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RESEARCH ARTICLE

Determination of *Colletotrichum capsici* toxins by gas chromatography mass spectrometry analysis

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ABSTRACT

Totally 20 islatesof *C.capsici* isolates were collected from different chilli growing areas of Tamil nadu, among them three namely, Cc1, Cc2 an Cc8 isolates were found to be more virulent the toxins were purified from those isolates and anaysed by GC-MS analysis .The toxic sample from isolates of *C. capsici* exhibited a few new peak signals in the chromatogram .The sharp peak of chromatogram of the three isolates toxins were 2 tert-butyl-4- isopropyl-5-methyl phenol, 2,4,6 trimethyl phenyl glyoxylate, 4-dimethylaminomethyl-3- cyclohexane-1-carboxylate. The purified toxic metabolites of C. *capsici* was found to show 100 per cent inhibition on germination of seeds of chilli, greengram, blackgram, redgram and paddy at 5 per cent concentration. The shoot as well as root lengths were also completely inhibited at 5 per cent concentration when compared to others.

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INTRODUCTION

Chilli (*Capsicum annuum*) is the fourth most important vegetable crop in the world and first in Asia, with world production approximately 122.34 million tonnes of fresh chilli and 2.8 tonnes of dry chilli in 2010 (Indian Horticultural Database). The most important producers and exporters of chilli include China, India, Mexico, Morocco, Pakistan, Thailand and Turkey. Demand for chilli in the world is increasing every year. Chilli is a very remunerative spice crop of the Indian subcontinent (Sharma *et al.*, 2005) and occupies an area of about 0.81 million ha (Suthin Raj and Christopher, 2009) which accounts for 25% of the world production (Chandra Nayaka *et al.*, 2009). In Tamil Nadu, chilli is cultivated on 49.0 thousand hectares with 31.8 thousand tonnes of production. Chilli not only meets domestic consumption but also helps in earning foreign exchange. Unlike other chilli-producing countries, about 90 per cent of the production (estimated over 10 lakh tonnes of chilli) in India is absorbed by the huge domestic market.India exports only about 1.5 lakh tonnes of chilli out of the total production of 7.5 lakh tones.

Chilli is attacked by several fungal, bacterial and viral diseases among them, anthracnose and powdery mildew are found to be the major diseases incurring heavy losses, if not cared. Anthracnose (fruit rot and die back) caused by *Colletotrichum capsici* (Syd. Butler and Bisby) is prevalent throughout the chilli growing areas of India. (Jeyalakshmi, 1996).

Microbial toxins are metabolites produced by plant pathogens (fungi, bacteria), which play a role in host pathogen interactions and in disease expression. They are low molecular weight substances produced by some pathogens which are capable of reproducing symptoms similar to that found in natural infections in plants (Bilgrami and Dube, 1976) According to Scheffer (1983) phytotoxins are a product of microbial pathogens, which should cause an obvious damage to plant tissue and must be known with some confidence to

be involved in disease development. Isolation of phytotoxic metabolites from many species of *Colletotrichum* have been reported by many authors (Ballio *et al.*, 1969; Dasgupta, 1986; Goddard *et al.*, 1979; Goodman, 1960; Grove *et al.*, 1966; Nair and Ramakrishnan, 1973; Narain and Das, 1970; Ohra *et al.*, 1995; Wang, 1986; Yoshida *et al.*, 2000).Several workers have reported production, purification and characterization of toxin produced by many species of *Colletotrichum*. Goodman (1960) studied the chemical nature of toxin produced by *C. fuscum* and found that it had polysaccharide and peptide fractions and named it as colletotin, colletotrichin and colletopyrone from *C. nicotianae* (Gohbara *et al*., 1978). Production of phytotoxin by the turmeric leaf spot pathogen, *Colletotrichum capsici*, and its involvement in disease induction was reported by Nair and Ramakrishnan (1973). Several phytotoxins are now known, beyond reasonable doubt, to be the determinant factor in pathogenesis and some can even act as reliable surrogates for pathogen that produce them. Amusa (1994) reported that the partially purified metabolites of *Colletotrichum* spp. induced necrotic lesion of varying sizes on leaves and stems of susceptible hosts. While the phytotoxic metabolites of *Colletotrichum graminicola*,*C. truncatum* and *C. lindemutianum* inhibited seed germination in respective host crops (Amusa, 1994). Results of seedling bioassay revealed that sorghum, millet, maize, cowpea and soybean seedlings treated with 100µg/ml of the toxic metabolites of the respective pathogens showed symptoms of blight and cessation of growth and the potency of the metabolites also decreased with increased dilution. Phytotoxic metabolites have been employed in screening crops for disease resistance (Wheeler and Luke, 1955; Hartman *et al*., 1986; Amusa, *et al*., 1994, Amusa, 1998). Considering the importance of phytotoxic metabolites in crop protection management's practices, this paper gives the use of phytotoxic metabolites of pathogen, *C.capsici* in plant disease management.

