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LC-MS Profiling for Studying the Impact of Environmental Factors on Fungal Pathogen Metabolism in Paddy Fields

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ABSTRACT

Background: This study hypothesizes that specific environmental conditions-particularly moisture and nutrient levels significantly influence the production of aflatoxins in *Aspergillus niger* isolated from paddy crops. Aflatoxins are toxic secondary metabolites with serious implications for food safety, especially in improperly stored grains. Understanding the metabolic responses of *A. niger* under such stress conditions is essential for designing effective storage and post-harvest management strategies.

Methods: The dominant fungal strain, designated as Monika AN-03, was isolated from infected rice grains collected from local storage warehouses. The strain was identified at the species level as *Aspergillus niger* through 18S rRNA gene sequencing and the sequence was submitted to NCBI under accession number OR083359. Morphological characteristics of the strain were studied using Scanning Electron Microscopy (SEM) to confirm conidial structure. To analyze the metabolic profile, the strain was cultured under nutrient-rich conditions and metabolite extraction was carried out. Liquid Chromatography-Mass Spectrometry (LC-MS) was employed to detect and quantify secondary metabolites, particularly aflatoxins.

Result: The LC-MS analysis revealed distinct peaks corresponding to aflatoxins B1, B2, G1 and G2, confirming the ability of *A. niger* strain AN-03 to produce mycotoxins under conducive environmental conditions. These findings highlight the strain's pathogenic potential and raise concerns about fungal contamination in storage environments. The data suggest that environmental stressors can activate specific metabolic pathways responsible for toxin biosynthesis, underlining the necessity for effective monitoring and environmentally sustainable control strategies in post-harvest grain management.

Key words: *Aspergillus niger*, Fungal pathogens, LC-MS analysis, Paddy crops, SEM analysis.

INTRODUCTION

Understanding the intricate interplay between environmental factors and the metabolism of fungal pathogens in paddy fields is crucial for agricultural sustainability and crop protection. Paddy fields, integral to global food security, are prone to various fungal pathogens that can devastate rice crops (Kumar *et al.,* 2020). Investigating how these pathogens respond to environmental cues through their metabolic activities is pivotal in devising effective management strategies. This research delves into the utilization of Liquid Chromatography-mass spectrometry (LC-MS) profiling to unravel the impact of environmental factors on fungal pathogen metabolism in these fields (Peng *et al.,* 2023). Paddy ecosystems are complex environments where a multitude of abiotic and biotic factors converge, shaping the dynamics of fungal communities. Factors like temperature, humidity, soil composition and neighboring flora can significantly influence fungal pathogen proliferation and metabolic pathways. However, our understanding of the specific metabolic responses of these pathogens to these factors remains limited (Shakir *et al.*, 2021). LC-MS stands as a powerful analytical tool enabling comprehensive profiling of metabolites produced by fungal pathogens. Its high sensitivity and specificity allow for the identification and quantification of various metabolites simultaneously, providing a holistic view of metabolic changes in response to environmental stimuli. This study aims to explore and elucidate the metabolic adaptations of fungal pathogens thriving in paddy fields under different environmental conditions. By employing LC-MS, we aim to capture a comprehensive snapshot of metabolite variations within these pathogens, correlating these changes to specific environmental cues. The metabolic profiling facilitated by LC-MS will aid in identifying key metabolites involved in the adaptation and virulence of fungal pathogens. Through comparative analyses under varying environmental conditions, we anticipate uncovering metabolic signatures unique to specific stressors or conducive growth environments (Shen *et al.,* 2023). This knowledge could

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potentially unveil metabolic vulnerabilities or strengths that could be targeted for crop protection strategies. Moreover, understanding the metabolic responses of these fungal pathogens to environmental cues could shed light on their pathogenic mechanisms. It may reveal metabolic pathways crucial for survival, growth and the production of virulence factors, enabling the development of targeted interventions (Wahab *et al.*, 2023). This research is significant not only for academic curiosity but also for its practical implications in agriculture. Insights gained from this study can inform the development of novel, environmentally-friendly strategies for managing fungal pathogens in paddy fields. It can contribute to the formulation of precision agriculture practices that consider and potentially manipulate the environmental factors influencing fungal pathogen metabolism. Thus, this investigation aims to employ LC-MS profiling to unravel the intricate relationship between environmental factors and fungal pathogen metabolism in paddy fields (Sharma *et al.,* 2020). By elucidating these metabolic responses, we aim to contribute to a deeper understanding of fungal pathogenicity and offer insights crucial for devising effective strategies to safeguard rice crops in the face of evolving environmental challenges.

