

## TOXIGENIC AND NON-TOXIGENIC FUNGAL METABOLITE SIGNATURES IN MAIZE CULTIVATED IN PAKISTAN

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### Abstract

The current study extends on previous work of fungal species isolation from corn cobs collected from National Agricultural Research Centre (NARC) Pakistan. The Ammonium test on Yeast Extract Sucrose Medium (YES) was used to screen out toxigenic and atoxigenic fungal strains. In present study, by means of Fourier Transform Infrared Spectroscopy (FTIR) and Gas Chromatography–Mass Spectrometry (GCMS), the toxigenic isolates such as *Aspergillus flavus*, *Penicillium chrysogenum*, and *Penicillium oxalicum*, and the non-toxigenic isolates of *Trichoderma harzianum* and *Aspergillus niger* associated with maize, were identified based on their metabolic profiles. Distinct functional groups were revealed in toxigenic and atoxigenic samples by FTIR. A broad spectrum of volatile and semi-volatile metabolites was identified by GCMS. FTIR, based on functional group chemistry, efficiently distinguished functional groups, while compound-level resolution of toxigenic versus atoxigenic metabolic pathways was provided by GCMS. Together both techniques showed that toxigenic isolates withstand metabolite signatures linked with toxin production and pathogenicity, however, non-toxigenic biocontrol fungi showed mild or antifungal metabolite profiles. This work verifies the usefulness of FTIR and GCMS as effective tools for risk assessment, rapid distinction and depiction of fungal communities connected with maize.

**Key words:** Mycotoxins; FTIR; Fungal metabolites, GCMS, Corn cobs

### Introduction

Maize is the most significant crop in Pakistan and its contamination with fungus triggers production of mycotoxin which impacts food safety and human health (Goda *et al.*, 2025). To mitigate mycotoxin contamination metabolic profiles of toxigenic and atoxigenic fungal species are crucial for developing strategies (Mandal *et al.*, 2024).

In Pakistan, due to several reasons such as human and animal health, food safety, agricultural economy, export quality and climate change, profiling of secondary metabolites of toxigenic and atoxigenic fungi is crucial. This profiling can help to develop targeted strategies for fungal management, enhance food safety and security, improve crop quality and yield, and support the agricultural export industry of country.

This study aims to provide insights into the metabolite signature of toxigenic and atoxigenic fungal metabolites in maize contributing to the development of diagnostic tools and strategies for reducing mycotoxin contamination in Pakistan maize crops through FTIR and GCMS analysis. The fungal isolates investigated in this study were isolated for our previous study where corn cob associated fungi were collected from the National Agricultural Research Centre (NARC), Pakistan. Ammonia vapor test on Yeast Extract Sucrose (YES) medium was used for screening of atoxigenic potential which resulted in the identification and classification of

toxigenic and atoxigenic based on their pigmentation response and metabolic characteristic (Khalid *et al.*, 2026). Although FTIR and GC-MS have been used separately for fungal characterization, there are still few investigations that combine the two methods to analyze toxigenic and atoxigenic fungal isolates linked to maize. Additionally, nothing is known about the metabolic fingerprints of fungal species linked to corn cobs in Pakistan. The use of FTIR and GC-MS in tandem to characterize and distinguish previously screened toxigenic (*Aspergillus flavus*, *Penicillium chrysogenum*, and *Penicillium oxalicum*) and atoxigenic (*Trichoderma harzianum* and *Aspergillus niger*) isolates from NARC, Pakistan, is what makes this study novel.

Contamination of fungus in maize considered a major threat to agricultural and public health because of ability of toxigenic species to produce harmful secondary metabolites, specifically aflatoxins and related mycotoxins. *Aspergillus*, *Fusarium* and *Penicillium* produce the most hazardous mycotoxin that threatens quality of grains and food safety (Atnafu, *et al.*, 2024; Syraji *et al.*, 2025). On the other hand, non-toxigenic species such as *Trichoderma harzianum* is considered as natural competitor as it restrains pathogenic fungi through enzyme secretion, mycoparasitic interactions, volatile production and control plant diseases (Meher *et al.*, 2021; Rush *et al.*, 2021). Globally, more than twenty commercial bio control agents have been produced (Xiao *et al.*, 2023; Khan *et al.*, 2021; Haggag *et al.*, 2014; Van *et al.*, 2018; Lahlali *et al.*, 2022).

Biological potential of fungi became clear due to characterizing the biochemical signatures of these fungi and it is easy to identify distinct metabolic profiles, detoxification capability, and potential roles in biocontrol through metabolomics (Alves *et al.*, 2025). Fourier-transform infrared spectroscopy (FTIR) considered as a powerful technique to identify functional groups linked with fungal cell walls, lipids, proteins, and secondary metabolites (Workman *et al.*, 2024; Jain *et al.*, 2025). High-resolution profiling of volatile and semi-volatile compounds is further provided by Gas chromatography–mass spectrometry (GCMS) (Stoppacher *et al.*, 2010; Li *et al.*, 2024; Song *et al.*, 2025).

Metabolites of non-toxicogenic species, such as *T. harzianum* and *A. niger* analyzed by GCMS revealed their biocontrol activity, antagonism and ability to control biosynthesis of toxins. A complete biochemical fingerprint that allows distinction between toxigenic and atoxigenic maize associated fungi are provided by integration of FTIR and GCMS data. These findings contribute to better understanding of fungal metabolic behavior and also set out as potential biomarkers for early detection and differentiation of pathogenic versus beneficial fungi in maize ecosystems.

## Methodology

**Preparation of crude extracts:** On Potato Dextrose Agar (PDA) plates, the fungus was cultivated in the dark at 28°C for 4–5 days. The isolates were cut from the culture plate that was actively growing and inoculated in 500 ml Erlenmeyer flasks with 150 ml of Potato Dextrose Broth (PDB). For 21 days at 27±1°C, the flasks were incubated in a BOD incubator. The endophyte's fermentation broth was filtered using filter paper to remove mycelia mats. To extract the filtrate at room temperature ethyl acetate was used three times. Extract was evaporated by means of rotary vacuum (Mahmud *et al.*, 2020; Kumar *et al.*, 2024).

