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# Elution, Biological Activity and Characterization of Phytotoxin Produced by Sugarcane Red Rot Pathogen *Colletotrichum falcatum*

**Velusamy Jayakumar[1*] • Seiya Tsushima[2] • Palaniyandi Malathi[1] • Rasappa Viswanathan[1]**

[1] Crop Protection Division, Sugarcane Breeding Institute, Coimbatore 641007, Tamil Nadu, India
[2] Natural Resources Inventory Center, National Institute for Agro-Environmental Sciences 3-1-3 Kannondai, Tsukuba, Ibaraki, 305-8604, Japan

*Corresponding author*: * **jkpath@rediffmail.com**

## ABSTRACT

An attempt was made to assess the chemical nature of the toxic compound(s) present in the culture broth of *Colletotrichum falcatum* by selection of only biologically active fractions at every stage of toxin elution and characterization. In liquid culture, *C. falcatum* produced toxic metabolites that are soluble in water, methanol and diethyl ether. The column eluted semi-purified form of secondary metabolites produced severe necrotic lesions on sugarcane leaves at a concentration of 5000 mg/l and the toxin was found to be host specific. Production of necrotic lesion size in sugarcane leaves increased with increase in concentration and time of toxin inoculation, i.e. severe necrotic lesions were noticed after 72 h at 10000 mg/l and after 96 h at 5000 mg/l. The biologically active column eluted fractions *viz*., RP1, RP2, RP3 and RP8 were analyzed with HPLC and GC-MS for structural characterization. The analysis of biologically active fractions identified seven toxic compounds, *viz*., $C_{12}H_{30}O_4$, $C_{16}H_{40}O_5$, $C_{21}H_{50}O_7$, $C_{17}H_{29}NO_5$, $C_{24}H_{62}O_6$, $C_{30}H_{50}O_4$ and $C_{15}H_{24}O$ in the metabolites of *C. falcatum*. Among the identified compounds $C_{17}H_{29}NO_5$ (methoxy phenylpyruvic acid) appeared consistently (at Rt 10.1 min of GC/MS analyzed TIC) in all the biologically active fractions tested. These seven identified compounds constitute phenolics, sugars, acids and few esters.

**Keywords:** Red rot disease, sugarcane, *Cf* toxin, HPLC, GC-MS, structural characterization
**Abbreviations: GC-MS**, Gas Chromatography Mass Spectrometry; **HPLC**, High Performance Liquid Chromatography; **NP**, Natural Phase; **NMR**, Nuclear Magnetic Resonance; **RP**, Reverse Phase; **Rt**, retention time; **Si**, silica; **TIC**, Total Ion Chromatogram

## INTRODUCTION

Red rot caused by *Colletotrichum falcatum* Went. is regarded as a disease of major importance in all the sugarcane growing countries and epidemics of this disease have been very common in India ever since it was reported. In India, the serious epidemics of red rot were noticed in 1901 in Madras, in 1906 in Bihar and in 1922 in Jammu. The disease incidence in northern parts of India was active and remained above 20% till 1955-56 and rose up to 50% in 1957-58 (Sarkar 1973). Yield loss ranging from 28 to 82% and a loss of 35% sugar recovery has been reported in sugarcane due to red rot disease (Ahmad *et al*. 1986) and in Southern states of India, nearly 100% loss of crop due to red rot has been reported (Viswanathan and Samiyappan 2000). Red rot infection on sugarcane not only reduces its yield attributes but also the juice qualities such as Brix value, sucrose content and purity (Kalaimani *et al*. 2000; Kumar *et al*. 2000; Pandey *et al*. 2000). The red rot disease affected canes recorded up to 75% reduced sucrose content than healthy canes (Viswanathan and Padmanaban 2008). Cultivation of several important commercial varieties was stopped due to their high susceptibility to this disease.

Olufolaji and Bamgboye (1986) found that severity of this disease was found to be associated with a toxic material produced by the pathogen. The toxin was reported to be soluble in water, ethanol, methanol, acetone, but very little in chloroform. They also reported that toxic metabolite was stable even at 121°C and produced characteristic red rot symptom in stem and leaf of sugarcane but not in other Graminaceous plants. In a similar experiment Mohanraj *et al*. (2003a) also found that the toxin reproduced some of the symptoms of the disease on sugarcane plants. The results of red rot toxin induced electrolyte leakage in sugarcane showed that the symptoms such as necrosis, disintegration and drying of tissues were also related with toxin (Mohanraj *et al*. 2003b). The association of the disease severity with the toxin production makes it an important factor to be considered in the management of pathogen. But the lack of knowledge on the chemical nature of the toxin is a constraint in developing a management strategy for this disease. There are diverse and increasing demands to determine such properties of the toxin, not only in pathological studies of host-parasite interactions, but also in the fields of modern agricultural biotechnology.

Utilizing toxin as a tool in the management of the pathogen has been successfully demonstrated in many crops. Disease resistant plants were successfully developed in rose-scented geranium, *Pelargonium graveolens* against leaf blight disease caused by *Alternaria alternate* by induction of disease resistance in callus cultures with toxin produced by pathogen (Saxena *et al*. 2008). Classification of chickpea genotypes as resistant or susceptible to *Fusarium* wilt based on seedling reaction to Fusaric acid, a phytotoxin was identified to be in agreement with the classification of genotypes based on wilt-sick plot or molecular marker experiments (Ravikumar and Ratna Babu 2007). Similarly in banana the host selective and non host selective toxins were successfully used to screen the shoot meristem, callus, cell suspension and protoplasts resistant to sigatoka disease (Okole 2010). Sheath blight (SB) toxin inhibition rate was correlated with resistance of rice plants to disease and successfully used in variety improvement of SB resistance in rice breeding programs (Zuo *et al*. 2009). Albicidin phytotoxins are pathogenicity factors in sugarcane leaf scald disease caused by *Xanthomonas albilineans*. A bac-

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**Table 1** Association of toxin with *Colletotrichum* spp.

| Crop | Pathogen | Toxin properties | References |
|---|---|---|---|
| Chilli pepper | *C. capsici* | Two metabolic products of pathogen, *viz*., acetylcolletotrichin and colletodiol were identified as toxin fractions | Grove *et al.* 1966 |
| Turmeric | *C. capsici* | Toxic fraction from culture filtrate produces leaf spot similar to pathogen | Nair and Ramakrishnan 1973 |
| Bean | *C. lindemuthianum* | Produces high molecular weight polysaccharides that produces symptoms on host at less than $10^{-7}$ g of glucose equivalents | Anderson 1978 |
| Tobacco | *C. nicotianae* | Three non host specific phytotoxic substances *viz*., colletotrichin, colletotrichin B and C were identified in culture filtrate of pathogen | Gohbara *et al.* 1978 |
| Alfalfa | *C. trifolii* | Polysaccharide toxic material produced by pathogen causes paling, desiccation, wilting and desiccation of excised leaves, shoots and seedling | Frantzen *et al.* 1982 |
| Coffee | *C. kahawae* | Partially purified culture filtrates were phytotoxic to coffee plant | Nyange *et al.* 1995 |
| Mango | *C. gloeosporioides* | Non host specific toxin produced by pathogen causes complete mortality of embryogenic cultures at 10% concentration. | Jayasankar *et al.* 1999 |
| Rubber tree | *C. acutatum* | Thermo stable, non host specific culture filtrate from pathogen was responsible for symptom production at a concentration of 700 µg dry mycelium mass/ml | Jayasinghe and Fernando 2000 |
| Mulberry | *C. dematium* | Four fractions of phytotoxins produced by pathogen are host nonspecific and play a role in fungal pathogenesis and disease development | Yoshida *et al.* 2000 |
| Yam | *C. gloeosporioides* | Non host specific, thermostable amide peptide toxin produced by pathogen was responsible for anthracnose symptom. | Abang *et al.* 2009 |
| Grapevine | *C. acutatum* | Culture filtrate and its ethyl acetate extracts are toxic to grapevine plant parts | Jang *et al.* 2011 |
| Tobacco | *C. capsici* f. *nicotianae* | Thermo, acid-base stable, non host specific and non-protein toxin is produce by the pathogen | Zhang *et al.* 2012 |

terium *Pantoea dispersa* that degrade the albicidin was identified as potential biocontrol agent to manage the leaf scald disease and a gene (*albD*) encoding esterase from this biocontrol agent was found to attenuate pathogenicity of *X. albilineans* and control the disease (Zhang and Birch 1997). Similarly it is understood that toxin produced by *C. falcatum* may play an important role in disease development and if it is proved as primary determinant of the disease then toxin can be used as a tool for developing strategy to manage this disease. Hence, a comprehensive work was undertaken to carry out a systematic approach on characterization of toxin metabolites produced by *C. falcatum* during disease development.

## Role of toxins in pathogenesis and disease development

Since Hutchinson's first toxigenic hypothesis of fungal plant diseases, there have been many reports of phytotoxins isolated from culture filtrates of pathogens. Over many years the evaluation of toxin in pathogenesis has resulted in many exciting results. The pathotypes of *Alternaria alternata* were reported to cause severe diseases on several fruit crops and in all that a host specific toxin was involved as primary determinant of disease, i.e., AM-toxin in apple, AK-toxin in Japanese pear, ACR-toxin in rough lemon and AF-toxin in strawberry were associated in pathogenesis of their respective fungus (Kohmoto 1992). Meehan and Murphy (1947) demonstrated that *Helminthosporium victoriae* produced a toxin in culture that specifically affected the oats derived from the cultivar 'Victoria' and later it was established that the identified toxin was found in strong association with southern corn leaf blight epidemic of USA. The association of phytotoxic metabolites with anthracnose disease caused by several species of *Colletotrichum* were been reported in many crops (**Table 1**). Apart from host selective toxins few non-host selective toxins also play a major role in pathogenesis, for e.g. macrosporin, a bioactive metabolite produced by a plant-pathogenic fungus *Stemphylium lycopersici*, was found to be play a part in leaf necrosis disease (Trigos *et al*. 2011). A review by Horbach *et al*. (2011) on infection strategies of plant pathogenic fungi describes in detail about the mode of action of various fungal toxins and their importance in pathogenesis.

