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In Vitro Callus Multiplication and Shoot Regeneration of Resistant Calli of Carnation cv. 'Raggio-de-Sole' against *Rhizoctonia solani* Kuhn

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ABSTRACT

In a procedure of *in-vitro* callus multiplication and shoot regeneration of resistant callus to *Rhizoctonia solani* Kuhn., it was observed that callus cultures were established only from leaf explants of carnation cv. 'Raggio-de-Sole' treated with 0.2% bavisitin for 6 min and 0.1% $HgCl_2$ for 2 min and not from petals or internodes segments. Pale, friable growth of callus was obtained after 40-45 days on MS basal medium containing 2.0 mg/l NAA and 0.5 mg/l 2,4-D and screened *in-vitro* for resistance to Millipore-filtered purified culture produced by the fungus *R. solani* for creating resistance to rot disease in carnation. Calli were challenged by different concentrations of culture filtrate of *R. solani*. A cell survival rate of 20.66% at a 20% selective dose of culture filtrate was achieved in unselected calli while at >25% concentration of culture filtrate, 100% calli died after 4 weeks. However, calli survived within a range of 40-93.33% subjected to 15-2% fungal culture filtrate indicating low survival rate at higher concentration and high survival rate at lower concentration. The selected calli multiplied better after screening on the callus multiplication medium. Regeneration of shoots from the resistant calli were obtained successfully after 4 weeks on MS medium containing 0.5 mg/l NAA and 0.5 mg/l IAA.

Keywords: fungus, MS medium, plant growth regulator, resistance, selective medium

INTRODUCTION

Carnation (*Dianthus caryophyllus* L.), belonging to the family Caryophyllaceae, is one of the most important cut flowers in the world and ranks second after rose attributed to quality flowers produce (Xia *et al*. 2006). Standard carnations are well known for their attractive, long-lasting and multicoloured blooms in the florist trade. India, too, has the potential for growing good quality carnations. The specific climatic requirements of the crop are met with at different places like Himachal Pradesh, West Bengal, Kashmir and Karnataka in any season. The mid hills of Himachal Pradesh offer a tremendous scope for its cultivation, where farmers can produce carnation flowers when these are not available in North Indian plains. Although carnations are attacked by a number of pathogens viz. fungi, bacteria and viruses, carnation stem rot caused by the fungus, *Rhizoctonia solani* Kuhn. (Martin 1926) is considered to be a serious problem in the world's carnation industry having resulted in huge losses. In carnation the disease was found to cause stem rot symptoms which are manifested as wilting and death of plants along with rotting of stem base (Wager 1931). This disease on carnation was firstly recorded in India from Solan District of Himachal Pradesh in 1991 (Meeta and Mathur 1991). The disease was also reported from parts of the world in destructive form such as South Africa, Paris, Italy, Israel, Egypt and Hawaii (Wager 1931; Moreau 1953; Garibaldi 1966; Elad *et al*. 1981; Sheir *et al*. 1982; Trujillo *et al*. 1988). The disease incidence in the field during the first season may reach 68-100% by keeping the soil wet for 45 days, as reported by Hadar *et al.* (1982). In Illinois, USA and Argentina, the lowest value was estimated at around 50% in cuttings and 20% annually under field condition (Peltier 1988; Wolcan *et al*. 1999). Complete resistance against the disease is lacking in existing genotypes according to the available literature. It has now been possible to obtain variants with disease resistance in carnation using *in-vitro* selection to toxins produced by pathogens (Mercuri *et al*. 1992; Sanjuan *et al*. 2001; Thakur *et al*. 2002; Mehta *et al.* 2007).

In-vitro culture is a promising tool for selection of resistant mutants. Therefore the use of resistant cultivars may help to reduce the incidence of *R. solani*. In this paper, we describe a protocol for callus multiplication and shoot regeneration of resistant calli of carnation cv. 'Raggio-de-Sole' against culture filtrate of *R. solani*.

