



P-ISSN: 2349-8528

E-ISSN: 2321-4902

[www.chemijournal.com](http://www.chemijournal.com)

IJCS 2021; 9(1): 3017-3021

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Received: 03-10-2020

Accepted: 11-12-2020

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## GC-MS analysis of metabolites from blast pathogen infected from foxtail millet

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DOI: <https://doi.org/10.22271/chemi.2021.v9.i1ap.11688>

**Abstract**

Foxtail millet blast caused by the fungal pathogen *Magnaporthe grisea* (Anamorph: *Pyricularia grisea*) is an important and most destructive disease. *M. grisea* is a ubiquitous plant pathogen with a wide host range. To understand the host-pathogen interaction system, a knowledge about the role of toxins in disease development is paramount. This may be helpful in development of a new resistant cultivar screening procedures. The crude toxin extracted from *Magnaporthe grisea* (TNFxm1) isolate was determined through Gas chromatography–mass spectrometry (GC-MS) technique. The main constituents were Glycerol and 1, 6 Dihydroxynaphthalene.

**Keywords:** *M. grisea* – GC/MS – crude toxin

**Introduction**

Foxtail millet (*Setaria italica* L.) is known as Italian or German Millet. It is one of the oldest cultivated crops in the world. Globally, it is the second most cultivated millet next to pearl millet. In India foxtail millet has been cultivated primarily in Karnataka, Andhra Pradesh, Rajasthan, Madhya Pradesh, Chhattisgarh, and Tamil Nadu in an area of about 1 million ha (Prasad 2017, Sharma *et al.*, 2014) [14]. In Tamil Nadu, it is cultivated in marginal and non-irrigated condition of mid and high hill areas. The yield limiting factors of the millets are fungal, bacterial, viral, and nematode diseases. The most important foliar diseases of foxtail millet are downy mildew, blast, smut and rust (Strange and Scott 2005). *Setaria* blast incited by the fungus *Pyricularia grisea* Sacc. (teleomorph: *Magnaporthe grisea* (T.T. Herbert) M.E. Barr) affected both forage and grain production (Sharma *et al.*, 2014) [14] and caused yield losses up to 60% (Karthikeyan and Gnanamanickam 2008) [15]. *M. grisea* is a foliar plant pathogen causing diseases on wide range of more than fifty plant species of Poaceae which are economically important agricultural crops including rice, wheat, barley, finger millet, pearl millet and foxtail millet (Talbot 2003, Tanweer *et al.*, 2015, Sharma *et al.*, 2013, Prabhu *et al.*, 1992) [20, 10, 15, 21]. Conidia/ spores of several phytopathogenic fungi proliferate under conducive environmental conditions only. (Staples and Hoch 1987, St Leger *et al.*, 1994) [18, 17]. The conidium has its own chemical inhibitor to suppress the growth of conidial germination and appressorium formation (Liu and Kolattukudy 1999) [6]. Plant pathogenic fungi could cause a serious yield loss and affected the quality of agricultural commodities. The various infectious mechanisms of plant pathogenic fungi could help to develop the novel management strategies. In current scenario, metabolomics has been widely exploited in the field of plant pathology. This is mainly focus on the function of the fungal metabolites and metabolic pathway during fungal growth and development, pathogenesis and interaction with plants. The easy identification and quantification of toxic metabolites were mainly through Fourier-transform infrared spectroscopy (FTIR) and Gas chromatography–mass spectrometry (GC-MS) techniques (Shanmugapackiam *et al.*, (2017) [13].

**Materials and Methods****Collection and isolation of the pathogen**

A total of 32 blast pathogen isolates were collected from the different foxtail millet growing areas of Tamil Nadu including hill areas. The disease infected leaves and sheath portions were collected and cut into small pieces and surface sterilized with 0.1 per cent mercuric chloride

solution for 60 seconds. Then the leaves were continuously washed with sterile distilled water for two times and dried in sterile filter paper. The affected tissues were placed on Potato Dextrose Agar medium and incubated at  $25 \pm 2$  °C for 7 days. Pure cultures of the fungi were obtained by single spore isolation method (Ou 1985) [8]. The identification of the pathogen was done by spore morphology.