Similar investigations in many plants-diseases association indicated that pathogenic abilities of fungus on whole or in part depend on their capacity to produce phytotoxins. The review of literatures on phytotoxin and its involvement in pathogenesis supports the statement of Gaumann (1954) that 'microorganisms are pathogenic only if they are toxigenic'.

## Characterization of toxin

The role of toxin in pathogenesis and its mode of action could be established only by quantitative use of chemically defined toxin preparations (Otani *et al*. 1991). 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 inactive alcohol ($C_{14}H_{20}O_6$) in the metabolic products of *C. capsici* (Grove *et al*. 1966). The molecular formulas 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) by thin layer chromatography, mass spectrometry and X-ray crystallographic analysis (Gohbara *et al.* 1978). 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). Application of modern chromatographic techniques *viz*., NMR and ESI showed the production of cochliodinol by the endophytic fungus *Chaetomium* spp. from woody plants (Debbab *et al*. 2009). In addition to these, associations of toxins in several other important diseases were analyzed and their structures were elucidated. Apart from their role in pathogenesis the secondary metabolite profiling (chemotaxonomy) has been shown to be of great value in classification of species in many genera and that has been successfully demonstrated to differentiate species of *Alternaria* (Frisvad *et al*. 2008).

## Toxin as a tool for disease management

Phytotoxic metabolites were successfully used in screening several crops for disease resistance. The toxin from *Helminthosporium oryzae*, brown leaf spot causing pathogen in rice was used to select resistant calli and that produced stable resistant plants (Vidyasekaran *et al*. 1986). Amusa (2000) screened cassava and yam for rsistance to anthracnose disease with toxin from *C. gloeosporioides* f.sp. *manihotis* and similar such attempts are being made in rice (Zuo *et al*. 2009), banana (Okole 2010) and several other crops. Detoxification acts as a self protection mechanism in plant pathogens and this mechanism of self protection against phytotoxins can be well utilized in the management of

disease (Durbin and Langston-Unkefer 1988). Utilizing the microorganisms to degrade phytotoxins and thereby protecting plants against toxigenic plant pathogens was well reviewed by Ouchi *et al*. (1989). Albicidins are a family of phytotoxins produced by *X. albilineans* that play a significant role in leaf scald disease development on sugarcane (Birch and Patil 1987). A translational product of *albA* and *albD* gene from *Klebsiella oxytoca* and *Pantoea dispersa* found to inactivate albicidin and subsequently, Zhang *et al*. (1999) successfully developed transgenic sugarcane plants expressing *albD* in which the transgenic plants were able to protect themselves from leaf scald disease. *Penicillium* sp., *Trichoderma* sp., *Ralstonia pickettii* and *Streptomyces* sp. were reported to degrade thaxtamin A, a phytotoxin produced by *S. scabies*, the casual agent of potato scab (Doumbou *et al.* 1998). A potent antagonist strain *P. fluorescens* Pf10 was reported to reduce the wilt disease in banana crop by the mechanism of detoxifying the toxin produced by *F. oxysporum* f.sp. *cubense* (Thangavelu *et al.* 2001). These microbes were also utilized as potent biocontrol agents to manage the disease by direct application. Head blight caused by *Fusarium graminearum* is a devastating disease of small grain cereal crops due to the production of trichothecene mycotoxin by the pathogen. Ohsato *et al*. (2007) developed transgenic rice plants with *Tri101* gene encoding trichothecene 3-*O*-acetyltransferase (the gene was present in *Fusarium* sp itself for self protection from toxin) and the transgenic plants were found resistant to toxin and the pathogen. These earlier reports indicate the scope of utilizing toxin in disease management.

The literature reports several methods to select plants with increased levels of disease resistance using toxin produced by plant pathogens as selective agents. Insensitivity to phytotoxins produced by plant pathogens can be used as marker for the early screening of segregating populations within conventional and non-conventional breeding programs. Olufolaji (2000) and Ramesh Sundar *et al*. (1999) utilized the semi-purified fraction of toxin produced by *C. falcatum* to screen the sugarcane clones resistance to red rot disease. Similarly, fusaric acid produced by several species of *Fusarium* has been used as selective agent to select tolerant callus of tomato, barley, pears and melon (Remotti *et al*. 1997; Shahin and Spivey 1986; Remotti and Loffler 1996; Hadrami *et al*. 2005). Horbach *et al*. (2011) report that identifying the link between morphogenesis and synthesis of toxins may allow developing novel strategies in plant protection and lead to designing transgenic pathogen-resistant plants exhibiting new defense mechanisms.

### Diseases of sugarcane versus toxin

It has been established that *H. sacchari* symptom production on sugarcane was associated with a toxin (Steiner and Byther 1971). The chemical nature of HS-toxin was identified as Sesquiterpene glucoside, which binds to 48000 daltons protein present in plasmalemma of susceptible sugarcane (Strobel 1973). Leaf scald caused by *Xanthomonas albilineans* is one of the most devastating diseases of sugarcane in many countries. During pathogenesis the pathogen produces a phytotoxin, albicidin that block DNA replication in sugarcane protoplastids (Birch and Patil 1985). The phytotoxin albicidin appear to be important in disease development as tox$^-$ mutant of *X. albilineans* fail to produce any disease.

Olufolaji and Bamgboye (1986) first reported the production of phytotoxic metabolites by *C. falcatum*. They found that the *C. falcatum* toxin (*Cf* toxin) was soluble in water, ethanol, methanol and acetone. According to these authors, the toxin eluted from *C. falcatum* produced peaks similar to that observed for some anthroquinones such as emodin and chrysophanol. Mohanraj *et al.* (1995) partially purified *Cf* toxin and showed that the toxin at high concentration induced red rot symptoms on sugarcane similar to that of the fungus. Similar observations were made by Ramesh Sundar *et al.* (1999) and the intensity of symptom increased with increase in concentration of the toxin (Mohanraj *et al.* 2002). Aamir Ali *et al*. (2007) used this semi-purified toxin fraction to screen the tissue culture plants in order to develop red rot resistant sugarcane clones. Therefore, the present study was undertaken to assess the chemical nature of the *Cf* toxin, which will be useful in developing management strategies of red rot disease in near future.

## MATERIALS AND METHODS

### *In vitro* toxin production

Fresh virulent culture of *C. falcatum* was grown on Petri plates and from that 8 mm mycelial discs were aseptically transferred to 250 ml Erlenmeyer flasks containing 100 ml of Czapek's liquid medium in which sucrose was substituted with host extract (as a carbon source), in such a way that 100 ml of the medium would contain extract from 30 g of young sugarcane stalk tissues prepared by homogenization and filtration. The pathogen was cultured in broth for 15 days 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 secondary metabolites of the filtrate were extracted and fractionated by the method described by Nair and Ramakrishnan (1973). The collected 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 thrice and the collected ether phase was brought to its 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 packed columns. The solvents used in the column are listed in **Table 2**.

### Si column

Silica (Si) natural phase column (Bond elute® 3cc/500 mg) was washed and then eluted with 15 ml of chloroform. Weighed quantity of crude toxin was transferred to the column and eluted with 6 ml of solvents as given in **Table 2**. The eluted compounds by each solvent fraction were collected separately and subjected to centrifugal evaporation at 40°C to evaporate the solvents and the samples were collected.

### C18 column

The reverse phase column (C18) (Bond elute® 3cc/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, eluted with 6ml of solvents and subjected to centrifugal evaporation as described above. For large-scale elution a column was packed with Analytical Bondesil - preparative grade 40 µm (Varian C18) part # 1221-3012 in the column tube with the dimensions of 2.2 cm diameter and 21 cm height with the column height of 4 cm. The samples were eluted as described above and the contents were filtered through cellulose acetate (0.45 µl 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.

**Table 2** Solvents used in the column.

| Fraction | Solvents |
|---|---|
| **Natural Phase (NP) column** | |
| NP 1 | 100% chloroform |
| NP 2 | 5% methyl alcohol + 95% chloroform |
| NP 3 | 10% methyl alcohol + 90% chloroform |
| NP 4 | 20% methyl alcohol + 80% chloroform |
| NP 5 | 40% methyl alcohol + 60% chloroform |
| NP 6 | 70% methyl alcohol + 30% chloroform |
| NP 7 | 100% methyl alcohol |
| **Reverse Phase (RP) column** | |
| RP 1 | 100% water |
| RP 2 | 20% methyl alcohol + 80% water |
| RP 3 | 40% methyl alcohol + 60% water |
| RP 4 | 60% methyl alcohol + 40% water |
| RP 5 | 80% methyl alcohol + 20% water |
| RP 6 | 100% methyl alcohol |
| RP 7 | Acetone |
| RP 8 | Ethyl acetate |
| RP 9 | Chloroform |

## Plant toxicity assay

### *1. Assay of crude toxin*

The toxicity assay of crude toxin was conducted as described by Steiner and Byther (1971) on detached and attached leaves and inter-nodal tissues of sugarcane. The crude toxin samples were dissolved in known quantity of de-ionized water to get the concentrations of 1, 100, 300, 500, 1000, 3000, 5000 and 10000 mg/l and used for bioassay.

### *2. Detached leaf bioassay*

Healthy sugarcane leaves of variety 'NiTn 10' cultivated in glass house were sampled and cut into small fragments with sterile scissors. The leaves were punctured with sterile needles just before inoculation of toxin fractions. The prepared toxin solution was mixed well by mechanical agitation and 50 µl samples in each concentration were placed on the punctured surface of leaf fragment. The leaves were maintained in a humid chamber at 28°C and observed at 24, 48, 72 and 96 h interval after inoculation of toxin for the symptom production. The reaction of leaves to different concentration of toxin was graded as described by Yoshida *et al*. (2000). The experiments were carried out with 4 replications and repeated 4 times to confirm the results.

| Grade | Diameter of necrotic lesion |
|---|---|
| - | No lesion |
| (+) | Less than 5 mm |
| + | 5 to 7 mm |
| ++ | 7 to 10 mm |
| +++ | More than 10 mm |

### *3. Attached leaf bioassay*

A 100 µl of toxin (of each concentration) was injected into the leaf spindle of intact plants grown in glass house condition. The plants were observed for symptom production at 24, 48, 72 and 96 h interval after inoculation and the reaction of leaves to toxin was graded as described above.

