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SHORT COMMUNICATION

In Vitro Selection of Groundnut Cell Lines from *Cercosporidium personatum* Culture Filtrates and Regeneration of Resistant Plants Through Cell Culture

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Cell suspensions derived from immature leaves of the groundnut (*Arachis hypogaea* L.) were cultured in the presence and absence of *Cercosporidium personatum* pathotoxic culture filtrates. Cell viability and reactions of cell lines were determined after exposure to various concentrations (25-100%, v/v) of the filtrates. Cell lines have been selected for resistance to the toxin(s) produced by *C. personatum*. Selected cell lines were used for plant regeneration on regeneration media containing *C. personatum* culture filtrates. Plant regeneration frequency was found to be low in long-term cultures, whereas it was high in short-term cultures. The selfed progeny of the plants regenerated from the resistant cell lines showed resistance to the pathogen in the field. Six out of 82 plants exhibited enhanced resistance in the R_2 generation. The culture filtrate stimulated callus proliferation as well as plant regeneration at lower concentrations, a response that could prove to be very useful for obtaining disease resistant plants through *in vitro* selection.

Keywords: *Arachis hypogaea*, cell suspension, *Cercosporidium personatum*, disease resistant plants, *in vitro* selection, pathotoxic culture filtrate

INTRODUCTION

The tikka late leaf spot disease (*Cercosporidium personatum*) results in extensive damage to the leaf area of groundnut (*Arachis hypogaea* L.) plants. When groundnut leaves are infected with *C. personatum*, brown spots spread rapidly and they eventually cause premature defoliation. The most economic and effective method of leaf spot control, however, would be to use an agronomically acceptable disease resistant variety. In the absence of resistance, leaf spot can be controlled by copper and sulphur based fungicides. The use of resistant cultivars is a more sustainable alternative method of control. However, groundnut plants with resistance to *C. personatum* have not yet been reported. Therefore, it is necessary to find approaches for obtaining groundnut plants resistant to this pathogen.

The *in vitro* selection of plant cells or tissues for resistance to fungal pathogens can be accomplished by using different screening agents, such as purified specific or non-specific toxins, crude extracts of pathogen cultures, or by co-culture with the fungus itself (Biondi *et al.*, 1991). Two methods are typically used to produce resistant plants from cell culture techniques: (1) *in vitro* selection of cell lines resistant to toxins produced by the pathogen, followed by regeneration of the plants from the resistant cell lines, and (2) screening of regenerated plants from unselected cell cultures and identifying resistant somaclonal variants. Many plant pathogenic fungi produce host-specific pathotoxins that are primary determinants in pathogenesis and induce typical disease symptoms in the absence of the pathogen (Goodman *et al.*, 1986). Toxic culture filtrates and purified toxins of pathogens have been used for *in vitro* selection, and disease-resistant plants were regenerated in various plant species (Hartman *et al.*, 1984; Binarova *et al.*, 1990; Vidhyasekaran *et al.*, 1990; Song *et al.*, 1994).

The fungus *C. personatum* is the causal agent of the groundnut tikka late leaf spot disease. Ramanujan (1982) studied the phytotoxic metabolites produced by *C. personatum* in culture filtrates. Two phytotoxic

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compounds, namely personatin and CP3 pigments (dothistromin and its derivatives), which almost certainly play an important role in pathogenesis, have been investigated. The pathogenicity of *C. personatum* probably results, however, in personatin affecting both respiration and permeability of leaf cells, but the pigment affects only permeability. For this reason, we preferred to use the pathotoxic culture filtrate of the fungus as the screening agent, rather than the purified toxin, in an attempt to select resistant calli of *A. hypogaea* L. (peanut or groundnut).

To date, no resistance to *C. personatum* has been found in the groundnut using conventional methods (Wells *et al.*, 1994). Therefore, an alternative biotechnological approach may be to use pathotoxic culture filtrates to screen cell lines for resistance to *C. personatum* by the *in vitro* selection technique. In the present study, we describe the selection and regeneration of groundnut plants resistant to pathotoxic culture filtrates of *C. personatum* and the evaluation of the regenerated plants for resistance to the pathogen in the field.

