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CELLULAR SELECTION AND PARTIAL CHARACTERISATION OF GLADIOLUS CELL LINES RESISTANT TO CULTURE FILTRATE OF *FUSARIUM OXYSPORUM*

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SUMMARY

The pedicel explants of Gladiolus cv. Amsterdam were cultured on MS medium supplemented with NAA (10 mg/l) and kinetin (0.5mg/l) for callus initiation. For multiplication of callus MS medium supplemented with 2, 4 D (2mg/l) was used. Different concentrations of culture filtrate of *Fusarium oxysporum* f.sp. *gladioli* were added into the culture medium for selection of resistant cell lines. Upon incubation for six weeks, the resistant clones were selected at 10% concentration of culture filtrate. The selected clones were cultured on medium devoid of culture filtrate for 3 weeks and then characterised using biochemical parameters. Selected cell lines revealed a higher protein content, phenol content, total sugar and reducing sugar content compared to non-selected cell lines. In the non-selected cell lines higher activities of phenylalanine ammonia lyase and tyrosine ammonia lyase were found.

Key words: **Cell lines, culture filtrate, *Fusarium*, gladiolus, resistance.**

INTRODUCTION

Gladiolus, a bulbous ornamental plant is grown for its cut flowers and garden display purpose. In India, gladiolus has emerged as one of the most important commercial flower crops. Himachal Pradesh, endowed with various climatic zones, has a great potential for successful cultivation of this ornamental crop. Among various diseases attacking gladiolus, *Fusarium* yellow is considered the most serious disease and is known to cause losses to the tune of 60-70%. The wilt pathogen causes premature yellowing and death of leaves and eventually corm destruction. Besides conventional breeding methods, *in vitro* cell selection and regeneration of plants from potential mutant cells can be used to develop plants with desirable trait. The cellular selection has been adopted to develop resistant cell lines by various workers in alfalfa, potato, barley and rice (Behnke 1979, Acrioni *et al.* 1987, Chawla and Wenzel 1987). Since, no such work has been reported in gladiolus, the present investigations were carried out to develop a technique for the *in vitro* selection of gladiolus cell lines resistant to *Fusarium oxysporum* f. sp. *gladioli*. The selected cell lines have been compared with non-selected cell lines with respect to a few biochemical parameters.

MATERIALS AND METHODS

Gladiolus cultivar Amsterdam was used as the experimental material. Freshly harvested cut spikes of the material were procured from Himachal Floritech and Hybrid Pvt. Ltd., Solan. The pedicel explant from the inflorescence were initially washed in running tap water for 20 minutes and then subsequently sterilised in 0.1% $HgCl_2$ for 4 minutes and then washed in sterile water a few times. In order to find out the optimum growth medium of the callus, the explants were inoculated on Murashige and Skoog (1962) medium supplemented with various concentrations of growth regulators viz., IAA (2mg/1), NAA (0.1-10mg/l), 2,4-D (0.5-2mg/l), kinetin (0.05-1

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mg/l) either alone or in combination for 15 days. The proliferated calli were separated from initial explants and sub-cultured on fresh MS medium supplemented with various concentrations of 2,4-D (0.5 - 2.5 mg/l) for 45 days. The growth of the callus was recorded at weekly intervals.

Isolation and assessment of culture filtrate

The pure culture of wilt pathogen (*Fusarium oxysporum* f.sp. *gladioli*) was isolated from infected corms of gladiolus obtained from the Department of Floriculture and Landscaping, UHF, Nauni Solan. The fungus was first grown on slants in test tubes and then inoculated in Petri plate for uniform growth. The fungal culture was maintained for 6-7 days at 25°C in an incubator, unitil uniform, fluffy, circular mycelial growth was obtained. The pure culture of fungus was cultured on liquid Richard's medium. From 10 days old culture of pathogen, the mycelial mat, microconidia, macroconidia and chalamydospores were removed through strainer and filtered through Whatman Filter paper 42, after centrifuging the suspension at 30,000 rpm for 15 minutes. The filter sterilised culture filtrate was obtained by passing through 0.22 μm Millipore filter.

