



Occurrence of toxigenic *Fusarium* spp. in paddy and sorghum

NEERA¹, N GOPALAN² and H S MURALI³

Food Biotechnology Division, Defence Food Research Laboratory, Siddharthanagar, Mysore 570 011

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ABSTRACT

A total of 45 cereal samples (35 paddy and 10 sorghum) were collected from Mandya and Mysore districts of Karnataka during March, 2019 to study the mycotoxigenic *Fusarium* strains present in the field and market samples. All samples were brought to DFRL, Mysore and screened for *Fusarium* presence by agar plating method on Selective Fusarium Agar (SFA) containing streptomycin (1 mg/ml) with incubation at $25 \pm 2^\circ\text{C}$ for 3–5 days. In the study, almost 10 different *Fusarium* species association was revealed with the collected cereal samples. *Fusarium* species were micromorphologically identified and further confirmed by PCR-based detection using ITS1 and ITS4 primers followed by sequencing. PCR studies confirmed that all the tested fungal isolates belonged to *Fusarium* spp. with the amplicon size of 590 bp. NCBI data base confirmed the sequence similarity of 99 % to the genus *Fusarium* and accession numbers were obtained by submitting the sequences to the GenBank. The study disclosed the diversity in phytopathogenic *Fusarium* spp. in paddy and sorghum growing in different agro-climatic regions of Mysore and Mandya districts of Karnataka, India.

Key words: Chemotyping, *Fusarium*, Paddy, PCR, Trichothecene

The genus *Fusarium* is saprophytic, plant pathogens and mycotoxin producers that infects grains, vegetables, seeds and are also associated with human and animal health hazards. They grow in tropical and temperate regions and mainly cause Fusarium head blight, crown rots and wilt formation in major cereal crops like wheat, barley, sorghum, triticale, rye, paddy and other food grains (Ferrigo *et al.* 2016). Diseases caused by *Fusarium* not only severely reduce crop yield, but also result in contamination of grain with undesirable high amounts of mycotoxins, a problem of world-wide importance. It is estimated that globally approximately 25% of cereal production and approximately 20% of plant production may be contaminated with mycotoxins (Eskola *et al.* 2019). Aflatoxins, trichothecenes (T-2, DON and Zearalenone) and fumonisins are the major mycotoxins that contaminate crop plants and, as a result, are of great importance to agricultural economics and in food and feed safety (Alshannaq and Yu 2017). They may co-exist in feeds, foods, and processed food products because some fungi have the ability to produce more than one mycotoxin, and/or more than one fungal species may colonize a substrate. Mycotoxins were responsible for the death of thousands of people in Russia during World War II causing alimentary toxic aleukia (ATA), affecting the hematopoietic system due to the ingestion of overwintered cereal grains colonized with *F. sporotrichioides* and *F. poae* which are known to produce T-2 toxin (O'Donnell *et al.*

2018). In India, ATA was reported during 1987 attributed to the consumption of moldy wheat. Also, during 1995, an outbreak of acute food-borne disease was reported in south India attributed to fumonisin affecting 1424 people due to consumption of contaminated sorghum and maize (Reddy and Raghavender 2008).

The study was carried out to create awareness and to generate data on prevalence, existence/incidence of mycotoxigenic *Fusarium* spp. in paddy and sorghum grains collected from the fields of Mysore and Mandya districts of Karnataka as these mycotoxins cause chronic health risks prevalent in India where the diets of the people are highly prone to mycotoxins due to poor grain harvesting and storage practices.

MATERIALS AND METHODS

Sample collection and isolation of *Fusarium*

For isolation of *Fusarium* spp., 35 paddy and 10 sorghum samples were collected from agricultural fields and local markets of Mysore and Mandya districts of Karnataka during March, 2019 and brought to the laboratory at DFRL, Mysore. The shrunk and discoloured seeds were selected and surface-sterilized with 1% sodium hypochlorite for 3 min and rinsed with sterile distilled water. The sterilized samples were placed onto water agar (Burgess *et al.* 1994) and a semi-selective medium for *Fusarium*, i.e. selective Fusarium agar (SFA) containing streptomycin (1 mg/ml) and incubated under a standard growth condition (Salleh and Sulaiman 1984). The resulting single-spored *Fusarium* colonies were transferred onto potato dextrose agar (PDA) for

*Corresponding author e-mail: neera@dfrl.drdo.in

morphological identification (Leslie and Summerell 2006).

