

IN VITRO SCREENING OF BLACK PEPPER (*PIPER NIGRUM* L.) FOR TOLERANCE TO *PHYTOPHTHORA CAPSICI*

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ABSTRACT

In vitro screening of black pepper (*Piper nigrum* L.) for tolerance to *Phytophthora capsici* was carried out using concentrated culture filtrate (CCF) of *P. capsici* and the results indicated that CCF induced quick electrolyte leakage from callus cells was similar to that from leaf tissues of Panniyur-1, Karimunda, Kalluvally, Cheriakani kkan and Balankotta. The calli after screening were successfully regenerated and the elongated shoots rooted and planted out. This technique might serve as one of the approaches in identification of tolerance/resistance to *P. capsici*.

INTRODUCTION

The wealth of genetic diversity available for black pepper (*Piper nigrum* L.) is now plagued with the dreadful *Phytophthora* foot rot disease. The conventional breeding programmes so far carried out could hardly induce any variability for resistance to this disease. Therefore, *in vitro* culture induced genetic variation constitute an important source of variability for the improvement of this crop and hence the search for somaclonal variants and isolation of tolerant/resistant plant type is of great significance.

Selection among somaclonal variants will be more effective if a selective agent is incorporated in *in vitro* callus/cell culture to discriminate the susceptible ones. The selective agent should be a determinant of the disease and should act at the cellular level for efficient selection.

Phytophthora capsici, the causal agent of *Phytophthora* foot rot disease is capable of producing toxic metabolites *in vitro* Lee (1973) observed that the symptoms produced by the inoculation of the toxic metabolites were the same as that of the fungus. The toxin produced by *P. capsici* is non specific (Sarma *et. al.*, 1991). Selection for resistance to non specific toxins is important because resistance to these toxins does not appear naturally and one would induce sources of resistance not currently available (Daub, 1986). The possibility of obtaining disease resistant plants by *in vitro* screening with non host specific phytotoxins had been demonstrated by Materne *et. al.* (1978) and Behnke (1979).

The present study was undertaken to assess whether the toxic metabolites produced by *P. capsici* act at the cellular level and the possibility of using this toxic metabolite for *in vitro* selection.

MATERIALS AND METHODS

Five cultivars of black pepper viz. Panniyur-1, Karimunda, Cheriakanikkadan, Balankotta and Kalluvally were used for the study. *In vitro* seedlings of the above cultivars were raised in sand + vermiculite medium.

Induction of calli

Stem and leaf explants of the above five cultivars were inoculated into Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) at half the concentration supplemented with sucrose 3%, IAA 1.0 mg l⁻¹ and BAP 1.0 mg l⁻¹ for induction of calli. After three weeks, the initiated calli were subcultured to the same modified MS medium for proliferation. Cream nodular calli grown in this subculture medium for two weeks were used for screening studies.

Preparation of culture filtrate

The stock isolate of *P. capsici* was maintained by periodic transfer on Carrot agar medium. Liquid cultures of the fungus were grown in 50 ml Ribeiro's medium (Ribeiro, 1978) by placing 10 mm culture disc of the seven day old cultures of the fungus and were shaken for 10 days in an orbital shaker cum incubator. Culture filtrate was harvested by subsequent filtration through two layers of muslin cloth and Whatman No.1 filter paper. The filtrate was concentrated to 1/10th of its volume so as to get the concentrated culture filtrate (CCF).

In vitro screening of leaves and calli

Different methods were used for *in vitro* screening (1) electrolyte leakage from leaves and calli, (2) growing calli in CCF incorporated modified MS solid media, (3) shaking callus pieces in CCF incorporated modified MS liquid media and growing them in CCF free media and (4) double layer culture technique.

