated calli (98.40%) at same concentration of CF.

Interaction among doses of gamma rays, concentrations of CF and cultivars revealed minimum survival (2.13%) of 50 Gy gamma irradiated calli of 'Tempo' grown in MS medium containing 20 per cent CF (Table 4.6 b). In case of 'Raggio-de-Sole' also, same combination of treatments resulted in minimum survival of calli (5.60%). In contrast, among different treatments of gamma rays and CF, maximum survival observed of 10 Gy gamma rays irradiated calli of 'Raggio-de-Sole' (99.20%) with 5 per cent CF in the medium was found to at par with 'Tempo' (98.42%) when unirradiated calli were grown in the same medium.

Discussion

Genetic variation in cells, tissues or plants is available, in order to have a reasonable chance for selection of the desired variant with a particular resistant trait (Bulk, 1991) [2]. To increase genetic variation, cells, tissues or plants are treated with mutagens so that plants with an enhanced tolerance to the disease could be selected (Sacristan, 1982) [25].

In present studies, *in vitro* selection was conducted at cellular level by growing callus on a toxic medium containing CF of the pathogen. At cellular level, the chances of selecting resistant cell lines are more over whole plant or tissue level. It may be due to more accumulation of phytoalexins, a biochemical resistant factor in cells *in vitro* (Latunde-Dada and Lucas, 1985; Davis and Hahlbrock, 1987; Chapell *et al.*, 1987; Parker *et al.*, 1988; Apostol *et al.*, 1989) [16, 5, 35, 22, 1], *In vitro* selection at cellular level have been reported earlier (Helgesen *et al.*, 1976; Deaton *et al.*, 1982; Miller *et al.*, 1984; Sun *et al.*, 1986) [12, 6, 20, 30].

For present studies, culture filtrate was used as selective agent. In many studies CF have been utilized as selective agent (Behnke, 1979, 1980; Scala *et al.*, 1984; Hartman *et al.*, 1984; Connell and Heale, 1986; Rotino *et al.*, 1987; McCoy, 1988; Toyoda *et al.*, 1989) [33, 34, 26, 11, 4, 24, 18,]. It contains toxic metabolites produced by the pathogen. Toxic metabolites which has a variety of compounds including peptides carbohydrates, glycopeptides and lipids (Jan *et al.*, 1993) [14], is active at the cellular level employed in cell culture (Hammerschlag, 1988) [10].

Results obtained revealed that 20 per cent of CF of *Fusarium oxysporum* f. sp. *dianthi* for both the cultivars of carnation has been found effective for selecting *Fusarium oxysporum* f. sp. *dianthi* insensitive calli. At the concentration beyond 20 per cent CF, the calli did not survive.

Resistance to *Pseudomonas syringae* pv. *tabaci* (Carlson, 1973) [36], *Xanthomonas campestris* pv. *campestris* (Mangal, 1999) [17] and *Fusarium oxysporum* f. sp. *gladioli* (Pathania

and Mishra 2003) [23] was also achieved through *in vitro* selection with the application of mutagenic treatment. In present studies, the growth of calli on toxic medium was inversely proportional to the CF concentration. Similar reports were given by (Chawla and Wenzel, 1987; Kumar *et al.*, 1996; Naik and Vendamurthy, 1997; Shukla *et al.*, 1997; Kanwar, 1997; Mangal, 1999; Thakur, 1999; Goel, 2000; Mehta, 2004; Sharma, 2005) [3, 37, 21, 29, 15, 17, 31, 38, 19, 28].

The duration of selection pressure i.e. the number of selection cycles used affects the outcome of *in vitro* selection experiments. In present studies, calli was selected after 3 selection cycles. At least two selection cycles are required to get resistant plants (Thanutong *et al.*, 1983) [38]. Discontinuous selection than prolonged carnation of the selection on toxic medium has resulted in better regeneration capacity (Chawla and Wenzel, 1987) [3]. Prolonged duration might lead to unwanted alterations e.g. elevated ploidy levels (McCoy *et al.*, 1982; Hartman *et al.*, 1984) [18, 11] and decreased regeneration ability (Thanutong *et al.*, 1983) [38]. In agreement to this, the short selection cycles of 20 days resulted more regenerate and healthy calli during this study.