### *4. Bioassay on internode*

Canes with 2 internodes were collected from healthy plants grown in glasshouse condition. A 5 mm diameter borehole was made in internode using a cork borer and 100 µl of toxin was injected into the borehole. The hole was closed with cane tissue and covered with parafilm. This procedure was followed for different concentrations of toxin, incubated for 3 days and the cane was cut open and observed for symptom production.

### *5. Bioassay of column eluted toxin*

The column eluted semi purified toxin fractions were tested for their toxicity on detached leaves of sugarcane as described for crude toxin bioassay. The toxin preparations of 100, 300, 500, 1000, 3000, 5000 and 10000 mg/l were used for the experiment. Based on the results of bioassay of semi-purified toxin, the fractions RP1, RP2, RP3 and RP8 were identified as biologically active fractions and utilized for characterizing physio-chemical and biological properties of toxin as described below.

The effect of toxin on production and progress of symptom at various time intervals were tested by detached leaf bioassay as described above. The concentrations of 100 to 10000 mg/l were placed on punctured leaf and symptom production was observed at 24, 48, 72 and 96 h after incubation time. The host specificity of toxin was examined by applying detached leaf bioassay technique on a wide variety of monocot and dicot plant leaves. A concentration of 3000 mg/l (both crude and semi-purified toxin) was used for this experiment and observed for symptom production at 96 h after incubation period.

## HPLC analysis

The column-separated biologically active samples i.e., RP1, RP2, RP3 and RP8 were used for HPLC study. The samples of 100 µl were drawn from each elution and the solvents were evaporated in vacuum with centrifugal evaporator. The samples were then dissolved with required quantity of 40% methanol to get the concentration of 10,000 mg/l. The HPLC was performed with a Waters 626 pump and 996-photodiode-array detector, equipped with a reversed-phase column (analytical column; Inertsil® ODS-3, 3 µm, 4.6 mm i.d., 250 mm length, GL Sciences Inc., Tokyo, analytical guard column; Inertsil® ODS-3, cartridge guard column E, 5 µm, 4 mm i.d., 20 mm length, GL Sciences Inc.) at 40°C. The eluent was fixed as 60% water + 40% methanol and the flow rate of HPLC system was fixed at 1 ml/min. Fifty µl of the samples were injected in to HPLC system and the spectrum was recorded with the photodiode array equipped with the HPLC system on-line. The samples were collected at specified interval and then subjected to centrifugal evaporation to evaporate the solvents.

The HPLC separated dry samples were re-dissolved with de-ionized water of required quantity to get various concentrations. Different concentrations of the toxin were subjected to detached leaf bioassay on sugarcane leaves. The experiment was repeated for each fraction and the results were confirmed. Based on biological activity the samples of HPLC purified toxin fractions were collected again on peak basis and subjected to bioassay to confirm the results again and then forwarded to GC/MS analysis.

## Gas Chromatography-Mass Spectrometry (GC/MS) analysis

### *1. Preparation of sample*

The samples of biologically active fractions were dried by centrifugal evaporation at 40°C and derivatised. 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 0.3 ml of hexane.

### *2. Analysis*

The TMS derivative of the methanolysis product was analyzed with GC/MS (QP- 5050, Shimadzu Corp., Kyoto) equipped with a capillary column (DB-1, 0.25 mm i.d., 30 m length, 0.25 µm thickness, 122- 1032, J&W) under the following conditions: injector, 250°C; interface, 320°C; injection, split- less mode with 30sec of sampling time; column temperature 80°C for the initial 2 min followed by an increase in temperature at a rate of 2°C/min up to 200°C and kept at the same temperature for 8 min. The total ion chromatogram was analyzed for each sample, the base peak of each mass spectrum was compared with the homologous chemical compounds in the National Institute of Standards and Technology (NIST) library through on-line to arrive at a conclusion on chemi-

**Table 3** Bioassay of crude toxin on sugarcane.

| Plant part used for bioassay | Concentration of toxin (mg/l) | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 100 | 500 | 1000 | 5000 | 10000 |
| Detached leaf | - | - | (+) | ++ | +++ | +++ |
| Attached leaves | - | - | - | (+) | + | ++ |
| Inter node | - | - | - | - | (+) | + |

(+) to +++ indicate the severity of necrosis in increasing order

cal nature of toxin fraction.

## RESULTS

### Elution of toxin and bioassay

In liquid culture, *C. falcatum* produced toxic metabolite(s). In the natural phase (NP) Si column, no elution was detected in 100% chloroform and a light yellow colored compound was noticed in eluted fraction of 100% methanol. In reverse phase (RP) C18 column elution it was found that the toxic fraction was soluble in water. The eluted component of RP1 appeared as dark yellow and the same was observed in RP2 and RP3. The elution of metabolites was reduced from RP4 to RP9.

The bioassay results of crude toxin are presented in **Table 3**. On detached leaf, the necrotic symptom appeared at a minimum concentration of 500 mg/l and the severe necrotic lesion (>10 mm) was noticed above 5000 mg/l (**Fig. 1**). On leaves attached to the plant the symptom production was less and delayed, i.e., even at 10000 mg/l only moderate necrotic symptom was observed. The symptom produced on the internode (cane) was highly dispersed and the grade observed was only + even at 10000 mg/l. In case of semi-purified toxin no symptom was noticed on sugarcane-detached leaves treated with NP1 to NP5 fractions (**Table 4**). The fraction eluted in NP7 (100% methanol) induced moderate necrosis (grade ++) at high concentrations. Production of severe necrotic lesions, a typical symptom produced by *C. falcatum* on sugarcane was noticed with bioassay of RP1 (water) fraction (**Fig. 2**). RP1 fraction initiated symptom on sugarcane leaves at 500 mg/l and severe necrosis was noticed at 5000 mg/l. The symptom production by toxin fraction was reduced with increase in concentration of methanol in solvent. The acetone fraction (RP7) did not induce any symptom while ethyl acetate fraction (RP8) induced red necrotic lesion.

The RP1 fraction induced necrosis at 1000 ppm 48 h after application, whereas none of the fractions of the toxin induced any symptom within 24 h after treatment (**Table 5**). The necrotic lesion size increased with increase in time and concentration. Severe necrotic lesions were noticed after 72 h at 10000 mg/l and after 96 h at 5000 mg/l. A similar trend was noticed in the RP2 fraction but moderate necrotic lesions (grade ++) appeared only after 72 h at 5000 mg/l. The symptom appearance in RP3 injected leaves was further delayed and only moderate necrotic lesion of ++ grade

**Table 4** Bioassay of semi-purified toxin on detached sugarcane leaves.

| Toxin fraction | Toxin concentration (ppm) | | | | | | | Remarks |
|---|---|---|---|---|---|---|---|---|
| | 100 | 300 | 500 | 1000 | 3000 | 5000 | 10000 | |
| **Natural phase column fraction** | | | | | | | | |
| NP 1 | - | - | - | - | - | - | - | - |
| NP 2 | - | - | - | - | - | - | - | - |
| NP 3 | - | - | - | - | - | - | - | - |
| NP 4 | - | - | - | - | - | - | - | - |
| NP 5 | - | - | - | - | - | - | - | - |
| NP 6 | - | - | - | - | - | - | + | - |
| NP 7 | - | - | - | - | (+) | + | ++ | - |
| **Reverse phase column fraction** | | | | | | | | |
| RP 1 | - | - | (+) | + | ++ | +++ | +++ | Severe necrotic lesions |
| RP 2 | - | - | (+) | + | ++ | ++ | ++ | |
| RP 3 | - | - | - | - | (+) | + | ++ | |
| RP 4 | - | - | - | - | - | - | + | |
| RP 5 | - | - | - | - | - | - | + | |
| RP 6 | - | - | - | - | - | (+) | + | |
| RP 7 | - | - | - | - | - | - | - | |
| RP 8 | - | - | - | - | (+) | ++ | ++ | Reddening |
| RP 9 | - | - | - | - | - | - | (+) | |
| Crude toxin | - | - | (+) | + | ++ | +++ | +++ | |

(+) to +++ indicate the severity of necrosis in increasing order

**Table 5** Effect of active fractions of toxin on symptom production in detached sugarcane leaves at different time intervals.

| Toxin fraction | Incubation time (h) | Concentration (mg/l) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 100 | 300 | 500 | 1000 | 3000 | 5000 | 10000 |
| RP 1 | 24 | - | - | - | - | - | - | - |
| | 48 | - | - | - | (+) | (+) | + | + |
| | 72 | - | - | (+) | + | ++ | ++ | +++ |
| | 96 | - | - | + | ++ | ++ | +++ | +++ |
| RP 2 | 24 | - | - | - | - | - | - | - |
| | 48 | - | - | (+) | (+) | (+) | (+) | + |
| | 72 | - | - | + | + | + | ++ | ++ |
| | 96 | - | - | + | + | ++ | ++ | ++ |
| RP 3 | 24 | - | - | - | - | - | - | - |
| | 48 | - | - | - | - | (+) | (+) | (+) |
| | 72 | - | - | - | - | (+) | + | + |
| | 96 | - | - | - | - | + | ++ | ++ |
| RP 8 | 24 | - | - | - | - | - | - | - |
| | 48 | - | - | - | - | (+) | (+) | (+) |
| | 72 | - | - | - | (+) | + | + | + |
| | 96 | - | - | - | + | ++ | ++ | ++ |

(+) to +++ indicate the severity of necrosis in increasing order

**Table 6** Formation of necrotic lesions by crude and semi purified active fractions of the toxin from *C. falcatum* on leaves of various plants.

| Plants | Reaction (96 h after incubation) | | | | | |
|---|---|---|---|---|---|---|
| | Toxin fraction (3000ppm) | | | | | Remarks |
| | Crude toxin | RP 1 | RP 2 | RP 3 | RP 8 | |
| Sorghum | + | - | - | - | - | Scorching of injured area in crude toxin treated leaves |
| Maize | - | - | - | - | - | - |
| Wheat | - | - | - | - | - | Mild chlorosis around the injured area in crude toxin treated leaves |
| Rice | (+) | - | - | - | - | Mild scorching of injured area |
| Oat | - | - | - | - | - | - |
| Barley | | | | | - | |
| Green millet | - | - | - | - | - | |
| Orchard grass | - | - | - | - | - | - |
| Tomato | | | | | - | |
| Japanese pear var. Kosui | - | - | - | - | - | - |
| Japanese pear var. Chojuroo | - | - | - | - | - | - |
| Carrot | - | - | - | - | - | - |
| Cabbage | - | - | - | - | - | - |
| Sugarcane | +++ | +++ | ++ | + | ++ | Severe necrotic lesions surrounded by yellow hallow |