MATERIAL AND METHODS

Toxin production by *C. capsici in vitro* :

The toxin was extracted using the method described by Nair and Ramakrishnan (1973). Three isolates of Cc1,Cc8 and Cc10 which were found during the survey were used for the toxin study. Eight mm mycelial discs of fresh cultures of *C.capsici* were aseptically transferred to 250 ml Erylenmeyer flasks containing 100 ml of Czapek's Dox broth in which sucrose was substituted with host extract (as a carbon source), so that 100 ml of the medium would contain extract from 30 g of matured leaf tissues prepared by homogenization and filtration. After 15 days of growth in a rotary shaker, the cultures were pooled, homogenized in a blender, filtered through Whatman No.1 filter paper and the filtrate was used as the source of pathogen toxin.

Fractionation of toxin :

The phytotoxic component of the culture filtrate was extracted by the method described by Nair and Ramakrishnan (1973). The filtrate was reduced to 1/10 volume in a rotary vacuum flash evaporator, mixed with equal volume of methanol, kept overnight and filtered through Whatman No.1 filter paper. The methanol was removed by vacuum evaporation at 40°C and the pH of the aqueous phase was adjusted to 3.5 using HCl. The aqueous phase was mixed with equal volume of diethyl ether and shaken for few minutes. The ether phase was separated and the aqueous phase was again mixed with diethyl ether and shaken for few minutes and the ether phase was collected. This process was repeated three times and the collected ether phase was brought to original volume by vacuum evaporation. To this ether phase, equal volume of Na_2CO_3 (5%)was mixed, shaken well and the ether phase was separated and evaporated to dryness. The air-dried powder form of extract served as crude toxin.

Purification of the toxin :

The crude toxin was eluted using different solvents (polar to non polar) through a packed columns as per the methods of Jayakumar (2004)

Si column :

Silica (Si) natural phase column (Bond eluteâ 3 cc/ 500 mg) was washed and then eluted with 15 ml of chloroform. Weighed quantity of crude toxin was mixed with methanol and chloroform mixture and transferred to the column and eluted with 6 ml of various combinations of methanol and chloroform mixture. The eluted compounds by each fraction were collected separately. The collected samples were subjected to centrifugal evaporation at 40°C to evaporate the solvents and the dried samples were collected.

C18 column :

The reverse phase column (C18) (Bond eluteâ 3 cc/ 500 mg) was washed with 15 ml methanol and then with equal volume of water for 3 times The weighed quantity of crude toxin was applied to the column and eluted with 6 ml of different solvents *viz*., methyl alcohol, chloroform, acetone, ethylacetate. Each eluted sample was collected separately and concentrated by centrifugal evaporation. For large scale elution, the column was packed with Analytical Bondesil-preparative grade 40µm (Varian C18) part # 1221-3012 in the column tube with the dimensions of 2.2 cm diameter and 21cm height with the column height of 4 cm. The samples were eluted as described above and the contents were filtered through cellulose acetate (0.45µl pore size) using DISMIC-3CP (Advantec®) disposable syringe filter unit to remove the inert matter. The collected samples were used as semi- purified toxin for further experiments.