**Fourier transform infrared (FTIR) spectroscopy:** FTIR was used to identify functional groups present in the ethyl acetate extract of the fungus. To record the FTIR spectra in the wavelength range of 4000–400cm<sup>-1</sup> SHIMADZU 8400 (FT-IR) spectrometer was used. The spectrum provided by FTIR was perceived as sample's biochemical "fingerprint" by measuring the vibrations of bonds within chemical functional groups (Prasher *et al.*, 2017).

**Gas chromatography-mass spectroscopy (GCMS):** The extracted sample was subsequently analyzed by using gas chromatography-mass spectroscopy (GC-MS) fitted with injector. The temperature of injector was regulated at 250°C together with ion source temperature at 280°C. The column temperature was set to increase from 110°C (isothermal for 2 min) to 200°C at 10°C/min, then increased to 280°C at 5°C/min and ultimately reaching at 280°C with 9 minutes isothermal. The injecting volume of sample was 1 µl having split flow ratio of 10:1. Helium was used as carrier gas at constant flow rate of 1ml/min. The mass spectra were taken at 75 eV ionization voltages with mass scan range of 45 to 450 Da. The GCMS analysis for the sample took a total of 36minutes to complete. The individual constituents separated from the plant samples were identified by comparison with the National Institute of Standards and Technology NIST database. The mass

spectroscopy patterns of unknown compounds were compared with known spectra of NIST library. Different compounds with their name, molecular weight and structures were determined (Prasher *et al.*, 2017).

## Results

In order to further understanding of the biochemical basis of toxigenicity and antagonistic activity, Fourier transform infrared (FTIR) spectroscopic and Gas Chromatography Mass Spectrometry (GC-MS) are used to characterize the volatile and semi-volatile of isolated fungi.

**Chemical fingerprinting by FTIR spectroscopy:** The most popular method for locating functional groups is FTIR analysis, which is also the main way to verify that test samples contain metabolites. Additionally, it aids in the identification of bioactive metabolites. The functional groups found in the secondary metabolites isolated from the fungal isolates were identified using FTIR spectroscopy. The types of bonds and biochemical makeup of bioactive compounds are revealed by the FTIR spectra.

Fourier Transform Infrared (FTIR) spectroscopy was used to characterize functional groups present in metabolites of toxigenic isolates of *Aspergillus flavus*, *Penicillium oxalicum*, and *Penicillium chrysogenum*. The spectra recorded in the mid-infrared region (4000–500 cm<sup>-1</sup>) indicate the absorption bands that correlate with structural vibrations of toxigenic and bioactive compounds. Unique spectral patterns exhibited by every isolate expressed its metabolic profile and capacity to produce mycotoxin or secondary metabolites.

Similarly, the spectra of a toxigenic fungi that are *Trichoderma harzianum* and *Aspergillus niger*, also possessed unique transmittance patterns indicating the existence of functional groups such as, carbonyl, hydroxyl, amine, and ester that are linked to bioactive metabolites.

**FTIR analysis of toxigenic fungi:** The functional group present in the metabolites of toxigenic *Aspergillus flavus* (Fig. 1 a) were recognized and compared with literature.

FTIR spectrum of *Aspergillus flavus* shows a discernible absorption at about 2920 cm<sup>-1</sup> because of C–H stretching, which is indicative of aliphatic hydrocarbons that are frequently found in fungal lipids and toxins. The presence of carbonyl (C=O) functional groups linked to lactone rings, which are indicative of aflatoxins, the extremely toxigenic and carcinogenic secondary metabolites generated by this species, is indicated by a sharp and strong peak located close to 1700 cm<sup>-1</sup>. N–O stretching, commonly seen in compounds containing nitroso or nitrate that are involved in mutagenic reactions, is represented by additional peaks in the region around 1500–1400 cm<sup>-1</sup>. The existence of nitrogen-based toxin components is further supported by the presence of C–N stretching peaks. The C–O stretching of ethers, alcohols, and polysaccharides, which is characteristic of fungal cell wall structures and oxygenated toxin derivatives, is correlated with strong absorptions between 1200 and 1000 cm<sup>-1</sup>. All things considered, these spectral characteristics clearly show the synthesis of compounds linked to aflatoxin that are mutagenic, carcinogenic, and hepato-toxicogenic.

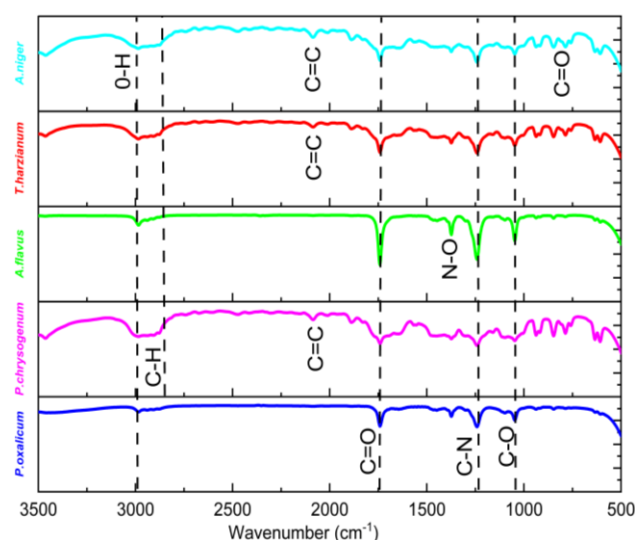


Fig. 1. FTIR spectra of (a) *Penicillium oxalicum* (b) *Penicillium chrysogenum* (c) *A. flavus* (d) *Trichoderma harzianum* (e) *Aspergillus niger*.

FTIR spectrum of *Penicillium oxalicum* (Fig. 1b) highlights the main absorption peaks and functional group indicating the presence of secondary metabolites involved in mycotoxin biosynthesis.