(+) to +++ indicate the severity of necrosis in increasing order

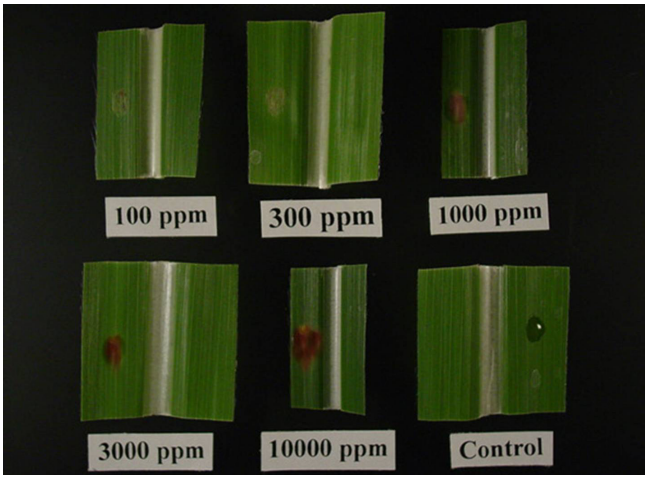
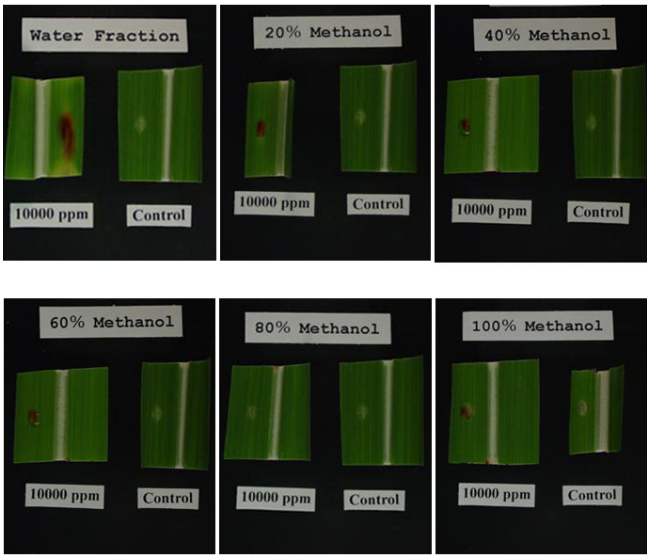



**Fig. 1 Bioassay of crude toxin on detached leaves of sugarcane.**



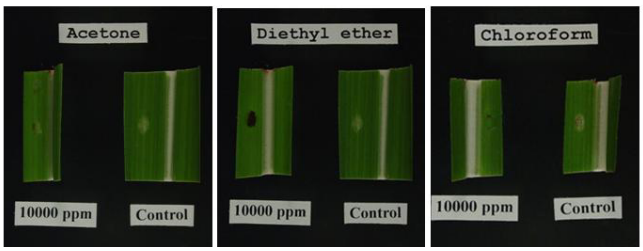




**Fig. 2 Bioassay of column eluted semi-purified toxin fractions on detached leaves of sugarcane.**

was noticed even after 96 h. On assessing the host range the crude toxin induced scorching on sorghum and mild chlorosis on wheat leaves, whereas the semi purified toxin fractions did not induce any symptom on the leaves of plants listed in **Table 6**.

## HPLC analysis and biological activity of toxin

The chromatogram depicted in **Fig. 3A** corresponds to the fraction RP1. A sharp peak was noticed at 2.931 min retention time (Rt) and few other significant signals were also noticed at Rt of 2.814, 3.346, 3.728 and 13.570 min. No spectrum was observed after 35min of retention time. In RP2, many peaks were observed in chromatogram (**Fig. 3B**). The peaks appeared between Rt of 3 to 4 min resembled that of RP1 peaks between Rt 2 to 3 min but the intensity was less. In RP2 signals were recorded at Rt of 3.144, 5.498, 9.482, 11.790, 12.705, 30.617 and 33.740 min. The spectrum peaks in RP3 resembled to that of RP2 chromatogram (**Fig. 3C**). The high intense peaks noticed in RP1 were very weak in RP3 chromatogram. The spectrum in Rt between 29 to 33 min was constantly noticed in all the fractions.

The results of bioassay of HPLC fractions on sugarcane-detached leaves are presented in **Table 7**. In HPLC elution of RP1, sample 3 induced severe necrotic lesions on sugarcane leaves (**Fig. 4**), which coincides with the spectrum appeared in time interval between 2 to 3 min. In RP2 chromatogram, sample 6, 15 and 16 induced the necrotic lesion to the grade of + and the remaining samples induced mild or no symptom. The sharp peaks (Rt 11 to 13) in chromatogram of RP2 did not produce necrotic lesions. In RP3 fraction, the sample 7 induced necrotic lesion to the grade of +, which did not coincide with any spectrum in the chromatogram of RP3. The remaining samples induced poor or no symptom on sugarcane leaves. Based on the above results another set of HPLC analysis was performed with same samples, in that the time intervals for collection of HPLC eluted fractions were fixed in such a way that the biologically active signals (peaks of each spectrum) were collected individually and completely. These biologically active samples were once again confirmed for plant toxicity and forwarded to GC-MS analysis.

## GC/MS analysis

The biologically active fractions of metabolites were analyzed and their total ion chromatogram (TIC) showed the presence of wide array of secondary metabolites. Every individual signals in TIC were analyzed in detail, compared with NIST chemical library and the results of signals relevant to the present study only are presented here.

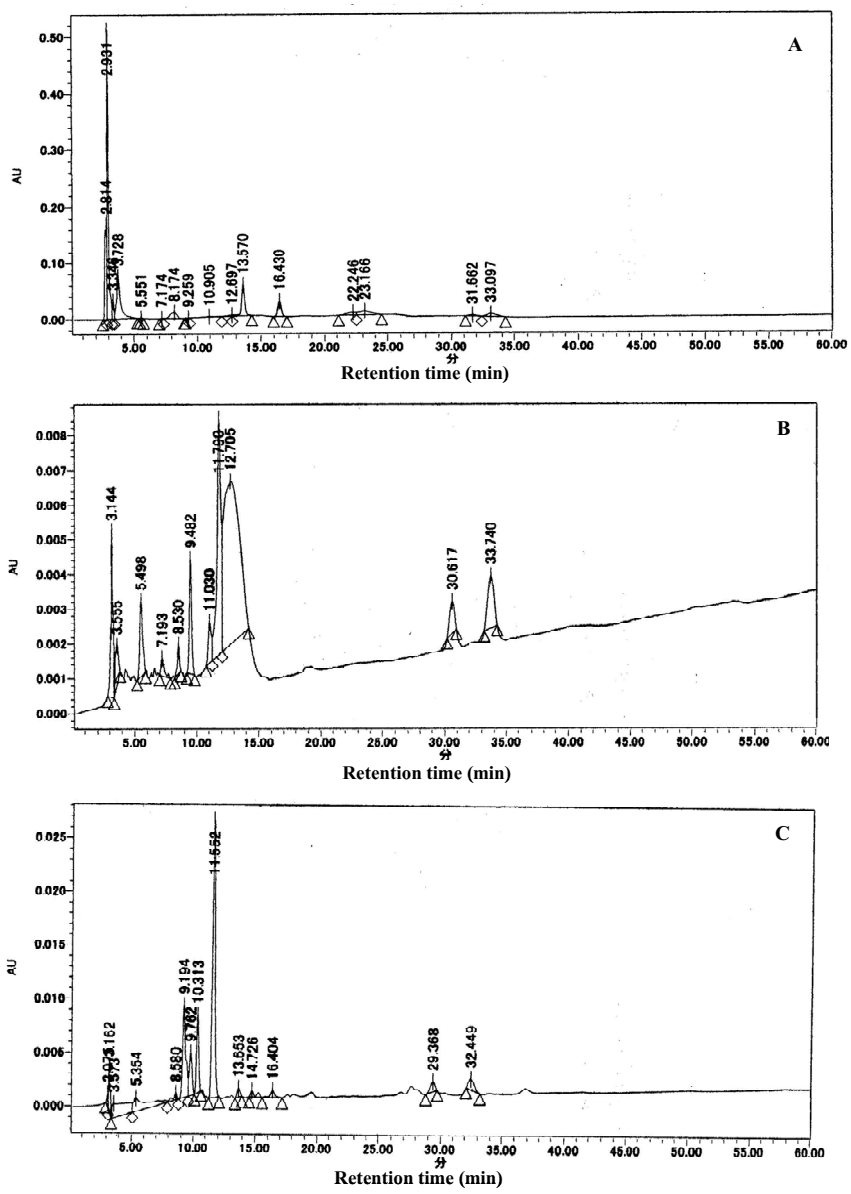



**Fig. 3 HPLC chromatogram of biologically active toxin fractions (A) RP1 (water fraction), (B) RP2 (eluted with 80% water + 20% methyl alcohol), (C) RP3 (eluted with 60% water + 40% methyl alcohol).**