MATERIALS AND METHODS

Plant Material

The groundnut (*A. hypogaea* L.) cv. VRI-2 susceptible to the tikka leaf spot disease was used in the study. Seed materials were obtained from Tamil Nadu Agricultural University, Coimbatore, India. Seeds were surface sterilized and germinated as previously described by Venkatachalam *et al.* (1994).

Callus Induction and Maintenance

Immature leaf explants were excised from axenically-grown 7-day-old seedlings and used for callus initiation. High frequency of callus induction was observed on an MS (Murashige and Skoog, 1962) medium containing B_5 vitamins (Gamborg *et al.*, 1968), NAA (2.0 mg/L), BAP (0.5 mg/L), 3% (w/v) sucrose, and 0.7% (w/v) agar. The pH of the medium was adjusted to 5.7 before autoclaving. The medium was autoclaved at 121°C for 15 min. All cultures were incubated at 24±2°C with a 16 h photoperiod (Philips cool-white fluorescent light, 80 $\mu Em^{-2}s^{-1}$). Subcultures of all materials were made at 4-week intervals.

Initiation of Cell Suspension Culture

Suspension cultures were established by transferring 1 g of friable callus to 25 mL of liquid medium (MS basal medium supplemented with 1.0 mg/L 2,4-D and 1.0 mg/L BAP) in 150 mL flasks. Cultures were shaken at 80 rpm and incubated under diffuse light (20 $\mu Em^{-2}s^{-1}$) and temperature regimes as described above. Suspension cultures were subcultured at 2 week intervals. Growth of cell suspension cultures was determined by counting cell clusters under a microscope.

Pathogen Culture

The fungal spores of *C. personatum* were obtained from the International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Hyderabad, India. Axenic cultures of the fungal isolates were maintained on a Czapek's dextrose agar (CDA) medium at 4°C in the dark and were routinely transferred to a fresh medium every 4 weeks (Fig. 1A).

Preparation of Culture Filtrate Media

To obtain pathotoxic culture filtrates, cultures were grown in 500 mL flasks containing 150 mL of MS liquid medium. Flasks were inoculated with 3 pieces of fungal mycelium using an inoculation loop and agitated at 80 rpm for 15 to 20 days. Uninoculated fungal culture media was similarly handled. Flasks showing no mycelial growth were discarded. The fungal culture media were initially filtered through Whatman filter paper No. 1 to remove spores and mycelia. The pH of the pathotoxic culture filtrates was adjusted to 5.7 with 0.1 N NaOH, and was filter sterilized with a 0.45 μm Millipore filter unit. For preparation of toxic media, filter-sterilized volumes of pathotoxic culture filtrate were added to the MS media after autoclaving to provide four treatment concentrations viz., 25, 50, 75 and 100% (v/v). A control without filtrate was the fifth treatment. The media were poured into petri dishes.

Selection of Resistant Cell Lines and Plant Regeneration

Long-term Selections

Selection of resistant cell lines was made by placing 2-week-old cell suspensions on a filtrate-containing (FC) medium and surviving cells were again transferred to a fresh FC medium. After cell lines demonstrated continued growth for an additional two subcultures, they were transferred to a fresh FC medium to determine if callus resistance was maintained on a toxic