#### Gas chromatography–mass spectrometry (GC-MS) technique

**Preparation of sample:** The toxin was isolated from the TNF<sub>x</sub>M1 isolate of *M. grisea*. The *in vitro* toxin was partially purified as per the procedure described by Samiyappan *et al.*, (2003). Erlenmeyer conical flasks containing 100 ml of potato dextrose broth were inoculated with 9 mm mycelial disc of two-week-old *M. grisea* isolate separately. After 20 days of growth under stationary conditions at laboratory temperature ( $25 \pm 1$  °C), the culture filtrates were pooled, filtered through three layers of cheese cloth under sterile conditions and concentrated *in vacuo* at 45°C using rotary evaporator to 10 per cent of its original volume. The condensed material was treated with equal volume of methanol and allowed to precipitate overnight at 4°C. Precipitates were removed by filtering through Whatman No. 1 filter paper. Methanol was evaporated *in vacuo* and the aqueous fraction was extracted three times with equal volume of ethyl acetate using separating funnel. The water fraction containing toxin was evaporated to dryness *in vacuo* at 40°C and dissolved in 10 ml of distilled water.

#### Detection of volatile compound from *M. grisea* toxin

The volatile compounds produced by the virulent isolates of *M. grisea* were analyzed through GC/MS (Thermo scientific Trace GC Ultra DSQ II) equipped with column (30mm × 0.25mm × 0.25µm) under the following conditions. Helium was used as carrier gas with a flow rate at 1ml per minute. One µl sample injection with pre injection of solvent by AI/AS 3000 Method with Split-less mode injection with 30 seconds of sampling time. The column temperature was maintained initially at 50°C at the increasing rate of 10°C/min, no hold was followed by increasing up to 200°C and kept at the same temperature for 2 minutes hold with surge pressure 3kPa and 220 base temperature at right SSL method and 250 base temperature at right ECD method with the Aux 1 MS transfer line at 250°C. The electron impact energy was 70eV, Julet line temperature was set at 2000 °C and the source temperature was set at 200°C. Electron impact (EI) mass scan (m/z) was recorded in the 45-450 aMU range. An ion mass spectrometer and OMA detector were used to monitor the eluted compounds. Compounds were identified by absorbance at nm over 10 to 25min (total analysis time 35min). Particular compounds structures were putatively identified and evaluated by comparing the molecular masses (m/z values) of the eluted compounds with literature data and standards.

#### Detached leaf bioassay

Healthy leaves of 20 days old foxtail millet plant cv. Co (Te) 7 were detached and chopped up into 5 cm pieces. Toxin was injected on the punctured leaf area placed on moistened filter paper in Petridishes. The Petridishes having crude toxin inoculated leaves were kept under incubator ( $25 \pm 2$  °C; 12 h of light and 12 h of darkness) for 5 days. The symptom development was assessed after 5 days. In control treatment the leaf was injected with sterile water only.

## Results and Discussion

### Morphological confirmation of *M. grisea*

The blast symptoms of foxtail millet collected from the infected leaf and sheath in various regions of Tamil Nadu were noticed in three stages (Plate 1.) *viz.*, circular to oval spots with grayish centre, greyish centre becomes dark brown and spots coalesce and necrosis of leaves.

The virulent pathogen isolate (TNF<sub>x</sub>M1) (Plate 2.) was employed for spore morphological study and the microscopic observation of the pathogen showed hyaline with septate mycelium. The conidia were produced abundantly on specialized stalks, called conidiophores. Pre-infection development in *M. grisea* exhibited three morphologies *viz.*, conidial formation, germ tube elongation and formation of appressorium. The conidia were usually three-celled and produced on the apex of a conidiophore. Infection process of blast pathogen primarily aided when conidia deposited on host tissues and germinated by producing a germ tube and an appressorium. The appressorium melanized structure, formed and from this an infection peg was developed which penetrated the tissue. (Plate 2.). The results are also in confirmation with (Meena, 2006) [7] and (Gashaw *et al.*, 2014) [3] who described the conidia of *M. grisea* as pyriform in shape, round base with narrow apex and produced 3 celled conidia with 2-3 septation.

