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IN VITRO PRODUCTION OF TOXIC METABOLITE(S) BY *PHYTOPHTHORA CAPSICI* AND PARTIAL PURIFICATION OF THE METABOLITE(S)

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Abstract : *Phytophthora capsici*, the causal organism of *Phytophthora* foot rot disease in black pepper produces toxic metabolite(s) under *in vitro* conditions. Maximum accumulation of toxic metabolite(s) was observed in shake cultures of 15 days incubation in Ribeiro's medium. The symptoms induced by toxic metabolite(s) were quite typical to symptoms of natural and artificial infection by the pathogen. The toxic metabolite(s) accumulated in the *in vitro* culture was found to be heat stable and non-specific. The toxic metabolite(s) could not be separated using organic solvent fractionation since it is present in the aqueous fraction of the culture filtrate. However, ion exchangers like Dowex 1 and Dowex 50 could be used for separating the metabolite(s) from the aqueous fraction.

Key words: Culture filtrate, ion exchangers, *Phytophthora capsici*, *Phytophthora* foot rot, toxic metabolite(s).

INTRODUCTION

Phytophthora foot rot disease of black pepper caused by *Phytophthora capsici*, is the most dreadful disease in all pepper growing tracts of India. The soil borne fungus infects all parts of the vine but the collar and root infections are most fatal and the infected vine succumbs in 10-20 days. Since none of the cultivated types of black pepper is resistant to the disease, studies were conducted to exploit tissue culture induced variation (somaclonal variation) for screening for resistance / tolerance to *Phytophthora* foot rot. *In vitro* production of toxic metabolite(s) of *P. capsici* and purification of the metabolite(s) were attempted in order to use the metabolite(s) as screening agent for *in vitro* callus screening against *Phytophthora* foot rot.

MATERIALS AND METHODS

In vitro production of toxic metaholite(s) by *P. capsici*

Influence of incubation period and type of culture on the accumulation of toxic metabolite(s) by P. capsici

Liquid cultures of *P. capsici* were initiated in 50 ml of Ribeiro's medium (Ribeiro, 1978) by placing 10 mm culture discs of the seven day old cultures of the fungus grown in potato dextrose agar medium. The cultures were given three different incubation periods viz., 5 days, 10 days and 15 days and two different growing conditions viz., shaking and stationary. The culture filtrate was collected by subsequent filtration through two layers of muslin cloth and Whatman No.1 filter paper. Half of the filtrate collected from each treatment was reduced to 1/10th of its volume to get the concentrated culture filtrate (CCF). The accumulation of toxic metabolite(s) in different treatments was assessed using leaf puncture bioassay with detached leaves of the cultivar Karimunda and the development of necrotic spots was observed.

Effect of autoclaving of concentrated culture filtrate on symptom development

Concentrated culture filtrates collected from shake cultures of three incubation periods were autoclaved at 1.06 kg cm^{-2} (15 psi) pressure for 20 minutes. The autoclaved CCF was bioassayed using detached leaves of the cultivar Karimunda.

Effect of dilution of concentrated culture filtrate on symptom development

Concentrated culture filtrate collected from shake cultures of ten day incubation period

was diluted with sterile distilled water to 25, 50 and 75 per cent v/v. The diluted culture filtrate was tested for the development of symptoms on detached leaves of the cultivar Karimunda and the symptoms were compared with that induced by 100 per cent v/v CCF.

Host specificity of concentrated culture filtrate on symptom development

In order to test the host specificity of the collected CCF, bioassay was done on detached leaves of crops like tomato, cinnamon, clove, amaranth, chillies, brinjal, *P. colubrinum, P. nigrum* and *P. longum*. Observations on development and nature of symptoms were recorded.

Partial purification of the toxic metabolite(s) of *P. capsici*

In order to separate the toxic metabolite(s) from culture filtrates of *P. capsici*. partial purification of the culture filtrate was attempted.

Separation of the toxic metabolite(s) by organic solvent fractionation

Six organic solvents viz., methanol, acetone, dichloroethane, diethyl ether, chloroform and ethyl acetate were tried to separate the toxic metabolite(s) from the concentrated culture filtrate. The solvent and aqueous layers were separated using a separating funnel. Both solvent and aqueous fractions were bioassayed using leaf puncture bioassay in the cultivar Karimunda.

Separation of the toxic metabolite(s) from concentrated culture filtrate by ion exchange chromatography

Strongly basic anion exchange resin Dowex 1 having dry mesh size 20 to 50 and strongly acidic cation exchange resin Dowex 50 with a dry mesh size of 100-200 were used. Five gram each of anion and cation exchange resins was suspended in distilled water overnight. On the **following** day. Dowex 1 was repeatedly washed with 1 *N* sodium acetate to make it in the basic form and Dowex 50 with 1 *N* HC1 to make it in the acidic form. After adjusting the pH of Dowex 1 to 6.5 and Dowex 50 to 5.5 glass columns of size 24 x 1.5 cm were prepared. Dowex 1 was further adjusted to pH 6.5 using acetate buffer and Dowex 50 with tris HCl buffer of pH 7.0.