MATERIALS AND METHODS

Establishment of callus cultures and multiplication

Commercial cv. 'Raggio-de-Sole' carnation imported from The Netherlands (Florance flora), which is reported to be susceptible to *R. solani*, was selected for callus establishment. High quality plants of this cultivar were procured from the Department of Floriculture, Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan. Leaves and internodal segments were used as explants for the establishment of callus cultures. The explants were surface sterilized with 0.2% bavistin (Indofil Chemical Co.) and washed 3-4 times with sterilized distilled water so as to remove the soil contaminants. Thereafter these were subsequently treated with 0.1% mercuric chloride solution for different time intervals and washed 3-4 times with sterilized distilled water. The treated explants were inoculated and cultured on modified Murashige and Skoog (1962) medium supplemented with different concentrations and combinations of plant growth regulators (PGRs) viz. α-naphthalene acetic acid (NAA), 2,4-dichlorophenoxy acetic acid (2,4-D), benzyl amino purine (BAP) and kinetin (Kn) for the establishment of callus cultures. All the cultures were kept in a culture room at 26 ± 2°C with a 16-hr photoperiod (40 W; 9.7 µmol/m^2/sec Philips). Chemical and reagents used throughout the study were of CDH (Central Drug House) origin.

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Fig. 1 'Raggio-de-Sole' showing stem rot symptoms under field conditions.

Preparation of culture filtrate

The fungus (*R. solani*) was isolated from infected stem of carnation cv. 'Raggio-de-Sole' showing stem rot symptoms under field conditions (**Fig. 1**). The pathogen was identified by the Department of Mycology and Plant Pathology of the University. The diseased stems, after washing under running water, were cut into small pieces (0.3-0.5 cm) and treated with 0.1% mercuric chloride for about 2-3 min. These pieces, after washing with sterile distilled water (2-3 times), were aseptically placed on PDA (potato dextrose agar) medium prepared as per the constituents mentioned by Tuite (1969) and incubated at 25°C for one week. The isolated axenic culture of *R. solani* was maintained (at 25°C in incubator for 10-15 days) on PDA medium in test tubes (10-15 ml in borosil tubes) for uniform growth and used in different experiments. The pure culture of *R. solani* was cultured in liquid Richard's medium to obtain culture filtrate (Tuite 1969). The inoculated Erlenmeyer flasks were kept in an orbital shaker incubator at 25°C and 100 rpm for about 30-35 days. The preparation of fungus culture filtrate was carried out in three distinct phases to remove mycelia (Nyange *et al*. 1997). The coarse filtration of fungal suspension was carried out through Whatman No. 42 filter paper following centrifugation at 10,000 rpm for 15 min and finally subjected to sterilization of culture filtrate through 0.22 µm. After filter sterilization, the culture filtrate was kept in the culture room for 48 hrs and stored in a refrigerator at 4°C. The supernatant was used for screening the cells for resistance against fungal toxin using various dilutions for testing the toxicity of the culture filtrate, small pieces of callus were first macerated mechanically to separate single cells and then put in culture filtrate and kept for 48 hrs. Macerated callus in Richard's medium was kept as control. After 48 hrs both samples (treated and control) were stained with 0.1% erythrocin B (which stains only dead cells) and then seen under light microscope for confirming its viability which indicated the toxicity of culture filtrate.

In-vitro selection of resistant callus

The actively growing callus were challenged to different concentrations of fungal culture filtrate by mixing the purified culture filtrate of fungus with sterilized molten MS medium to obtain several concentrations (v/v): 0, 2, 4, 6, 8, 10, 15, 20, 25%. About 20 ml of medium, after thoroughly mixing with culture filtrate, was poured in pre-sterilized Petri dishes under aseptic conditions. The MS medium used was supplemented with standardized concentrations of PGRs used for maintenance of the callus. Callus was cut into small pieces of about 20 mg each and then inoculated into

