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Toxin Produced by *Colletotrichum falcatum* Causing Red Rot of Sugarcane

R. Saikia[1]*, P. Azad[2] and D. K. Arora[1]

[1]*Laboratory of Applied Mycology, Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi 221005, India*
[2]*Division of Life Sciences, Institute of Advanced Study in Science & Technology, Khanapara, Guwahati 781022, India*
[1]*Present address: National Bureau of Agriculturally Important Microorganisms (NBAIM), Kusmau, P. O. Kaithuli, Mau, Mau Nath Bhanjan, U.P.- 275101, India*

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Toxin produced by *Colletotrichum falcatum* Went, the incitant of red rot of sugarcane was isolated, purified and assayed to determine host specificity and identify its chemical nature. The toxin was found to be not host specific as it inhibited germination of various seeds (gram, greengram, blackgram, pea, cowpea, rice and sugarcane) as well as different seedlings viz. tomato, coriander, pea and rice. The toxin consists of two distinct fraction - one fraction having R_f value at 0.36 producing identical red rot lesion when inoculated at leaf midrib of sugarcane, and the other having R_f value at 0.72 not showing any red rot lesion. Chromatogram of high performance liquid chromatography (HPLC) of the red rot lesion causing fraction showed a sharp peak at 1.62 min of retention time (RT), and spectral analysis indicated the presence of following chemical groups- C-H, C=O, C-N, $-CH_3$, $-CH_2$, $-CH(CH_3)$ and molecular mass of the compound was 203. (M^+, $C_{11}H_{11}N_2O_2$)

KEY WORDS: ***Colletotrichum falcatum*, Red rot of sugarcane, Toxin**

Red rot of sugarcane caused by *Colletotrichum falcatum* Went. is a seed transmissible, old disease with worldwide distribution where sugarcane is being cultivated. It is responsible for 12.0 to 41.5% of yield loss with normal ranges (Sandhu *et al.*, 1969) and the loss of sugar content in terms of total carbohydrate was as high as 12.0~60.0% (Saikia and Azad, 2002) but in epidemic spreads it reaches up to 100%. This dreadful disease is mainly due to lack of resistant varieties of sugarcane and still a major threat in subtropical areas. In India the disease attacks standing canes, often in epidemic form, and causes huge losses to the cultivators and the millers (Chona, 1980). The disease is commonly known as "Cancer of Sugarcane" (Agnihotri, 1990). Localized epidemics occur almost every year. The primary infection of the disease is through infected setts, and the secondary infection is from soil, water, wind and sporulating masses at the nodes affecting the adjacent standing cane (Muthukumarsamy *et al.*, 2000). Chemical control of the disease is a mere failure, and a biomolecular mediated control and induced resistance is the only way left.

Toxins produced by pathogenic organisms became a major subject of interest to study the biochemical and molecular mechanisms involved in disease development (Yoder, 1981). These studies thus help biological control of the disease and induced resistance. The search for toxins in the host-pathogen systems is stimulated not only for better understanding the pathogenic process but also for potential use in identifying resistant cells or protoplasts for tissue cultures or screening and breeding of disease resistance (Lamari and Bernier, 1989). Toxins are generally products of the pathogen, host, or host-pathogens interaction, directly act on living host protoplasm to influence the course of disease development or symptom expression even at very low concentrations (Orolaza *et al.*, 1995; Yoder, 1980). Most microbial toxins are not host-specific to their hosts (Lamari and Bernier, 1989; Mitchell, 1984) but several phytopathogenic fungi produce host-specific toxins (Effertz *et al.*, 2002; Strelkov *et al.*, 1999). However, several nonspecific toxin-producing pathogens also produce host-specific toxin (Vidhyasekaran *et al.*, 1986).

Thus the study of toxin in respect of red rot development becomes indispensable. *In vitro* production of toxic metabolites by this fungus was demonstrated by Chandrika *et al.* (1984) and Viswanathan *et al.* (2000). There are also reports on isolation and partial purification of toxic metabolites from *Colletotrichum* species (Higgins and Ingham, 1981; Hong *et al.*, 2000). New bioactive metabolites produced by *Colletotrichum* spp. and their structures were elucidated by Hong *et al.* (2000). Olufolaji (2000) isolated and partially purified toxic metabolites from *C. falcatum* and reported that movement of the toxin was towards apical region of the plant parts. The possible roles of toxin produced by *C. falcatum* in pathogenesis and in induction of electrolytes leakage which is a measure of host susceptibility in sugarcane leaves, intern-

*Corresponding author <E-mail: ratulsaikia@yahoo.com>

ode and callus, were also investigated (Sundar *et al.*, 1999). However, very little is known about the contribution of toxin in disease development, seed germination, tolerance, chemical compositions etc. So this work aims to isolate, purify and assay to identify the active chemical ingredient(s) produced by *C. falcatum* and also to determine to their host specificity.

