Regeneration of late leaf spot-resistant groundnut plants from *Cercosporidium personatum* culture filtrate-treated callus

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Cotyledon callus cultures of groundnut (*Arachis hypogaea* L.) derived from tikka late leaf spot disease-susceptible and resistant genotypes were exposed to various concentrations of fungal culture filtrate (FCF) of *Cercosporidium personatum*, the causal fungal agent of tikka late leaf spot disease. Fresh weight and cell viability of calli were determined after exposure to various concentrations of FCF. Sensitive calli have failed to increase in fresh weight and lost viability after exposure to media containing the FCF whereas insensitive calli retained growth and maintained viability similar to controls, viz. calli not exposed to FCF. Insensitive calli were selected by culturing on growth medium containing various concentrations of the FCF. Resistant calli obtained by selection survived three subcultures under the same conditions and were used for plantlet regeneration. Regenerated plants when transferred to soil–sand mixture in plastic cups and subsequently shifted to field conditions, set few viable seeds. Plants of R_2 generation exhibited enhanced resistance to tikka late leaf spot disease.

THE plant tissue culture technology has potential application in the development of disease-resistant plants in various crops. Further, the establishment of a host–pathogen interaction *in vitro* permits screening and selection for disease resistance at cellular level[1]. In recent years, pathotoxins (fungal elicitors) have been

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identified as agents useful for induction of disease resistance and selection of plants in cell culture, and the problems involved have been reviewed in detail[2]. Subsequently, many investigators have used toxins (crude or partially purified), crude extracts of mycelium, and culture filtrates (crude or partially purified) in selecting disease-resistant plants in several species[3–10]. Compounds have been isolated from a host of fungal species that trigger most or at least some of the plant defense reactions. These compounds classified as elicitors were covered in a recent review and various aspects related to their functions discussed[11]. Phytotoxins have been identified from a broad spectrum of pathogens, but their actual role in pathogenesis remains poorly understood[11].

Among the causal agents of infectious diseases of crop plants, phytopathogenic fungi play a dominant role not only by causing devastating epidemics, but also through the less spectacular, although persistent and significant annual crop yield losses that have made fungal pathogens a serious economic factor attracting the attention of farmers, plant breeders and scientists alike[11]. The tikka late leaf spot disease of groundnut, caused by the fungus *Cercosporidium personatum*, is almost co-existent with the crop and contributes to significant losses in yield throughout the world[12]. Chemical control has still been the major approach to minimize the loss of yield but the development of resistant cultivars through genetic manipulation would open up new avenues to supplement conventional methods[13]. Ramanujam[14] studied the phyto-toxic compounds, personatin and CP_3 pigments (dothistromin and its derivative) that seem to play an important role in pathogenesis. The pathogenicity of *C. personatum* probably results in personatin affecting both respiration and permeability of leaf cells, while the pigment affects only permeability. For this reason, we used the fungal culture filtrate (FCF) as the selection agent, rather than the purified toxin, in an attempt to select resistant callus lines of groundnut. Plant tissue culture systems allow control of the environment, thereby eliminating confounding results incited by other pathogens and facilitate observation of the interaction at the tissue or cellular level. Tissue culture further facilitates screening a large number of genotypes year-round within a relatively small space. A protocol that has been widely used for the isolation of disease-resistant lines is to grow callus in the presence of a FCF. Studies so far carried out did not encompass groundnut (*Arachis hypogaea* L.) which is an important oil-seed crop.

Of the two groundnut cultivars chosen for the present study, VRI-2 is susceptible whereas TMV-7 is resistant to the tikka late leaf spot disease. Seeds of these cultivars were obtained from Tamil Nadu Agricultural University, Coimbatore and were surface sterilized, rinsed and germinated as previously described by Venkatachalam *et al.*[15]. Callus cultures were initiated from sliced cotyledonary nodal segments of the seedlings. Cotyledonary segments were transferred to Murashige and Skoog (MS)[16] medium containing B_5 vitamins[17], naphthaleneacetic acid (NAA) (1.5 mg/l) and kinetin (0.5 mg/l), 3% (w/v) sucrose and 0.7% (w/v) agar. The pH of the medium was adjusted to 5.8 before adding agar and sterilized at 121°C with 1.1 kg cm^2 pressure for 15 min. Cultures were maintained under 16 h photoperiod at 24 ± 2°C with 80 $\mu E\ m^{-2}\ s^{-1}$ light intensity. Proliferating callus cultures were transferred to fresh MS medium containing the same supplements at four weeks interval.