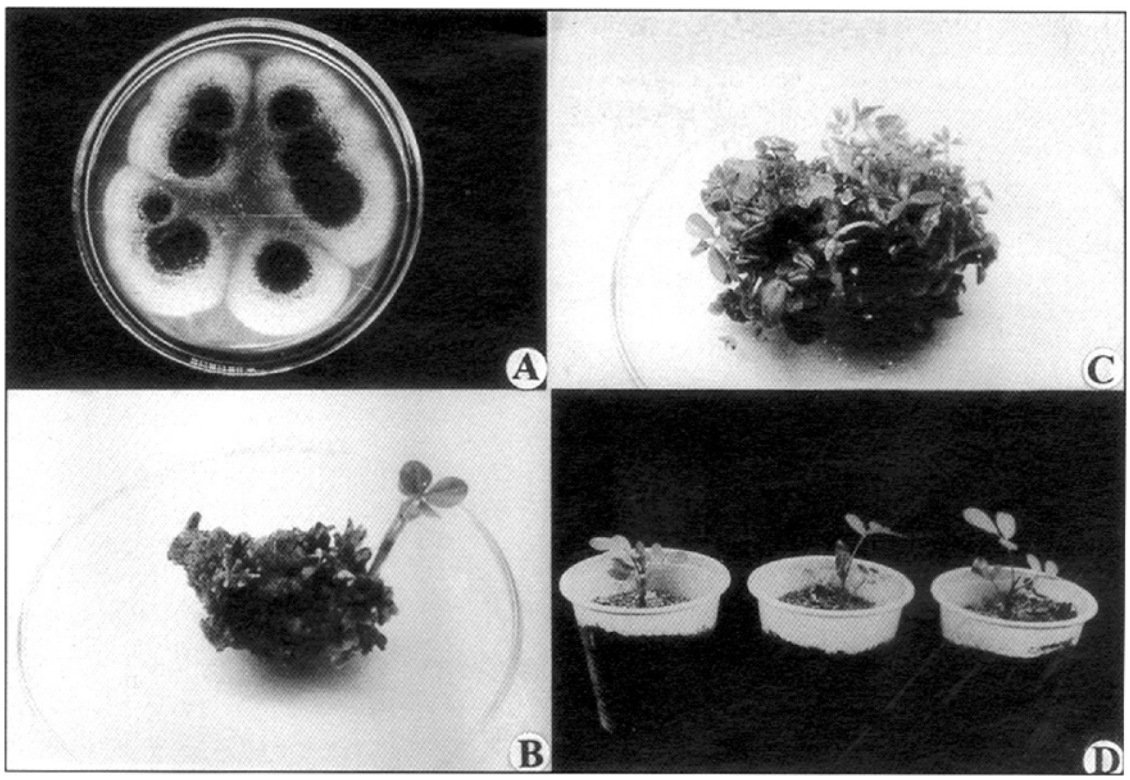

Fig. 1. Selection and regeneration of groundnut plants resistant to pathotoxic culture filtrate of *C. personatum*. A, *C. personatum* fungus pure culture; B, Toxin-resistant callus; C, Shoot bud regeneration; D, Regenerated plants established in plastic cups.

medium. Long-term selections were maintained on toxic medium for a minimum of 3 months. The viability of the callus clumps was determined by a 2,3,5-triphenyl tetrazolium chloride (TTC) assay (Postlethwait and Nelson, 1957). Regeneration was then attempted by transferring calli to a shoot regeneration medium containing MS salts, NAA (0.5 mg/L), and BAP (2.0 mg/L). Developing shoot buds were transferred to 250 mL flasks containing 100 mL of MS medium and grown for 2-3 weeks under light conditions as described previously. When shoots attained 2-3 cm height, they were separated and transferred to a rooting medium (MS salts containing IBA at 2.0 mg/L and kinetin at 0.5 mg/L). Rooted plantlets were established in plastic cups filled with sterilized red soil and sand at a 1:1 ratio and kept for a week in the controlled temperature. After acclimatization they were transferred to the field.

Short-term Selection

Selection of short-term resistant cell lines was made in the same manner as long-term except for 3-4 weeks. Calli were placed on an FC medium and after 4 weeks, the calli were used for plant regeneration on a MS medium. Plantlets were grown and maintained as previously described for long-term selection.

Testing Plant Resistance to Pathogens

The R_2 progeny of 82 plants were tested with fungal spore suspensions for infection response. The plants in the field were inoculated with a *C. personatum* spore suspension using a sprayer. In susceptible plants, brown leaf spots appeared on the leaves within 2-3 days, and typical symptoms of tikka disease were present in the inoculated plant 15-21 days after treatment. Regenerated plants exhibited enhanced resistance as judged by Raguchander *et al.* (1990). Reactions of individual regenerants to *C. personatum* were classified into six groups based on number of leaf spots as follows: highly resistant (0-10%), resistant (11-20%), moderately resistant (21-40%), moderately susceptible (41-50%), susceptible (51-60%), and highly susceptible (61-100%).