Plant toxicity assay :

Effect of purified toxin on seed germination **:**

The toxic effect of the purified toxin on seed germination was determined by testing seeds of various crops such as greengram, blackgram, redgram, paddy and chilli. The seeds were soaked for 12 hrs in the purified solution of 0.1, 0.5, 1.0, 2.0, 5.0 % w/W, plated on filter paper (20 seeds/plate) and kept for germination and seeds soaked in distilled sterile water served as control and the experiment was conducted with three replicates. The seeds were observed daily upto7 days for germination and statistical analysis of the data were conducted according to the method described by Snedecor and Cochran (1967).

*Effect of purified toxin on the growth parameters of seedlings***:**

Thirty seeds of greengram, blackgram, redgram, paddy and chilli were soaked in 10 ml each of 0.1, 0.5,1,2.5, 5% concentration of purified toxin for 1 h. Later they were spread on moistened blotting paper and each treatment was replicated thrice containing thirty seeds. Equal number of healthy seeds were soaked in sterile distilled water, which served as control. Observations on the shoot and root length were recorded as per the method given by Vincent (1947).

Gas chromatography mass spectrometry (GC/MS) studies for eluted toxin (Jayakumar, 2004) :

Preparation of samples **:**

The samples were derivatised as follows before subjecting to GC/MS. The samples obtained after purification were dried by vacuum centrifugal evaporation at 40°C. After complete drying, 0.05 ml of pyridine, 0.10 ml of hexamethyldisilazane, and 0.05 ml of chlorotrimethylsilane were added to the tube and incubated for 20 min at 80°C. The reactant (TMS derivative) was dried with N_2 gas and extracted with 0.3ml of hexane.

Analysis :

The TMS derivative of the methanolysis product was analysed with GC/MS (QP- 5050, Shimadzu Corp., Kyoto) equipped with a

Column type	:	TR 5 - MS capillary standard non - polar column
Column length	:	30 m
Carrier gas	:	Helium
Flow rate	:	1 ml per min.
Oven temperature	:	Initial 80 ^{0}C Final 250 ^{0}C
Rate of temperature change	:	8 ^{0}C per 10 min.
Injection temperature	:	80 ^{0}C
Mass range	:	50 to 650 amu (atomic mass unit)

The total ion chromatogram was obtained for each sample. The base peak of each spectrum was compared with the base peak of the chemical components in the NIST library through on-line. The chemical structure of the homologous compounds was compared with the chromatogram of samples.

RESULTS AND DISCUSSION

The results in the present investigation are summarized here with and discussed for their significance :

Toxin production by *C.capsici* :

Studies on total ion chromatogram of toxins byGas chromatography Mass spectrometry **:**

The results from the present study indicated the toxic fractions varied according to the nature of the isolates (Fig. 1 – 3). The isolate Cc1 was found to produce the toxic fraction C14C22O (2 tert-butyl-4- isopropyl-5-methyl) (Table 1). The toxic fraction C12 H 21 N0 2 (2,4,6trimethyl phenyl glyoxylate) was produced by Cc8 (Fig. 3) where as the toxic fraction C12 H14 N03 (4-dimethylaminomethyl-3-cyclohexane-1-carboxylate) was seen in Cc10 (Fig. 2). These fractions may act on host to produce symptom. The presence of several fractions of toxin in still cultures of pathogen has already been reported.

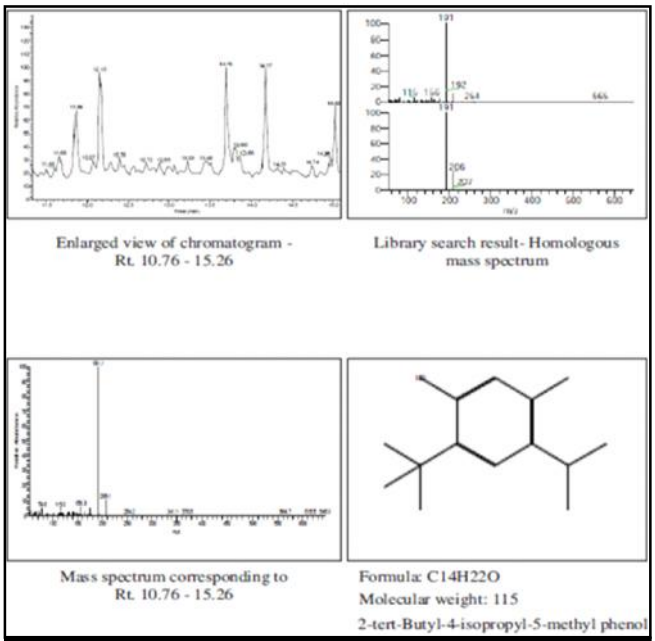

Fig. 1 : GC-Ms analysis of purified toxin of *C.capsici* (Cc1)

