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Identification of *Aspergillus Fumigatus* using Molecular Techniques

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Abstract

Plant pathogenic fungi belong to the Kingdom Fungi comprising of over 100,000 registered species grouped into about 4,300 genera. Many of these fungi infect a variety of cereals particularly stored condition. A study was conducted to evaluate the occurrence of Plant pathogenic fungi associated with stored *Sorghum vulgare* grains. The fungal was subjected to the fungal load for exact identification using the molecular biology technique. The fungal isolates were isolated from the collected grains. Results revealed that Genus *Aspergillus fumigatus* was the most frequent isolate which are known to produce Aflatoxins. A combination of many different isolates was present on the grains. The molecular identification method using Polymerase Chain Reaction (PCR) helped in the identification of fungi at the species level with precision and in the least possible time. In conclusion, the isolated species were identified morphologically as *Aspergillus fumigatus*. The fungal samples were then identified through molecular techniques by DNA sequencing which was identified as *Aspergillus fumigatus*. The molecular techniques used in this study, has added great benefits to the process of distinguishing between similar species of fungi in comparison with the classical techniques. Furthermore, there is a need for having a National Level, Germplasm collection centre and detailed database of all the naturally occurring post-harvest Plant pathogenic fungi for easy intervention and timely overcoming of situations in case of any emergency.

Keywords: Fungi Identification, *Aspergillus fumigatus*, Molecular techniques, Plant pathogens

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Introduction

The name "Fungi" is a Greek word and simply represents the most popular member of the group known as Mushroom. Although Fungi are not easily defined in a single term by most mycologists, they can be described as a diverse congregation of eukaryotic, achlorophyllous, chemo-organotrophic, spore-bearing living organisms that were formerly regarded as plants. They were therefore initially grouped with algae in the division "thallophyta" probably because of their enormous similarities, especially in reproductive behaviours.[1] However, they possess many differences from the typical plants hence, in the five-kingdom classification; the fungi are separated and grouped into a separate kingdom (kingdom fungi), comprising of about 50,000 to 100,000 registered species usually grouped into about 4,300 genera.[2]

Cereals and derived products represent an important nutrient source for mankind world-wide. In addition, Sorghum is being cultivated in Karnataka during *kharif* (area 3.20 lakh ha) and *rabi* (area 12.19 lakh ha) seasons, meeting the dual need of grain as well as fodder. There is a large area under late *rabi*/summer irrigated sorghum, which has higher productivity (4 tonnes/ha). In southern Karnataka, forage sorghum is gaining popularity. The demand for sorghum stover and fodder (green) is increasing and hence dual purpose cultivars are preferred. The common cereals which are used include maize, sorghum and millet. Unfortunately, cereals are naturally contaminated with fungi in the field, during drying, processing, transportation and subsequent storage and it may be difficult to completely prevent mycotoxins formation in contaminated commodities, particularly those that are produced in tropical and subtropical climates, in countries where high temperature and humidity promote the growth and proliferation of fungi.[3] Thus, they are often colonized by fungi, including species from the genus *Aspergillus*, *Penicillium* and *Fusarium*, which cause significant reductions in crop yield, quality and safety due to their ability to produce mycotoxins. Mycotoxins commonly occurring in cereals and cereal products include zearalenone, fumonisins, trichothecenes (as deoxynivalenol and T2-T2), ochratoxin and aflatoxins.[4] It was reported that 25-50% of harvested world crops have been contaminated with mycotoxins.[5] A number of surveys have been carried out to identify a general pattern of toxigenic fungi and mycotoxins contamination in crops that are dried prone to contamination.

