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In Vitro Selection and Regeneration of Asiatic Hybrid Lily Resistant to Culture Filtrate of *Phytophthora cactorum*

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ABSTRACT

Callus cultures derived from bulbscale segments of asiatic lily 'Alaska' susceptible to *Phytophthora cactorum* were successfully used for *in vitro* selection for resistance to this pathogenic fungus. Resistant cell lines were selected by culturing calli on growth medium containing various concentrations of culture filtrate of *P. cactorum.* Resistant calli obtained after two cycles (30 days/cycle) of selection were used for plant regeneration. No variation was observed in the morphological characters in the selected regenerates. 40% of the plants raised from one year-old bulbs had acquired resistance against the pathogen.

Keywords: cell selection, foot rot, *in vitro, Lilium,* regeneration
Abbreviations: 2,4-D, 2,4-dichlorophenoxy acetic acid; **BA**, 6-benzyladenine; **NAA**, α-naphthalene acetic acid

INTRODUCTION

Lilium belongs to the family Liliaceae and is a native of Northern Hemisphere, which is widely distributed in China, Japan, Siberia, South Canada and extended into the United States. It is one of the most leading cut flowers and ranked fourth in the international flower trade (Anon 1996). Among the various types of *Lilium*, asiatic, oriental and *L. longiflorum* hybrids are the major lilies popular in the worldwide floral trade. Though the demand of lily flowers has increased considerably during the last decades, the main limiting factor in large-scale cultivation is its susceptibility to a number of pathogens including fungi, bacteria and viruses. The fungal diseases are the most troublesome worldwide and are responsible for reducing the yield and quality of flowers (John *et al*. 2006).

Phytophthora cactorum is one of most important pathogens in number of crops (Rosati *et al*. 1990; Mezzetti *et al*. 1993; Goel *et al*. 2007). It causes foot rot disease in *Lilium* under field conditions resulting up to 36.8% yield loss in India (Anon 2000). The fungal diseases in *Lilium* are controlled by using a variety of fungicides (Jana and Roy Choudhury 1989), however, these chemicals are expensive and not eco-friendly. To overcome this problem, attempts have been made to induce resistance through conventional breeding approaches, however, the development and detection of resistant varieties and clones is a time-consuming and expensive (Garibaldi and Gullino 1987; Liu *et al*. 2005). It is therefore, imperative that novel methods such as mutagenesis, *in vitro* cell selection and genetic transformation with desirable genes be applied to induce variability needed for introducing specific traits to the available germplasm. In the present investigation an attempt was made to induce variability for resistance to *Phytophthora cactorum* using *in vitro* selection in lily hybrid cultivar 'Alaska'.

MATERIALS AND METHODS

Explant source and preparation

Vernalized bulbs of asiatic lily hybrid 'Alaska' were obtained from the Department of Floriculture and Landscaping, University of Horticulture and Forestry, Solan, India. Scales were separated and washed in running tap water for 30 min. The basal and apical portions of the scale were trimmed and the middle portion of the outer scales (3-4 mm) was used as explant. These were again washed in 1% Tween-20 (v/v) solution, followed by washings in distilled water for three times and surface sterilized by dipping the explants in 0.1% mercuric chloride solution for 10 min and then washing 3-4 times in sterile distilled water.

Culture medium and culture conditions

The sterilized explants were cultured with the abaxial side down on solidified MS (Murashige and Skoog 1962) medium containing 3% (w/v) sucrose and supplemented with 2 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D). The pH of the medium was adjusted with 1 N HCl and/or 1 N NaOH to 5.8 prior to adding 0.8% (w/v) Difco bacto agar. Medium was dispensed in 30 ml aliquots into 100 ml Erlenmeyer flasks (Borosil Bombay, India), which were plugged with non-absorbent cotton plugs. Medium was autoclaved at 1.1 kg cm^{-2} for 15 min at 121°C. The cultures were maintained under 16-h light provided with white, cool fluorescent tubes (50-60 µmol/m^2/s, 40W each, Philips)) at a temperature of 24 ± 2°C. The cultures were transferred at four weeks interval to fresh medium with the same composition.

Maintenance and multiplication of the pathogen culture

The pure cultures of *P. cactorum* (local isolates) were obtained from the Department of Mycology and Plant Pathology, University of Horticulture and Forestry, Solan. The isolates were multiplied at 25°C on PDA (200 g/l potato, 20 g/l dextrose, 20 g/l agar). The pH of the PDA medium was 6.0–6.5. After inoculation, the cultures were incubated at 25°C in dark for four days until a uniform fluffy mycelial growth was obtained. The stock cultures were maintained at 4°C on the same medium.