Cell plating and selection medium

The selection media were prepared by mixing the filter sterilised culture filtrate with sterilised MS medium containing 2,4-D (2mg/l) with following concentration of phytotoxin 0, 2.5, 5, 7.5, 10, 12.5 and 15.0 per cent volume by volume. The medium thoroughly mixed with culture filtrate was dispensed in sterilised Petri dishes aseptically. Cell clumps of about 1 mm in diameter were cultured on the selective medium. Petri plates were sealed with parafilm and incubated at 25±2°C with 16-h photoperiod. The observations were recorded after 45 days of incubation (Table 1).

Preparation of ethanol extract and estimations

One gram of callus tissue, both selected and non-selected was boiled in 20 ml of 80% ethanol for 10 minutes. The supernatant so obtained was evaporated to dryness and then dissolved in 2 ml of ethanol (20%). The total sugar, reducing sugars and phenols were estimated following the method described by Dubois *et al.* 1956, Nelson 1944, Bray and Thorpe, 1954, respectively. The extraction was done in three replications and estimations in duplicate.

Preparation of enzyme extract and enzyme assay

The callus tissue both selected and non-selected was homogenised in 5 ml of 0.1 M Boric acid-Borax buffer (pH 8.8). This crude extract was centrifuged at 12,000 rpm for 10 minutes. The clear supernatant was used for enzyme assays. Phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL) were assayed as described by Prakash *et al.*(1994). A change of 0.001 in OD was described as one enzyme unit (EU). The estimations were done in three replications. The soluble protein content in the above enzyme extract were estimated as described by Lowry *et al.* (1951).

RESULTS AND DISCUSSION

Surface-sterilised pedicel explants from cut spikes were cultured on the callus initiation MS medium supplemented with 10mg/l NAA and 0.5mg/1 kinetin. Callus initiation was achieved within 14-15 days. Optimum growth of the callus was obtained on MS+2,4 D (2mg/l). The callus was of nodular type and cream yellow in colour. A change in the growth regulator composition proved to be advantageous showing excellent callus growth (Bajaj *et al.* 1985, Jain *et al.* 1996). Substitution by 2, 4-D (2mg/l) was effective for callus multiplication in gladiolus (Bajaj *et al.* 1985). NAA and kinetin were found to be effective for callus multiplication in Gladiolus cultivar 'American Beauty' by Jain *et al.* (1996). This difference may be attributed to the inherent genetic character of the specific cultivars.

During the course of present investigations single selection method was adopted for screening of resistant cell lines. This method is justified due to the fact that prolonged selection duration may lead to detrimental alteration e.g. elevated ploidy levels (Hartman *et al.* 1984) and decrease in regeneration ability (Thanutong *et al.* 1983). Toxicity of the culture filtrate of *Fusarium oxysporum* f. sp. *gladioli* tested by mungbean radicle length inhibition technique was found to be 49.5 per cent. Cell clumps about 1 mm in diameter was exposed to the increasing concentrations of culture filtrate (i.e. 0, 2.5, 5, 7.5, 10, 12.5 and 15%) in the medium. Upon incubation for 45 days, the cells, which could tolerate these

concentrations of culture filtrate, grew to form cell clones (Table 1). Increasing concentrations of culture filtrate beyond 7.5 per cent, inhibited the cell growth adversely. A complete growth inhibition was observed in the medium containing 12.5 and 15 per cent of the culture filtrate. All cells turned dark brown and died on these media. Except 4 cell clumps, most of the cell clumps showed no further growth on the selective medium containing 10 per cent of the culture filtrate. In the present studies 10 per cent of the culture filtrate was found optimum for selection of tolerant cell lines. Later on, the selected clones (at 10 per cent level), were subcultured on the selective medium and then transferred to the callus multiplication medium for proliferation (3 weeks) and biochemically characterised. Although a purified toxin may have been a more appropriate selective agent, our partially fractionated culture filtrate proved effective. A number of other investigators have also obtained successful selections using culture filtrate to select resistant cell lines in many crop species (Acrioni *et al.* 1987, Hammerschlag 1988, Malepszy and El-Kazzaz 1990).