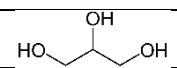
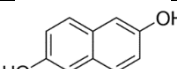
The toxic compounds from *M. grisea* were analyzed through GC/MS to detect the secondary metabolites responsible for pathogenicity. Several compounds have been identified and the compound identity was confirmed through NIST library 2005 AMDIS software programme. Identification of major biotic constituent present in the *M. grisea* isolates would help to acquire the mode and source of infection and further it will help to develop effective disease management strategies. In the present study, the major toxic compounds (glycerin and 1, 8 dihydroxynaphthalene) were identified through GC/MS (Table 1) (Plate 3).

Both the compounds were involved in the formation of appressorium and acted as a virulence factor. This result has been concordance with Wheeler (1983) [22] who found that 1,8 dihydroxynaphthalene was highly helpful in the formation of black or brown colour melanin layer in the appressorium. Melanin acted as a semi permeable layer to allow the solute. In the melanin biosynthesis pathway, the compound namely 1, 8 dihydroxynaphthalene acted as an intermediate, underwent polymerization and finally yielded the compound DHN-melanin. Foster *et al.*, (2017) [2] discussed the role of appressorium in which glycerin was a major solute and created turgour pressure of about 8.0 MPa for the infection in rice. This finding is in accordance with our findings that the similar compound was detected in the analysis. Cessna *et al.*, (2000) [1] proved that the oxalic acid is a major pathogenicity factor for *Sclerotinia sclerotiorum*. Hussaini *et al.*, (2011) [4] reported that the oxalic acid compound was detected in the GC/MS analysis of ripe tomato fruits inoculated with *Aspergillus niger*. They further proved that the oxalic acid compound was the key factor for pathogenicity.

Toxic metabolites have the ability to produce typical blast symptoms on the leaves (Plate 4). Symptom was observed on 7 days after inoculation. The infected leaves were removed and re-isolated for the toxic compounds from the spots, which were similar to the original toxin isolated from the *M. grisea*. The results of the present study was in agreement with the earlier work of Shanmugapackiam *et al.*, (2017) [13] who reported that the crude toxin extracted from *M. grisea* could produce symptoms on detached leaf of finger millet.



**Table 1:** Compounds identified from leaf blast causing *M. grisea* through GC/MS under *in vitro*

S. No	Name of the compound	Molecular formula	Structure	Mol. Weight (g/mol)	Peak area (%)	RT	Metabolic activity	Reference
1.	Glycerin	C <sub>3</sub> H <sub>8</sub> O <sub>3</sub>		92.09	7.47	3.175	Necessary mechanical force for invasion	(Foster <i>et al.</i> , 2017) [2]
2.	1,8 Dihydroxy naphthalene	C <sub>10</sub> H <sub>8</sub> O <sub>2</sub>		160.17	27.44	22.76	Involved in the formation of melanin layer	(Wheeler, 1983) [22]



Circular to oval spot with grayish centre (Initial stage)



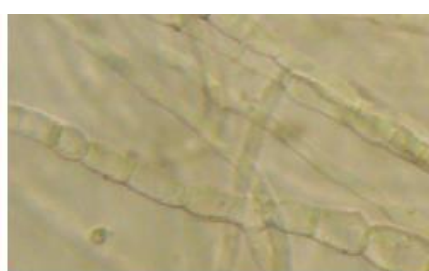
Grayish centre becomes dark brown (Sporulation stage)

Spots coalesce and necrosis of leaves (Final stage)

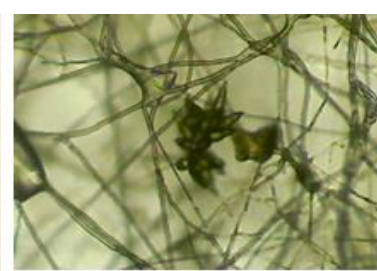
**Plate 1:** Different stages of leaf blast symptom