Cereals are attacked by a number of fungi at every stage of development.[6] Reported that fungi cause about 50-80% damage to farmers' grain during the storage period or when conditions are favorable for their development resulting in significant loss both quantitatively and qualitatively. In addition, fungi produce mycotoxins which are hazardous to man and animals. Various reports have shown yield losses of up to 67%.[7-9,10] The storage fungi damage the grains in several ways; they reduce the germinability, produce undesirable odour and kernel discoloration, depletion in seed viability, hardness, colour, size and shape, grain weight and various biochemical parameters; protein, carbohydrate and vitamins decrease the food value and also produce toxins injurious to the health of consumers.[11,12]

The present study was designed to molecular level identification using PCR technique for identification of different isolates from a group of isolates parasitizing upon the sorghum. Culture characteristics, direct microscopy and histopathology have been the foundation for identification of fungal infection for many decades. Every fungal species is unique.

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Therefore, every description of a fungal species is also unique. The morphological, physiological, ecological, and molecular diversity in fungi means that descriptions and illustrations differ from one taxonomic group to another. But sometimes identification of fungi by colony characteristics and direct microscopy does not assure the species as spores of fungi in same genus looks alike and also some fungi produce different types of colonies. However, to overcome this, DNA sequencing of fungi is done to identify fungi at species level.

Materials and Method

Reagents used

- 1% aqueous sodium hypochlorite
- 10% sodium hypochlorite solution
- Insta Gene™ Matrix Genomic DNA isolation kit
- 1% agarose gels
- Ethidium bromide
- Distilled water

Samples collection

Samples of *Sorghum vulgare* were purchased randomly from the two major grain markets from Karnataka state. The samples were packed in polyethylene bags, brought to the laboratory and labelled accordingly for further processing.

Sample Preparation

The grains were surfaced sterilized by dipping into 0.2% aqueous sodium hypochlorite solution for 1 min, followed by three successive rinses in sterile distilled water. The grains were blotted dry in between sterile plated on Potato Dextrose Agar (PDA) at the rate of 25 grains per plate and incubated at a temperature of 25 ± 2°C for 2 days. Mixed growth was then sub-cultured to obtain axenic cultures (Figure 1).

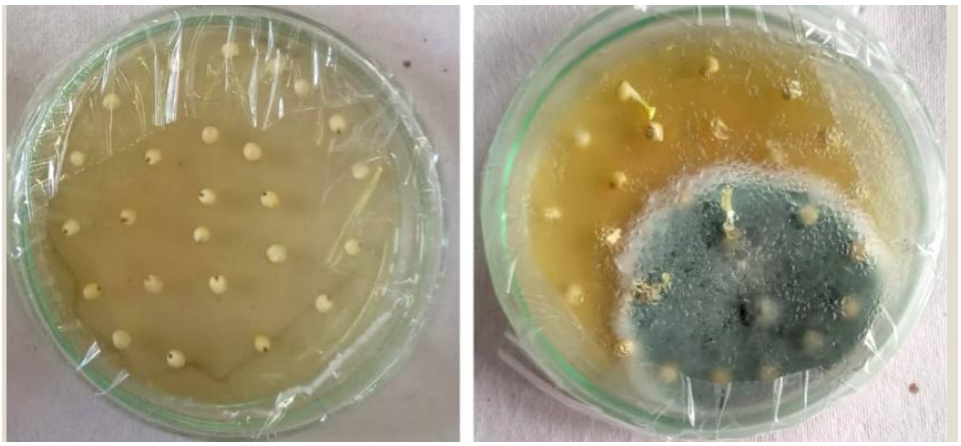

Figure 1: Plate showing *Sorghum vulgare* infected by *Aspergillus fumigatus*

Morphological Identification of Fungal Isolate

The isolated fungi were identified according to colony morphology and microscopic examination as described by Barnet and Hunter,[13] Nelson et al.,[14] Pitt and Hocking,[15] Leslie

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and Summerall.[16] The fungi was observed under 40 x and under scanning electron microscope (600x).