Table 1. Selection of cell lines resistant to culture filtrate of *Fusarium oxysporum* f. sp. *gladioli*

Per cent concentration of culture filtrate	Growth behaviour of cell lines after 45 days		Percentage survival of cell lines
	Callus colour	Growth of callus	
0.00	Yellowish green	++++	100
2.50	Yellowish	+++	80
5.00	Yellowish brown	++	60
7.50	Brownish yellow	—	only 5 clones survived
10.00	Colonies turned brown	—	only 3-4 clones survived
12.50	Dark brown	—	100% inhibition
15.00	Dark brown	—	100% inhibition

++++ : Normal growth ++ : Inhibition
+++ : Satisfactory growth — : Complete Inhibition

Compared to control, the selected clones revealed higher soluble proteins, phenols, total and reducing sugars (Table 2). However, the activity of phenylalanine ammonia lyase and tyrosine ammonia lyase was found to be the three folds higher in the non-selected as compared to the selected cell lines. There are numerous reports indicating that disease resistance depends on the ability of host to synthesise proteins, which associates resistance response to gene activation. Kumar *et al.* (1996) reported that *Fusarium oxysporum* tolerant cell lines of pea had a higher protein content than the non-selected cell lines when expressed on g^{-1} fresh weight basis.

Table 2. Comparison of the levels of biochemical parameters and enzyme activities in the control and selected clones of gladiolus resistant to culture filtrate of *Fusarium oxysporum* f. sp. *gladioli*

Biochemical parameters	Control	Selected clones	CD (0.05)
Phenols (mg g^{-1} fresh weight)	0.57	1.03	0.04
Total sugar (mg g^{-1} fresh weight)	1.69	1.81	0.09
Reducing sugar (mg g^{-1} fresh weight)	1.34	1.57	0.06
Soluble protein (mg g^{-1} fresh weight)	14.52	20.64	4.41
Phenylalanine ammonia lyase EU min^{-1} g^{-1} fresh weight	20.00	7.67	0.93
Tyrosine ammonia lyase EU min^{-1} g^{-1} fresh weight	27.67	12.67	1.31

1EU=0.001 change in OD

The synthesis of phenols, phytoalexins and lignins are three key factors responsible for disease resistance in higher plants (Friends *et al.* 1981, Hahlbrock and Sheel 1989). Tyrosine ammonia lyase and phenylalanine ammonia lyases are the main enzymes responsible for synthesis of phenols. After infection by pathogen, an important host enzyme β-1, 3 glucanase is induced which convert non-toxin glycosides to toxin phenols that are inhibitory to the pathogen (Hilderbrand and Schorth 1964). The higher levels of phenols in the selected clones (Table 2) contribute toward its resistance to *Fusarium* wilt. Accumulation of phenols in resistant variety cell clones than the susceptible one has been reported by other worker (Sharma *et al.* 1983, Prakash *et al.* 1994). A relationship between resistance and phenolic content was

explained by suggesting that in susceptible variety the fungus gets enough time for growth before phenol content reaches a level inhibitory to fungus, whereas in resistant variety higher accumulation of phenols in initial stage restricts the growth of fungus. In *Prunus persica* the level of phenol was reported to increase in the advance of the fungus *Cytospora lencostoma* (Wisniewski and Bogle 1984). However, in the present study the activity of phenylalanine ammonia lyase and tyrosine ammonia lyase was found to be three folds higher in the non-selected cell lines than the selected clones. Prakash *et al.* (1994) reported that on culture filtrate free medium the activities of PAL and TAL were found to be significantly higher in callus cultures of WR-315 (resistant variety) and C235 (susceptible variety) than in resistant cell lines FC-3 of chickpea. Addition of culture filtrate in the medium resulted in faster increase in the activities of these enzymes in susceptible genotypes than in the resistant cell lines despite its low phenolic content. This puts a question mark on the regulatory role of PAL and TAL in disease resistance via phenolic synthesis. Hence, the enzyme PAL and TAL cannot be directly related to defense mechanism. Non-involvement of PAL in phenolic synthesis has also been reported Prakash *et al.* (1994). This suggests the presence of an alternate pathway like shikimic acid or acetate pathway for phenolic synthesis. Shikimic acid is synthesised from carbohydrates and during the present investigations the level of total sugars and reducing sugars were significantly higher in the selected clones.

The results so obtained suggest that Fusarium wilt resistant gladiolus plants could be produced by regenerating plants from cultured callus that is insensitive to culture filtrate (toxin metabolite produced by *Fusarium oxysporum* f. sp. *gladioli*). Resistant clones can be characterised biochemically and be used to regenerate resistant plants.

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