Materials and Methods

Fungal strain. A highly virulent strain, *C. falcatum* RsK1 (*Cf*RsK1) was obtained from the Culture Bank of Life Science Division, Institute of Advanced Study in Science & Technology (IASST), Guwahati-781022, India.

Extraction of toxin. Toxin from the strain *Cf*RsK1 was isolated following the method as described by Naik and Vedamurthy (1997). The fungus was grown in host extract medium containing 250 g of sugarcane extract, 2.0 g $NaNO_3$, 1.0 g K_2HPO_4, 0.5 g $MgSO_4$, $7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4$, $7H_2O$ and 1000 *ml* distilled water, incubated 30±1°C for 15 days. Then the culture broth was filtered, and the mycelial biomass was homogenized with five volumes (w/v) of sterile distilled water. The homogenates were centrifuged at 3500 g for 30 min. The supernatant was reduced to one tenth of the original volume by evaporating at 100°C on a water bath. An equal volume of methanol was added to the reduced volume with constant stirring, and the mixture was kept overnight at 4°C. Methanol was removed by distillation, and the filtrate was taken into a separating funnel at pH adjusted to 3.5. An equal volume of diethyl ether was added and shaken well. The ether phase was separated and mixed well with equal volume of 5% aqueous solution of sodium carbonate, separated again and the aqueous phase was discarded. The ether solution was evaporated at 40°C on the water bath and stored in desiccators at 4°C for further assays.

Effect on seed germination. The crude extract on seed germination was determined by testing seeds of various crops such as gram, greengram, blackgram, pea, cowpea, rice and sugarcane. Seeds were soaked for 12 h in the crude solution of 0.1, 0.5, 1.0, 2.0 and 5.0% (w/w), plated on filter paper (20 seeds/plate) and kept for germination and seed soaked in distilled sterile water served as the control and the experiment conducted with three replicates. The seeds were observed daily for 7 days for germination and statistical analysis of the data were conducted according to the method described by Snedecor and Cochran (1967).

Effect on seedlings. Bioassay was performed with seedlings of tomato, coriander, pea and rice. Twenty days-old seedlings for each crop were put into test tubes, so that the seedling roots were dipped into the crude extract solution were observed for development of wilting symptom. Bioassay of this crude extract was also carried out on midrib of detached leaf of sugarcane (30 days old). The crude extract solutions of 100 *µl* was applied minutely after injuring surface of midrib with sterilized syringe. Crude extract extracted from sterile host extract medium and sterile distilled water served as control. The leaves were observed under microscope at 1, 3, 6, 12, 24, 36, 48 and 72 h interval.

For toxicity test, 20 days old tomato seedling was treated in test tubes containing different concentrations (0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0%) of crude extract solutions. Wilting symptom was observed every 30 min.

Purification and characterization of the crude extract. Five *ml* of crude extract was put in a 32×1.4 cm column of silica gel (80~120 mesh) and eluted by petroleum ether, petroleum ether+chloroform (5 : 4), ethyl acetate and methanol. Elutes were collected and evaporated in a vacuum. Then spotted on silica gel thin layer chromatography (TLC) plates using petroleum ether+chloroform (5 : 4) and ethyl acetate as solvent. Spots were developed by iodine vapour and observed under UV light (254 nm). Two distinct fractions were collected by TLC (R_f value 0.36 and 0.71). The fraction having R_f value at 0.36 showed red rot disease lesion when inoculated on leaf midrib of sugarcane, while the other fraction (R_f value 0.71) did not produce any symptom. This disease-causing fraction was further purified by HPLC. The HPLC system used was Waters (Millipore) 510 pump, 680 gradient controller with a 746 data module. The separation was performed in reverse phase by injecting 10 *µl* of the sample in a Nucleosil C18 column, using an isocratic system of water-acetonitrile (60 : 40). The detector's wavelength was fixed at 250 nm.