Preparation of culture filtrate

The culture filtrate (CF) was prepared by incubating 4 mm^2 of the fungal mycelium in 200 ml Clarke medium (Clarke 1966). The pH of the medium was maintained at 6.6–7.2 and incubated at 25°C in

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the dark. After 20 days, the fungal cultures were filtered through Whatman filter paper no. 1 and the filtrate was centrifuged at 15,000 × *g* for 20 min at 25°C while maintaining the pH at 5.6. The supernatant was then filtered through Whatman filter paper no. 42 and finally through sintered glass filter (G-5 grade, 0.25 µm pore size) to produce the transparent CF.

Analysis of toxicity of CF

The calli mass, obtained from the middle portion of the outer scale, was divided into small pieces and macerated with 0.5% macerozyme (Sigma-Aldrich, Bangalore, India) for 30 min in dark. The cell clumps were separated through a nylon sieve (Millipore, India) (64 µm pore size). After washing, the macerated cells were incubated in Clarke's medium and undiluted CF, separately. After 48-h, cells were stained with 0.1% erythrocin B (which stains only dead cells) and observed under a light microscope (Olympus, India).

Cell selection

One hundred pieces of callus (15 mg/callus) in five Petri-plates, each plate having 20 pieces for each culture filtrate treatment were inoculated on MS medium containing 0, 5, 10, 12.5, 15, 17.5, 20, 22.5 or 25% (v/v) CF and 2 mg/l 2,4-D. In the control cultures, CF was replaced by an equal volume of Clarke medium. Cultures were inoculated at 25°C under 16-h light with light intensity of 50-60 µmol/m^2/s. Light green calli were obtained from selection medium containing 20% CF, and the highest concentration of CF at which only few calli survived were subcultured onto the same selection medium for two more cycles (30 days/cycle) to produce resistant calli. The latter were multiplied on callus multiplication medium (2 mg/l 2,4-D) without CF.

Regeneration of bulblets

The selected calli were inoculated on MS basal medium containing 2 or 3 mg/l 2,4-D, 0.2 or 0.6 mg/l α-naphthalene acetic acid (NAA) and 1 or 1.2 mg/l 6-benzyladenine (BA) to regenerate bulblets. The regenerated bulblets were multiplied by separating the individual bulblets on the same medium.

Bulblet storage

The individual bulblets were separated and transferred to MS medium without plant growth regulators. When the leaves had dried, bulblets obtained from each of the control and selected lines were taken out of the culture vessels, washed thoroughly and dried at room temperature. The bulblets were treated with Bavistin (Indofil, Bombay, India) (0.1%, w/v) and stored at 2°C in coco peat.

In vivo testing against the pathogen

One year-old bulbs (one bulb per pot; control and selected) were transferred to plastic pots (10 cm diameter) filled with sterile sand and soil mixed in a 1:1 ratio. Three replications with 25 bulbs in each were maintained for the screening of resistant cell lines. After the bulbs sprouted, these were subjected to infection under *in vivo* conditions by adding freshly prepared (10 bits of mycelium, 5 mm each bit, were mixed with 100 ml sterile distilled water and concentration of suspension with 40 sporangia/ml) suspension. 40 ml of the spore suspension was added evenly to each pot. After one month, disease severity was recorded visually according to 0-4 rating scale with 0, no symptoms (highly resistant); 1, yellowing of 1-5 upper leaves (resistant); 3, yellowing of 10-15 upper leaves (moderately resistant); 3, yellowing of more than 50% leaves, drooping of leaves and blackening of basal portion of stem (susceptible) and 4, crown collapse, complete wilting (highly susceptible). The scale was devised just to record the comparative reaction of the different plants to the spore suspension of the pathogen.

Statistical analysis

Three replications with 10 explants in each replication were maintained for each treatment and the data were analysed statistically using a completely randomized design (Gomez and Gomez 1984). The statistical analysis based on mean values per treatment was made using the technique of analysis of variance. The comparative LSD multiple range test ($P \leq 0.05$) was used to determine differences between treatment means.

RESULTS AND DISCUSSION

The bulbscale segments cultured in MS medium containing 2 mg/l 2,4-D gave best callus growth. Repeated subculturing was necessary to obtain friable callus required for the selection of resistant lines. Testing the macerated single cells incubated in CF and in Clarke's medium with 0.1% erythrocin B showed that all cells incubated in CF did stain after 48 h, thus proving the toxicity of CF to the lily cells. The percentage survival of callus cultured on media containing CF at various concentrations decreased with an increase in CF concentration, reaching 100% mortality at 25% CF (**Table 1**). Cell selection was, therefore, carried out at 20% CF to effect the maximum selection pressure that would allow the recovery of resistant lines. These results suggest that the CF contains toxin(s) produced by *P. cactorum* and, thus, it may be used as an effective screening agent in a cell selection program. A number of previous studies have also found that toxins present in the CF were able to inhibit cell growth and that the cells of the host species were sensitive to the toxins than those of the non-host plants (Binding *et al*. 1970; Hartman *et al*. 1984; Venkatachalam *et al*. 1998). Many attempts have been made since then to apply such selection schemes for crop improvement (Thakur *et al*. 2002; Rosati *et al.* 1990; Ganesan and Jayabalan 2006).