The fungal isolates of *C. personatum* were obtained from International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India. Axenic cultures of the fungal isolates were maintained on Czapek's dextrose agar (CDA) medium at 20 ± 2°C in the dark, and were routinely transferred to fresh medium every two weeks. Fungal culture filtrates of *C. personatum* isolates were prepared from liquid cultures and the inoculum used was obtained from 4-day-old stock cultures. Czapek's liquid medium (100 ml) in 250 ml Erlenmeyer flask was autoclaved at 121°C with 1.1 kg cm^2 pressure for 15 min. Each flask of sterile medium was inoculated with 5 mm dia mycelial plugs. Liquid cultures were incubated at 24 ± 2°C in the dark on a rotatory shaker (80 rpm) for two weeks. Later the fungal cultures were passed first through filter paper to remove the mycelium, then filter sterilized using a membrane of 0.45 µm pore size. The pH of the FCF was adjusted to 5.8 with 1N HCl. To avoid thermal degradation of toxic compounds in the FCF, it was added to the autoclaved medium at 40°C.

The FCF was added to MS medium at different concentrations, viz. 0, 25, 50, 75 and 100% (v/v). The media were dispensed into sterile petri plates. Control plates contained either the uninoculated FCF-containing medium or sterile distilled water at the same concentrations. Calli (about 500 mg) were placed and incubated in the dark at 24 ± 2°C for 60 days. Fresh weights were obtained from 10 plates per treatment and each experiment was repeated thrice. The viability of the callus clumps was determined by 2,3,5-triphenyl-tetrazolium chloride (TTC)[18] assay.

The resistant calli were subcultured onto fresh media with FCF after 60 days of culture initiation. Plant regeneration was achieved on MS medium containing B_5 vitamins, 0.5 mg/l NAA and 2.0 mg/l 6 benzylaminopurine (BAP). Regenerated shoots were rooted on MS medium containing IBA or NAA (2.0 mg/l) and kinetin (0.2 mg/l). Plantlets were established initially in plastic cups containing red soil and sand in the ratio 1:1. Plants recovered from both culture filtrate-sensitive (susceptible) and culture filtrate-insensitive (resistant) calli were transferred to the field for further evaluation. A suspension of spores of the fungus was sprayed on these plants

and disease severity was rated using the rating scale of Raguchander *et al.*[19], based on the proportion of diseased leaf tissue. Disease symptoms and plant growth stage were evaluated at least once a week for eight weeks. Reactions of individual regenerants to *C. personatum* were classified into six groups based on the percentage of diseased leaf area 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%).

Cotyledonary nodal segments cultured on MS medium containing NAA (1.5 mg/l) and kinetin (0.5 mg/l) gave the best response in terms of callus growth. Repeated subcultures were necessary to obtain homogeneous callus lines that were friable and greenish. The fresh weight of the callus was tested with various concentrations of FCF (0, 25, 50, 75 and 100% v/v). Table 1 shows the inhibition of tissue growth (as percentage of control weight) of susceptible cultivar grown on media at different concentrations of FCF. Final fresh weight of callus decreased as FCF concentration increased. The intensity of growth inhibition depended on the concentration of FCF used. Growth inhibition was less severe with 25% FCF concentration. Callus growth was also reduced significantly ($P < 0.05$) compared with control when 50 or 75% of the FCF was added. At higher concentration (100%), growth was significantly ($P < 0.05$) affected when compared with other FCF concentrations. The callus fresh weights of the resistant cultivar were not significantly ($P < 0.05$) decreased as a result of increased concentration of the FCF in the medium. The fresh weights as well as the growth, on the other hand, increased significantly ($P < 0.05$) in the presence of FCF. Similar results were reported in elm also by Pijut *et al.*[8].

Van Asch *et al.*[20] reported that the callus of the control treatment and of that grown at the lowest concentration of fungal toxin looked healthy and increased in size considerably whereas the calli treated with the highest toxin level showed a brown colour with no visible growth. We demonstrate that a reduction in the callus growth of a susceptible cultivar occurs when the culture medium is amended with a cell-free FCF. This decrease in callus fresh weight was the result of the presence of toxic metabolites in the FCF. The dose-dependent inhibition of growth of callus cultures caused by the FCF was very similar. Earlier, the toxic effect of the culture filtrate of *Fusarium oxysporum* on *in vitro* grown cultures of alfalfa[6], tobacco[21], muskmelon[22] and that of *Ceratocystis ulmi* on elm[8] had been investigated. The effect of toxin from culture filtrates of *Alternaria solani* on the growth of cell suspensions of 'host' and 'non-host' crop species has been examined by Handa *et al.*[23]. They found that the toxin-inhibited growth and that the cells of the 'host' species were more sensitive to the toxin than those of 'non-hosts'.