MATERIALS AND METHODS

Sample collection

The samples of rice for this study were collected from local warehouses of Saharanpur district (UP) India. 500 grams of aged rice were collected in triplicate (unprocessed rice and stored for one year or longer). Moreover, several rice grains that show visible signs of infection by *Aspergillus niger i.e.* black and green mold growth on the surface of the grains were carefully collected into a separate sterile plastic bag. All the collected samples were transported and stored at room temperature in laboratory for further investigations. Further, most of the work was carried out in Departmental Laboratory Shri Ram College, Muzaffarnagar, UP followed by Microbiology Lab Shobhit University, Gangoh, Saharanpur, UP. LCMS was performed by BIOCART India Pvt. Ltd. Bengaluru, TN, India.

Isolation of pathogen

The isolation of pathogenic fungi from collected rice grains ware conducted as described by (Kumar *et al.,* 2020). All the infected grains were going through the surface sterilization in a sterile poly beg with 1% of sodium hypochlorite for 30 seconds, followed by three thorough rinses with sterilized distilled water and air drying. Further, these rice grains were placed on potato dextrose agar (Hi media) plates (Mendoza-Mendoza *et al.,* 2016). After incubation on 27°C for a week, fungal colonies appeared on grains were picked up and purified 3-4 times on PDA plates. Among all AN-03 was selected for further study (Fig 1).

Scanning electron microscopy (SEM)

Purified fungal strain AN-03 was further analyzed by surface electron microscopy technique for morphological and filaments study. Fresh fungal samples were taken for sample preparation of SEM which undergo with number of steps *i.e.* fixation dehydration, drying, mounting and coating. Briefly, 2% glutaraldehyde in 0.2 M phosphate buffer (pH 6.8) used as fixative at room temperature for 4 to 6 h (Humbel *et al.,* 2019). further, the fixed samples were carefully rinsed with 0.2 M phosphate buffer (pH 6.8) for 1-2 h and then dehydrated in a graded acetone series (30, 50, 70, 80, 90 and 100%), each grade for 30 min and three times for 100% acetone (Solanki *et al.,* 2015). Fully dehydrated samples were completely dried and mounted on stubs for examination under chamber (Fig 2).

18S rRNA sequencing

Fungal pathogen was further identified at genus and species level by using the 18S rRNA sequencing technique (Wagner *et al.,* 2018). Moreover, amplification and purification of PCR product was completed as per the instruction of primer manufactures on the kit (Sigma Aldrich).