In the *Penicillium oxalicum* FTIR spectrum shows the presence of hydroxyl and carboxyl functionalities, which are characteristic of organic acids like oxalic acid produced by this species, is confirmed by the strong O–H stretching band visible at about  $3400\text{ cm}^{-1}$ . The C–H stretching from aliphatic groups is represented by the absorptions at about  $2920\text{ cm}^{-1}$ , which suggests the presence of lipid-like components or long-chain secondary metabolites. The strong peak at about  $1700\text{ cm}^{-1}$  indicates the presence of carbonyl stretching, which is frequently connected to lactones, quinone structures, and acidic metabolites that are linked to cytotoxicity and tissue corrosion. Peaks in the  $1500\text{--}1400\text{ cm}^{-1}$  range show C–N stretching, emphasizing nitrogenous compounds, whereas the  $1200\text{--}1000\text{ cm}^{-1}$  range shows C–O stretching vibrations of alcohols, polysaccharides, and esters. All of these indicators point to the production of oxidative and corrosive metabolites by *P. oxalicum*, and the potential for oxalic acid production may increase the toxicity and pathogenicity of grains that have been stored.

The FTIR spectrum of *Penicillium chrysogenum* (Fig. 1c) showed a broad absorption band in the region around  $3400\text{ cm}^{-1}$  that corresponds to O–H stretching vibrations, indicating the presence of hydroxyl groups, which are generally connected to secondary metabolites that possessed antimicrobial and cytotoxic properties. The absorption band in the region around  $2920\text{--}2850\text{ cm}^{-1}$  correlates to C–H stretching of aliphatic chains indicating the existence of lipid-like components in the fungal extract. The clear and sharp absorption around  $1700\text{ cm}^{-1}$  corresponds to C=O stretching of carbonyl groups which is attributes of esters, aldehydes, and lactones present in a variety of bioactive fungal metabolites. The additional peaks observed between  $1500$  and  $1400\text{ cm}^{-1}$ , represented C–N functional vibrations suggesting nitrogen-containing

substances such as alkaloids, peptides and potential penicillin precursors.

Finally, noticeable C–O stretching exhibits the fingerprint region between  $1200$  and  $1000\text{ cm}^{-1}$ , indicating the presence of polysaccharides, oxygenated toxins and carbohydrates. When combined, these functional groups show that the fungus produces a number of bioactive substances with cytotoxic properties.

Different transmittance patterns among the toxigenic fungal isolates are shown in the comparative FTIR spectra (Fig. 1) confirming variation in their structural and biochemical compositions (Table 1).

**FTIR analysis of atoxigenic fungi:** The relative FTIR spectrum (Fig. 1d) of atoxigenic fungal isolate *Trichoderma harzianum* exhibit sharp transmittance patterns, displaying differences in their structural and biochemical makeup (Table 1). Remarkable absorption peaks were present in *T. harzianum*'s FTIR spectrum that correlated to significant functional groups linked with secondary metabolites of fungus. A broad and intense band at about  $3400\text{--}3500\text{ cm}^{-1}$  indicating O–H stretching vibration. This shows the presence of hydroxyl groups from alcohols or phenolic compounds, which are frequently associated with antioxidant activity. In volatile antifungal metabolites alkynes or unsaturated compounds are present, exhibiting C≡C stretching, that corresponds the absorption peak nearly  $2100\text{--}2200\text{ cm}^{-1}$ . The biological activity of carbonyl groups from esters, carboxylic acids, or amides compounds was affirmed the strong band around  $1650\text{--}1740\text{ cm}^{-1}$  assign to C=O stretching.

The additional bands observed nearly  $1400\text{--}1550\text{ cm}^{-1}$  suggesting C–N stretching, corresponds amide or amine functional groups characteristic of peptide-like metabolites. A indicated the presence of alcohols, esters, or ether linkages presented by a peak close to  $1050\text{--}1200\text{ cm}^{-1}$  indicating C–O stretching. These functional groups verify that *T. harzianum* produces a wide range of bioactive metabolites, including esters, phenolic compounds and fatty acids that possess antifungal and biocontrol properties. Atoxigenic species with potential antioxidant qualities are supported by the preponderance of O–H and C–O vibrations indicating a rich composition of non-toxic secondary metabolites.

The FTIR spectrum of atoxigenic fungal isolates of *Aspergillus niger* show potential bioactive compounds and functional group composition (Fig. 1e). FTIR spectrum of *A. niger* also showed various functional groups presenting a number of distinctive absorption bands. A broad absorption band at about  $3400\text{ cm}^{-1}$  confirmed the presence of hydroxyl groups, possibly from alcohols or phenols represented the O–H stretching vibration. Aliphatic chains of fatty acids and hydrocarbons were observed by sharp peak near  $2900\text{ cm}^{-1}$  was related to C–H stretching. An absorption band at about  $2100\text{--}2200\text{ cm}^{-1}$  indicated the presence of alkyne or unsaturated compounds suggesting C≡C stretching. The presence of amide or proteinaceous compounds was confirmed by the strong peak at  $1650\text{--}1740\text{ cm}^{-1}$ , which correlated with C=O stretching, and the peaks at  $1550\text{--}1400\text{ cm}^{-1}$ , which correlated with C–N stretching. Alcohols, esters, and ethers are known for their C–O stretching, which was demonstrated by another

noteworthy band at 1050–1200  $\text{cm}^{-1}$ . The ability of *A. niger* to produce oxygenated compounds, including alcohols, esters, and amides, is demonstrated by its FTIR profile and is consistent with a toxigenic secondary metabolism. Strong O-H and C-O vibrations and a low number of reactive. The carbonyl absorption may be associated with metabolites related to aflatoxin biosynthetic pathways and support the idea that *A. niger* is a biochemically active but atoxigenic strain, which is consistent with its established use as a safe industrial strain and biocontrol.

#### GCMS analysis of toxigenic *Aspergillus flavus*:

Metabolites of *Aspergillus flavus* are identified by GCMS. The peaks and retention time is presented in the chromatograph (Fig. 2a) and different major and minor bi compounds are detected (Table 2).

**3-Docosenamide (erucylamide):** is a long-chain fatty-acid amide and in the current profiles the most abundant individual metabolite ( $\approx 32\%$ ). High abundance indicates it is a major secondary metabolite of this isolate of *A. flavus* and probably indicates extensive lipid metabolism. Fatty-acid amides like erucylamide have been shown to possess in vitro antimicrobial and cytostatic activity and can modify membrane properties and cell signaling and, accordingly, their presence could be implicated in local cytotoxicity during grain infection and indirectly with metabolic conditions favorable for biosynthesis of aflatoxins.

