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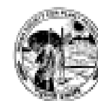

IN VITRO SELECTION AND BIOCHEMICAL CHARACTERIZATION OF CARNATION (*DIATHUS CARYOPHYLLUS* L.) CALLUS CULTURE TOLERANT TO *ALTERNARIA DIANTHI*

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SUMMARY

For the establishment of callus cultures of carnation (*Dianthus caryophyllus* L.), internodal segments were used as explants. Maximum percentage of callus induction was obtained on Murashige and Skoog (MS) medium supplemented with 2.0 mg/l naphthalene acetic acid (NAA) and 2.0 mg/l kinetin. Whereas, maximum shoot regeneration was obtained on medium supplemented with 2.0 mg/l 6-benzyladenine (BA) and 0.5 mg/l NAA. Well developed root system was obtained on liquid MS medium supplemented with 2.0 mg/l indole–3-butyric acid (IBA) and 0.2 per cent activated charcoal. Calli were subjected to different concentrations of culture filtrate of *Alternaria dianthi* and cell survival at 15 per cent selective dose of culture filtrate was 11.67 per cent. Selected calli showed significantly higher levels of biochemical constituents viz., total sugars, phenols, reducing sugars and proteins as compared to non-selected calli. Banding patterns of isozymes esterase, peroxidase and polyphenol oxidase were different in selected and non selected calli. *In vitro* shoot regeneration from selected calli was obtained on MS medium supplemented with 2.0 mg/l thidiazuron (TDZ).

Key words: **Biochemical constituents, callus culture, carnation, culture filtrate, isozyme, organogenesis**

INTRODUCTION

Carnation (*Dianthus caryophyllus* L.) is one of the most important cut flower of the world and ranks next to rose (Laurie *et al.* 1968). In India, the specific climatic requirements of the crop are met in Himachal Pradesh, West Bengal, Kashmir and Karnataka in one season or other. With increase in its cultivation, the limiting factors for the production of quality flowers are also increasing simultaneously. Carnation is attacked by a number of pathogens viz. fungi, bacteria, viruses and among the various pathogens attacking this crop, *F. oxysporum, F. roseum and Alternaria dianthi* are known to cause heavy losses in yield and quality of flowers. *A. dianthi* is known to cause leaf blight disease of carnation, which initially produces purple small circular and later light brown to black spots on carnation leaves, stem, petioles including flowers giving blightened appearance (Fig. 2A). In Solan district, A.*dianthi* causes 28 % loss in the yield and quality of flowers (Chandel and Bhardwaj 2001). Fungicides, herbicides and pesticides used commercially for the management of the diseases are expensive and are causing environmental hazards. *In vitro* culture is the promising tool for the selection of resistant mutants. The use of resistant cultivars may help to reduce the incidence of the diseases or pathogens attack. A selection criterion used widely till date is the growth of the callus in the presence of culture filtrate or toxin of pathogen (Sacristan 1982)

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MATERIAL AND METHODS

The present investigations were carried out in the Department of Biotechnology, Dr. Y S Parmar, UHF, Nauni, Solan with the objective to select the cell lines resistant to *A. dianthi* in carnation cv. Tempo using internodal segments of size 0.2 to 0.5 cm from stem cutting as explants. These explants were thoroughly washed under running tap water and surface sterilized with 0.1 % mercuric chloride solution for 2 min and were washed with sterilized distilled water 2 to 3 times to remove the traces of mercuric chloride. MS salts (macro and micro), vitamins supplemented with 100 mg/l, mesoinositol, 3 % sucrose and 0.8 % agar-agar were used as basal medium (Murashige and Skoog 1962).

Surface sterilized internodal segments were cultured on MS medium containing various concentrations and combinations of plant growth regulators viz., NAA (1.0-2.0 mg/l), kinetin (1.0-2.0 mg/l) and 2, 4-D (0.5-2.0 mg/l) and incubated under 16-h photoperiod at 26+2 ^{o}C for the induction and establishment of callus. After the establishment of callus culture, fresh green callus was cut off into small pieces and sub-cultured on the same nutrient medium for further multiplication. After sufficient multiplication of callus, it was transferred to shoot regeneration medium. *In vitro* regenerated shoots were transferred to rooting medium supplemented with activated charcoal and different concentrations of IAA and IBA. *In vitro* raised plantlets were hardened and further maintained in glass- house.