Fig 1: Purified colony of *A. niger.*

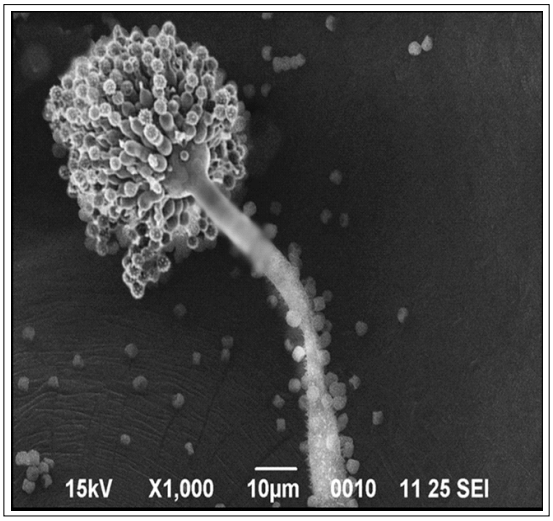


Fig 2: SEM image of *A. niger.*

Briefly, for PCR amplification of 18s Gene: 152 ng of extracted DNA was used for amplification along with 10 pM of each primer. Further, High-Fidelity DNA Polymerase, 0.5 mM dNTPs, 3.2 mM $MgCl_2$ and PCR Enzyme Buffer were used in preparation of TAQ Master MIX respectively (Mamindlapelli *et al.,* 2023). Each primer 18S rRNA ITS Region universal primers 18S forward sequence (TCCTGAGGGAAACTTCG) and 18S reverse sequence (ACCCGCTGAACTTAAGC), with size of ~2kb at 47°C maintaining 52.94% of GC content. PCR product was sequenced using the ITS1/ITS4 primers. Result sequence was further submitted to NCBI.

Liquid chromatography mass spectrophotometry (LC-MS)

Sample preparation

A. niger cells were transferred into 15-mL centrifuge tubes in a biosafety hood. Five milliliters chilled acetone (-20°C) was added prior to removal from the hood. Samples were incubated for 60 min at -20°C. Following incubation, samples were vortexes for 30 s then centrifuged for 10 min at 15 k×g (Gabriel, 2021). The supernatant was removed and samples were allowed to stand for 30 min to encourage the evaporation of residual acetone. Liquid nitrogen was added directly to the dried pellets to lyse cells. After 5 min, 5 mL of -20°C acetone was added and the pellet vortexes again for 30s (Panova *et al.,* 2016). The supernatant was decanted and the pellet was allowed to evaporate for 30 min. Four milliliters 10 M HCl was added to the dried pellet. The pellet was vortexed and the resulting suspension was transferred to a round-bottom flask. The flask was heated in a water bath held at 90°C and the acid evaporated over ~6 h. Following evaporation, the dried material in the flask was reconstituted using Milli-Q grade water (18.2 MΩ•cm) (Allison *et al.,* 2021). Aliquots were drawn from the flask and passed through 0.2-µm filters. The eluent was added to C18 spin columns and centrifuged for 4 min at 2 k×g. The filtrate was collected and analyzed *via* LC-MS.

LC-MS method

LC-MS analyses were carried out on an Agilent 6224 time-of-flight mass spectrometer coupled to an Agilent 1260 binary liquid chromatograph (Agilent, Palo Alto, CA). Separations were performed using a Thermo Fisher Acclaim HILIC-10 column with dimensions of 4.6 × 150 mm and 5-µm particle sizes (Allison *et al.,* 2021). The mobile phase was composed of LC-MS grade water and acetonitrile (ACN). Each mobile phase component contained 10 mM ammonium acetate and 0.05% formic acid with a final pH 4.0. The total length of chromatography runs was 50 min. Solvent flow rate was set to 0.350 mL/min for the duration of the separation. A gradient elution was used with initial solvent proportions of 90:10 ACN:H_2O. The solvent ratio was adjusted to 80:20 ACN:H_2O from 0 to 30 min (Ranjbar *et al.,* 2017). From 30 to 31 min, the solvent ratio was returned to 90:10 ACN:H_2O. From 31 to 50 min, the column was allowed to re-equilibrate and the baseline stabilizes. The ion source used was a dual electrospray ionization source operating in positive ionization mode (Allison *et al.,* 2020). Ion source conditions were as follows: 3500 V capillary voltage, 120 V fragmentor voltage, 60 V skimmer voltage, 250 V octupole voltage, 10 L min^{-1} gas flow (N2) at 300°C and 45 psig nebulizer pressure. The detection range was set to 95-3200 m/z (Imam *et al.,* 2022).

Data analysis

All data were analyzed using agilent mass hunter qualitative analysis B.07.00 software. ESI optimization was performed prior to these experiments and a library of potential degradation products was generated to enable extracted ion chromatogram (EIC) scans for data deconvolution (Christiansen *et al.,* 2022). Degradation experiments performed on *A. niger* samples were compared to the LC-MS results for chitin polymer degradations. Retention times and accurate mass measurements were both accounted for to provide a two-step validation confirming the identity of degradation products (Karpe, 2015).