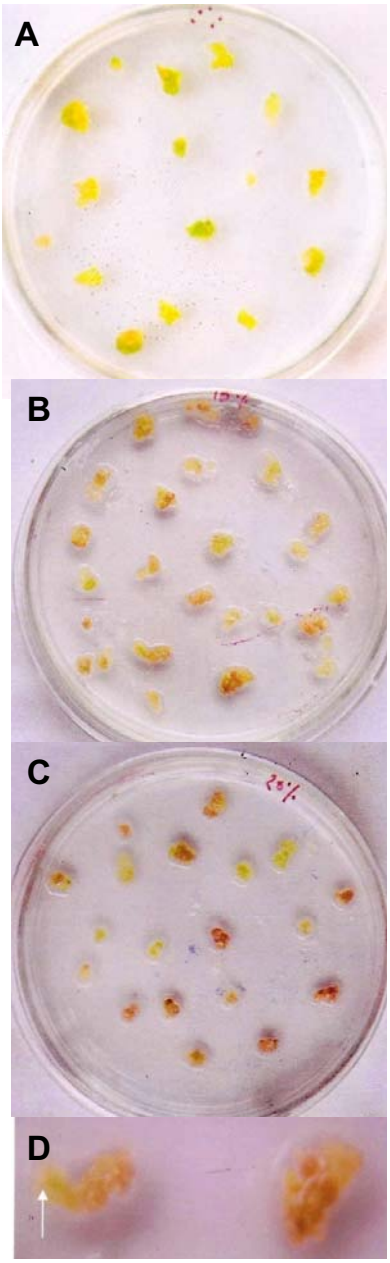

Fig. 2 Callus multiplication of resistant calli of carnation cv. 'Raggio-de-Sole'. (**A**) Callus on normal MS + 2.0 mg/l NAA and 0.5 mg/l 2,4-D; (**B**) Callus on selective medium with of culture filtrate of *Rhizoctonia solani* Kuhn; (**C**) Callus on selective medium with 20% of culture filtrate of *R. solani*; (**D**) Magnified view of selected callus on medium with 20% culture filtrate.

selective media of different concentrations under a laminar flow chamber. The growth of the cells was monitored by their ability to divide and form colonies. The highest concentrations of culture filtrate at which calli survived was recorded. The pale yellow colour of callus depicted the presence of living cells whereas dark brown colour indicated dead cells (**Fig. 2**). The surviving calli were further sub-cultured on callus maintenance medium (MS basal + 2.0 mg/l NAA and 0.5 mg/l 2,4-D) then transferred to the same medium at 20% concentration of the selective dose of culture filtrate of the fungus. The resistance capacity of finally selected callus cultures was compared with previously non-selected callus cultures (control). Surviving callus cultures were separated for shoot formation only when same batch of culture filtrate caused greater than 88% survival rate under control to maintained the regeneration potential of calli.

Selection of calli and shoot regeneration

After screening the calli for resistance against the fungal culture filtrate, the selected (resistant) calli were multiplied on the callus multiplication medium containing different concentrations of 2,4-D and NAA. The multiplied calli after 3-4 weeks were finally transferred to shoot regeneration medium containing MS basal medium supplemented with different concentration of auxins and cytokinins.

Experimental design and statistical analyses

Data was analysed by the χ^2 test using SPSS version 16.0.

RESULTS AND DISCUSSION

Induction of callus and differentiation

For the establishment of callus cultures from explants, leaf, internodal and petal segments (0.5-0.7 cm in size) of carnation cv. 'Raggio-de-sole', those explants which were treated with 0.2% bavistin for 6 min followed by treatment with 0.1% $HgCl_2$ for 2 min resulted in the survival of 91.44% uncontaminated cultures, whereas leaf explants exposed to 10 of bavistin (0.2%) and 1 min of $HgCl_2$ (0.1%) died (**Table 1**). Shorter durations increased the percentage of contaminated cultures, although exposure of explants to 4 min in 0.2% bavisitin and 2 min in 0.1% $HgCl_2$ resulted in 74.94% of uncontaminated leaf explants. Only young leaf explants, and not petal and internodal segments, formed calli on MS medium supplemented with various concentrations and combinations of different PGRs viz. NAA, 2,4-D, Kn and BAP. Callus formation was maximum on MS medium containing 2 mg/l NAA + 0.5 mg/l 2,4-D. About 10-14 days were required for callus initiation and 40-45 days for full growth of the callus. The callus formed from the cut ends of the initial explants was separated and subcultured onto fresh medium of the same composition i.e. MS basal medium containing 2.0 mg/l NAA + 0.5 mg/l 2,4-D. This medium was subsequently used for the multiplication and maintenance of the callus culture (**Fig. 2**).