In vitro selection and biochemical characterization: Pure culture of *A. dianthi* was isolated from infected leaves of carnation plants, maintained and multiplied on Potato Dextrose Agar (PDA) medium (Fig. 2B). To carry out pathogenicity test, mycelial suspension of fungus was applied on leaves of carnation plants for development of disease symptoms under high humid conditions in glass- house. For preparation of culture filtrate, small bits (5 mm) of mycelium were inoculated in liquid Richard's medium, incubated at 25 ^{o}C for 15-20 days. The crude culture filtrate was purified following filtration through ordinary filter paper, Whatman filter paper, sintered glass filter (G-S grade) and finally the pure culture filtrate was used for selective medium preparation for the selection of calli resistant to *A. dianthi.*

The small pieces of macerated callus (approx 1-3 mm) were cultured on selective medium prepared by mixing pure culture filtrate of *A. dianthi* with sterilized molten MS medium (v/v) to obtain the concentrations of 5, 7.5, 10, 12.5, 15, 17.5, 20, 25 % respectively and monitored for the growth of callus. A control containing MS medium without culture filtrate was also prepared. Calli showing resistance to culture filtrate was isolated and sub-cultured on fresh MS medium devoid of culture filtrate for 4 weeks to check the stability for resistance. Selected calli showing resistance to culture filtrate was multiplied and used for biochemical characterization.

Biochemical characterization of selected and non selected calli: For biochemical characterization, ethanol extraction was carried out by boiling 1 gm of selected and non selected callus in 80 % ethanol for 10 min, at room temperature. Supernatant was evaporated; residue was dissolved in 20 % ethanol and centrifuged. The clear supernatant was used for estimations. Total soluble sugars were estimated according to the method described by Dubios *et al.* (1956). Reducing sugars were estimated according to Nelson (1944). Total phenols were estimated according to method described by Bray and Thorpe (1954) and total soluble proteins were estimated after trichloro acetic acid (TCA) precipitation as described by Lowry *et al.* (1951)

For isozyme studies, three enzymes i.e. esterase (EC 3.1.1.2), peroxidase (EC 1.11.1.7) and polyphenol oxidase (EC 1.10.3.2) were investigated. For extraction of enzyme, 1 g of selected and non selected calli were crushed in pre-chilled pestle and mortar using extraction buffer as described by Parafitt *et al.* (1986) .The homogenate was spun at 10,000 rpm for 20 min at 4 ^{0}C to remove cellular debris. The clear supernatant was used for separation of enzyme by polyacrylamide gel electrophoresis at 4 ^{0}C using bromo phenol blue (0.2 %) as a tracking dye. Gel buffer and tray buffer were prepared as per the method described by Scandalios (1969). The electrophoresis was carried out at constant current of 50 mA till the tracking dye reached the end of slab gel and gels were removed. Esterase and

polyphenol oxidase were stained as described by Kuhns and Fretz (1978). Polyphenol oxidase isozymes were stained as described by Kaushal Kanika (1997), using catechol and sulphanilic acid as staining reagents. Locations of isozymes were specified by relative mobility (Rm) values.

In vitro plant regeneration from selected calli: Selected calli were sub-cultured on different concentrations and combinations of BAP, IAA and TDZ. Shoot regeneration from callus was obtained only on MS medium supplemented with 2.0 mg/l TDZ (Fig. 2D).

Statistical analysis: The data recorded for different parameters was subjected to completely randomized design (Cochran and Cox 1963). For biochemical constituents, experiments were done in three replicates with duplicates.

RESULTS AND DISCUSSION

Callus initiation on cultured internodal segments started within 7-10 days from cut ends of the explants. All the media showed some degree of callus initiation. Maximum callus induction (92 %) was obtained in MS medium supplemented with 2.0 mg/l NAA and 2.0 mg/l kinetin (Fig. 1A). For callus multiplication, callus was cut into small pieces and sub cultured on fresh MS medium .The callus multiplication was also obtained on callus induction medium (Fig. 1B). Callus induced on MS medium supplemented with 2, 4-D alone or in combination with NAA was friable, light green and less in amount as obtained on best callus induction medium. Garg (1995) also reported callus formation from leaf explants of carnation on all combinations of MS medium containing equal concentrations of NAA and kinetin ranging from 0.5-2.0 mgl/1. Ruffino *et al.* (1990) reported callus induction from fragments of *in vitro* multiplied carnation plantlets on MS medium supplemented with 1.0 mg/l BA and 1.0 mg/l NAA. These observations led clearance to the earlier observation made by Mubarack *et al.* (1991), they also reported different concentrations of 2, 4-D and NAA for callus induction from carnation leaf segments. In present studies however, less amount of callus was obtained in 2, 4-D alone and in combination with NAA.