A decrease in cell viability (as measured with the tetrazolium assay) of the susceptible calli was observed when callus cultures were exposed to the FCF (Table 2). As the concentration of FCF was increased in the culture medium, a significant ($P < 0.05$) decrease in cell viability was noted. The FCF decreased the cell viability (sensitive lines) right from the lowest concentration and complete inhibition was recorded at 100% of the FCF concentration. Culture filtrate-insensitive (resistant) calli, nevertheless, maintained normal cell viability. Therefore, this screening procedure may also be useful in performing cellular selection for putative tolerance to the toxin. Similar results were also recorded in elm by Pijut *et al.*[8].

Culture-filtrate sensitive (susceptible) as well as insensitive (resistant) calli were transferred to MS medium supplemented with 0.5 mg/l NAA and 2.0 mg/l BAP for shoot bud regeneration. Elongated shoots (> 3 cm long) were rooted on MS medium containing IBA or IAA. The rooted plantlets were subsequently transferred to soil–sand mix in plastic cups (Figure 1) and later established in the field where 60–70% of them survived and resumed growth to maturity. The progeny of 144 plants was tested with pathogen inoculation for their response to infection. Eighty five plants were tested from the resistant cultivar and 59 from susceptible cultivar. When screened under artificial conditions, 12 plants exhibited high resistance in both the cultivars.

Table 1. Effect of different concentrations of fungal culture filtrate of *C. personatum* on the fresh weight increase of groundnut callus after six weeks of incubation

Filtrate (% v/v)	Callus fresh weight (g)	
	Susceptible	Resistant
0	2.56 ± 1.2^a	2.65 ± 1.4^a
25	2.12 ± 1.1^b	2.80 ± 1.3^b
50	1.58 ± 0.9^c	2.97 ± 1.6^c
75	0.81 ± 0.4^d	3.26 ± 1.5^d
100	0.51 ± 0.2^e	2.83 ± 1.2^b

Means followed by same letter are not statistically different according to Duncan's new multiple range test ($P < 0.05$).

Table 2. Effect of *C. personatum* culture filtrate on cell viability (TTC assay) in susceptible (sensitive) and resistant (insensitive) calli of groundnut

Filtrate (% v/v)	Susceptible (sensitivie)	Resistant (insensitive)
0	0.69 (100)^a	0.68 (100)^a
25	0.58 (84.1)^b	0.65 (95.6)^{ab}
50	0.40 (57.9)^c	0.68 (100)^a
75	0.21 (30.4)^d	0.64 (94.1)^{ab}
100	0.00 (0.0)	0.67 (98.5)^a

Figures in parentheses indicate percentage of control of cell viability. Means followed by same letter are not statistically different according to Duncan's new multiple range test ($P < 0.05$).