Molecular identification of fungal isolate[16,17]

Extraction of DNA

Genomic DNA was isolated by using the Insta Gene™ Matrix Genomic DNA isolation kit (Catalog # 732-6030)

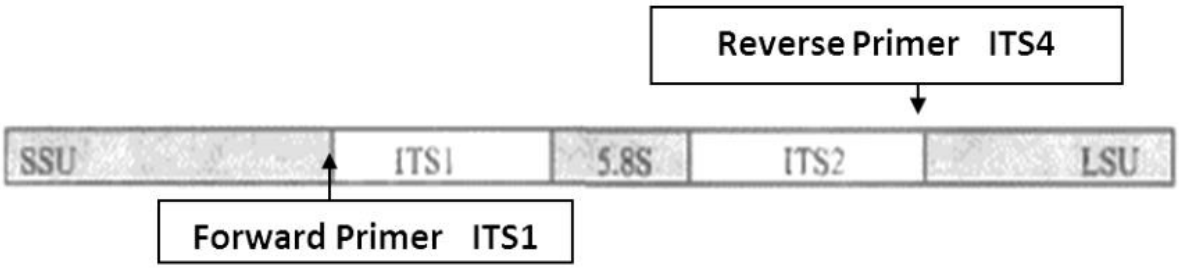

Figure 2: Ribosomal gene organization and target region amplified

Table 1: Primer Details: Ribosomal RNA ITS Region Universal primers

ITS Primer for Fungi	Sequence Details	Amplicon size (bp)
Forward PrimerITS1	GGAAGTAAAAGTCGTAACAAGG	620bp
Reverse Primer ITS4	TCCTCCGCTTATTGATATGC	

Polymerase Chain Reaction:

Target gene fragment was amplified using Thermo Scientific Veriti Thermal Cycler PCR Protocol: DNA fragments are amplified using 1 µl of template DNA in 10 µl of total PCR reaction mixture using ITS1F/ITS4R primers (50 pmol) and 30 amplification cycles with following program (Table 2).

Table 2: Amplification protocol

Initial denaturation	95°C for 5 min
Denaturation	95°C for 1 min
Annealing	55°C for 30 sec
Extension	72°C for 1 min
Final Extension	72°C for 7 min

Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using the 1492R ITS1F/ITS4R primers. Sequencing reactions were performed using a ABI PRISM® BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq®DNA polymerase (FS enzyme) (Applied Biosystems). Single-pass sequencing was performed on each template using18S rRNA gene

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universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were re suspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Results

The isolated fungi were identified according to colony morphology and microscopic examination (Figure 3), and observed under scanning electron microscope (Figure 4).

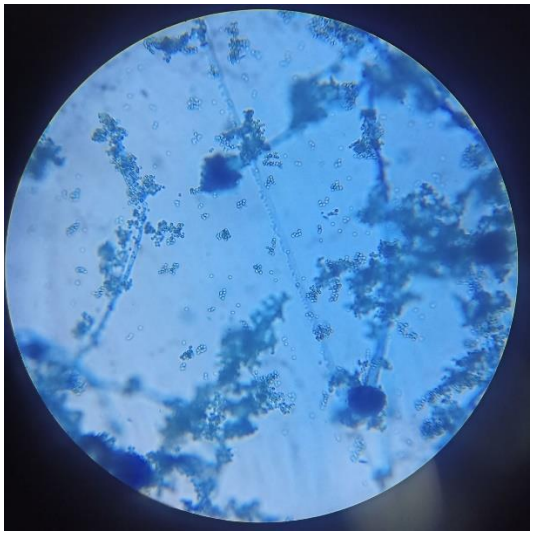

Figure 3: *Aspergillus fumigatus* observed under compound microscope (40x)