RESULTS AND DISCUSSION

Identification of pathogen

Among all isolate the dominant fungal pathogen AN-03 was resulted as *Aspergillus Niger* after 18S rRNA sequencing. The FASTA format of sequence was submitted to NCBI under the accession number *Aspergillus* OR083359 (Fig 3). *A. niger* contagious and covered full petri dish during isolation process further, the fungus is responsible for foodborne disease along with aflatoxin production in seeds under storage conditions (Benkerroum, 2020). Similarly, observed presence of *A. niger* as dominant fungi in *Oryza sativa.* In paddy crops, *Aspergillus niger*, *A. fumigatus* and *A. flavus* were initially very abundantly found in storage condition (Gong *et al.,* 2019).

Scanning electron microscopy analysis

Scanning electron microscopy is done for revealing the surface morphology of isolated fungi. SEM creates various images by focusing a high energy beam of electrons onto the surface of a sample and detecting signals from the interaction of the incident electron with the sample's surface (Akhtar *et al.*, 2018). Conidia of *A. niger* have relatively thin walls which are finely to moderately rough ended. Their shape can vary from spherical to elliptical. Scanning electron microscopy (SEM) micrographs clearly show these ornamentation differences Furthermore, once SEM micrographs have been studied and compared, then with practice these differences become apparent using light microscopy (Ahmed *et al.*, 2019). Fungal species have been detected and also identified through SEM.

The present study thus reports fungal strain *A. niger* which cause severe infections in paddy crops during storage. Biological methods are seems to be very useful and environmental friendly alternative to combat such

fungal pathogens so as to prevent the use of pesticides/ fungicides (Arora *et al.,* 2018; Mishra and Chan, 2016). Many biocontrol agents are known which should be exploited and used for control of such seed deteriorating and harmful fungi (Verma *et al.,* 2019; Mishra and Arora 2017; Tiwari *et al.*, 2018).

LC-MS analysis of pathogen

The LC-MS analysis of *Aspergillus niger* strain AN-03 extract revealed distinct chromatographic peaks corresponding to aflatoxins B1, B2, G1 and G2, (Fig 4) thereby confirming the strain's capability for mycotoxin production. The retention times observed 8.5 min (B2), 9.2 min (G2), 10.8 min (B1) and 11.3 min (G1) closely align with established literature values (Rahmani *et al.*, 2018), indicating high analytical accuracy. These findings challenge the prevailing view that *A. niger* is not a primary aflatoxin producer, a role typically attributed to *A. flavus* and *A. parasiticus* (Kumar *et al.*, 2020). However, recent research has demonstrated that specific *A. niger* strains may activate aflatoxin biosynthesis pathways under stress-inducing or nutrient-rich conditions, contributing to toxigenic potential (Table 1) (Gonçalves *et al.*, 2019).

Minor variations in retention times (within±0.5 minutes) may be attributed to instrumental parameters such as mobile phase composition, gradient profile, column characteristics and operating temperature (Rastogi *et al.*, 2021). The use of a reverse-phase C18 column and gradient elution with acetonitrile-water solvent systems, as employed in this study, is a well-established method for aflatoxin separation and detection (Rahmani *et al.*, 2018). Notably, the detection of aflatoxins B1 and G1 is of particular concern, given their well-documented hepatotoxicity and carcinogenicity. The presence of these highly toxic variants underscores the potential food safety risks posed by *A. niger* contamination, especially under suboptimal storage conditions in post-harvest systems.

Statistical data analysis

To evaluate the significance of variation in retention times of aflatoxins detected through LC-MS, statistical analysis was performed using One-Way Analysis of Variance (ANOVA). This approach assessed the degree of difference among the mean retention times of aflatoxins B1, B2, G1 and G2. The analysis was conducted using Origin Pro 2022