Since Hutchinson's first toxigenic hypothesis of fungal plant diseases proposed in 1913, there have been many reports of phytotoxins isolated from culture filtrates of pathogens. It has been established that *Helminthosporiumsacchari* symptom production on sugarcane was associated with toxin

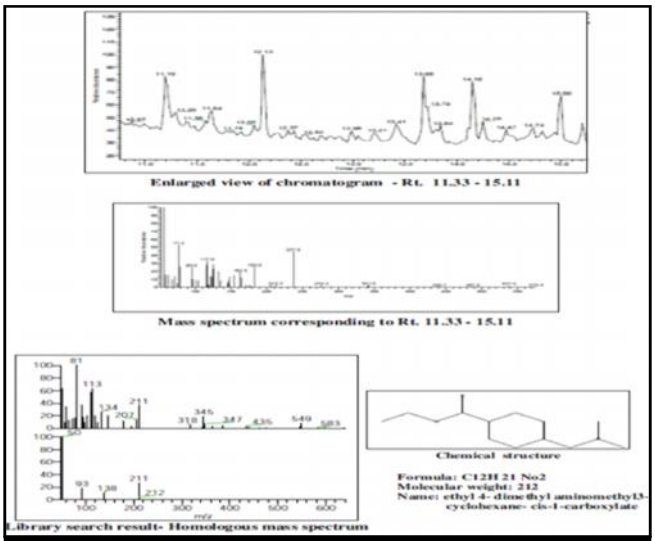

Fig. 2 : GC-Ms analysis of purified toxin of *C.capsici* (Cc10)

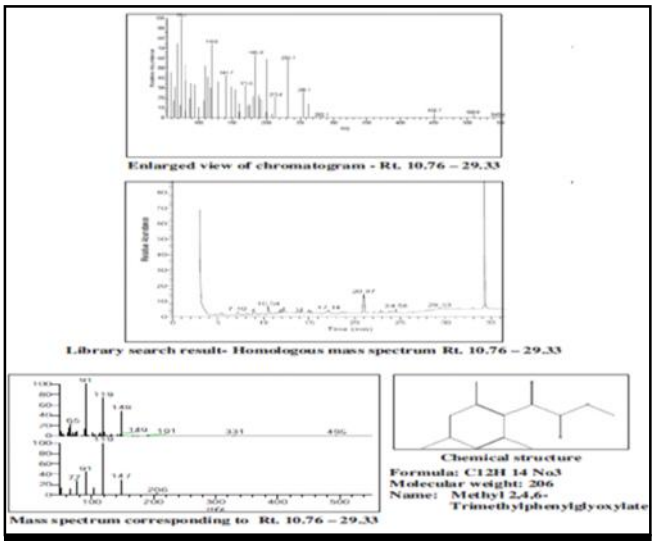

Fig. 3 : GC-Ms analysis of purified toxin of *C.capsici* (Cc8)