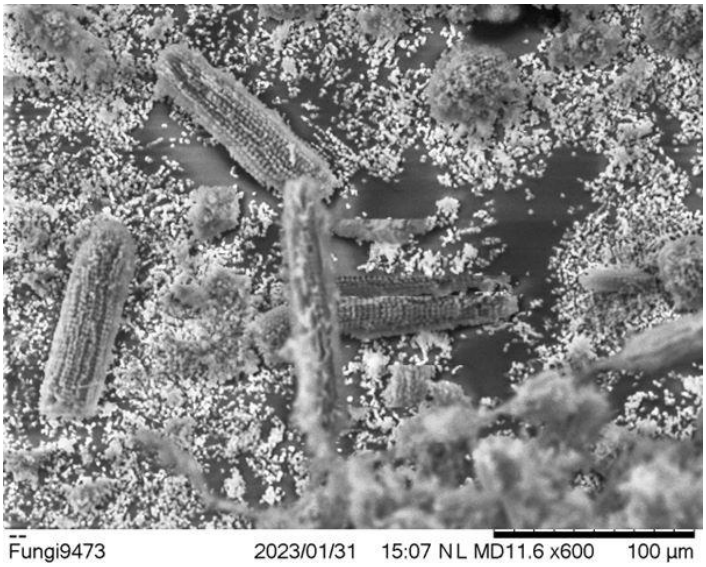

Figure 4: *Aspergillus fumigatus* observed under scanning electron microscope

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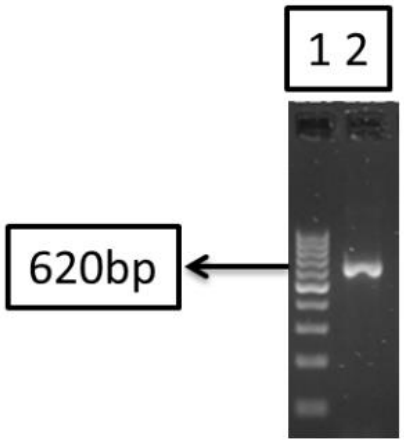

Figure 5: PCR Amplicon of ITC 1-4
Lane 1 – Molecular ladder, Lane 2-AF

Forward Sequence

>0323_099_001_PCR_SLS_0323_01_FORWARD_D04.ab1

CGTAGTGACTGCGGAAGGACATTTACCGAGTGAGGGCCCTTCTGGGTCCAACCTC
CCACCCGTGTCTATCGTACCTTTTGCTTCGGCGGGCCCGCCGTTTCGACGGCCGCC
GGGGAGGCCTTGCGCCCCCGGGCCCGCGCCCGCCGAAGACCCCAACATGAACGC
TGTTCTGAAAGTATGCAGTCTGAGTTGATTATCGTAATCAGTTAAAACTTTCAACA
ACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAAT
GTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTG
GTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCACGGCTTGT
GTGTTGGGCCCCCGTCCCCCTCTCCCGGGGGACGGGCCCGAAAGGCAGCGGCGG
CACCGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCTGCTCTGTAGGCCCGG
CCGGCGCCAGCCGACACCCAACTTTATTTTTCTAAGGTTGACCTCGGATCAGGTAG
GGATACCCGCTGAA CTTAAGCATATC

Reverse sequence
>0323_099_002_PCR_SLS_0323_01_REVERSE_D04.ab1
TTGCGATCCTACCTGATCCGAGGTCAACCTTAGAAAAATAAAGTTGGGTGTCGGCT
GGCGCCGGCCGGGCCTACAGAGCAGGTGACAAAGCCCCATACGCTCGAGGACCG
GACGCGGTGCCGCCGCTGCCTTTCGGGCCCGTCCCCCGGGAGAGGGGGACGGGG
GCCCA
ACACACAAGCCGTGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCCCCCCG
GA
ATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGCAAT
TCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCC
GTTGTTGAAAGTTTTAACTGATTACGATAATCAACTCAGACTGCATACTTTCAGAA

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CAGCGTTCATGTTGGGGTCTTCGGCGGGCGCGGGCCCGGGGGCGCAAGGCCTCC
CCGGCGGCCGTCGAAACGGCGGGCCCGCCGAAGCAACAAGGTACGATAGACACG
GGTGGGAGGTTGGACCCAGAGGGCCCTCACTCGGTAATGATCCTTCCGCAGGTTC
ACCTACG GAAACCTTGTTACACTTTTTTTTTCTTCCAA