**Colony growth of *M.grisea***



**Hyaline mycelium (40x)**



**Conidial germination (10x)**



**Conidia with conidiophore (10x)**

Conidia  
Conidiopho



**Conidia with melanized appressorium (40x)**

Appressorium  
Germ tube  
Conidium

**Plate 2:** Morphological characters of *M. grisea*

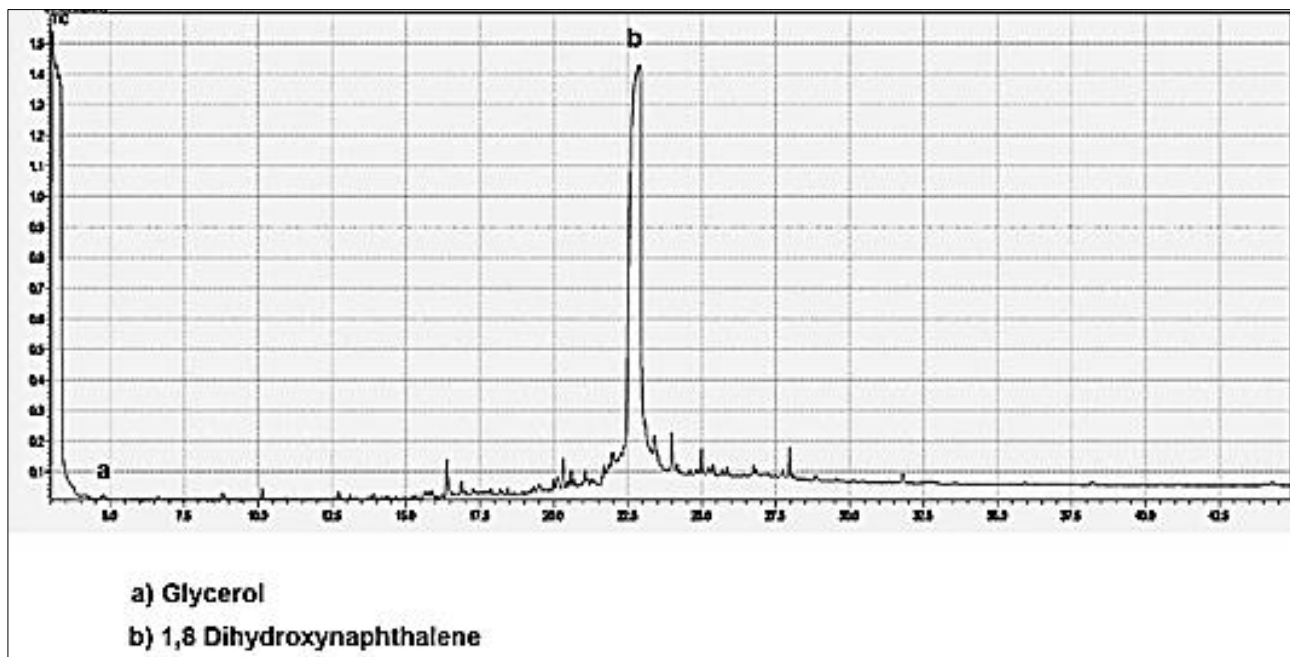


Plate 3: GC-MS chromatogram of toxic compounds from leaf blast causing *M. grisea*

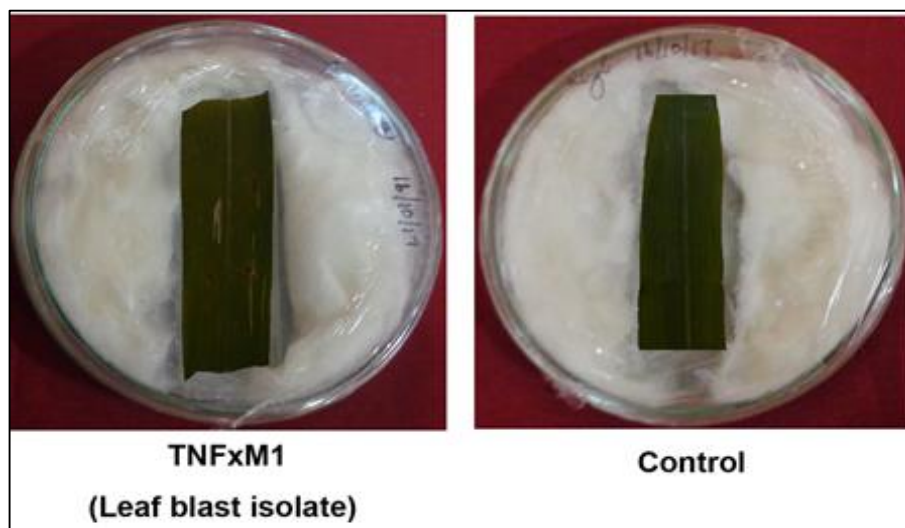


Plate 4: Symptoms produced by crude toxin on foxtail millet leaves (cv. Co (Te) 7)

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