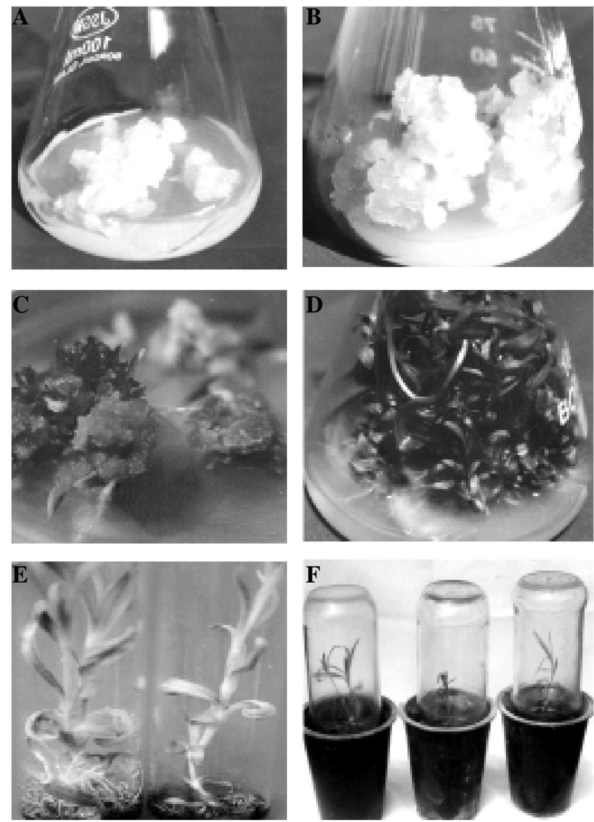

Fig. 1. (A-F). *In vitro* plant regeneration in carnation (*Dianthus caryophyllus* L. cv. Tempo): A) Callus induction from internodal segment explants, B) Callus multiplication, C) *In vitro* shoot regeneration from callus on shoot regeneration medium, D) *In vitro* shoot multiplication, E) Root regeneration in *in vitro* developed shoot and formation of complete plantlets, F) Hardening of *in vitro* raised carnation plants

For shoot regeneration, calli pieces (0.5 cm approx. in dia) were cultured on MS medium supplemented with 9 different combinations of BAP and NAA. The average number of shoots per explant varied significantly in different media, but maximum number of shoots per explant i.e. 22.50 was obtained on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA (Fig. 1C). Kozak and Hempel (1979) reported maximum shoot multiplication on MS medium containing 0.5 mg/l BA and 0.1 mg/l NAA. Higher concentrations of cytokinins viz., BAP, Kinetin, TDZ than auxins viz. NAA, IAA is required for the induction of shoots from callus, nodal, petal and leaf explants (Roest and Bokelmann 1981, Frey and Janick 1991, Mubarack *et al.* 1991, Garg 1995).

After a desirable level of shoot multiplication was achieved (Fig. 1D), elongated shoots were transferred to different rooting media. Root initiation started after about 10 days of transfer in rooting media and well developed root system was obtained within 5 weeks (Fig. 1E). Maximum average number of roots per shoot (13) was obtained in MS liquid rooting medium containing 2.0 mg/l IBA and 0.2 % activated charcoal. The maximum root length (5.7 cm) was also observed on the same MS liquid medium. Pathania (1988) and Garg (1995) also reported rooting of carnation shoots on full strength MS solid medium supplemented with 2.0 mg/l IBA. In contrast, Ghosh and MohanRam (1986) achieved rooting on semi-solid and liquid B_5 medium containing 1.5 % sucrose with or without auxins.

After development of root system, in *vitro* raised plantlets were gently taken out from the culture tubes, washed thoroughly under running tap water and treated with 1.0 per cent bavistin for 10-15 min. Plantlets were transferred to plastic pots containing sterilized mixture of soil: FYM (3:1) and covered with jars to maintain high humidity (Fig. 1F). The percent survival after one month of transplanting was 60. The hardened plants were shifted to earthen pots after one month.