Two ml of CCF was applied to Dowex 1 and fractions of 2.0 ml were collected from the column using acetate buffer of pH 6.5 to separate the toxic metabolite(s) bound to the resin. Fractions having toxic metabolite(s) were pooled and applied to Dowex 50 for further purification and concentration. Two ml fractions were collected from the column using tris HCl buffer of pH 7.0 to separate the toxic metabolite(s) bound to the cation exchange resin.

RESULTS AND DISCUSSION

In vitro* production of toxic metabolite(s) by *P. capsici.

Influence of incubation period and type of culture on accumulation of toxic metabolite(s) by Phytophthora capsici

The effects of period of incubation and type of culture on the accumulation of toxic metabolite(s) in culture filtrates of *P. capsici* were assessed based on the capacity of the culture filtrate to form lesions on detached leaves of the cultivar Karimunda. The average diameter of lesions formed 60 h after inoculation of culture filtrate is presented in Table 1.

The collected culture filtrate when bioassayed without reducing the volume did not produce

Table 1. Influence of incubation period and type of culture on the accumulation of toxic metabolite(s) by *Phytophthora capsici*

Treatments		Concentrated or not	Development of symptoms	*Diameter of lesion 60 h after inoculation, cm
T_1	5 day incubation shaking	Concentrated	+	0.563
T_2	5 day incubation shaking	Not concentrated	-	-
T_3	5 day incubation stationary	Concentrated	-	-
T_4	5 day incubation stationary	Not concentrated	-	-
T_5	10 day incubation shaking	Concentrated	+	1.575
T_6	10 day incubation shaking	Not concentrated	-	-
T_7	10 day incubation stationary	Concentrated	-	-
T_8	10 day incubation stationary	Not concentrated	-	-
T_9	15 day incubation shaking	Concentrated	+	1.600
T_{10}	15 day incubation shaking	Not concentrated	-	-
T_{11}	15 day incubation stationary	Concentrated	-	-
T_{12}	15 day incubation stationary	Not concentrated	-	-
CD (0.05)				0.088
SEm ±				0.027

* Average of four replications; + indicates development of symptoms
(Culture filtrate was reduced to 1/10th of its volume. Leaf puncture bioassay was done in the cultivar Karimunda)

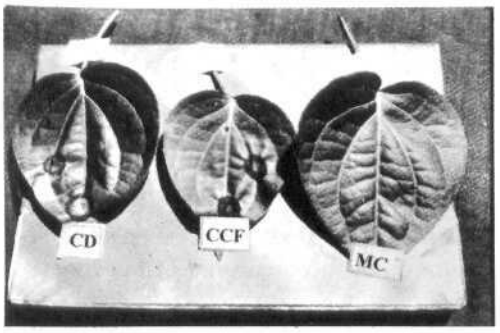

Plate 1. Comparison of symptoms induced by concentrated culture filtrate (CCF), culture disc (D) of *Phytophthora capsici* and medium control (MC)

symptoms in any of the treatments. But when the volume of the culture filtrate was reduced to 1/10th and bioassayed, symptoms were observed in shake cultures for the different incubation periods (Plate 1). The highest accumulation of toxic metabolite(s) was noticed in 15 days incubation which recorded a lesion diameter of 1.60 cm 60 h after inoculation. However, accumulation of toxic metabolite(s) in 10 day incubation and 15 day incubation was found to be on par. The concentrated culture filtrate from shake cultures of *P. capsici* was found to produce the same symptoms as natural and artificial infection on detached leaves of the cultivar Karimunda thereby fulfilling the criteria prescribed for a phytotoxin by Graniti (1972). *In vitro* production of toxic metabolite(s) by *P. capsici* was also reported from the studies conducted by Lee (1973), CPCRI (1979) and Vilasini (1982). The production of *in vitro* phytotoxin in shake cultures of *P. nicotianae* var *parasitica* was reported by Ballio *el al.* (1972). The advantage of giving agitation is that fermentation will proceed at a faster rate

Table 2. Effect of autoclaving (1.06 kg cm $^{-2}$ or 15 psi for 20 min) of concentrated culture filtrate on development of symptoms

Treatments		Autoclaved or not	*Diameter of lesion 60 h after incubation, cm
T_1	5 clay incubation shaking culture	Autoclaved	0.600
T_2	5 day incubation shaking culture	Not autociaved	0.563
T_3	10 day incubation shaking culture	Autoclaved	1.625
T_4	10 day incubation shaking culture	Not autoclaved	1.575
T_5	15 day incubation shaking culture	Autoclaved	1.637
T_6	15 day incubation shaking culture	Not autoclaved	1.600
CD (0.05)			0.0813
SEm			0.027

*Average of four replications

probably because shaking allows more rapid diffusion of oxygen through the culture medium and media constituents (Shaw, 1981).