In-vitro selection of resistant callus

The incubated callus cultures along with fungal culture filtrate showed maximum (93.33%) calli survival at minimum (2%) concentration of culture filtrate. At 25% concentration of culture filtrate, 100% of the cells died in unselected calli whereas in selected cell cultures 20.66% survival of calli was observed at 20% concentration (**Table 2**). Cell survival of carnation cvs. 'Feynoord' and 'Cabaret' varied between 0.5 and 2.0% in one month at 15% of culture filtrate of *Fusarium oxysporum* f.sp. *dianthi* (Thakur *et al.* 2002). Callus that was tolerant to 20% of culture was further multiplied on callus initiation and maintenance medium. The culture was passed through three cycles of selection on this medium. In each selection cycle, the number of surviving calli decreased and finally selected calli grew better on medium with fungal toxin. The possibility of using either pathotoxin or partially purified culture filtrate of the pathogen as a screening agent for disease resistance *in-vitro* was first tested by Carlson (1973) for wild fire of tobacco caused by *Pseudomonas syringae* pv. *tabaci*. This is also true for most of the *Fusarium* spp. filtrates used successfully in selection of alfalfa, tomato and carnation callus cultures (Hartman *et al.* 1984; Shahin and Spiney 1986; Arcioni *et al.*1987; Mosquera *et al.* 1999). Various other researchers also reported cell selection based on toxins/culture filtrate of serious pathogens, e.g. *Fusarium oxsporum* f.sp. *dianthi* (pathotype II and IV) (Mercuri *et al*. 1992; Sanjuan *et al*. 2001), *Alternaria dianthi* (Mehta *et al*. 2007), *Helminthosporium sativum* (Ling *et al.* 1985; Rines and Luke 1985; Chawla and Wenzel 1987), *Phoma lingam* (Sacristan 1982; Sjodin and Glimelius 1989), *Phytopthora infestans* (Behnke 1979, 1980; Rosati *et al.* 1990), *Cercosporidium personatum* (Venkatchalam *et al.* 1998) in carnation, rice, oat, wheat, *Brassica* spp., potato and groundnut crops, etc.

Table 1 Effect of surface sterilants on percentage survival of leaf explants of carnation cv. 'Raggio-de-Sole' after 15 days.

Sterilants used		Percentage of uncontaminated leaf explants after 15 days
0.2% bavistin (min)	0.1%$HgCl_2$ (min)	
2	2	33.33
4	2	74.94
6	2	91.44
8	2	41.66
10	1	0.00

The χ^2 stastistic for the data above is 0.0000024 with 4 degrees of freedom

Table 2 Different concentrations of culture filtrate of *Rhizoctonia solani* Kuhn. on percentage survival of callus cultures of carnation cv. 'Raggio-de-Sole' after 4 weeks.

Concentration (%) of fungal culture filtrate	% Survival of calli
Control (without CF)	100.00
2	93.33
4	86.66
6	80.00
8	66.66
10	53.33
15	40.00
20	20.66
25	0.00

The χ^2 stastistic for the data above is 0.0000029 with 8 degrees of freedom

Multiplication of selected calli and shoot regeneration

The selected calli grew better on MS medium containing 2 mg/l NAA + 0.5 mg/l 2, 4-D and after 2-3 subcultures the desired calli were transferred to basal MS medium containing 2.0 g/l of activated charcoal. Calli cultured on this medium turned green from pale yellow within 1 week's time; within 15 days, the colour changed to dark green. Finally the calli appeared green, fragile, soft in texture and roots started appearing on the callus surface.

Formation of adventitious buds were observed within 10 days on callus cultured on MS medium supplemented with 0.5 mg/l NAA + 0.5 mg/l IAA. After about 4 weeks, shoots were derived from the adventitious buds of calli on MS medium supplemented with NAA and IAA each at 0.5 mg/l. Plantlet regeneration was achieved by Ishida and Adachi (1988) on MS medium with BA (1 mg/l) + NAA (1 mg/l) from friable and nodular ginger (*Zingiber officinale*) cv. 'Ohshoga' and maintained for 1-1.5 years on MS medium with 2,4-D (2 mg/l). Similarly embryogenic cell mass in gladiolus differentiated successfully into green leafy structures and subsequently to entire plantlets forming both shoots and roots from normal and selected cell lines (Gupta *et al.* 2006).

IMPLICATONS AND FUTURE PERSPECTIVES

From the present studies it can be concluded that culture filtrate of *Rhizoctonia solani* Kuhn was injurious to the survival of callus of carnation beyond 25 per cent. About 20-40 per cent survival rate of the callus was recorded at 15 to 20 per cent concentration of culture filtrate. However resistant plants could be obtained at 20% concentration of culture filtrate of the fungus for mass propagation and for use in breeding programme.

Leaf was found to be the best explant for the establishment of callus cultures and maximum callus growth was obtained on MS medium supplemented with 2.0 mg/l NAA and 0.5 mg/l 2,4-D. Maximum regeneration was obtained on MS + 0.5 mg/l NAA and 0.5 mg/l IAA.

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