Electrolyte leakage from leaves and calli

Leaves of the five cultivars were collected from field grown vines and leaf tissues for electrolyte leakage studies were prepared as described by Vidhyasekaran *et. al.* (1986). The procedure followed by Vidhyasekaran *et. al.* (1990) was used for the preparation of calli for electrolyte leakage studies. Calli/leaves infiltrated with 3 ml of concentrated Ribeiro's medium served as the control. Conductance of ambient solutions was measured in μ mhos at intervals of ten minutes with a century model CK 701 conductivity bridge using a dip type cell ($K = 0.964$).

Growing calli in CCF incorporated modified MS solid media

Concentrated culture filtrate was incorporated to the modified MS media at 7.5% v/v and autoclaved (The toxic metabolite was found to be stable on autoclaving). Callus pieces (5 - 10 mm) were inoculated into the medium and incubated for a period of three weeks in a culture room at 25 \pm 2°C provided with a photoperiod of 12 h. The necrosis of the calli was assessed and the percentage of necrotic calli was calculated. Modified MS medium to which concentrated Ribeiro's medium was added to the same volume as that of CCF served as control.

Shaking calli pieces in CCF incorporated modified MS liquid media and growing them in CCF free media

Calli (less than 5 mm size) were transferred to liquid modified MS medium containing CCF at 10% v/v and were shaken for 48 h in an orbital shaker cum incubator. The calli were then transferred to the modified MS solid medium free of CCF and necrosis of the calli were noted. Callus pieces shaken in modified MS liquid medium to which concentrated Ribeiro's medium was added at the same volume as that of CCF served as control.

Double layer culture technique

The double layer technique utilised in microbiology (Lepoivre *et. al.*, 1986) was modified. Jars containing the Ribeiro's medium with toxic metabolites was over layered with modified MS medium each at a thickness of 20 mm and were incubated for a period of seven days for the diffusion of the toxic materials. Three weeks old cream nodular calli (5 - 10 mm) were inoculated on the upper medium at the rate of 10 calli/jar. The toxin sensitivity was assessed on the basis of necrosis of the calli three weeks after inoculation. Callus pieces inoculated on double layer without the fungus served as the control.

Shoot regeneration from calli, rooting, hardening and planting

Callus mediated organogenesis and successful establishment of plantlets in black pepper were reported by Nazeem *et. al.*, (1993). Surviving calli after screening by the different methods mentioned above were transferred to modified MS medium for regeneration. The regenerated cultures were transferred to elongation medium containing ½ MS salts supplemented with IAA 0.1 mg l⁻¹ and BAP 0.2 mg l⁻¹. The elongated shoots were rooted in ½ MS media containing sucrose 2% and IBA 1.0 mg l⁻¹. The rooted plantlets were hardened and planted out.

RESULTS AND DISCUSSION

Concentrated culture filtrate prepared by the above method was found to produce typical symptoms of *P. capsici* as confirmed by detached leaf bioassay. Even after autoclaving it was giving the same symptoms showing its thermostable nature.

Electrolyte leakage from leaves and calli

When calli and leaves were infiltrated with CCF produced by *P. capsici*, electrolyte leakage

was induced both from leaves and calli, ten minutes after the treatment which increased with increase in time intervals. The highest leakage was observed 20 minutes after the treatment, thereafter the difference in leakage showed a decreasing trend. The electrolyte leakage after 20 minutes was taken to evaluate the response of different cultivars.

The electrolyte leakage from leaves of the cultivars under study is presented in Table I. The highest leakage of electrolytes was observed for Panniyur-1 followed by Karimunda, Balankotta, Cheriakanikkadan and Kalluvally. Panniyur-1 registered 78 per cent more leakage of electrolytes as compared to Kalluvally. This indicated that Panniyur-1 is most susceptible to CCF followed by Karimunda. Kalluvally and Cheriakanikkadan are showing tolerance to CCF. The tolerance of Kallyvally and Cheriakanikkadan to *Phytophthora capsici* and the high susceptibility of Panniyur-1 and Karimunda to the fungus was reported by Kuch and Khew (1980) and Sarma *et. al.* (1988). This suggests the possibility of utilising CCF for *in vitro* screening. For comparison of *in vitro* screening results and the reaction of *P. capsici* on genotypes separate experiments need to be carried out.