Table 1 : Effect of purified toxin of *C.capsici* on seed germination

Crop	Toxin (per cent)	Per cent seed germination	Per cent inhibition over control *
Greengram	0.1	25	70.93 (57.53)d
	0.5	19	77.90 (61.96)c
	1	16	80.99 (50.94)b
	2.5	12	85.74 (67.78)a
	5	–	–
Control		84.20	
Blackgram	0.1	28	62.16 (52.00)d
	0.5	24	67.56 (55.24)c
	1	20	72.97 (58.63)b
	2.5	13	82.43 (65.20)a
	5	–	–
Control		74.00	
Redgram	0.1	26	67.50 (55.24)d
	0.5	25	68.75 (55.98)c
	1	22	72.5 (58.37)b
	2.5	19	76.25 (60.80)a
	5	–	–
Control		80.00	
Paddy	0.1	25	67.16 (55.0)d
	0.5	22	71.10 (57.48)c
	1	20	73.73 (62.51)b
	2.5	18	76.35 (60.87)a
	5	–	–
Control		76.14	
Chilli	0.1	19	76.25 (60.8)d
	0.5	14	82.50 (65.27)c
	1	16	80.00 (63.43)b
	2.5	10	87.50 (69.30)a
	5	–	–
Control		80.00	

*Values are the mean of four replications.
The values in parentheses are arcsine transformation.
Means followed by a common letter are not significantly different at the 5 % level by DMRT.

production (Steiner and Byther, 1971). Isolation of phytotoxic metabolites from many species of *Colletotrichum* have been reported by many authors (Ballio *et al.,* 1969; Dasgupta, 1986; Gohbara *et al.,* 1978; Goddard *et al.,* 1979; Goodman, 1960; Grove *et al.,* 1966; Nair and Ramakrishnan, 1973; Narain and Das, 1970; Ohra *et al.,* 1995; Wang, 1986; Yoshida *et al.,* 2000).

The first attempt on characterization of toxin produced by *C. falcatum* was made by Olufolaji and Bamgboye (1986). They found that the toxin was soluble in water, ethanol, methanol and acetone but very little in chloroform. According to these authors, the thermostable toxin secreted by *C. falcatum* produced peaks similar to that observed for some anthroquinones such as emodin and chrysophanol. The spectrophotometric studies of toxin extracted from internodal tissues produced similar peaks as that of semipurified toxin isolated from fungal culture filtrate (Ramesh Sundar *et al.,* 1999).

Several workers have reported production, purification and characterization of toxin produced by many species of *Colletotrichum*. Goodman (1960) studied the chemical nature of toxin produced by *C. fuscum* and found that it had polysaccharide and peptide fractions and named it as colletotin. The chromatographic and other chemical studies revealed the presence of acetylcolletotrichin, a phytotoxic terpenoid ($C_{28}H_{42}O_7$) and biologically inactivealcohol ($C_{14}H_{20}O_6$) in the metabolic products of *C. capsici* (Grove *et al*., 1966). The molecular formulae of three toxic substances from *C. nicotianae* were determined as $C_{28}H_{42}O_7$, $C_{29}H_{12}O_8$ and $C_{29}H_{42}O_8$ (colletotrichin, colletotrichins B and C, respectively)

Table 2 : Effect of purified toxin on the growth parameters of seedlings

Crop	Toxin (per cent)	Root length (cm)	Per cent inhibition over control *	Shoot length (cm)	Per cent inhibition over control*
Greengram	0.1	6.42	8.28 (16.64)^{d}	8.00	20.00 (26.57)^{d}
	0.5	5.20	25.71 (30.46)^{c}	7.60	24.00 (29.33)^{c}
	1	4.20	40.0 (39.23)^{b}	5.30	47.00 (43.28)^{b}
	2.5	3.80	45.71 (42.53)^{a}	4.00	60.00 (50.77)^{a}
	5	–	–	–	
Control		6.00		10.00	
Blackgram	0.1	6.00	14.28 (22.14)^{d}	9.6	4.00 (11.54)^{d}
	0.5	4.00	42.85 (40.86)^{c}	8.40	16.00 (23.58)^{c}
	1	3.80	45.71 (42.53)^{b}	6.40	36.00 (36.87)^{b}
	2.5	3.40	51.42 (45.8)^{a}	5.60	44.00 (41.55)^{a}
	5	–	–	–	
Control		7.00		10.00	
Redgram	0.1	3.60	10.00 (18.43)^{d}	6.00	11.76 (20.00)^{d}
	0.5	3.40	15.00 (22.79)^{c}	5.90	13.23 (21.30)^{c}
	1	3.33	16.75 (23.54)^{b}	4.30	36.76 (37.29)^{b}
	2.5	3.20	20.00 (26.57)^{a}	4.00	41.17 (39.87)^{a}
	5	–	–	–	
Control		4.00		6.80	
Paddy	0.1	3.22	19.50 (26.21)^{d}	6.4	5.71 (13.81)^{d}
	0.5	3.20	20.00 (26.57)^{c}	5.22	25.42 (30.26)^{c}
	1	2.80	30.00 (33.21)^{b}	4.00	42.85 (40.86)^{b}
	2.5	2.20	45.00 (42.13)^{a}	3.40	51.42 (45.80)^{a}
	5	–	–	–	
		4.00		7.00	
Control					
Chilli	0.1	5.6	6.60 (14.89)^{d}	8.00	20.00 (26.57)^{d}
	0.5	4.28	28.60 (32.33)^{c}	7.60	24.00 (29.33)^{c}
	1	3.20	46.60 (43.05)^{b}	5.30	47.00 (43.28)^{b}
	2.5	2.20	63.30 (52.71)^{a}	4.00	60.00 (50.77)^{a}
	5	–	–	–	
Control		6.00		8.00	