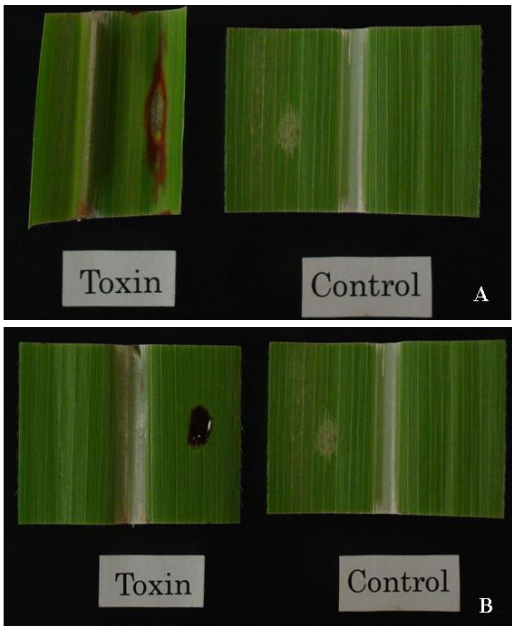



**Fig. 4 Bioassay of HPLC eluted toxin on detached leaves of sugarcane (A) RP1 fraction 2, (B) RP 8.**

## Analysis of total ion chromatogram of RP1-fraction 1

The total ion chromatogram (TIC) of RP1-fraction 1 presented in **Fig. 5A** shows the signals of chemical compounds present in the toxin fraction. The mass spectrum corresponding to Rt 12.70-12.75 min had base peak of 73 (**Fig. 5B**). The homologous search from NIST library identified the empirical formula of closely related compound as $C_{12}H_{30}O_4Si_3$, i.e., ester of methyl propanoic acid, which had base peak of 73 and other high intense peaks at spectrum of 147, 189 and 292 mass numbers. The chromatogram peak at Rt 18.9 to 18.933 min showed the mass spectrum with base peak of 73 and other intense peaks at 147 and 292 mass numbers (**Fig. 5C**). Based on base peak, total number of peaks and other intense peaks the chemical compound was identified as TMS derivative of 2,3,4-trihydroxybutyric acid ($C_{16}H_{40}O_5Si_4$). Another intense peak in RP1 fraction chromatogram was identified at Rt 24.233 to 24.283 min. The corresponding mass spectrum had base peak at 73 and other intense peaks at mass numbers of 103, 147, 217 and 292 (**Fig. 5D**). The library search result identified the compound as 2-keto-1- gluconic acid ($C_{21}H_{50}O_7Si_5$). Another peak in RP1 fraction 1 was noticed at Rt 25.950 to 26.0 and the corresponding mass spectrum had base peak of 73 and other peaks were noticed at 147, 245 and 345 mass numbers (**Fig.**

**Table 7** Formation of necrotic lesions by HPLC purified fractions on detached leaves of sugarcane.

| Sample No. | Reaction (96 h after incubation) | | |
|---|---|---|---|
| | RP1 | RP2 | RP3 |
| 1 | - | - | - |
| 2 | - | - | - |
| 3 | +++ | (+) | (+) |
| 4 | + | - | (+) |
| 5 | (+) | (+) | - |
| 6 | - | + | (+) |
| 7 | - | (+) | + |
| 8 | - | (+) | (+) |
| 9 | - | (+) | (+) |
| 10 | (+) | (+) | - |
| 11 | - | - | (+) |
| 12 | (+) | (+) | - |
| 13 | - | (+) | - |
| 14 | (+) | - | - |
| 15 | - | + | (+) |
| 16 | - | + | (+) |
| 17 | - | (+) | (+) |
| 18 | (+) | (+) | - |
| 19 | - | - | - |
| 20 | - | - | (+) |
| 21 | (+) | - | - |
| 22 | - | - | - |
| 23 | (+) | (+) | (+) |
| 24 | (+) | - | - |
| 25 | - | - | - |
| 26 | - | (+) | - |
| 27 | - | (+) | (+) |
| 28 | - | - | - |
| 29 | - | (+) | - |
| 30 | - | - | - |
| 31 | - | - | - |
| 32 | - | (+) | - |
| 33 | - | - | - |
| 34 | (+) | (+) | (+) |
| 35 | - | (+) | (+) |
| 36 | (+) | - | - |
| 37 | - | - | - |
| 38 | - | - | - |
| 39 | - | - | - |
| 40 | - | (+) | - |
| 41 | - | (+) | (+) |
| 42 | - | - | - |
| 43 | - | - | - |
| 44 | - | - | - |
| 45 | - | - | - |
| Control | - | - | - |

(+) to +++ indicate the severity of necrosis in increasing order

**5E**). In NIST107 library, the homologous mass spectrum with entry number 98935 had all similar peaks except mass number 345 and identified the formula as $C_{17}H_{40}O_6Si_4$ i.e., ester of pentane dioic acid. The other peaks seen in total ion chromatogram of RP1 were identified as contaminants and the property of those compounds are not discussed here.

## Analysis of total ion chromatogram of RP1-fraction 2

The **Fig. 6A** represents the TIC of HPLC-fraction 2 of RP1 toxic component. The chromatogram showed several signals and the property of reliable peak is as follows. The signal noticed at Rt 10.1 to 10.167 min was intense among the prominent peaks of TIC. The base peak of corresponding mass spectrum was 292.95 and had intense peaks at mass number 73 and 205 (**Fig. 6B**). The empirical formula of this mass spectrum was established as $C_{17}H_{29}NO_5Si_2$. The entry no 90300 in NIST107 library was found close to sample with mass spectrum of 73. The homologous compound had aromatic ring and it was identified as TMS derivative of pyruvic acid (**Fig. 6C**).

## Analysis of total ion chromatogram of RP1-fraction 3

The peaks in TIC of fraction 3 varied from fraction 2 except for Rt. 10.117 to 10.183. The mass spectrum corresponding to the given Rt had base peak at 73 and high intense peaks at 205 and 293 (**Fig. 7A** and **7B**). A signal at Rt 28.633 to 28.667 min was identified as useful candidate (**Fig. 7C**) with base peak of 117.05. The homologous spectrum identified the corresponding chemical as esters of hexadecanoic acid/palmitic acid ($C_{19}H_{40}O_2Si$). An another signal corresponding to Rt 27.717 to 27.767 min was identified with base peak of 73 and other intense peaks with mass numbers of 103, 147, 205 and 319 (**Fig. 7D**). The chemical formula was identified as $C_{24}H_{62}O_6Si_6$ and the homologous spectrum in NIST library identified it as glucitol.

## Analysis of total ion chromatogram of RP 2-fraction 1

The TIC of toxin sample RP2-fraction 1 is presented in **Fig. 8A**. The signal of spectrum at Rt 10.083 to 10.183 min had base peak of 293.05 and sharp peaks at 73 and 205 mass numbers (**Fig. 8B**). This mass spectrum is exactly similar to the mass spectrum recorded in RP1- HPLC fraction 2 with the Rt of 10.100 to 10.167. The mass spectrum corresponding to Rt 17.667 to 17.683 min had base peak of 73.05 and intense peak of 267 mass number (**Fig. 8C**). A similar mass spectrum was observed in NIST library with a new type of compound, p-trimethylsilyl oxyphenyl-(trimethylsiloxy) trimethyl silylacrylate ($C_{18}H_{34}O_4Si_3$). The remaining signals in this TIC were identified as noise.

## Analysis of total ion chromatogram of RP 2-fractions 2, 5 and 8

The TIC of RP2-fraction 2 presented in **Fig. 9A** showed a signal at Rt 10.1 that was the same signal those observed in other samples and this fraction also showed some new signals other than those recorded in earlier fractions. The chromatogram with Rt 26.06 to 26.117 was related to a new mass spectrum that had base peak at mass number 149 (**Fig. 9B**). The search result identified this compound as ester of benzene dicarboxylic acid/phthalic acid. The chromatogram of Rt 36.767 to 36.8 had mass spectrum with base peak of 149 (**Fig. 9C**). This spectrum was similar to the previous one but the intensity of other peaks varied. The homologous mass spectrum and the chemical compound related to the peak were similar to the previous one with little variation in structure. The related chemical compound was identified as di-isooctyl ester of 1, 2-benzene dicarboxylic acid/phthalic acid and the chemical formula was established as $C_{30}H_{50}O_4$. The TIC of fractions 5 and 8 had the signals around Rt 10.1 min, which is similar to the one found in other fractions and no other significant peaks were identified in the chromatogram.

## Analysis of total ion chromatogram of RP 3

The signals in TIC of RP 3 varied from other fractions (**Fig. 10A**). The peak at Rt 10.1 that consistently occurred in other fractions also seen in this fraction but its intensity was weak. The chromatogram of Rt 23.833 to 23.883min had base peak at 73.05min in its mass spectrum (**Fig. 10B**). The other high intense peaks noticed in this spectrum were 129 and 147 and the chemical formula was identified as $C_{10}H_{24}O_2Si_2$. The chromatogram of Rt 41.40- 41.45 min was found associated with mass spectrum that had base peak at mass number 69 ($C_{25}H_{42}$) (**Fig. 10C**). The homologous mass spectrum was identified as eicosapentaene.

## Analysis of total ion chromatogram of RP8

The sample RP8 that induced reddening on cane leaves showed a few new signals in the chromatogram (**Fig. 11A**).