Table 1: Effect of *Fusarium oxysporum* f. sp. *dianthi* culture filtrate (CF) in MS medium on percent survival of *in vitro* derived gamma ray irradiated calli of 'Tempo' and 'Raggio-de-Sole after 20 days of inoculation

Concentration of CF (%)	Dose of Gamma Rays (Gy)							Cultivars	
	Control	10	20	30	40	50	Mean	Tempo	Raggio-de-Sole
Control	100.00 (89.43)	100.00 (89.43)	100.00 (89.43)	100.00 (89.43)	100.00 (89.43)	100.00 (89.43)	100.00 (89.43)	100.00 (89.43)	100.00 (89.43)
5	98.41 (83.08)	98.40 (83.13)	95.62 (78.01)	93.40 (75.27)	89.29 (70.96)	82.26 (65.78)	93.00 (76.04)	93.64 (76.53)	92.36 (75.55)
10	89.40 (71.75)	85.63 (67.89)	83.61 (66.16)	80.37 (63.71)	79.90 (63.46)	71.48 (57.74)	81.74 (65.12)	79.67 (63.37)	83.81 (66.87)
15	61.40 (51.62)	59.60 (50.55)	56.30 (48.63)	52.33 (46.34)	40.32 (39.41)	31.53 (34.11)	50.25 (45.11)	47.29 (43.26)	53.20 (46.86)
20	19.82 (26.33)	17.25 (24.44)	15.10 (22.80)	11.73 (19.98)	8.20 (16.60)	3.87 (11.04)	12.66 (20.20)	14.29 (21.29)	11.03 (19.11)
25	0.00 (0.26)	0.00 (0.26)	0.00 (0.26)	0.00 (0.26)	0.00 (0.26)	0.00 (0.26)	0.00 (0.26)	0.00 (0.26)	0.00 (0.26)
Mean	61.50 (53.74)	60.15 (52.62)	58.44 (50.88)	56.31 (49.17)	52.95 (46.69)	48.29 (43.06)	-	55.81 (49.04)	56.73 (49.68)

Figures in parenthesis are arc sine transformed values

$CD_{0.05}$:	
Doses of gamma rays = (0.71)	Doses of gamma rays x Concentrations of Culture Filtrate = (1.72)
Culture Filtrate = (0.71)	Concentrations of Culture Filtrate x Cultivars = (1.00)
Cultivars = (0.41)	

Table 2: Interaction effect of *Fusarium oxysporum* f. sp. *dianthi* culture filtrate, (CF) cultivars and doses of gamma rays on per cent survival of *in vitro* derived irradiated calli after 20 days of inoculation

Concentrations of CF (%)	Tempo						Raggio-de-Sole					
	Doses of gamma rays (Gy)						Doses of gamma rays (Gy)					
	Control	10	20	30	40	50	Control	10	20	30	40	50
Control	100.00 (89.43)	100.00 (89.43)	100.00 (89.43)	100.00 (89.43)	100.00 (89.43)	100.00 (89.43)	100.00 (89.43)	100.00 (89.43)	100.00 (89.43)	100.00 (89.43)	100.00 (89.43)	100.00 (89.43)
5	98.82 (84.10)	97.60 (81.59)	95.20 (77.43)	94.20 (76.31)	90.40 (72.00)	85.60 (68.02)	98.00 (82.05)	99.20 (84.97)	96.04 (78.58)	92.60 (74.23)	88.18 (69.19)	80.12 (65.53)
10	85.60 (67.73)	82.20 (65.05)	83.20 (65.81)	76.60 (61.09)	76.60 (61.09)	70.00 (56.81)	93.20 (75.77)	89.06 (70.73)	84.08 (66.51)	80.33 (63.68)	83.20 (65.83)	72.96 (58.68)
15	57.73 (49.46)	56.00 (48.45)	53.40 (46.95)	50.80 (45.46)	38.60 (38.39)	27.20 (31.43)	65.06 (53.78)	63.20 (52.66)	59.20 (50.30)	53.87 (47.22)	42.04 (40.42)	35.86 (36.78)
20	23.60 (29.06)	20.43 (26.87)	17.20 (24.47)	13.20 (21.29)	9.20 (17.66)	2.13 (8.40)	16.04 (23.60)	14.08 (22.01)	13.00 (21.13)	10.27 (18.86)	7.20 (15.55)	5.60 (13.69)
25	0.00 (0.26)	0.00 (0.26)	0.00 (0.26)	0.00 (0.26)	0.00 (0.26)	0.00 (0.26)	0.00 (0.26)	0.00 (0.26)	0.00 (0.26)	0.00 (0.26)	0.00 (0.26)	0.00 (0.26)

Figures in parenthesis are arc sine transformed values

$CD_{0.05}$

Doses of gamma rays x Concentrations of Culture Filtrate x Cultivars = (2.43)

Conclusion

Per cent survival of calli on toxic medium was inversely proportional to the concentration of culture filtrate (CF). Minimum survival (12.66%) was obtained at 20 per cent CF and beyond this concentration, calli did not survive. Calli was selected after 3 discontinuous selection cycles of CF.

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