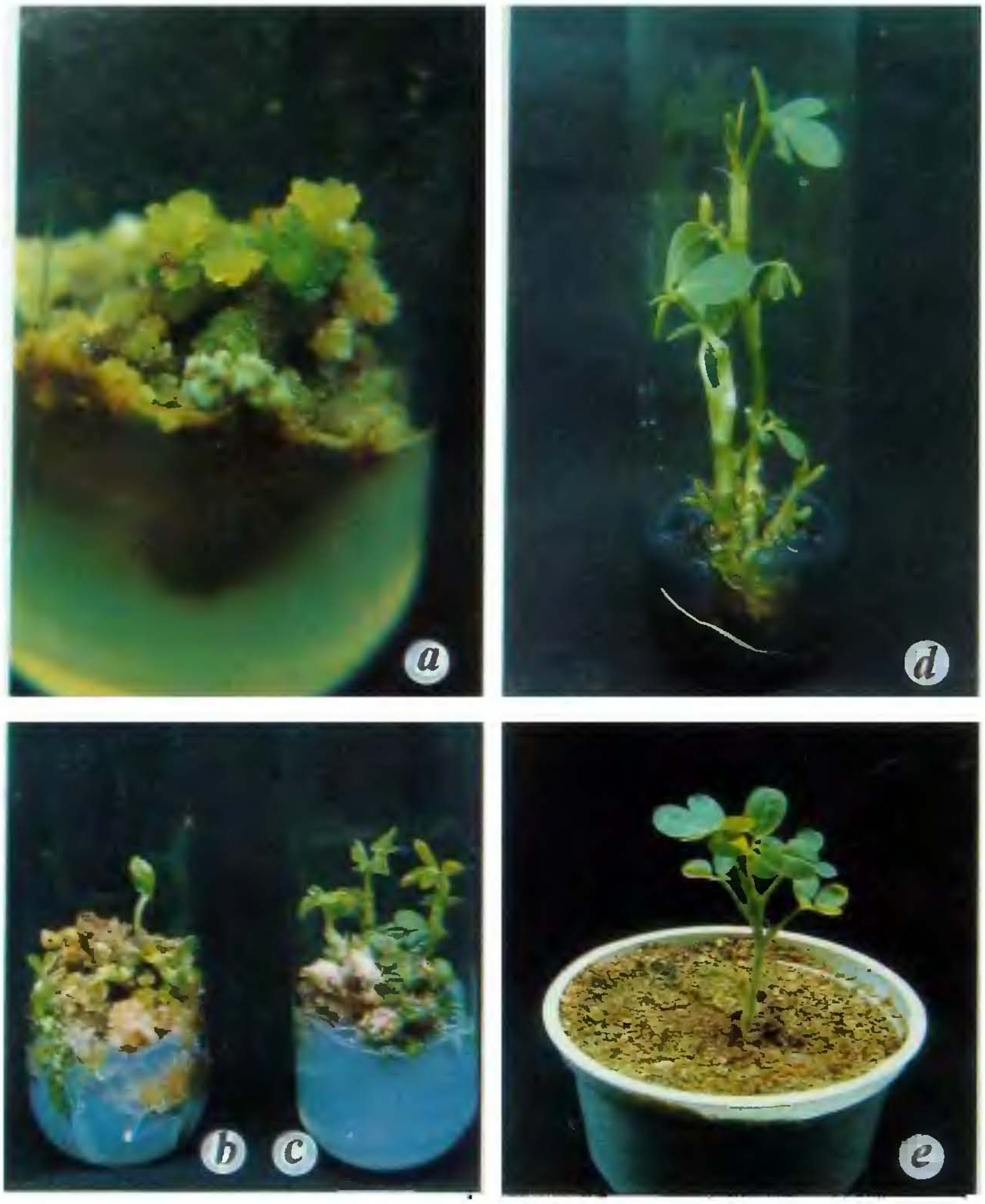

Figure 1. Isolation of callus and regeneration of plants from fungal culture filtrate-treated cotyledon callus cultures of groundnut. *a*, Vigorously growing culture filtrate-insensitive calli; *b*, Shoot bud initiation from culture filtrate-sensitive calli; *c*, Regeneration of plants from culture filtrate-insensitive calli; *d*, Regenerated shoot with roots; *e*, Regenerated plant established in soil–sand mix.

Results are shown in Table 3. About 16.9% and 21.2% of the progeny of susceptible and resistant cultivars respectively have acquired resistance to *Cercosporidium* late leaf spot disease whereas none of the control plants of these cultivars showed resistance. Seeds of the resistant R_1 generation gave a progeny (R_2) that exhibited enhanced resistance when subjected to pathogen inoculation. Our results clearly indicate that the resistance shown by these plants was heritable. This approach of generating disease resistance raises a question whether the trait acquired is due to a mutation or somaclonal variation. Many plant species earlier have been selected for disease resistance using culture filtrates[9,24–26] or partially purified toxins[27,28]. No phenotypic variation was reported from the progeny of selected regenerants. Selection of disease-resistant plants without other apparent mutations has been accomplished in rice by short-term exposure of calli to *Helminthosporium oryzae* toxin followed by regeneration on toxin-free medium[27]. The present study helps us conclude that the sensitivity of the cultured cells to the FCF of *C. personatum* is related to the susceptibility of the groundnut regenerants to the pathogen and the selection system using the FCF of *C. personatum* produced tikka late leaf spot disease-resistant plants. The present study further demonstrates recovery of resistant plants to a specific disease in groundnut through tissue culture technology. Similar reports were published in other crop species. Potato plants resistant

Table 3. Disease response to the pathogen (*C. personatum*) in plants regenerated from fungal culture filtrate-treated groundnut cell cultures

Filtrate (%)	No. of plants tested	Disease reaction spectrum					
		HR	R	MR	MS	S	HS
Susceptible cultivar							
0 (control)	15	–	–	2	2	6	5
25	14	–	1	3	3	3	4
50	17	2	2	2	2	3	6
75	13	2	3	2	1	2	3
100	–	–	–	–	–	–	–
Total	59	4	6	9	8	14	18
Resistant cultivar							
0 (control)	14	–	–	1	3	4	6
25	16	1	2	2	3	4	4
50	20	2	3	3	3	4	5
75	18	2	2	4	3	3	4
100	17	3	3	4	3	2	2
Total	85	8	10	14	15	17	21

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

to *Fusarium oxysporum*[24], tobacco resistant to *Alternaria alternata*[5] and rice resistant to *Pyricularia oryzae*[13] have been identified among regenerants from cells which showed resistance to the culture filtrate of the respective pathogens.

Although some of the more general elicitors of fungal cultures such as oligo-*N*-acetylglucosamines and oligo-galacturonates are active in several plants, others appear to be species-specific including groundnut. The most extensive data are available for two different elicitors from *Phytophtora sojae*[29]. A glycoprotein derived from culture filtrates of this fungus induces many defense reactions in suspension-cultured cells. In the most common model, elicitor activity is explained by its binding to a specific cell surface-localized plant receptor that initiates a defense-related transduction cascade.

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