Since CCF was inducing quick electrolyte leakage from the calli, its activity at cellular level was indicated. Cellular level activity of cercosporin and *Helminthosporium oryzae* toxin in rice was reported by Batchvarova *et al.* (1992) and Vidhyasekaran *et. al.* (1990) respectively. Electrolyte leakage from pepper calli exhibits the same trend as in the case of leaf tissues (Table I). The highest leakage was shown by Panniyur-1 followed by Karimunda and Balankotta. The calli induced from Cheriakanikkadan and Kalluvally showed the lowest leakage of electrolytes. A similar response for leaves and calli to *Pyricularia oryzae* toxin was observed in rice by Velazhahan *et. al.* (1992). The present result also indicated that the level of tolerance to *Phytophthora capsici* in the whole plant level under field conditions

Table I. Comparison of electrolyte leakage from leaves and calli (μ mhos)

Electrolyte leakage 20 minutes after the treatment

Cultivar	Leaves		Calli	
	Mean electrolyte leakage	Leakage over control	Mean electrolyte leakage	Leakage over control
Panniyur-1	357.30	321.63	194.33	179.00
Karimunda	242.33	197.00	139.00	128.00
Kalluvally	200.33	169.00	98.66	81.00
Cherrikanikkadan	209.66	178.33	100.66	83.33
Balankotta	216.67	186.00	121.66	101.66

reported earlier (Sarma *et. al.*, 1991) showed correlation to the level of expression at the callus phase. The similarity in the disease reaction between calli and whole plants was reported in eucalyptus to *Phytophthora cinnamomi* (Mc Comb *et. al.*, 1987), in alfalfa to *Phytophthora megasperma* (Miller *et. al.*, 1984) and in pines to *Phytophthora cinnamomi* (Jang and Tainter, 1990). This response suggests the potential use of callus cultures for understanding the host-pathogen interactions at cellular level.

To confirm this the same isolate of *P. capsici* should be tested on test genotype and their calli in order to get precise comparison to attribute the same level of tolerance/resistance in both *in vitro* and *in vivo* systems.

Growing calli in CCF incorporated modified MS solid media

The performance of calli of different cultivars at 7.5% v/v CCF in modified MS solid media was studied with respect to their growth and survival of regenerated cultures (Table II). The highest percentage of necrotic calli was

observed in Panniyur-1 followed by Karimunda, Balankotta, Cherrikanikkadan and Kalluvally. Better response in the survival of regenerated cultures was observed in Kalluvally, Cherrikanikkadan and Balankotta as compared to Panniyur-1 and Karimunda. Similar difference in response of cultured cells of host cultivars that have different levels of resistance to the pathogen was reported in studies with culture filtrates of *Alternaria solani* (Handa *et al.*, 1982) and *Verticillium* sp. (Hartman, 1985) in potato.

Shaking calli pieces in CCF incorporated modified MS liquid media and growing them in CCF free media

Comparison of the performance of calli from different cultivars to shaking in CCF at a concentration of 10% v/v in the modified MS liquid media is presented in Table III. Callus pieces after shaking, when inoculated into CCF free media, good percentage of calli put forth growth in the initial culture. But the nature of calli produced was different from those in the CCF incorporated modified MS solid media and control. Of the few regenerated calli healthy

Table II. Growth of calli and survival of regenerated cultures of different cultivars in CCF incorporated (@ 7.5% CCF v/v) modified MS media

Cultivar	Treatment	% of necrotic calli	% of growing calli	Survival % of regenerated cultures (5th subculture)
Kalluvally	CCF	21.11	78.89	90.00
	Control	0	100.00	95.00
Cherianikkadan	CCF	37.37	62.63	70.00
	Control	0	100.00	90.00
Balankotta	CCF	43.00	57.00	60.00
	Control	0	100.00	100.00
Karimunda	CCF	51.60	48.40	25.00
	Control	0	100.00	45.00
Panniyur-1	CCF	54.44	45.56	0
	Control	0	100.00	20.00