**Pentadecanoic acid:** or pentadecylic acid is an odd-chain saturated fatty acid widely distributed in toxigenic fungi like *Aspergillus flavus* and the second largest peak with retention time 23.6 and peak area ( $\sim 30\%$ ) is reported herein. In toxigenic *A. flavus*, pentadecanoic acid was

linked with heightened lipid peroxidation and oxidative stress, which are the primary inducers of aflatoxin biosynthesis.

**Tetradecanamide:** is a mid-chain fatty-acid amide present here in significant abundance ( $\sim 17\%$ ). Fatty-acid amides and their N-acyl lipid analogues are bioactive lipids often implicated in causing membrane perturbation, signaling and cytotoxicity dose-dependently. In fungal extracts they are usually found together with other lipid metabolites that provide precursors for polyketide/mycotoxin biosynthetic routes; thus tetradecanamide is plausibly both a regulator of microbial interaction as well as a constituent of the general toxigenic environment created by aflatoxigenic *A. flavus*.

**9-Octadecenal:** (an unsaturated long-chain aldehyde) is a chemically reactive electrophile. Aldehydes can form covalent adducts with lipids and proteins, causing oxidative stress and cellular damage. Although its peak area is moderate ( $\sim 5\%$ ), reactive aldehydes when combined with aflatoxins can increase oxidative damage and protein dysfunction in affected tissues and thereby become toxigenologically significant even though in lower abundance.

**Hexadecane:** is a linear alkane that is commonly present in fungal VOC profiles. Alkanes are generally of low systemic toxigenicity with environmental exposures but can act as membrane-interacting hydrophobic compounds and aspirational risks in high concentrations. In fungal metabolite mixtures they are mainly indicative of lipid catabolism and contribute physical/chemical complexity to the extract but are not the dominant toxigenic. They can, however, influence the persistence of compounds and physical interactions with host tissue.

**Table 1. Characteristic IR absorptions of toxigenic (*P. oxalicum*, *P. chrysogenum*, *A. flavus*) and atoxigenic (*T. harzianum*, *A. niger*).**

|                                    |                                                                                                                                                                                               |
|------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| ( <i>Penicilliumoxalicum</i> )     | C–N stretch, C–O stretch, N–H bend, –C=O– stretch, O–H stretch, H–bonded                                                                                                                      |
| ( <i>Penicillium chrysogenum</i> ) | C–Br stretch, C–N stretch, C–O stretch, C=O stretch, –C≡C– stretch, O–H stretch, H–bonded                                                                                                     |
| ( <i>Aspergillus flavus</i> )      | C–N stretch, C–O stretch, N–O symmetric stretch, C–C stretch (in–ring), C–H bend, C–C stretch (in–ring), N–H bend, N–O symmetric stretch, –C≡N– stretch, –C≡C– stretch, O–H stretch, H–bonded |
| ( <i>Trichoderma harzianum</i> )   | C–N stretch, C–O stretch, N–O symmetric stretch, N–H bend, –C=C– stretch, C=O stretch, O–H stretch, H–bonded                                                                                  |
| ( <i>Aspergillus niger</i> )       | C–N stretch, C–O stretch, C–N stretch, N–H bend, N–O asymmetric stretch, C–C stretch, C–H rock, –C≡C– stretch, C–H stretch, O–H stretch, H–bonded                                             |

**Table 2. Metabolites of *Aspergillus flavus* identified by GCMS.**

| S. No. | Compound name                    | Molecular formula                      | Retention time (min) | Area (%) | Molecular weight |
|--------|----------------------------------|----------------------------------------|----------------------|----------|------------------|
| 1.     | 13-Docosenamide, (Z)-            | $\text{C}_{22}\text{H}_{43}\text{NO}$  | 27.5                 | 32.27    | 337              |
| 2.     | Pentadecanoic acid               | $\text{C}_{15}\text{H}_{30}\text{O}_2$ | 23.6                 | 30.16    | 242              |
| 3.     | Tetradecanamide                  | $\text{C}_{14}\text{H}_{29}\text{NO}$  | 25.5                 | 17.10    | 227              |
| 4.     | 9-Octadecenal                    | $\text{C}_{18}\text{H}_{34}\text{O}$   | 24.5                 | 4.93     | 266              |
| 5.     | Hexadecane                       | $\text{C}_{16}\text{H}_{34}$           | 22.6                 | 1.19     | 226              |
| 6.     | Phthalic acid, butyl octyl ester | $\text{C}_{30}\text{H}_{50}\text{O}_4$ | 21.9                 | 0.56     | 474              |
| 7.     | 2-Methyltetracosane              | $\text{C}_{25}\text{H}_{52}$           | 22.6                 | 0.84     | 352              |
| 8.     | 1-Octadecene                     | $\text{C}_{18}\text{H}_{36}$           | 20.80                | 0.55     | 252              |
| 9.     | Heneicosane                      | $\text{C}_{21}\text{H}_{44}$           | 16.8                 | 0.31     | 296              |
| 10.    | 2,4-Di-tert-butylphenol          | $\text{C}_{14}\text{H}_{22}\text{O}$   | 14.45                | 1.04     | 206              |