The calli selected on CF-containing medium were transferred to bulblet regeneration medium. The maximum percentage calli (75%) regenerating bulblets was recorded in medium containing 0.6 mg/l NAA and 1.2 mg/l BA (**Fig. 1; Table 2**). The percentage calli producing bulblets on each medium differed significantly with each other. Although the bulblets were produced on all media, the maximum average number of bulblets (4.86 with 12 leaves) was produced on medium containing 0.6 mg/l NAA and 1.2 mg/l BA.

The regenerated bulblets were stored at 2°C for further growth and development and screened for resistance under *in vivo* conditions. Only 40% of plants raised from one

Table 1 Cell line selection against *P. cactorum* culture filtrate.

CF concentration (%)	Survival of callus (%)
0 (control)*	100.00 (90.00)
5	86.16 (68.34)
10	77.78 (61.97)
12.5	69.44 (56.49)
15	52.78 (46.60)
17.5	19.33 (25.98)
20	5.53 (11.18)
22.5	0.00 (00.00)
25	0.00 (00.00)
$LSD_{0.05}$	(7.05)

Figures in parentheses are arc sine transformed values.
*100 pieces of callus (15 mg/callus FW) were kept for each CF treatment.

Table 2 Effect of growth regulators on calli regenerating bulblets, number of bulblets and number of leaves per bulblet*.

Treatment (mg/l)			Calli regenerating	№ of bulblets/	№ of leaves/
2,4-D	BA	NAA	bulblets (%)	callus	bulblet
2	0	0	61.00 (51.36)	2.03	3.33
3	0	0	57.33 (49.22)	1.83	6.33
0	1	0.2	74.25 (59.51)	2.79	10.62
0	1.2	0.2	56.83 (48.93)	2.36	7.33
0	1	0.6	62.24 (52.09)	2.90	7.67
0	1.3	0.6	75.50 (60.38)	4.86	12.00
$LSD_{0.05}$			(0.85)	0.53	2.1

Figures in parentheses are arc sine transformed values.
*Three replications with 10 explants in each.

The methodology adopted to obtain the above-mentioned results has been represented as a flow chart (**Fig. 1**).

Bulbscale explant (3-4 mm)
↓3 weeks
Callus induction
↓7 weeks
Callus multiplication
(MS basal medium + 2 mg/l 2.4-D)
↓6 weeks
Selection medium
(MS + 2 mg/l 2,4-D + 20% CF)
↓
Selection of surviving cells
↓
Multiplication of surviving cells
(MS + 2 mg/l 2,4-D)
↓
Transfer to bulblet regeneration medium
(MS + 0.6 mg/l NAA + 1.2 mg/l BA)
↓2 weeks
Storage of bulblets at 2°C
↓
Bulbs transferred to pots (10 cm diameter)
filled with sterile sand and soil (1:1)
↓One year
In vivo testing

Fig. 1 Flow chart showing various steps to produce resistant plants.

Table 3 *In vivo* testing of *Lilium* for resistance against *P. cactorum*.

***Lilium* bulbs**	**Bulbs[a] inoculated**	**Groups[b]**				
		0	1	2	3	4
Control	25	NIL	NIL	NIL	10	15
Selected	25	3	7	10	5	NIL

[a]Mean of three replications.
[b]Described in Materials and methods.

year-old bulbs showed resistance against *P. cactorum* (**Fig. 2**), whereas none of the controls showed resistance (**Table 3**), thus showing a strong link between *in vitro* selection and *in vivo* resistance. Several other workers have also reported the expression of resistance at the plant level in number of host-parasite interactions (Behnke 1980; Hartman *et al*. 1984; Shahin and Spivey 1986).

In the present investigation an attempt was made to develop a protocol for Asiatic hybrid lily 'Alaska' resistant to *P. cactorum* through screening of callus cell lines against culture filtrate of the pathogen. Only 40% of plants developed from the one year-old selected bulbs showed resistance to the pathogen.

Fig. 2 One-year old bulbs of *Lilium*. (**A**) Selected and (**B**) control plants, 30 days after inoculation with sporangia suspension of *P. cactorum* (bar = 0.5 cm).

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