Pathogenicity test: Before using the fungal strain for present investigation, its pathogenicity was tested. Freshly prepared mycelial suspension of pathogen was gently applied on the leaves of carnation plants. Control was also maintained. Symptoms on infected plants started appearing after 14 days. First one or two yellowish brown spots appeared on leaves, followed by stem (Fig. 2A). Whereas, in case of control plants no such symptoms appeared.

Toxicity of culture filtrate: Different culture conditions viz. temperature, composition of medium etc. are some of the factors which may change the pathogen from virulent to avirulent form therefore, it become necessary to access the toxicity of culture filtrate. Ninety five per cent of treated cells viewed under light microscope stained with Ivan's blue dye whereas untreated cells were not stained. In present studies, 11.67 % survival of calli was obtained on selective dose of 15 % of *A. dianthi* culture filtrate. Selvapandiyan *et al*. (1988) reported 50 % survival of susceptible cell lines on the selective medium containing 27 % v/v of culture filtrate of *F. oxysporum.*

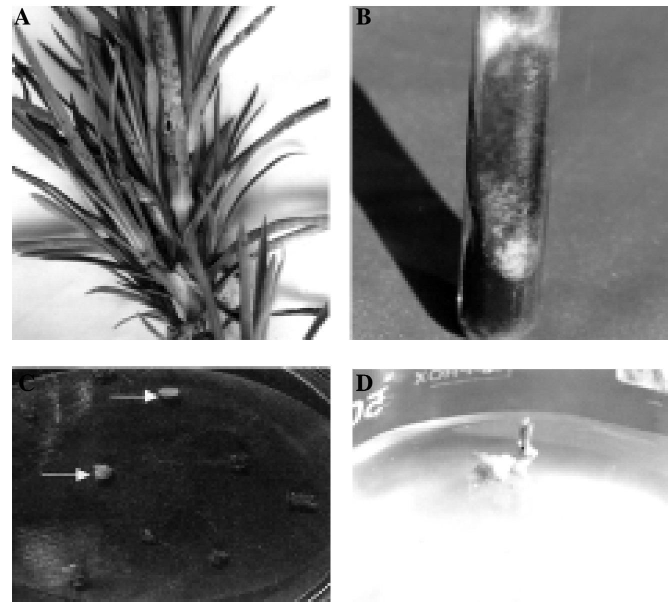

Fig. 2. (A-D). A) Symptoms of leaf blight in field grown plants, B) Culture of *Alternaria dianthi* on Potato dextrose agar medium, C) *In vitro* cell selection at 15% culture filtrate, D) *In vitro* shoot regeneration from resistant calli

The percent survival of the calli decreased steadily with the increase in concentrations of culture filtrate in the selective medium i.e. increasing the concentration to 17.5 % resulted in no survival of the calli, none of the calli survived. At 15 % culture filtrate, calli turned light brown in 10-12 days and after 20 days they turned to dark brown but few cells started growing out of these dead calli (Fig. 2C). The selective doses of culture filtrate of *F. oxysporum* was found to be 27 % in tobacco (Selvapandiyan *et al* 1988), 5-10 % in cucumber (Malepszy and El-Kazzaz 1990), 12.5 % in chick pea (Prakash *et al*. 1994) and 15 % in carnation (Thakur *et al.* 2002).

After one month, selected calli on 15 % culture filtrate were transferred to normal MS medium devoid of culture filtrate for 4 weeks. These calli started growing on media. To again check the stability for resistance, the calli were re-cultured on the same selective medium showing 100 % survival and multiplication of the selected callus was done on normal MS medium standardized for callus multiplication.

Biochemical characterization of selected and non-selected calli: Selected and non selected calli were characterized on the basis of phenols, reducing sugars, total sugars and proteins. In the present investigations, higher phenol content was found in the selected calli than non selected calli. Phenols in the selected and non-selected calli were found to be 0.474 and 0.330 mg/g fresh weight, respectively. The synthesis of phenols, phytoalexins and lignins are the three key factors responsible for disease resistance in higher plants (Hahlbrock and Sheel, 1989). Accumulation of phenols in resistant variety cell lines have been reported in tomato, chickpea, apple root stock MM 106, gladiolus etc. (Bhatia *et al.* 1972, Prakash *et al.* 1994, Goel 2000, Kanwar *et al.* 2003 and Sharma 2005).