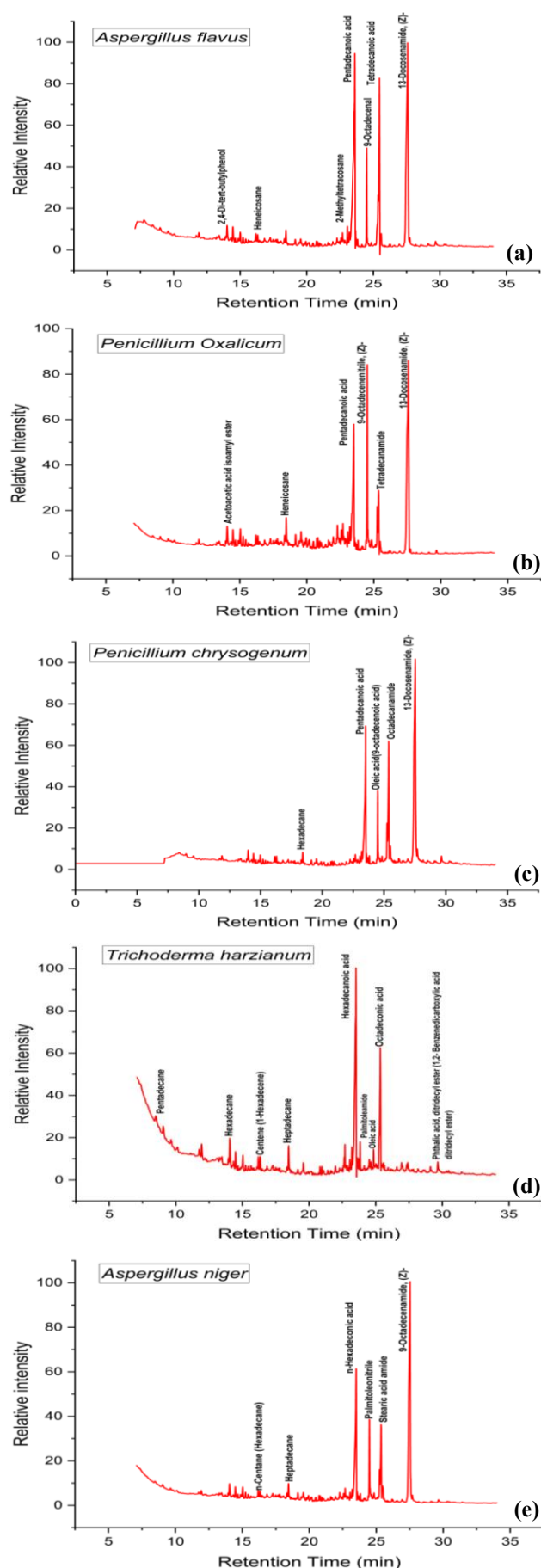


Fig. 2. GCMS profile of (a) *Aspergillus flavus* (b) *Penicillium oxalicum* (c) *Penicillium chrysogenum* (d) *Trichoderma harzianum* (e) *Aspergillus niger*.

An ester of the **phthalate** class, at even low abundances (~0.6%), is of toxicological concern because numerous phthalate esters are endocrine disruptors with animal model reproductive/hepatic toxicities. There are two qualifications: (1) phthalates occasionally originate from laboratory plastics (analytical contamination), so procedural blanks must be taken; (2) if indeed fungal-produced, trace amounts would represent a chronic toxicogenic burden when added to aflatoxin exposure.

**2-Methyltetracosane:** is a branched long-chain alkaneparaffin hydrocarbon. These high molecular weight hydrocarbons are common fungal VOC or extract components and are in the main ecological/metabolic markers (surface activity, hydrophobic interactions, possible antioxidant roles), rather than direct systemic poisons. Some phytochemical studies, however, report free-radical scavenging or mild bioactivities for such alkanes.

**1-Octadecene:** is a low-abundance unsaturated hydrocarbon (alkene). Unsaturated hydrocarbons are oxidizable to reactive intermediates and could thus be indirectly involved in oxidative stress mechanisms. It is likely one of the complex mixture of volatiles here; as an individual compound, it is a low-abundance constituent but could be participating in redox chemistry that intersects with aflatoxin-catalyzed oxidative processes.

**Heneicosane:** is a long-chain alkane found in trace amounts. Like other alkanes in the profile, it is mostly a marker of lipid catabolism and VOC makeup. It makes little direct contribution to mammalian toxicity at environmental levels, but is part of the toxicogenic signature of a strain and possesses potential ecological effects on spore adhesion or insect interaction.

**2, 4-DTBP:** is a phenolic secondary metabolite that is widely reported among fungi and other organisms. It is antimicrobial, antioxidant and cytotoxic in nature and may modulate oxidative balance and cell signaling. 2, 4-DTBP in a toxicogenic *A. flavus* extract may respond to aflatoxin-mediated oxidative stress pathways and to the overall biological activity and toxicogenicity profile, even though a minor peak (~1%).

**GCMS analysis of toxicogenic *Penicillium oxalicum*:** Metabolites of *Penicillium oxalicum* are identified by using GCMS and obtain chromatograph (Fig. 2b) while different compounds are also examined (Table 3).

The table shows the GCMS analysis of *Penicillium oxalicum* metabolites, where eight chemical compounds can contribute to the toxicogenic or cytotoxic nature of this fungal isolate.

The primary metabolite, **13-docosenamide (Z)-** with high abundance (30.54%) suggests it's a major metabolite that has a fundamental role in the toxicogenicity along with **9-octadecenitrile (Z)-**(11.8%), **Pentadecanoic acid** (15.6%), and **tetradecanamide** (8.6%) possess membrane disruption activity that leads to cell lysis.

The occurrence of **phthalic acid octylebutyle ester** (2.18%), a recognized endocrine disruptor also suggests potential harmful consequences on metabolic and hormonal functions.

Other compounds such as **olean-18-ene** (0.95%) and **heneicosane** (2.08%) also play a role in cytotoxicity.

Overall, these metabolites provide evidence that *P. oxalicum* is able to generate a variety of toxigenic and bioactive compounds that are likely to have adverse effects on living cells, making it a potential risk when lined with infested maize kernels.

Thus, the *Penicillium oxalicum* maize kernel isolate possesses toxigenic potential and this may result in health problems if the infected maize is ingested or not well stored.

**GCMS analysis of toxigenic *Penicillium chrysogenum*:** Secondary metabolites of *Penicillium chrysogenum* are identified by using GCMS and obtain chromatograph (Fig. 2c) while different compounds are also examined (Table 4).

GCMS analysis of the *P. chrysogenum* extract revealed various bioactive fatty acid derivatives such as 13-docosenamide (Z) - (39.8%), pentadecanoic acid (21.64%), **octadecanamide** (17.8%), **oleic acid** (5.8%), and **hexadecane** (18.4%). These chemicals are known to possess high cytotoxic activity.