Statistical Analysis

Results were analysed by analysis of variance (ANOVA). Effects of the main variables (pathotoxins,

callusing, shooting, and rooting) and their interactions on resistance were examined. Observations of repeated experiments were subjected to analysis of homogeneity of variance and pooled accordingly. Duncan's New Multiple Range Test was used to determine significance of differences among treatment means.

RESULTS AND DISCUSSION

Selection of Resistant Cell Lines

Efficient callus induction was observed on an MS medium supplemented with NAA (2.0 mg/L) and BAP (0.5 mg/L) from immature leaf explants. Cells grown in the suspension culture were plated onto a filtrate containing (FC) medium at an initial density of 3,000 cells/mL. Cultures were used for cell viability test by TTC assay after 30 days of the culture. A decrease in cell viability (as measured with the TTC assay) in both the long-term and short-term cultures was observed when callus cultures were exposed to the culture filtrate (Table 1). As the concentration of the culture filtrates was increased in the medium, a significant decrease in cell viability was noted; however, the cell viability was completely lost in 100% FC media (Table 1). The percentage of cell viability negatively correlated with culture filtrates dut to accumulation of toxins in medium. A similar result was also reported by Pijut *et al.* (1990) in *Ulmus americana*.

In long-term selections, cells appeared as small green callus clumps surrounded by masses of necrotic cells after 4 weeks of growth an FC medium. After two subcultures, putative resistant cell lines were transferred to a fresh FC medium at 4 week intervals. After 4 months, stable resistant cell lines capable of growth on an MS medium in a manner comparable to the control medium was isolated and used for plant regeneration. In short-term selections, cells were immediately subcultured after culture initiation. After 4 weeks (two subcultures) on MS media containing different concentrations of culture filtrates, putative resistant callus lines were isolated and used for plant regeneration (Fig. 1B). The frequency of resistant callus formation increased with increasing the culture filtrate concentrations in both the long-term and short-term cultures. The resistant callus induction frequency ranged from 6.6 to 22.1% in the long-term cultures, whereas it ranged from 9.8 to 27.5% in the short-term cultures (Table 2). The frequency of callus development varied due to the presence of toxins in the medium. The stimulatory effect of the culture filtrates on callus growth is illustrated in Table 2. Similarly, Yu *et al.* (1990) obtained increased frequency of callus on a medium containing *Verticillium albo-atrum* culture filtrates in alfalfa.

Table 1. Effect of pathotoxic culture filtrates from *C. personatum* on cell viability of long-term and short-term cultures of groundnut

Filtrate conc. (%)	Cell viability (%)	
	Long-term culture	Short-term culture
0	100.0a	100.0a
25	83.0a	88.5a
50	64.6ab	69.2ab
75	36.9b	42.5b
100	–	–

Values within column with the same letter are not significant at the 1% probability level according to the Duncan's New Multiple Range Test.

Regeneration of Groundnut Plants Resistant to Pathotoxic Culture Filtrates

Toxin-resistant cell lines (calli) were isolated from both long-term and short-term cultures and were

Table 2. Selection of toxin resistant groundnut calli from long-term and short-term cultures

Filtrate conc. (%)	No. of calli cultured		No. of calli showed resistance		Resistant calli frequency (Percentage # mean ± SD)	
	LTC	STC	LTC	STC	LTC	STC
0	45	51	3	5	6.6±2.44b	9.8±2.08b
25	63	56	10	8	15.8±4.08ab	14.2±3.10ab
50	72	65	18	13	25.1±5.88a	20.0±2.34a
75	69	68	19	15	27.5±4.55a	22.1±1.55a
100	–	–	–	–	–	–

LTC, Long-term culture; STC, Short-term culture.
Values within column with the same letter are not significant at the 1% probability level according to the Duncan's New Multiple Range Test.