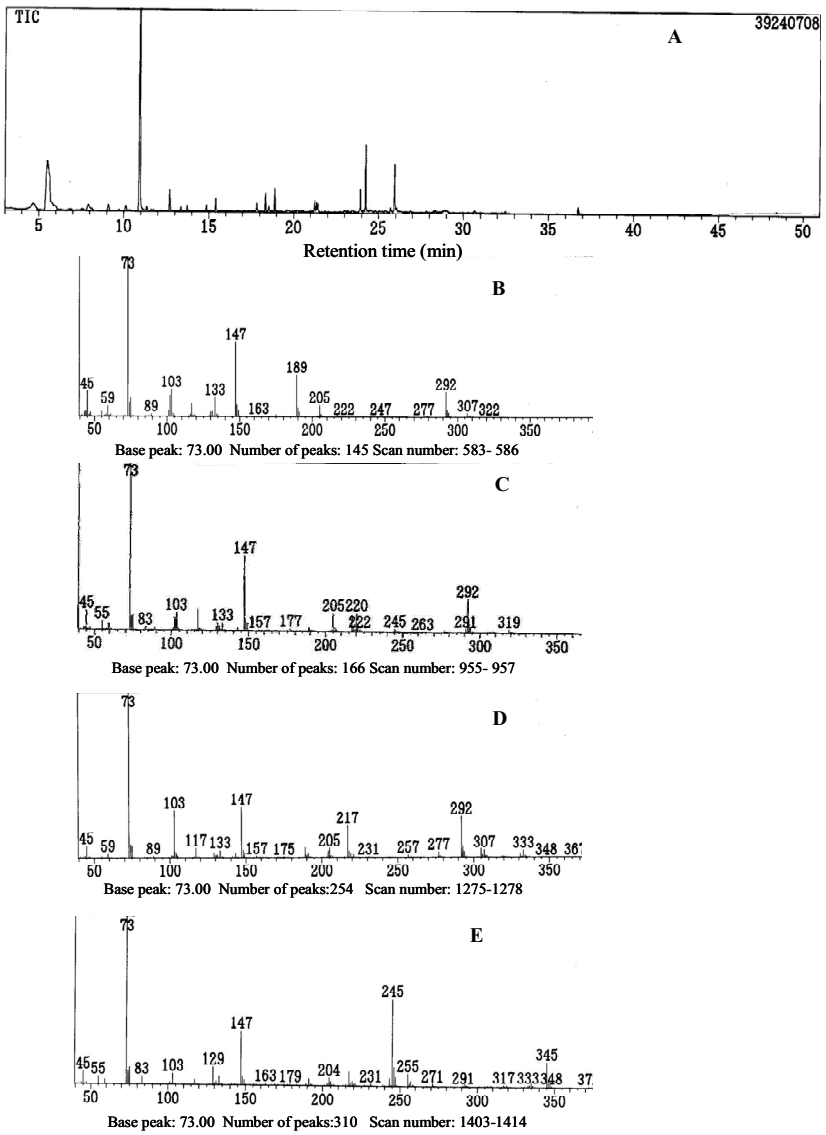



**Fig. 5 Total ion chromatogram and mass spectra of active signals in sample RP1- HPLC fraction 1.** (**A**) TIC of RP1- HPLC fraction 1, (**B**) Mass spectrum corresponding to the signal at Rt 12.700-12.750, (**C**) Mass spectrum corresponding to the signal at Rt 18.900-18.933, (**D**) Mass spectrum corresponding to the signal at Rt 24.233-24.283, (**E**) Mass spectrum corresponding to the signal at Rt 25.950- 26.000.

The sharp peak of chromatogram at Rt 16.483 to 16.533 min had the base peak at 205.1 mass number ($C_{15}H_{24}O$) (**Fig. 11B**). The chemical compound related to the spectrum was identified as butylated hydroxytoluene-phenol. The peaks at Rt 28.733 to 28.767 and 34.333 to 34.367 min had similar mass spectrum with little variation in intensity of signals (**Fig. 11C** and **11D**). In both, the mass spectrum had the base peak of 95.15 ($C_{15}H_{24}$) and the corresponding homologous spectrum was identified as neoclovene in NIST library search result.

## DISCUSSION

### Involvement of *Cf* toxin in pathogenesis and disease development

Production of toxin by *P. tucumanensis* (sexual stage of *C. falcatum*) in still cultures has already been reported by Olufolaji and Bamgboye (1986) and several other researchers but till date no attempts were made to analyze the chemical nature of this toxic metabolite. In the present study, a systematic and a successful attempt was made to assess the chemical nature of the toxic compound(s) present in the culture broth of *C. falcatum* through selection of only biologically active fractions at every stage of toxin elution and characterization. *C. falcatum* produced toxic metabolites in still culture, the crude and column eluted semi-purified form of toxin produced characteristic symptom of red rot disease at 5000 mg/l. The semi-purified toxin fraction induced symptom at 500 mg/l and caused severe necrotic lesions similar to red rot disease at 5000 mg/l at 96 h after injection. These results indicate that the quantity of toxic metabolite produced by *C. falcatum* in still cultures is very low and that supports the earlier findings of Mohanraj *et al*. (1995).

The toxin was soluble in water, methanol and diethyl ether and insoluble in chloroform and acetone. However, the metabolite fraction eluted in 80% methanol fractions did not induce any characteristic symptom on sugarcane leaf. It was due to the fast elution of most of toxic fractions in the initial stage of elution itself. Olufolaji and Bamgboye (1986) earlier reported that the toxin produced by *C. falcatum* was soluble in water, ethanol, methanol and acetone and Mohanraj *et al*. (2002) also confirmed the solubility of the toxin fraction in water and methanol.

The results of bioassay on 12 other plant species show that the eluted toxin fraction is host specific. The induction of mild scorching in sorghum and rice plants by crude toxin may be due to presence of some contaminants, which is confirmed by the fact that the semi-purified toxin did not produce any symptom on non-host plants while its toxicity to host plant was retained. Olufolaji and Bamgboye (1986) also reported the host specific nature of *C. falcatum* toxin wherein they observed characteristic symptom production

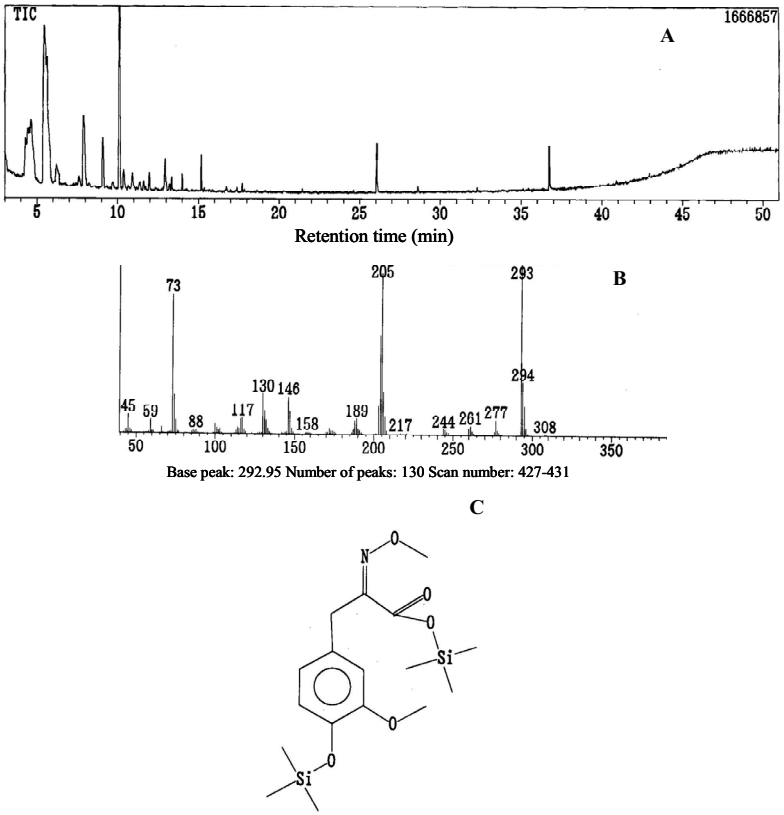



**Fig. 6 Total ion chromatogram and mass spectra of active signals in sample RP1-HPLC fraction 2.** (**A**) TIC of RP1- HPLC fraction 2, (**B**) Mass spectrum corresponding to the signal at Rt 10.100 10.167, (**C**) Homologous chemical structure corresponding to the above mass spectrum (molecular formula: $C_{17}H_{29}NO_5Si_2$; Compound: 4-hydroxy-3-methoxyphenyl pyruvic acid methoxime, di-TMS; molecular weight: 383).

in sugarcane 24 h after toxin treatment, while in *Sorghum vulgare*, *Zea mays*, *Pennisetum purpureum*, *Cynodon nlemfuensis* and *Oryza sativa* no symptoms formed. Mohanraj *et al.* (1995) also confirmed the host specificity of *C. falcatum* toxin.

## HPLC analysis of toxin

Analysis of chromatogram, contour plot and biological activity of HPLC purified fractions revealed the presence of many fractions that are individually toxic to sugarcane, i.e., the signals of RP1-fractions 2 and 3, RP2-fractions 2, 5 and 8 were highly active and also induced reddening and scorching symptom on detached leaves of sugarcane. Presence of many toxic fractions in a metabolite produced by a pathogen has been reported already, for e.g. HPLC analysis of toxic substances produced by *C. dematium* revealed the presence of four toxic fractions in the extract isolated from anthracnose lesions (Yoshida *et al.* 2000).

## GC-MS analysis of toxin

The GC/MS analysis of biologically active fractions has given the mass spectrum and empirical formulae of chemical compounds in the sample and their properties are discussed here. The chemical formula $C_{12}H_{30}O_4$ is closely related to the base peak and formula of ester of propionic acid. The compound is also called as methyl acetic acid or propionic acid. This is soluble in water, alcohol, chloroform and ether but solubility varies when reacted with the other compounds. Some of the propionates are used as mold inhibitors in bread and this compound was also reported to be present in milk as a product of bacterial fermentation (Anonymous 1956). This result indicates that the compound $C_{12}H_{30}O_4$ may be of a candidate toxin fraction. The chemical compound closely related the empirical formula $C_{16}H_{40}O_5$ is trihydroxy butyric acid. It is also called as erythronic acid or threonic acid, a derivative of erythrose/ threose (Pollock and Stevens 1965b). These compounds are highly soluble in water and ethanol and solubility varies when combined with other chemicals. Erythrose in reaction with phenol forms several compounds resembling antibiotics and other products of microbial origin (Pollock and Stevens 1965a). Erythroskyrine is one such compound produced by *Penicillium islandicum* Sopp. (Stevens 1969). Albicidins are a family of antibiotic based phytotoxins produced by *X. albilineans* that play a significant role in leaf scald disease of sugarcane (Birch and Patil 1987). Hence the identified chemical compound may of active toxic substance. The water and alcohol soluble chemical compound 2-keto-1-gluconic acid is related to the formula $C_{21}H_{50}O_7$. The chemical compound is also called as glyconic acid/dextronic acid/glycogenic acid/1, 2, 3, 4, 5-pentahydroxy caproic acid. They originated from bacteria and used in preparation of pharmaceutical (Anonymous 1956). A wide variety of moulds and acetobacter were reported to produce this compound and high yield of this compound was recorded in the metabolites produced by *Aspergillus niger* (Pollock and Stevens 1965a). The chemical compound related to the formula $C_{17}H_{40}O_6$ was identified as ester of pentanedioic acid. This chemical compound was identified as synthetic in nature (Pollock and Stevens 1965b) and may not be relevant to the present study.