*Values are the mean of four replications.

The values in parentheses are arcsine transformation.

Means followed by a common letter are not significantly different at the 5 % level by DMRT.

by thin layer chromatography, mass spectrometry and X- ray crystallographic analysis (Gohbara *et al.,* 1978). The results of the present study are in accordance with the above reports.

Grove *et al.* (1966) characterized the toxic fraction of *C. capsici* as $C_{28}H_{42}O_7$ and $C_{14}H_{20}O_6$. Three phytotoxic substances of formula $C_{28}H_{42}O_7$ and $C_{14}H_{20}O_9$ and $C_{29}H_{42}O_8$ were identified in the metabolites of *C. nicotianae* (Gohbara *et al.,* 1978). Contrary to the findings of the present study Olufolaji and Bamgboye (1986) reported the chemical nature of toxin of *C. falcatum* as anthroquinonesie, emodin and chrysophanol. They also reported that the toxic fraction was highly soluble in water. Though the chemical structure of the toxin fractions isolated in the present study did not confirm with the findings of Olufolaji and Bamgboye (1986), the solubility pattern is in agreement with the earlier findings (Mohanraj *et al*., 1995).

Ballio *et al*. (1969) reported the production of lycomarasmic acid (aspergillomarasmin B) by *C. gloeosporioides*. The toxin produced by *C. lagenarium* was characterized as 2- pyruvoylaminobenzamide(Kimura *et al*., 1973). High-pressure liquid chromatography studies of toxin produced by *C. dematium* showed the presence of four toxic fractions in the extract obtained from anthracnose lesions (Yoshida *et al.,* 2000). As a consequence, the development of an effective phytotoxin for use in plant disease control will require a comprehensive understanding of the pathogen(s) involved including its virulence and the biology of the target host plant.

Effect of purified toxins on seed germination and seedling growth parameters :

Effect of purified toxins on seed germination :

The purified toxic metabolites of *C.capsici* was found to inhibit the germination in the seeds of chilli, greengram, blackgram, redgram and paddy to the extent of 100 per cent at 5 per cent concentration. In addition, they also exhibited greater inhibitions on the root and shoot lengths of the seeds. (Table 1 and 2). These findings are in agreement with Islam and Maric (1980), Robeson and Strobel (1984) and Lu *et al.* (1987) who also demonstrated inhibition of shoot elongation and root elongation in sesame. The toxigenic potential of culture filtrate of *Alternaria macrospora* on cotton was studied by Ramegowda and Naik (2008). A preliminary indication of toxin production by any fungus *in vitro* is usually provided by a number of bioassay methods *viz.*, bioassay methods of plant cultures, seed germination bioassay and root and shoot elongation bioassay (Anahosur, 1976). The purified toxin obtained from isolate of *Alternaria sesami*, was subjected to above bioassay methods to know the toxigenic potentiality of *A. sesami*. Bhaskaran and Kandaswamy (1978) also reported toxicity of culture filtrate of *A. helianthi* on sunflower. Mahabaleshwarappa (1981) also made similar observation while working with *A. carthami* on safflower.

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