>contigous sequence
CTGCGGAAGGATCATTACCGAGTGAGGGCCCTCTGGGTCCAACCTCCCACCCGTG
TCTATCGTACCTTGTTGCTTCGGCGGGCCCGCCGTTTCGACGGCCGCCGGGGAGG
CCTTGCGCCCCCGGGCCCGCGCCCGCCGAAGACCCCAACATGAACGCTGTTCTGA
AAGTATGCAGTCTGAGTTGATTATCGTAATCAGTTAAAACTTTCAACAACGGATCT
CTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTG
CAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCG
GGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGG
GCCCCCGTCCCCCTCTCCCGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCG
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CAGCCGACACCCAACTTTATTTTTCTAAGGTTGACCTCGGATCAGGTAGG

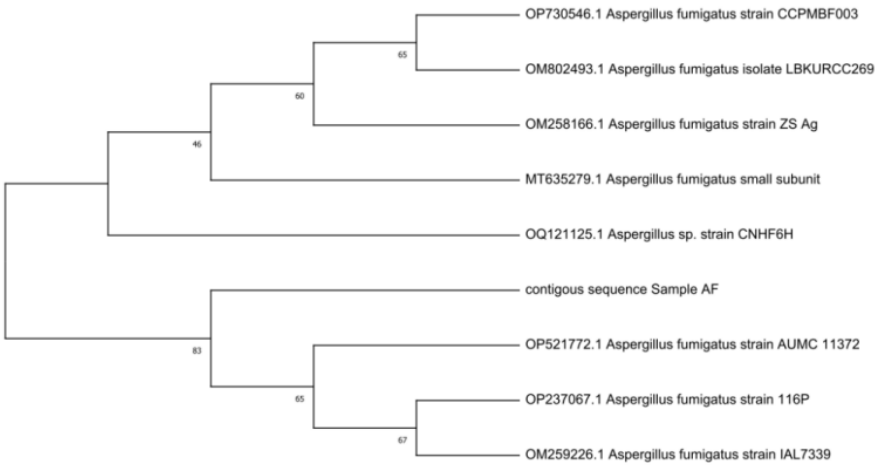

Figure 6: Phylogenetic tree of fungal isolate of *Aspergillus fumigatus*

Discussion

Storage fungi associated with grain sample were isolated and identified. The results showed that all the Sorghum grains obtained from the two markets from Karnataka state were infested with various degrees of fungi. Among *Aspergillus* species, the strains identified were *Aspergillus fumigatus* strain which are known to produce aflatoxins and the results have demonstrated the invasion of grains with mycotoxin producing fungi sold in markets. These results are similar to works of Kutama and Aliyu, 2008[19] who isolated three fungal genera from local groundnut samples sold in markets[20] who isolated six *Aspergillus* strains from millet and sorghum. The pathogens were present in all cases of the disease. The same pathogens were isolated from the diseased host and regrown in pure culture when inoculated into a healthy grain sample of millet and sorghum the pathogen from the pure culture caused

the same disease. The same pathogen was re-isolated from the new host and shown to be the same as the originally isolated pathogen. Fungal isolates were amplified on the basis of their molecular characteristics. Sequence analysis of the nuclear-encoded rDNA showed significant alignments (100%) identity in isolate for *Aspergillus fumigatus*. The fungal organisms isolated from the grain samples in this study are known to be spoilage organisms associated with many agricultural products including cereals, fruits and nuts.[21] These fungi might have colonized the grains during production in the field, transportation or storage. The variation in the frequency of their occurrence may have reflected differences in the inoculum density in the area or the prevailing environmental conditions favoring the growth of the fungi.

Conclusion

The fungal species were isolated from *Sorghum vulgare* samples obtained from the two prominent grain markets from Karnatak state. The isolated species were identified morphologically as *Aspergillus fumigatus*. The fungal samples were then identified through molecular techniques by DNA sequencing which was identified as *Aspergillus fumigatus*. The molecular techniques used in this study, has added great benefits to the process of distinguishing between similar species of fungi in comparison with the classical techniques.

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