Analytical procedure. The compound was partially characterized through ultraviolet visible (UV/Vis) spectro-

Table 1. Effect of the crude extract on seed germination

Seeds	Inhibition of germination (%)					
	Control	0.1%	0.5%	1.0%	2.0%	5.0%
Gram	0	0	3±0.2	23±3.0	67±4.2	100
Greengram	0	0	2±0.3	15±2.5	50±5.0	100
Blackgram	0	0	5±0.2	25±3.5	63±3.0	100
Pea	0	0	4±0.5	24±2.0	60±5.2	100
Cowpea	0	0	3±0.2	33±2.5	78±4.3	100
Rice	0	0	3±0.2	33±2.5	78±4.3	100
Sugarcane	0	7±1.3	67±4.3	100	100	100

Critical difference=16.85 for crude extract concentration; 15.38 for seed germination; 6.88 for crude extract concentration×seed germination; critical difference computed at P=0.05; ±=standard deviation.

photometer (Shimadzu-1601) with $CHCl_3$ as the reference, Infrared (IR) spectroscopy (Perkin Elmer 237B) with $CHCl_3$ as solvent, Varian 60 MHZ Nuclear Magnetic Resonance (NMR) spectroscopy ($CDCl_3$; with Tetra methyl silane (TMS) as the internal reference) and gas chromatography-mass spectroscopy (GC-MS). Five μl of the sample was injected through gas chromatography (GC) column (Chemito-8500, capillary column, 25 mm× 0.1 mm dia). At the time of injection, the column was rapidly heated to 200°C and the temperature programmed to 270°C. Mass spectroscopy (MS) was operated in the chemical ionization mode with isobutane as the reagent gas.

Results and Discussion

The crude extract isolated from *C. falcatum* was not host specific because it inhibited the seed germination and growth of seedlings of the different non-host plant (Table 1). More than 50% of the seed did not germinate at 2% concentration except sugarcane sett which did not germinate at 1.0% concentration. Crude extract concentration at 0.1% could inhibit the germination of sugarcane sett, and more than 50% inhibition was recorded at 0.5% concentration. The crude extract completely inhibited the germination of all the seeds at 5.0% concentration. The crude extract also induced wilting symptom in the seedlings like tomato, coriander, pea and rice. Among them, tomato seedlings were highly susceptible and required 18 min for wilting (Fig. 1). It appeared that accumulation of toxin in the host caused the severe symptom. This also indicated that the toxin is playing a main role in the disease development. For sugarcane plants, injected with 0.2% of crude extract into leaf midrib developed a typical symptom of red rot within 48 hr. The injected area turned water soaked, later developed reddish lesion and affected tissues were soften at 48 hr. The tissue treated with the crude extract was localized towards the cell protoplasm, but other remains were intact. However, the control plant treated with sterile liquid medium or distilled sterile water showed no such lesion except for the injection scar clearly indicating that the toxin is capable of causing red rot lesion. Toxicity test exhibited that this crude extract was active even up to 0.2% concentration, but required more time (415 min) to cause wilting (Fig. 2). At higher concentrations, the crude extract required less time to cause wilting and toxicity was proportional to the concentration.

In column chromatography, crude extract yielded two distinct fractions; a fraction eluted by petroleum ether+ chloroform (5 : 4) having R_f value at 0.36 showing red rot disease lesion in sugarcane, while the other fraction eluted by ethyl acetate, R_f value at 0.71 not showing any red rot

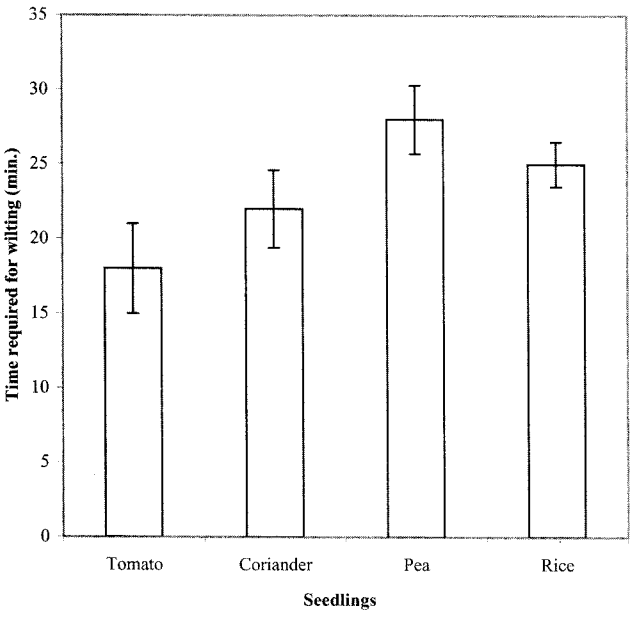

Fig. 1. Wilting sympotom of different crop seedlings with crude extract treatment.