Fatty acids such as oleic acid have the ability to trigger oxidative stress, membrane disruption and apoptosis in mammalian and plant cells. The fatty acid amides and acids present in *P. chrysogenum* extract are accountable for cytotoxic activities. Their higher concentrations may

cause oxidative stress, lipid-induced toxigenicity and cell death, thus potentially being toxigenic to host organisms such as, humans, animals and plants upon exposure. Disruption of lipid metabolism and cell membrane integrity caused by amide derivatives such as steramide and erucamide resulting in cell death at higher doses. Hexadecane, the hydrocarbons character of the compounds is responsible to overall toxigenicity by intruding with enzymatic and respiratory function.

A hydrocarbon that is a linear-chain hydrocarbon and toxigenic to aquatic organisms and causes cell membrane damage. In biological organisms, it can accumulate and disrupt cell respiration and enzyme functions. It is moderately rated cytotoxic and may be a contributor to overall toxigenicity of the fungal extract.

Thus while these metabolites increase the competitiveness of *P. chrysogenum* in survival, they are also potential cytotoxins with food safety and human health implications.

**GCMS analysis of atoxigenic *Trichoderma harzianum*:**

Gas chromatography mass spectrometry of ethyl acetate extract of *Trichoderma harzianum* culture filtrate revealed the presence of ten major compounds with varying retention times and peak areas (Table 5) and chromatograph (Fig. 2d).

**Table 3. Secondary metabolites analyzed by GCMS from *Penicillium oxalicum*.**

| S. No. | Compound name                                                                       | Molecular formula                              | Retention time (min) | Area (%) | Molecular weight |
|--------|-------------------------------------------------------------------------------------|------------------------------------------------|----------------------|----------|------------------|
| 1.     | 9-Octadecenitrile, (Z)-                                                             | C <sub>18</sub> H <sub>33</sub> N              | 24.5                 | 11.8     | 263              |
| 2.     | 13-Docosenamide, (Z)-                                                               | C <sub>22</sub> H <sub>43</sub> NO             | 27.5                 | 30.54    | 337              |
| 3.     | Pentadecanoic acid                                                                  | C <sub>15</sub> H <sub>30</sub> O <sub>2</sub> | 23.5                 | 15.6     | 242              |
| 4.     | Tetradecanamide                                                                     | C <sub>14</sub> H <sub>29</sub> NO             | 25.4                 | 8.6      | 227              |
| 5.     | Olean-18-ene                                                                        | C <sub>30</sub> H <sub>50</sub>                | 22.6                 | 0.95     | 410              |
| 6.     | Phthalic acid, butyl octyl ester, (1,2-Benzenedicarboxylic acid, butyl octyl ester) | C <sub>20</sub> H <sub>34</sub> O <sub>4</sub> | 21.9                 | 2.18     | 334              |
| 7.     | Heneicosane                                                                         | C <sub>21</sub> H <sub>44</sub>                | 18.5                 | 2.08     | 296              |
| 8.     | Acetoacetic acid isoamyl ester                                                      | C <sub>9</sub> H <sub>16</sub> O <sub>6</sub>  | 14.04                | 1.93     | 172              |

**Table 4. Secondary metabolites of *Penicillium chrysogenum* analyzed by GCMS.**

| S. No. | Compound name                    | Molecular formula                              | Retention time (min) | Area (%) | Molecular weight |
|--------|----------------------------------|------------------------------------------------|----------------------|----------|------------------|
| 1.     | 13-Docosenamide, (Z)-            | C <sub>22</sub> H <sub>43</sub> NO             | 27.5                 | 39.8     | 337              |
| 2.     | Pentadecanoic acid               | C <sub>15</sub> H <sub>30</sub> O <sub>2</sub> | 23.5                 | 21.64    | 242              |
| 3.     | Octadecanamide (stearamide)      | C <sub>18</sub> H <sub>37</sub> NO             | 25.4                 | 17.8     | 283              |
| 4.     | Oleic acid (9-octadecenoic acid) | C <sub>18</sub> H <sub>34</sub> O <sub>2</sub> | 24.4                 | 5.8      | 282              |
| 5.     | Hexadecane                       | C <sub>16</sub> H <sub>34</sub>                | 18.4                 | 1.27     | 226              |

**Table 5. Metabolites identified by GCMS from *Trichoderma harzianum*.**

| S. No. | Compound name                                                                    | Molecular formula                              | Retention time (min) | Area (%) | Molecular weight |
|--------|----------------------------------------------------------------------------------|------------------------------------------------|----------------------|----------|------------------|
| 1.     | Hexadecanoic acid (Palmitic acid)                                                | C <sub>16</sub> H <sub>32</sub> O <sub>2</sub> | 23.5                 | 32.49    | 256              |
| 2.     | Octadecanoic acid                                                                | C <sub>18</sub> H <sub>36</sub> O <sub>2</sub> | 25.3                 | 14.01    | 284              |
| 3.     | Eicosane                                                                         | C <sub>20</sub> H <sub>42</sub>                | 24.8                 | 8.72     | 282              |
| 4.     | Palmitoleamide                                                                   | C <sub>16</sub> H <sub>32</sub> O <sub>2</sub> | 23.8                 | 2.58     | 256              |
| 5.     | Oleic acid                                                                       | C <sub>18</sub> H <sub>34</sub> O <sub>2</sub> | 25.0                 | 2.47     | 282              |
| 6.     | Phthalic acid, ditridecyl ester (1, 2-Benzenedicarboxylic acid ditridecyl ester) | C <sub>34</sub> H <sub>58</sub> O <sub>4</sub> | 29.6                 | 1.47     | 530              |
| 7.     | Heptadecane                                                                      | C <sub>17</sub> H <sub>36</sub>                | 18.4                 | 2.83     | 240              |
| 8.     | Cetene (1-Hexadecene)                                                            | C <sub>16</sub> H <sub>32</sub>                | 16.1                 | 2.58     | 224              |
| 9.     | Hexadecane                                                                       | C <sub>16</sub> H <sub>34</sub>                | 14.0                 | 3.10     | 226              |
| 10.    | Pentadecane                                                                      | C <sub>15</sub> H <sub>32</sub>                | 9.6                  | 0.96     | 212              |