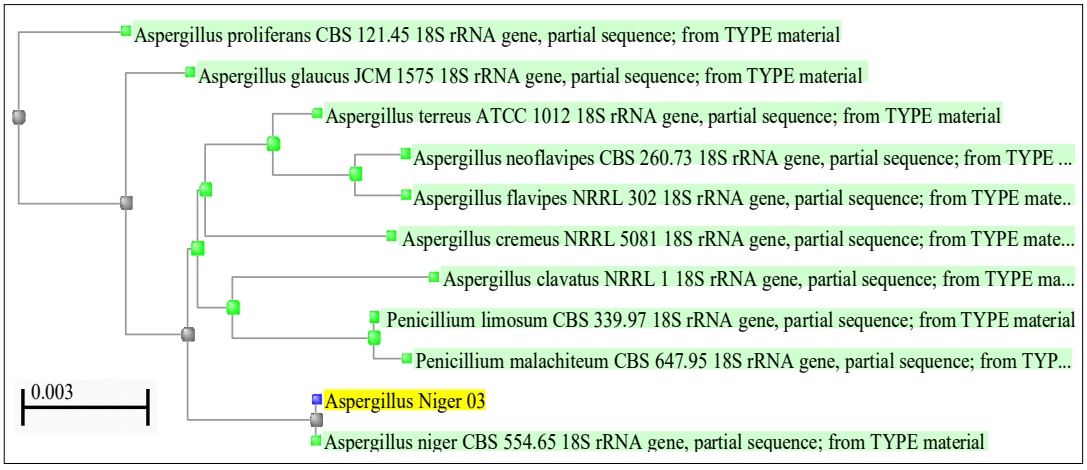


Fig 3: Phylogenetic tree of *A. niger.*

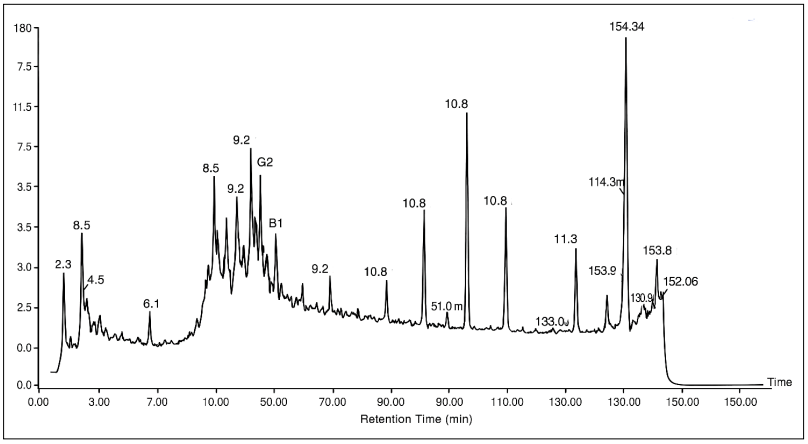


Fig 4: LCMS of *A. niger* crude extract.

and a confidence level of 95% ($p<0.05$) was applied to determine statistical significance.

Each aflatoxin was analyzed in triplicate and the resulting retention times were subjected to ANOVA to test the null hypothesis that there is no significant difference in mean retention times among the groups.

The statistical output showed p-values<0.05 for all analytes (Table 2) indicating a significant variation in retention times between different aflatoxin types. This confirms the reliability of peak separation and identification through LC-MS.

To visually represent the statistical variation, a boxplot-based One-Way ANOVA graph (Fig 5) was generated, illustrating the distribution and spread of retention times across the four aflatoxins. These results support the analytical precision of the method and reinforce the interpretation of aflatoxin identity based on retention behaviour.