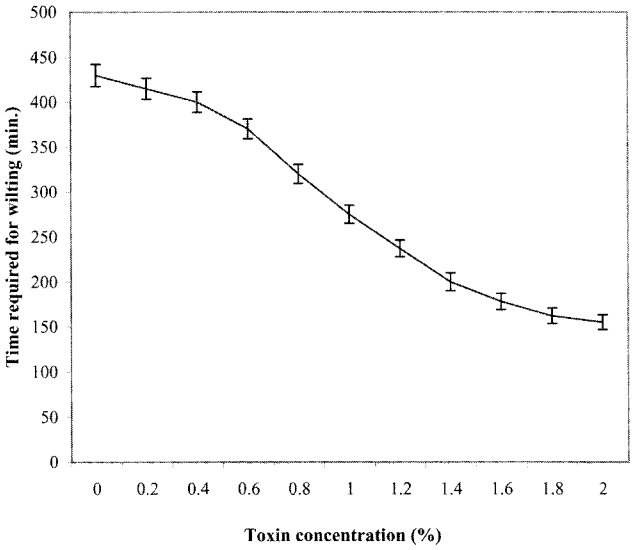

Fig. 2. Time required for wilting of tomato seedlings with crude extract solution.

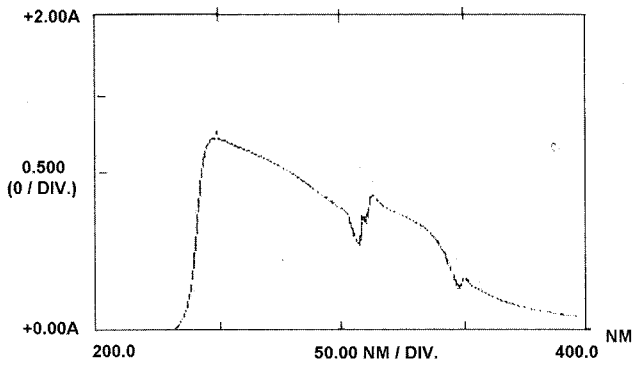

Fig. 3. UV/Vis spectrum of a toxin isolated from *Colletotrichum falcatum*.

disease symptom. Chromatogram of HPLC exhibited a sharp peak at the retention time 1.62 min (data not shown) and this was the disease causing fraction. When this fraction was collected and further inoculated, it produced red rot lesion.

UV/Vis spectrum (Fig. 3) showed a broad peak at 249.6 nm indicating the presence of $\overset{|}{C}$=O group. The IR spectrum (Fig. 4) showed an absorption band at 2900 cm^{-1} for C-H stretching, 2875 cm^{-1} for C-H stretching bending, and 1750 cm^{-1} for presence of $\overset{|}{C}$=O group, may be - $\overset{O}{\overset{||}{C}}$ - OR group. The absorption band also at 1700 cm^{-1} is an indication of $\overset{|}{C}$=O group, 1500 cm^{-1} for C-N stretching, 1450 cm^{-1} and 1300 cm^{-1} for C-H stretching deformation. The ^{1}H-NMR spectrum (Fig. 5) exhibited a peak at 3.85 ppm