**Table 6. Identification of secondary metabolites of atoxigenic *A. niger* analysed by GCMS.**

| S. No. | Compound name                       | Molecular formula                              | Retention time (min) | Area (%) | Molecular weight |
|--------|-------------------------------------|------------------------------------------------|----------------------|----------|------------------|
| 1.     | 9-Octadecenamide, (Z)-              | C <sub>18</sub> H <sub>35</sub> NO             | 27.5                 | 38.8     | 281              |
| 2.     | n-Hexadeconic acid                  | C <sub>16</sub> H <sub>32</sub> O <sub>2</sub> | 23.5                 | 20.28    | 256              |
| 3.     | Palmitoleonitrile                   | C <sub>16</sub> H <sub>29</sub> N              | 24.4                 | 7.08     | 235              |
| 4.     | Stearic acid amide (octadecanamide) | C <sub>18</sub> H <sub>37</sub> NO             | 25.4                 | 12.43    | 283              |
| 5.     | Heptadecane                         | C <sub>17</sub> H <sub>36</sub>                | 18.4                 | 2.12     | 240              |
| 6.     | Eicosane                            | C <sub>20</sub> H <sub>42</sub>                | 22.6                 | 1.46     | 282              |
| 7.     | n-Cetane (Hexadecane)               | C <sub>16</sub> H <sub>34</sub>                | 16.3                 | 0.41     | 226              |

The compounds that are identified are fatty acids, hydrocarbons, esters, and amides which are known to responsible for important antifungal, antioxidant and biocontrol activities.

The most abundant compound identified was **Hexadecanoic acid (palmitic acid)** which made up 32.49% of the total area at retention time of 23.5 minutes. Palmitic acid is a saturated fatty acid commonly reported in *Trichoderma* species and is known to possess significant antifungal and antibacterial activity by disturbing cell membrane stability and inhibition of spore germination of phytopathogens.

The second most common metabolite was **Octadecanoic acid (stearic acid)** with 14.01% of the total peak area. It is another saturated fatty acid that has also been found to have antimicrobial and antioxidant activities, contributing to the oxidative stability of fungal cells and blocking lipid peroxidation.

**Eicosane**, a hydrocarbon chain (C<sub>20</sub>H<sub>42</sub>) of larger chain length and retention time of 24.8 minutes and relative abundance of 8.72% was previously researched as a volatile antifungal substance involved in the inhibition of mycelium growth and germination of spores of toxigenic *Aspergillus flavus* and *Fusarium verticillioides*.

Another prominent compound, **Palmitoleamide** (2.58%), is a membrane-altering unsaturated fatty acid amide that participates in signaling processes capable of mediating *Trichoderma*'s communication with roots or competition with other fungi within the rhizosphere.

**Oleic acid** (2.4%), an unsaturated fatty acid, has wide-ranging antifungal and antioxidant activities. The ester compound Phthalic acid, dodecyl ester (**1,2 - Benzenedicarboxylic acid dodecyl ester**) was also found with a retention time of 29.6 minutes and an area percentage of 1.47. Even less frequent, phthalic acid esters are commonly associated with antimicrobial, antioxidant, and plant growth-stimulating activity as signaling molecules that potentially influence microbial interactions in the rhizosphere.

Hydrocarbons like **Heptadecane** (2.83%), **Hexadecane** (3.10%), **Pentadecane** (0.96%), and **Cetene** (1-Hexadecene, 2.58%) were also identified. They are classical long-chain alkanes and alkenes of *Trichoderma* volatiles and are accountable for the antagonism of fungi by fumigant action, reducing aerial spore and mycelia fragment viability of the inoculable fungi.

As a whole, GC-MS findings confirm that *T. harzianum* produces an intricate mixture of bioactive volatile compounds with speculative antifungal, antioxidant, and signaling functions. Dominance by fatty acids and hydrocarbons indicates that these metabolites

have a central role to play in modulating the biocontrol potential of *T. harzianum* towards toxigenic fungi of maize by inhibiting growth, spore formation, and suppression of mycotoxin biosynthesis.

GC-MS profiling of *Aspergillus niger* extract revealed the occurrence of diverse bioactive volatile and semi-volatile compounds, with predominantly fatty acid derivatives, hydrocarbons, and amides. The putative mycotoxigenic compound and other metabolites present in *A. niger* are responsible for its antifungal and antioxidant activity, hence playing the role of an atoxigenic competitor against toxigenic fungal species in maize kernels. Details are given (see figure 2 e and table 6).

The highest intensity compound that was detected was 9-Octadecenamide, (Z)-, at a peak area of 38.8% at a retention time of 27.5 minutes. The compound, otherwise known as oleamide, is an amide of a long-chain fatty acid that has been said to exhibit high antifungal and antibacterial activity by interfering with membrane permeability and mycelial growth inhibition.

In-Hexadeconic acid, a long-chain saturated fatty acid, is one of the major metabolites produced by *Aspergillus niger* under GCMS profile. The relatively larger peak area (20.28%) indicates its high density and suggests its key role in the organism's physiological and ecological functions, particularly in antifungal, antibacterial and antioxidant activities.

The stearic acid amide (octadecanamide) compound appeared with a frequency of 12.43%, confirming the amide-type fatty compound biosynthesis.

Some volatile hydrocarbons such as cyclic alkanes such as Heptadecane (C<sub>17</sub>H<sub>36</sub>) Eicosane (C<sub>20</sub>H<sub>42</sub>), and n-Cetane (Hexadecane, C<sub>16</sub>H<sub>34</sub>) also present at moderate level and play inhibitory and signaling functions.

## Discussion

This study provides both compound level resolution (from GCMS) and functional group level insight (from FTIR) producing a strong biochemical characterization of toxigenic and non-toxigenic maize associated fungi. Differences in the chemical signatures of the toxigenic (*A. flavus*, *P. chrysogenum*, *P. oxalicum*) and non-toxigenic (*T. harzianum*, *A. niger*) isolates were presented by FTIR spectroscopy. In toxigenic strains strong carbonyl (C=O) absorption was observed which represent a high abundance of ketone, ester and carboxyl functional groups, correlated with polyketide synthesis, active lipid metabolism and mycotoxin related precursor biosynthesis. This shows agreement with other researches that describe metabolically active and toxin-producing species have

complex secondary metabolite pools (such as fatty acids and polyketides) and higher lipid turnover (Scarpari *et al.*, 2014). On the other hand, predominance of aliphatic C–H stretches and decreases carbonyl intensity in *T. harzianum* and *A. niger* presents a higher percentage of structural biomolecules, such as cell wall polysaccharides and proteins than polyketide toxins, supporting their atoxigenic biocontrol aligned metabolism. Bioactive compounds of *T. harzianum* identified by GCMS analysis are dodecane, decane, octane, 1-monocyclohexene, and alcohols, all known for antifungal activity. This takes into account its strong antagonistic effect, as prevailed from our bioassay data in the form of growth inhibition values. Atoxigenic metabolic nature of *A. niger* was attributed by simple alkanes, organic acids, and terpenoid derivatives.