used for shoot regeneration. Shoot bud regeneration was obtained from the resistant calli on an FC medium containing 2.0 mg/L of BAP and 0.5 mg/L of NAA. After this step, surviving shoot bud clumps were continuously subcultured on FC media containing growth regulators every 2 weeks, at least 3 selection cycles, to select putatively resistant shoots to the culture filtrates (Fig. 1C). Regeneration frequency was also increased with increase in the filtrate concentration. The frequency of shoot bud regeneration ranged from 8.3 to 36.3% from long-term culture calli and it ranged from 15.6 to 42.3% for short-term culture calli (Table 3). More than 50% of the calli did not grow on the regeneration medium with FC. In the present study, the culture filtrate stimulated shoot bud regeneration. The frequency of shoot bud regeneration also varied depending on the concentration of filtrates used and exposure time (Venkatachalam and Jayabalan, 1995). A similar stimulatory effect of the culture filtrate on shoot bud regeneration had been reported earlier in alfalfa by Hartman *et al.* (1984) and Yu *et al.* (1990). Lynch *et al.* (1991) described that the culture filtrate of *Alternaria solani* stimulated shoot bud regeneration in potatos. Regenerated plants were successfully transferred to plastic cups (Fig. 1D) and later established in a field.

Disease Resistance of the Progeny of Regenerated Plants

Seeds from 6 R_1 plants in both the long-term and short-term selection systems were planted in the field and 80-85% of the seeds germinated, producing 82 R_2 plants. The 82 progeny of R_2 plants were tested for infection by *C. personatum* pathotype groundnut to ascertain whether their resistance was heritable. The results are shown in Table 4. About 30.3% of the progeny of 33 the plants regenerated from long-term cultures were resistant to tikka late leaf spot disease, whereas approximately 14.2% of the progeny of the 49 plants from short-term cultures were resistant. There was no resistance in the control plants. The disease resistance was found to be high in the long-term callus derived regenerants, whereas it was found to be low in short-term cultures (Venkatachalam, 1996). Similar results were also reported by Hartman *et al.* (1984) in alfalfa. Song *et al.* (1994) produced resistant plants to a host specific pathotoxic culture filtrate of *Septoria glycines* in the soybean. The

Table 3. Plant regeneration frequency from selected long-term and short-term toxin resistant calli of groundnut

Filtrate conc. (%)	No. of calli plated		No. of calli responded		Shoot bud regeneration frequency (Percentage # mean ± SD)	
	LTC	STC	LTC	STC	LTC	STC
0	48	51	4	8	8.3±3.02b	15.6±4.40bc
25	54	58	9	14	16.6±4.32ab	24.1±2.28b
50	56	61	17	20	30.3±7.02a	32.7±5.47ab
75	55	59	20	25	36.3±5.14a	42.3±4.89a
100	–	–	–	–	–	–

LTC, Long-term culture; STC, Short-term culture.
Values within column with the same letter are not significant at the 1% probability level according to the Duncan's New Multiple Range Test.

Table 4. Response to pathogen (*C. personatum*) in plants regenerated from long-term and shor-term cultures of groundnut

Filtrate conc. (%)	Long-term culture							Short-term colture						
	No. of plants screened	Disease resistance spectrum						No. of plants screened	Disease resistance spectrum					
		HR	R	MR	MS	R	HS		HR	R	MR	MS	R	HS
0	4	–	–	1	1	1	1	8	–	–	–	3	2	3
25	6	1	–	1	1	1	2	9	–	–	1	3	2	3
50	11	1	1	1	2	3	3	15	1	1	1	4	3	5
75	12	2	1	1	3	2	3	17	1	1	1	4	4	6
100	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Total	33	4	2	4	7	7	9	49	2	2	3	14	11	17

HR, Highly resistant; R, Resistant; MR, Moderately resistant; MS, Moderately susceptible; S, Susceptible, HS, Highly susceptible.

present study clearly indicates that the resistance shown by these regenerated plants was heritable, and it can be transmitted to offspring through sexual reproduction. Further genetic analysis of the progeny is needed to clarify this point. In the near future we will investigate how pathotoxic culture filtrate resistant plants behave against an infection with *C. personatum*. The aim of this program is to obtain plants with a general field resistance against this pathogen.

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