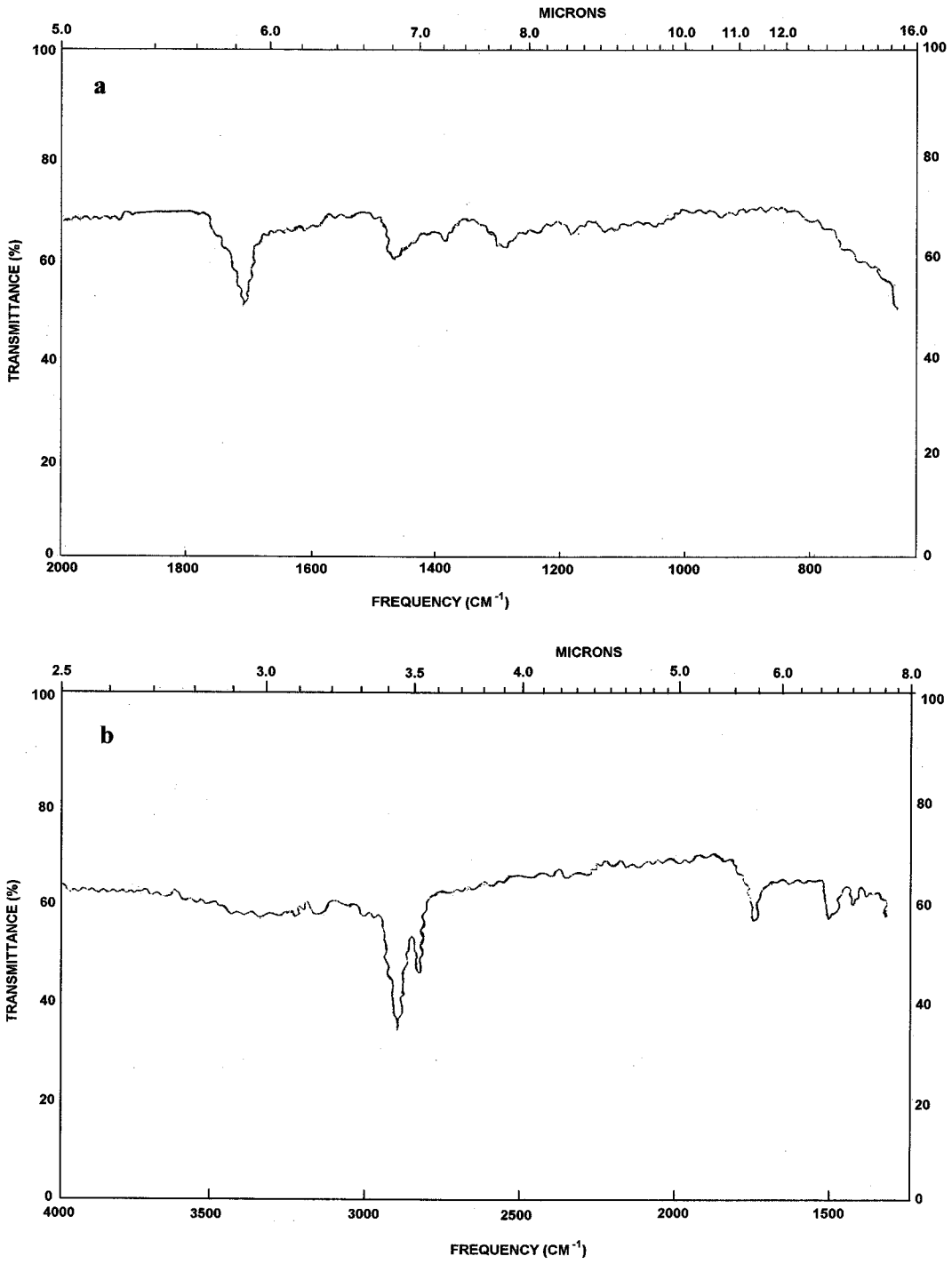

Fig. 4. IR spectrum of a toxin isolated from *Colletotrichum falcatum.*

CH_3
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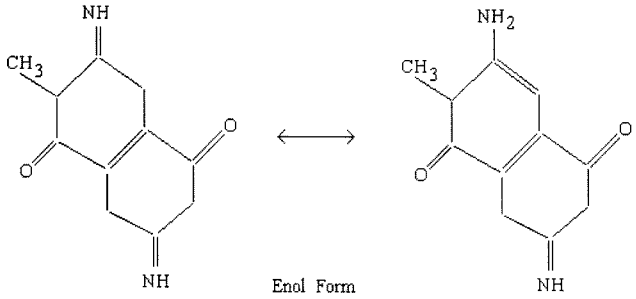
for 1H, -CH, 1.85 ppm for S, 3H, $-CH_3$, 1.25 - 1.6 ppm for S + M, 5H, CH_3, $-CH_2$, 1.0 ppm for S, 3H, $-CH_3$. GC-MS indicated that molecular mass of this compound was 203 (M^+; data not shown). From these data the molecular formula and structure could be evaluated as below-



($C_{11}H_{11}N_2O_2$)

A molecule can be considered as a toxin if the molecule produced by the pathogen causes damage to the host (Scheffer, 1983). The toxic compound isolated from *C. falcatum* RsK1 met these conditions - this compound produced a red rot lesion in sugarcane similar with the symptom produced by the pathogen. Crude extrct as well as purified toxin also produced a similar red rot symptom on the same susceptible genotype.