One highly active fraction with empirical formula of $C_{17}H_{29}NO_5$ was identified in RP1-HPLC fraction 2. The structure of was identified as methoxy phenylpyruvic acid. The compound is reported to be soluble in ethanol, ethyl acetate and hot chloroform and sparingly soluble in boiling water (Pollock and Stevens 1965b). Pyruvic acid is an important intermediate in protein and carbohydrate metabolism of biological system (Anonymous 1956). The toxicity of this compound on sugarcane is yet to be established; however this compound in reaction with other fractions may produce toxic substance. Pringle and Braun (1960) characterized the victoxinine, a toxin from *H. victoriae* as $C_{17}H_{29}NO$ and this empirical formula is closely related to the chemical identified in the present study. Considering the structure, i.e., location of single nitrogen atom close to the

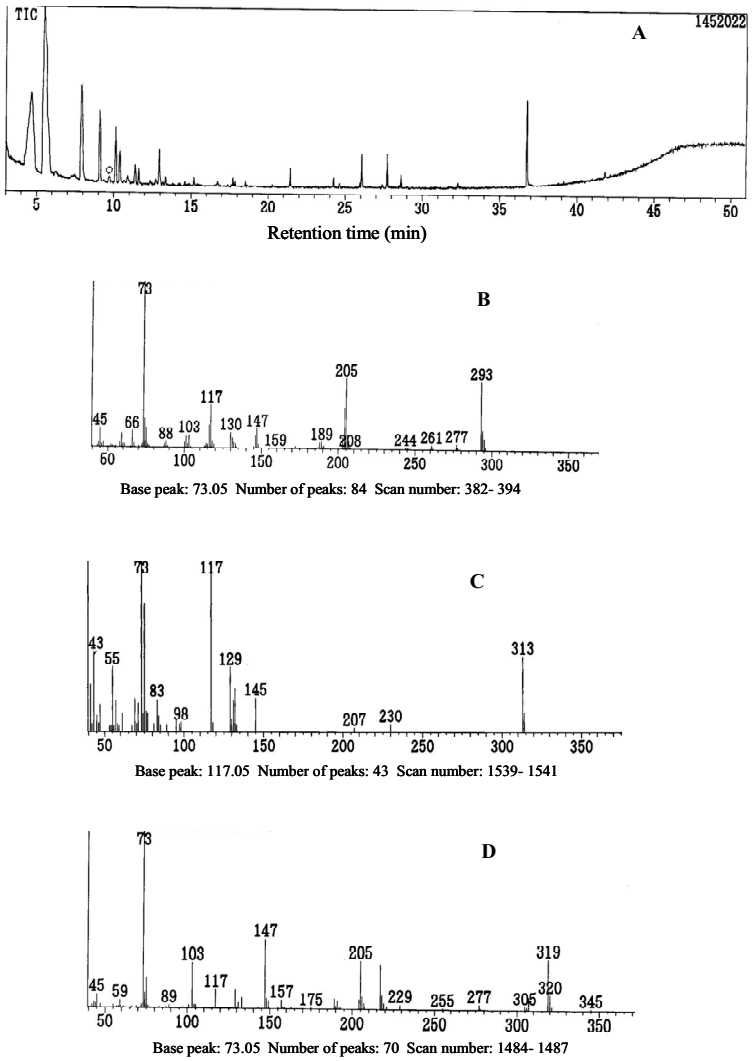



**Fig. 7 Total ion chromatogram and mass spectra of active signals in sample RP1-HPLC fraction 3.** (**A**) TIC of RP1-HPLC fraction 3, (**B**) Mass spectrum corresponding to the signal at Rt 10.117-10.183, (**C**) Mass spectrum corresponding to the signal at Rt 28.633-28.667, (D) Mass spectrum corresponding to the signal at Rt 27.717-27.767.

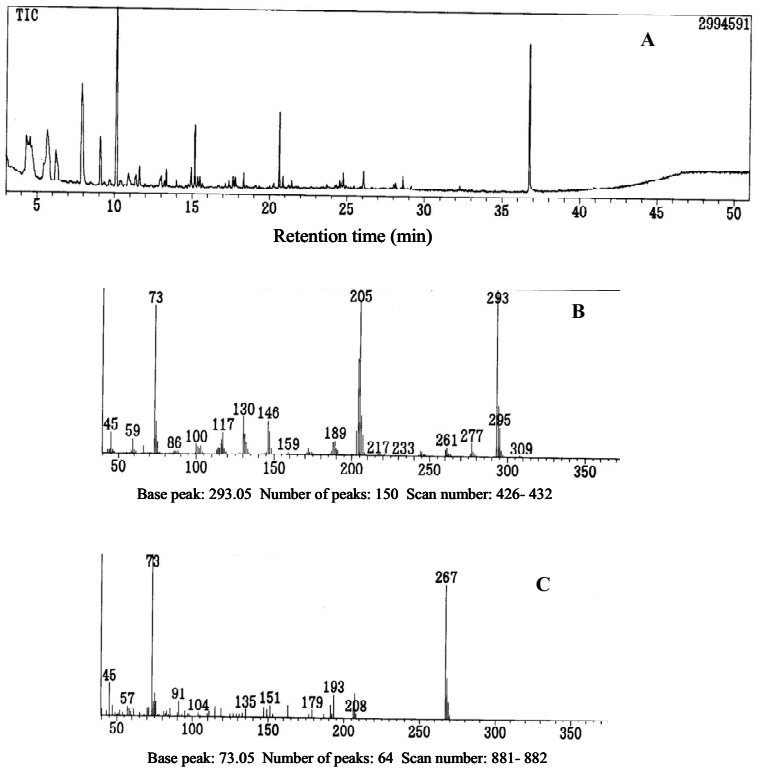



**Fig. 8 Total ion chromatogram and mass spectra of active signals in sample RP2- HPLC fraction 1.** (**A**) TIC of RP2- HPLC fraction 1, (**B**) Mass spectrum corresponding to the signal at Rt 10.083-10.183, (**C**) Mass spectrum corresponding to the signal at Rt 17.667-17.683.

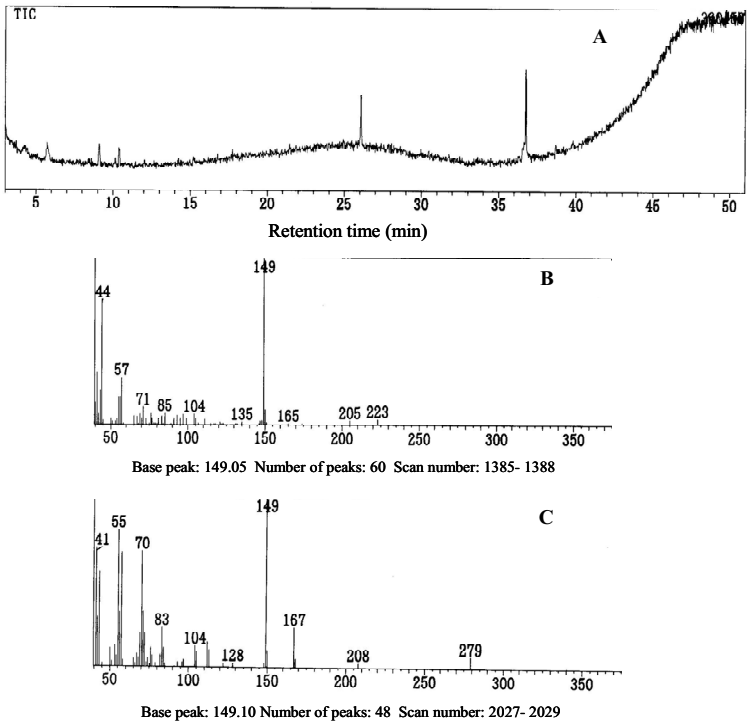



**Fig. 9 Total ion chromatogram and mass spectra of active signals in sample RP2- HPLC fraction 2.** (**A**) TIC of RP2- HPLC fraction 2, (**B**) Mass spectrum corresponding to the signal at Rt 26.060-26.117, (**C**) Mass spectrum corresponding to the signal at Rt 36.767-36.800.

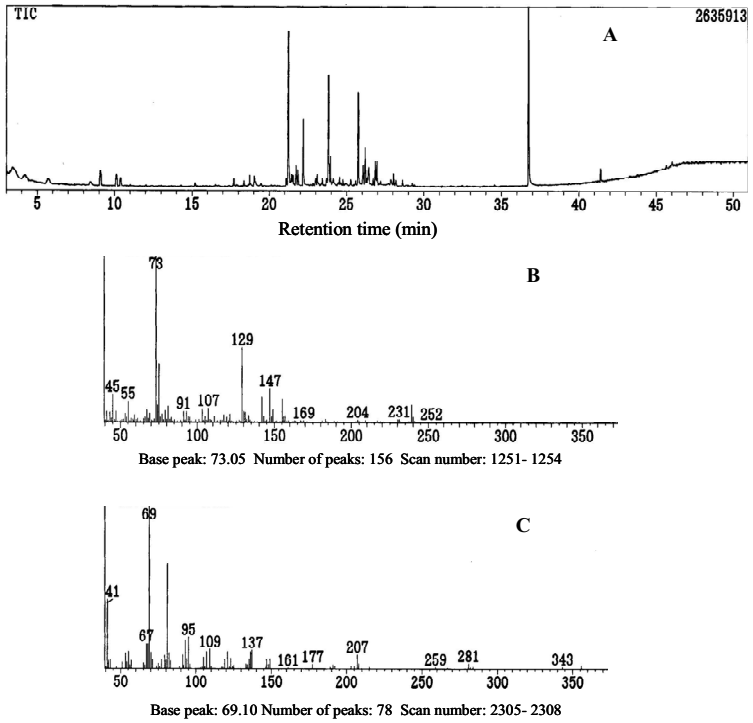



**Fig. 10 Total ion chromatogram and mass spectra of active signals in sample RP3.** (**A**) TIC of RP3, (**B**) Mass spectrum corresponding to the signal at Rt 23.833-23.883, (**C**) Mass spectrum corresponding to the signal at Rt 41.400-41.450.

double bond in both the compounds and changes in number of oxygen atom and presence of Si were due to TMS derivatisation process, it can be concluded that these two compounds may be closely related with each other. The consistent appearance of signal of this chemical compound (Rt 10.1 min) in GC/MS analysis of all active fractions tested and its toxicity in bioassay further confirms that it may be a potential candidate of toxin.