Effect of autoclaving of concentrated culture filtrate on symptom development

The autoclaved CCF gave typical symptoms as that of CCF without autoclaving in all three incubation periods suggesting the thermostable nature of the toxic metabolite(s) (Table 2). Even though the autoclaved CCF exhibited a slightly better symptom compared to CCF without autoclaving, both the treatments were on par at all the three incubation periods. Heat stability of the toxic metabolite(s) produced by *P. megasperma* var. *sojae* was reported by Paxton (1972) and *P. cinnamomi, P. palmivora* and *P. megasperma* var. *sojae* by Keen (1975).

Plate 2. Comparison of symptoms induced by CCF and fractions from DW 1 and DW 50

Effect of dilution of concentrated culture filtrate on symptom development

Highly significant variation was observed in lesion diameter when inoculated with CCF at different dilutions (Table 3). The highest lesion diameter of 1.59 cm was exhibited by 100 per cent CCF followed by 75 per cent CCF v/v (1.03 cm), 50 per cent CCF v/v (0.80 cm) and 25 per cent CCF v/v (0.51 cm). So the activity of CCF was found to decrease at higher dilutions of CCF.

Host specificity of CCF on symptom development

Concentrated culture filtrate produced symptoms on all the crops to which it was inoculated showing that the toxic metabolite(s) present in CCF was found to be non-host specific in nature. Symptoms were observed as brown spots on leaves of black pepper, long pepper, *P. colubrinum*, chillies, clove and brinjal, light brown spots in cinnamon and amaranth and brownish black spots in tomato. Since CCF induced symptoms in all the crops to which it was inoculated the toxic metabolite(s) produced by *P. capsici* is non-specific. The non-specific mature of the toxin produced by *P. capsici* was also reported by Sarma *et al.* (1991).

Table 3. Effect of dilution of concentrated culture filtrate on symptom development

Treatments		*Diameter of lesions 60 h after incubation, cm
T_1	CCF at 25% v/v	0.513
T_2	CCF at 50% v/v	0.800
T_3	CCF at 75% v/v	1.025
T_4	CCF at 100% v/v	1.587
	CD (0.05)	0.0689
	SEm ±	0.022

*Average of four replications

Partial purification of the toxic metabolite(s) of *P. capsici*

Separation of the toxic metabolite(s) by organic solvent fractionation

When the aqueous and solvent fractions were separately bioassayed, symptoms were found to produce only in aqueous fractions showing that the toxic metabolite(s) could not be separated using organic solvents. The presence of toxic metabolite(s) in aqueous fraction of the filtrate of *P. megasperma* var. *sojae* causing *Phytophthora* root and stem rot of soybean was reported by Paxton (1972). The presence of phytotoxic metabolite(s) in aqueous fraction of other *Phytophthora* species viz., *P. cinnamomi, P. palmivora* and *P. megasperma* var. *sojae* was also reported by Keen *el al.* (1975).

Separation of the toxic metabolite(s) from concentrated culture filtrate by ion exchange chromatography

Out of the 13 fractions collected from Dowex 1, typical symptoms were observed in 7th, 8th and 9th fractions as evident by leaf puncture bioassay. Further purifications and concentration of these fractions could be effected in Dowex 50. Leaf puncture bioassay of the 18 fractions collected from Dowex 50 showed typical symptoms in 7th, 8th and 9th fractions. The intensity of lesion development in fractions collected from Dowex 50 was more than that from Dowex 1 which was due to the concentration effect in Dowex 50 column (Plate 2).

The study revealed that *P. capsici* could produce toxic metabolite(s) under *in vitro* conditions. The toxic metabolite(s) present in aqueous fractions of the culture filtrate could be separated using Dowex 1 and Dowex 50 ion exchangers. The CCF and the fraction collected from Dowex 1 and Dowex 50 showed the same symptoms which indicated the selective inhibition of the toxic metabolite(s) at the specific site and effectiveness of the inhibitor. The thermostable nature of the toxic metabolite(s), its presence in aqueous fraction and its specific activity whether purified or not give the assumption that toxic metabolite(s) present in culture filtrate of *P. capsici* may be mycolaminaran (water soluble fi 1-3 glucan) as reported in other *Phytophthora* spp. by Keen *et al.* (1975). However, much more effort is needed to characterise and quantify the toxic metabolite(s).

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