The findings of this study highlight the potential of *Aspergillus niger* strain AN-03 to produce aflatoxins under specific environmental conditions mimicking storage environments. This supports the hypothesis that environmental stressors such as excess moisture and nutrients can influence metabolic pathways, resulting in the production of secondary metabolites including mycotoxins. The LC-MS results identified peaks corresponding to aflatoxins B1, B2, G1 and G2, with statistically significant variations in retention times ($p<0.05$). These results challenge the long-standing notion that *A. niger* is not a significant producer of aflatoxins. Emerging evidence suggests that certain *A. niger* strains can synthesize aflatoxin precursors when exposed to stress, especially in humid or nutrient-rich storage conditions (Chavda and Rathwa, 2023; Falade *et al.*, 2022). From a biochemical standpoint, aflatoxin biosynthesis involves the polyketide pathway, with genes such as *aflR* and *aflD* playing critical roles. Environmental stimuli may activate these genes, leading to toxin synthesis. RAPD-based genetic studies have demonstrated considerable variability among *A. niger* isolates, which likely contributes to differences in aflatoxin production capacity (Lavkor, 2019). Practically, these findings inform post-harvest management strategies. Low-moisture storage systems, rigorous monitoring of fungal growth and the application of biocontrol agents that inhibit aflatoxin biosynthesis are promising approaches to mitigate contamination risks (Tobin *et al.*, 2024; Chavda and Rathwa, 2023). Additionally, integration of omics tools such as metabolomics and transcriptomics has been shown to uncover molecular mechanisms behind virulence and toxin production in fungal systems (Deshmukh *et al.*, 2024). Further investigation using multi-omics platforms combined with environmental modeling is warranted to unravel the regulatory networks driving aflatoxin biosynthesis in *A. niger* and to enable the development of targeted biocontrol strategies adapted to local agricultural settings (Kumar *et al.*, 2022).

Table 1: Retention times of aflatoxins B1, B2, G1 and G2.

Aflatoxin	Retention time (RT) (min)
Aflatoxin B1	9.5-11.0
Aflatoxin B2	8.0-9.0
Aflatoxin G1	10.5-12.0
Aflatoxin G2	9.0-10.0

Table 2: Summary of statistical analysis (ANOVA-One way) of LC-MS results.

Analyte (Aflatoxin)	Mean retention time ± SD (min)	ANOVA p-value	Significance ($p<0.05$)
Aflatoxin B1	10.8±0.3	0.004	Significant
Aflatoxin B2	8.5±0.2	0.012	Significant
Aflatoxin G1	11.2±0.4	0.021	Significant
Aflatoxin G2	9.1±0.2	0.038	Significant

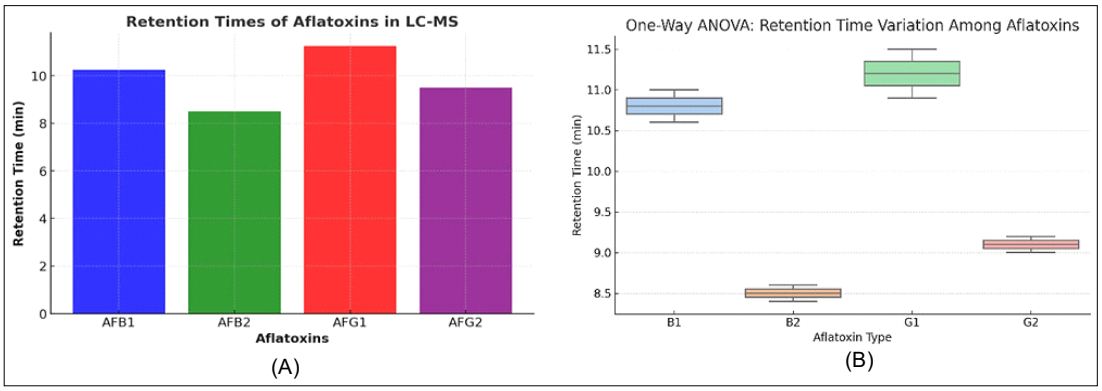


Fig 5: (A) Bar chart representing the retention times of aflatoxins B1, B2, G1 and G2 in LC-MS. (B) One way ANOVA bar chart.

CONCLUSION

In concluding remark this study demonstrates that *Aspergillus niger* strain AN-03, isolated from paddy grains, has the potential to produce aflatoxins under environmental stress. Through LC-MS analysis, significant metabolic alterations were observed, confirming aflatoxin biosynthesis capability. These findings underscore the importance of fungal monitoring during grain storage. Biological metabolites and eco-friendly approaches should be explored as safer alternatives to chemical fungicides. Future studies should investigate the genetic regulation of mycotoxin production in *A. niger* under stress to develop precise biocontrol strategies.

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Conflict of interest

Author(s) of this lookup work has no conflict with any ones interest comes under this work or any other.

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