In RP1-HPLC fraction 3 the first chemical compound $C_{19}H_{40}O_2$ was identified as hexadecanoic acid. Decanoic acid is also called as capric acid and is present in vegetable oils. The second signal peak related to $C_{24}H_{62}O_6$ was identified as glucitol (sorbitol). The chemical identified was a sweet sugar in fruits of several plants (Pollock and Stevens 1965c). The sugar was found soluble in water, slightly soluble in methanol, ethanol, acetic acid, and phenol and almost insoluble in other organic solvents (Anonymous 1956). The direct phytotoxicity of this compound was not reported so far, so it may be a breakdown product from toxin. The toxin from *Rhizoctonia solani* is reported to possess sugar fractions such as glucose and mannose (Vidhyasekaran *et al.* 1997).

In the sample RP2-fraction 1 the chemical compound related to $C_{18}H_{34}O_4$ was identified as trimethylsilyl acrylate.

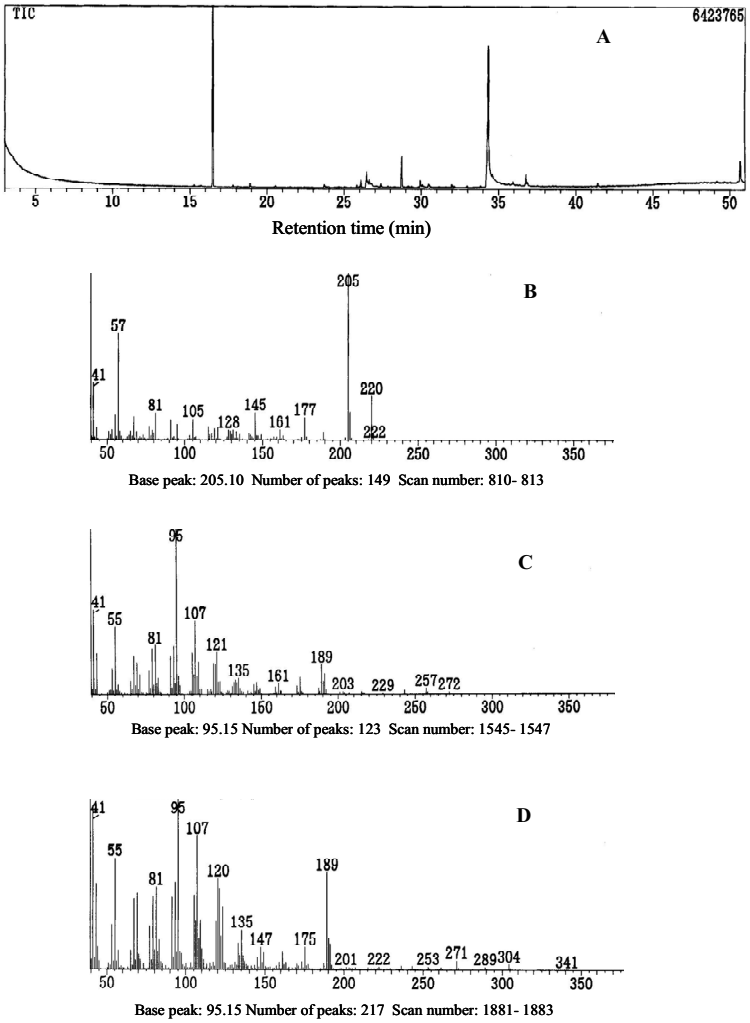



**Fig. 11 Total ion chromatogram and mass spectra of active signals in sample RP8.** (**A**) TIC of RP8, (**B**) Mass spectrum corresponding to the signal at Rt 16.483-16.533, (**C**) Mass spectrum corresponding to the signal at Rt 28.733-28.767, (**D**) Mass spectrum corresponding to the signal at Rt 34.333-34.367.

The properties indicated that this compound is synthetic and do not posses any property relevant to the present study (Stevens 1971). The mass spectral analysis of RP2 (fraction 2) showed signals at two different Rt and the associated compound was identified as $C_{30}H_{50}O_4$ corresponding to benzene dicarboxylic acid (phthalic acid). The chemical was reported to be soluble in alcohol and useful in medicine (Anonymous 1956). Few chemical compounds with empirical formula $C_{30}H_{50}O_4$ were also reported in plants and other organisms (Stevens 1971). The homologous compound priverogen B was reported from root and rhizome of *Primula veris* L. and Spergulagenin A from *Mollugo spergula*. The chemical pyxinic acid was reported as constituent of Lichen *Pyxine endochrysina* (Stevens 1969). This chemical can be considered as a useful candidate fraction of toxin.

The chemical compound related to the formula $C_{10}H_{24}O_2$ is 3, 8-dioxa-219-disiladec-5-ene, 2, 2, 9, 9-tetramethyl, which is synthetic and not relevant to the present study (Pollock and Stevens 1965c). The search result identified the chemical compound related to $C_{25}H_{42}$ as pentamethyl eicosapentaene. The chemical nature of this compound was identified as synthetic (Pollock and Stevens 1965a) and not related to the present study.

The signal at Rt 16.483-16.533 min in sample RP8 is related to the chemical $C_{15}H_{24}O$ and was identified as butylated hydroxy toluene. The chemical was reported to be completely soluble in water and is related to the phenolic compound 2,4-D, that is known for herbicidal property (Anonymous 1956). The presence of this phenolic compound in many fungal toxins was known. The compound $C_{15}H_{24}$ was related to neoclovene, an acid-catalysed rearrangement product of caryophyllene. This compound was reported as intermediary compound in metabolic products of few plants, but its toxicity was not proved (Stevens 1969). The presence of silica in chemical formula of many identified compounds was due to TMS derivations reaction in GC/MS analysis.

## Characterization and identification of toxin

Based on the above analysis it was identified that the metabolites of *C. falcatum* has seven possible toxic fractions, *viz*., $C_{12}H_{30}O_4$, $C_{16}H_{40}O_5$, $C_{21}H_{50}O_7$, $C_{17}H_{29}NO_5$, $C_{24}H_{62}O_6$, $C_{30}H_{50}O_4$ and $C_{15}H_{24}O$. These fractions constitute phenolic compounds, sugars, acids and few esters and these seven compounds individually or in combination may constitute the toxic substance that play a major role in red rot disease development. Similar to the present findings, presence of several fractions of toxin in still cultures of pathogen has already been reported. Grove *et al*. (1966) characterized two fraction of toxin i.e., $C_{28}H_{42}O_7$ and $C_{14}H_{20}O_6$ from *C. capsici* and Gohbara *et al.* (1978) identified three phytotoxic substances ($C_{28}H_{42}O_7$ and $C_{14}H_{20}O_9$ and $C_{29}H_{42}O_8$) in the metabolites of *C. nicotianae*. Soledade *et al*. (2008) also identified several fractions of maculansin, a phytotoxin produced by *Leptospheria maculans*. In the present study among the identified chemical compounds $C_{17}H_{29}NO_5$ appeared consistently (Rt 10.1 min) in all the biologically active fractions tested and the same was closely matched with victoxinine, a toxin from *H. victoriae*. Further investigation on this fraction will elucidate the structure of *Cf* toxin and reveal the closeness of these two toxins.

Contrary to the findings of the present study Olufolaji and Bamgboye (1986) reported the chemical nature of toxin of *C. falcatum* as anthroquinones i.e., emodin and chrysophanol. Chemically the compound emodin is insoluble in water and crysophanol is sparingly soluble in water and alcohol and highly soluble in chloroform (Anonymous 1956). Whereas in the same report Olufolaji and Bamgboye (1986) reported the eluted toxic fraction as highly water soluble but concluded the compounds as anthroquinones that are originally insoluble/sparingly soluble in water. The conclusion of these authors may be wrong by another way also as they characterized the toxin only based on UV spectrophotometer analysis, which is not a confirmatory chromatogramphic technique. Though the chemical nature of the toxin fractions isolated in the present study did not confirm with the findings of Olufolaji and Bamgboye (1986), the solubility pattern, host range, other physical parameters are in agreement with their finding and also few other earlier reports (Mohanraj *et al*. 1995, 2002).

## CONCLUSIONS

We conclude that the red rot pathogen *C. falcatum* produces toxic metabolites in still culture that are soluble in water, methanol and diethyl ether. The semi-purified form of toxin produces characteristic symptom of red rot disease at a concentration of 5000 mg/l and are host specific. The purified and biologically active portion of culture extracts of *C. falcatum* has seven toxic compounds with chemical formula of $C_{12}H_{30}O_4$, $C_{16}H_{40}O_5$, $C_{21}H_{50}O_7$, $C_{17}H_{29}NO_5$, $C_{24}H_{62}O_6$, $C_{30}H_{50}O_4$ and $C_{15}H_{24}O$. These identified seven compounds are directly toxic to sugarcane plants and we assume that they may also act in combination during pathogenesis and red rot disease development. Among these seven compounds $C_{17}H_{29}NO_5$ (methoxy phenyl pyruvic acid) occurred repeatedly in all biologically active fractions that produced severe scorching on sugarcane leaves and is considered to play a major role in red rot pathogenesis. Further elaboration of this experiment with other chromatographic techniques like NMR, IR spectrum, X-ray crystallography is in progress, which will elucidate the structure of *Cf* toxin and there by help the scientists to understand plant-pathogen interaction and management of red rot.

## CONCLUDING REMARKS

The prospective studies on the flavonoid pathway give us evidence that TFs are the most promising molecular tools to engineer plant secondary metabolite pathways as they allow a few transformation events to coordinately regulate the expression of genes and cellular differentiation involved in the biosynthesis of a metabolite. However, it was inferred that ORCAs by themselves were unable to control all the genes of the pathway and hence required additional TFs which could be specifically involved in the control of genes of secologanin and/or vindolin synthesis. This will open the way to screen for bacteria strains compatible to *C. roseus* material able to regenerate a plant. From the above discussion, it could be inferred that acquition of more knowledge about TFs regulating TIAs biosynthetic pathway genes, development of a more acceptable system for genetic transformation and efficient conversion of transformed cells to full fledged plants will greatly facilitate in modelling *C. roseus* for improved production of TIAs and thus could be a boom to the pharmaceutical industry.

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