In the current study, FTIR analysis revealed toxigenic strains (*A. flavus*, *P. chrysogenum*, *P. oxalicum*) presented strong absorption peaks around 3300–3400  $\text{cm}^{-1}$ , corresponding to –OH and N–H stretching displaying high metabolic activity and protein turnover. Absorption peak around 1640–1740  $\text{cm}^{-1}$  showed enhance carbonyl (C=O) stretching aligned with ketone, elevated ester and lipid-derived metabolites linked with active mycotoxin biosynthesis. While, non-toxicogenic *T. harzianum* and *A. niger* presented aliphatic C–H stretching at 2850–2920  $\text{cm}^{-1}$ , and reduced C=O intensity, correspond their production of fatty acid derived volatiles and cell wall polysaccharides preferably than polyketide-derived toxins. Such functional group are in consistent with previous findings where metabolic attenuation in atoxigenic strains corresponds to lower oxidative stress signatures and alter lipid utilization (Scarpari *et al.*, 2014; Maggio *et al.*, 2005).

The dominance of long chain alkanes, fatty acid methyl esters, aromatic derivatives, and bioactive alcohols are exhibited by toxigenic species which act as precursors or side-products for biosynthesis of mycotoxin. Among others, *flavus* generated hexadecane, heptadecane, benzene derivatives, octadecane, and methylated alkanes, in agreement with previous literature attributing these volatiles to aflatoxin-related metabolic pathways of the fungus (Josselin *et al.*, 2021; Moore *et al.*, 2023; Song *et al.*, 2020). A variety of esters, aldehydes, and hydrocarbons were produced in the VOC profiles of *P. chrysogenum* and *P. oxalicum*, demonstrating active fatty-acid  $\beta$ -oxidation, a key supporting mechanism for polyketide toxin formation.

Numerous compounds derived from fatty acids, such as hexadecanoic acid and long-chain esters/amides, are probably connected to the supply of acetyl-CoA and malonyl-CoA through  $\beta$ -oxidation, which are essential substrates for polyketide synthase pathways of aflatoxin biosynthesis (Uka *et al.*, 2020).

Collectively, our GC–MS results indicate that *A. flavus* isolates of corn seed produce a metabolically diverse range of compounds that both promote the biochemical biosynthesis of aflatoxins (via fatty-acid derived acetate building blocks) and introduce secondary bioactive metabolites with probable toxigenicological relevance. These findings accord with prior biochemical studies linking lipid metabolism to aflatoxin biosynthesis (Watanabe *et al.*, 1996; Chanda *et al.*, 2009; Lin *et al.*, 2026) and broaden the risk profile of contamination beyond quantitation of aflatoxins alone. In reality, the presence of

bioactive amides/phenolics and fatty-acid precursors in aflatoxigenic cultures highlights the need for combined toxigenicological analysis, blurring chromatographic aflatoxin analysis with individual analyses or bioassays on other fungal metabolites to evaluate fully the risk to animal and human health posed by contaminated maize. Atoxigenic isolates like *Aspergillus niger* and *Trichoderma harzianum*, on the other hand produced saturated hydrocarbons and fatty acid derivatives, including palmitoleonitrile, stearic acid amide, crocetane, 2-octyldecan-1-ol, and n-cetane, which are familiar to possess inert or antifungal properties. Interestingly, several metabolites, such as long-chain alcohols and phthalates, were found in both groups, indicating a capacity.

## Conclusion

*Aspergillus flavus* isolates from maize seeds produce several metabolites linked to aflatoxin production in addition to other bioactive chemicals that may increase toxigenicity, according to GC–MS research. This demonstrates that the risk of fungal contamination is not just related to aflatoxins; toxigenicological analysis should also take other metabolites into account. On the other hand, atoxigenic isolates like *Trichoderma harzianum* and *Aspergillus niger* mostly produced fatty acid derivatives and saturated hydrocarbons, which are often less toxigenic and may have antifungal qualities.

The five fungal isolates recovered from substrates linked with maize have different biochemical and metabolic profiles, according to FTIR and GC–MS studies. The isolates were successfully distinguished by FTIR using distinctive functional groups, such as O–H, C–H, C=O, C–N, and C–O vibrations, which represent differences in their secondary metabolic activity and biochemical makeup. Compound-level characterization was also made possible by GC–MS, which revealed metabolite fingerprints unique to each species. While *Penicillium chrysogenum* and *P. oxalicum* were dominated by docosenamide, pentadecanoic acid, octadecanamide, oleic acid, and 9-octadecenitrile, indicating active secondary metabolism, *Aspergillus flavus* was characterized by abundant 13-docosenamide, pentadecanoic acid, tetradecanamide, and 9-octadecenal. On the other hand, metabolites with antibacterial and antagonistic properties, including hexadecanoic acid, octadecanoic acid, oleic acid, 9-octadecenamide, and octadecamide, were generated by the atoxigenic isolates *Trichoderma harzianum* and *Aspergillus niger*. By combining quick functional-group fingerprinting with thorough metabolite identification, the combined use of FTIR and GC–MS improved discriminatory performance. In post-harvest maize systems, our integrated metabolic profiling approach shows promise as a quick screening tool for differentiating potentially hazardous fungal contaminants from beneficial fungi. It may also support future biocontrol initiatives and food safety monitoring. However, isolates' geographic origin, ambient variables, and culture conditions can all affect their metabolic profiles. In order to assess the biocontrol capability of *T. harzianum* and *A. niger* and validate the reliability of FTIR-based diagnostic markers, more validation utilizing larger fungal samples and a variety of growth circumstances is necessary.

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