Table III. Growth, regeneration and survival of healthy cultures after shaking in CCF incorporated liquid modified MS media @ 10% v/v

Cultivar	Treatment	% of growing calli	% of necrotic calli	% of regeneration (2nd sub culture)	Survival % of healthy cultures (5th subculture)
Cherianikkadan	CCF shaking	80.95	19.05	5.88	0
	Control shaking	100.00	0	11.11	0
Balankotta	CCF shaking	66.67	33.33	7.14	0
	Control shaking	100.00	0	16.66	0
Kalluvally	CCF shaking	90.00	10.00	5.20	20.00
	Control shaking	100.00	0	12.50	10.00
Karimunda	CCF shaking	60.00	40.00	13.33	0
	Control shaking	100.00	0	50.00	0
Panniyur-1	CCF shaking	52.49	47.06	0	0
	Control shaking	100.00	0	0	0

plantlets could be produced only from Kalluvally. Karimunda registered an early and high induction of shoots initially which in subsequent cultures became weak and could not be carried over. The better survival and regeneration of Kalluvally compared to other cultivars may be due to its tolerance to CCF as evidenced by the electrolyte leakage studies. Vidhyasekaran (1990) also obtained a lower percentage of regeneration and survival when shaking in toxin incorporated liquid media was adopted for callus screening in rice against *Helminthosporium oryzae*.

Double layer culture technique

Kalluvally and Cheriakaniakkadan were better compared to other cultivars with respect to percentage of growing calli (Table IV). Double layer without the toxic metabolite registered higher percentage of growing calli for all the cultivars. Better shoot regeneration was obtained for Kalluvally followed by Karimunda and Cheriakanikkadan. Even though a better

shoot induction was observed for Karimunda in initial culture (20%) the shoots regenerated became weak in subsequent cultures. The better survival of Kalluvally after screening in the double layer with fungus may be due to its tolerance to the toxic metabolites of the fungus. Ahmed *et. al.* (1991) regenerated wheat plants showing more tolerance to *Fusarium* spp. than the original cultivars adopting the double layer culture technique for screening the calli.

The results of the present study suggested the possibility of *in vitro* screening as one of the approaches for detection of resistance/tolerance of pepper cultivars to *P. capsici*.

The cultures regenerated from the screened calli were successfully elongated, rooted, hardened and planted out as mentioned earlier. However, the regenerants planted out needs further testing with fungal cultures of *P. capsici* to know its level of tolerance/

Table IV. Growth, regeneration and survival of healthy cultures in double layer culture

Cultivar	Treatment	% of growing calli	% of necrotic calli	% of regeneration (1st sub culture)	Survival % of healthy cultures (5th sub culture)
Kalluvally	DLF	72	28	40	30
	CDL	90	10	0	0
Cheriakanikkadan	DLF	70	30	13.33	0
	CDL	100	0	0	0
Panniyur-1	DLF	5	95	0	0
	CDL	90	10	0	0
Balankotta	DLF	60	40	0	0
	CDL	70	30	0	0
Karimunda	DLF	55	45	20	0
	CDL	90	10	0	0

DLF - Double layer with fungus
CDL - Control double layer

resistance. Since the pathogen is producing a non specific toxin, further tests with fungal inoculation will give the correct picture of the level of tolerance/resistance. Moreover, the possibility of unstable resistance due to epigenetic changes cannot be ruled out in this context.

ACKNOWLEDGEMENTS

Authors acknowledge Dr. Y.R.Sarma, Principal Scientist, National Research Centre for Spices, Calicut and Dr. P. Vidyasekharan, Professor, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore for the help and guidance rendered during the course of this study.

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