Toxin isolated from *C. falcatum* was not found host specific as it inhibited seed germination of different crops (gram, greengram, blackgram, pea, cowpea and rice). The toxin isolated from *C. falcatum* and this toxin producing red rot lesion in sugarcane has confirmed the pathogenicity on sugarcane by the fungus due to the influence of phytotoxin. In respect of host specificity, inhibition of seed germination of some specific crops including sugarcane sett was also reported by Chandrika *et al.* (1984). They also observed that the toxic activity was high up to 1 : 5 dilutions and it gradually decreased thereafter with increased dilution. Naik and Vedamurthy (1997, 1998) reported that the toxin levels beyond 0.1% could delay in callus proliferation, and growth of the callus was completely inhibited with 0.5% toxin concentration. Sundar *et al.* (1999) had also isolated toxin from this fungus and it had been established that disease symptoms on the host (sugarcane) could be obtained by using partially purified toxin. They also isolated a toxin from the red rot infected inter nodal tissues of the susceptible variety CoC67, gave similar peaks to the partially purified toxin in spectrophotometric studies indicating the possible role of the toxin in pathogenesis.

Over all findings suggested that i) the toxin produced by *C. falcatum* is not host specific; ii) playing a main role in the red rot disease; iii) consisting of two distinct fractions; and having chemical groups of C-H, $\overset{|}{C}$=O, C-N, $-CH_3$, $-CH_2$, $-\overset{|}{C}H$ and molecular mass of the compound is 203 (M^+, $C_{11}H_{11}N_2O_2$).

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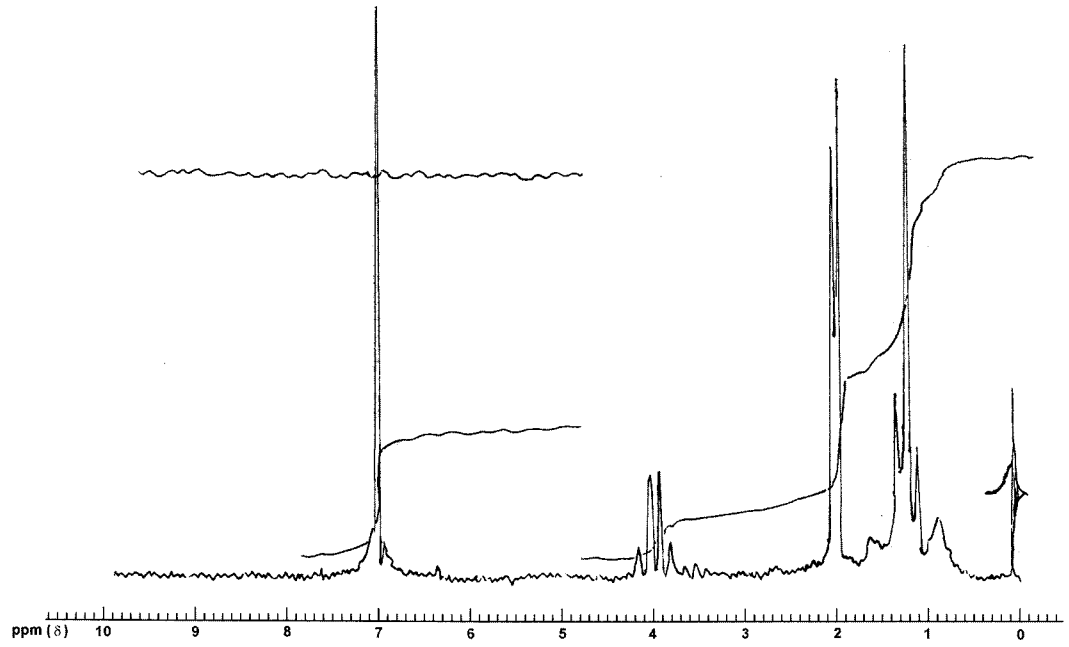

Fig. 5. ^{1}H-NMR spectrum of a toxin isolated from *Colletotrichum falcatum.*

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