Table 1: Comparison of levels of biochemical constituents in control and *in vitro* selected calli of carnation (*Dianthus caryophyllus*) against *Alternaria dianthi*

Biochemical parameters (mg g^{-1} fresh wt.)	Treatment		t-value
	Control	Selected	
Total sugars	2.27+0.26	1.55+0.20	3.58
Reducing sugars	2.14+0.18	1.44+0.31	4.20
Soluble proteins	9.50+1.48	6.17+2.10	3.63
Phenols	0.47+0.06	0.33+0.10	0.80

Each value is mean of six replication, t-table value at 10 degree of freedom and at 1 % level of significance is 3.17 and 5 % level of significance is 2.23

The level of total sugar and reducing sugars were significantly higher in the selected calli. Reducing sugars in selected calli was 2.19 mg/g fresh weight whereas in non selected calli it was 1.44 mg/g fresh weight. Total sugars in selected calli were 2.27 mg/g fresh weight whereas in non selected calli this was 1.55 mg/g fresh weight. An alternate pathway like shikimic acid or acetate pathway is involved for phenol synthesis. Shikimic acid is synthesized from carbohydrates.

The protein content was found to be significantly higher in the selected calli than in non selected calli in the present studies. Protein in selected and non-selected calli was found to be 9.5 and 6.16 mg/g fresh weight, respectively. Higher protein contents in selected calli has been reported in barley, chickpea, apple root stock MM 106 and gladiolus (Chawla and Wenzel 1987, Kumar *et al.* 1996, Kanwar *et al.* 2003 and Sharma 2005)

Isozyme studies: Under present studies three isozymes viz., esterase, peroxidase and polyphenol oxidase were studied in selected calli and different banding pattern was observed in selected and non selected calli with different Rm values (Fig. 3). In selected calli two isozyme bands of esterase were present with a relative mobility 0.25 and 0.40, whereas in non selected calli only one band was present with a relative mobility 0.40. While for peroxidase enzyme, only one band was present at a relative mobility of 0.20 in selected calli, whereas in non selected calli two bands were present with a relative mobility 0.29 and 0.41, respectively. Two bands were present in selected calli for polyphenol oxidase with a relative mobility of 0.41 and 0.25, whereas in non selected calli only one band was present with a relative mobility 0.20. It has been reported that the increase in enzyme activities in many diseased tissue may be due to enzyme protein synthesis triggered by parasite attack (Shaw 1963) or the activation of inactive enzymes (Farkas and Lovrekovich 1965). There are numerous reports suggesting that the isozyme pattern of a number of enzymes get changed due to infection by pathogens (Sala *et al.* 1990).

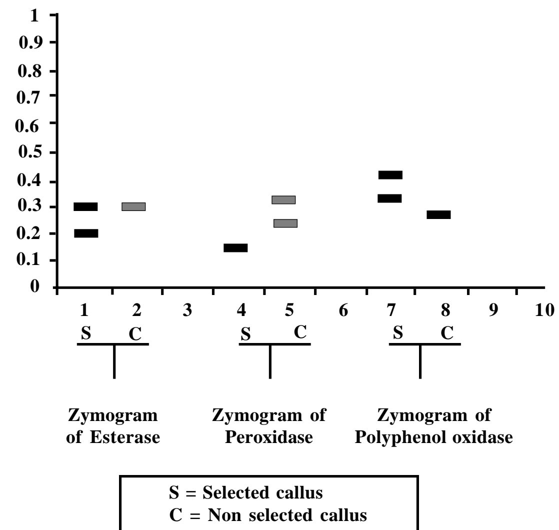

Fig. 3. Zymogram of esterase, peroxidase and polyphenol oxidase of selected calli

In vitro shoot regeneration from selected callus was obtained only on MS medium supplemented with 2.0 mg/l TDZ. It was observed that the selected cells failed to regenerate on the normal standardized medium. Similarly Arcioni *et al*. (1987) reported that the regeneration capacity was greatly reduced in selected calli of *Medicago sativa* resistant to culture filtrate of *F. oxysporum* f. sp. Medicaginis. The above standardized protocol could be further used to obtain disease resistant plants of carnation.

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