Morphological identification of *Fusarium*

The isolated fungi were identified according to colony morphology and micromorphology of fungal isolates examined by light microscopy with lactophenol cotton blue staining (Watanabe 2002). Single-spore cultures of *Fusarium* spp. were grown at 25°C for 5 days on PDA for the morphology identification of the colony.

Isolation of total cell DNA from fungal isolates

For DNA extraction, mycelia from potato dextrose agar plate were transferred to potato dextrose broth and incubated at 30°C for 3 days with constant agitation. After 3 days of incubation, a small pinch of mycelium was collected into sterile 1.5 mL Eppendorf tube and squeezed with micropestle and 500 µL of lysis buffer (100 mM Tris HCl, 50 mM EDTA, 150 mM NaCl and 1× SDS) was added and kept in water bath for 5 min at 60°C. The solution was mixed and incubated on ice for 10 min and then centrifuged at 12000g for 5 min. Four hundred microlitres of the supernatant was transferred to a new microcentrifuge tube and an equal volume of chloroform was added and again centrifuged at 5000g for 3 min, a 350 µL aqueous solution was transferred to a new microcentrifuge tube and to this a 300 µL of cold isopropanol was added to precipitate the DNA. The resulting pellet was washed with 70% ethanol and air dried. The DNA was dissolved in 50 µL of Tris-EDTA and stored at -20 °C.

PCR amplification

The DNA obtained was amplified using primers ITS-1 (5-TCC GTA GGT GAA CCT GCG G-3) and ITS-4 (5-TCC TCC GCT TAT TGA TAT GC-3) as described by Singha *et al.* (2016). The PCR conditions followed were initial denaturation at 94 °C for 4 min, denaturation at 94°C for 1 min, annealing at 48°C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 5 min.

Phylogenetic analysis of *Fusarium* species

The PCR amplicons of all *Fusarium* spp. were sequenced at Xcelris Genomics, Ahmedabad. The sequences were confirmed with NCBI, BLAST database for the identity of the isolates based on previously published database sequences. The nucleotide sequences of the related taxa with their accession numbers were obtained from the GenBank database available at NCBI (Table1). The similarity values of the ITS gene sequences were calculated from the multiple alignments. The software CLUSTAL X software as used to construct a phylogenetic tree. The stability of the relationships was assessed by a bootstrap analysis of 1000 data sets. The same sequence reads were deposited at NCBI and obtained accession numbers for each representative *Fusarium* spp.

RESULTS AND DISCUSSION

Cereal contamination with trichothecene producing

Table 1 Accession no. of the *Fusarium* isolates and the source of isolation

Isolate	Accession no.	Sample
<i>Fusarium oxysporum</i>	MN871796	Paddy
<i>Fusarium verticilloides</i>	MN871797	Paddy
<i>Fusarium verticilloides</i>	MN871798	Paddy
<i>Fusarium chlamydosporum</i>	MN871799	Paddy
<i>Fusarium equiseti</i>	MN871800	Paddy
<i>Fusarium solani</i>	MN871801	Paddy
<i>Fusarium fujikuroi</i>	MN871802	Paddy
<i>Fusarium chlamydosporum</i>	MN871803	Paddy
<i>Fusarium oxysporum</i>	MN871804	Paddy
<i>Fusarium oxysporum</i>	MN871805	Sorghum

Fusarium spp. is a global concern reported from various parts of the world. Many studies have been undertaken to understand the diversity, incidence and management of *Fusarium* species and their mycotoxins in cereals, food and feed. Being heat stable, the existence of *Fusarium* mycotoxins has been established in whole cereal supply chain from the farmer to the customer, providing problems to every stakeholder.

Isolation and identification of *Fusarium*

The collected samples of paddy and sorghum were screened for *Fusarium* presence on selective *Fusarium* agar (SFA) containing streptomycin (1 mg/ml). Ten *Fusarium* isolates were obtained based on their micromorphology (Fig 1). All the 10 *Fusarium* isolates were amplified using ITS primers with an amplicon size of 590 bp. Individual *Fusarium* species was identified based on the differences in ITS gene. The sequences of the Fungal strains were deposited in the GenBank database and the following accession numbers were obtained: MN871796 for *F. oxysporum* DFRP1, MN871797 for *F. verticilloides* DFRP2, MN871798 for *F. verticilloides* DFRP3, MN871799 for *F. chlamydosporum* DFRP4, MN871800 for *F. equiseti* DFRP5, MN871801 for *F. solani* DFRP6, MN871802 for *F. fujikuroi* DFRP7,



Fig 1 Macroconidia of *Fusarium verticilloides* grown on Potato Dextrose agar.

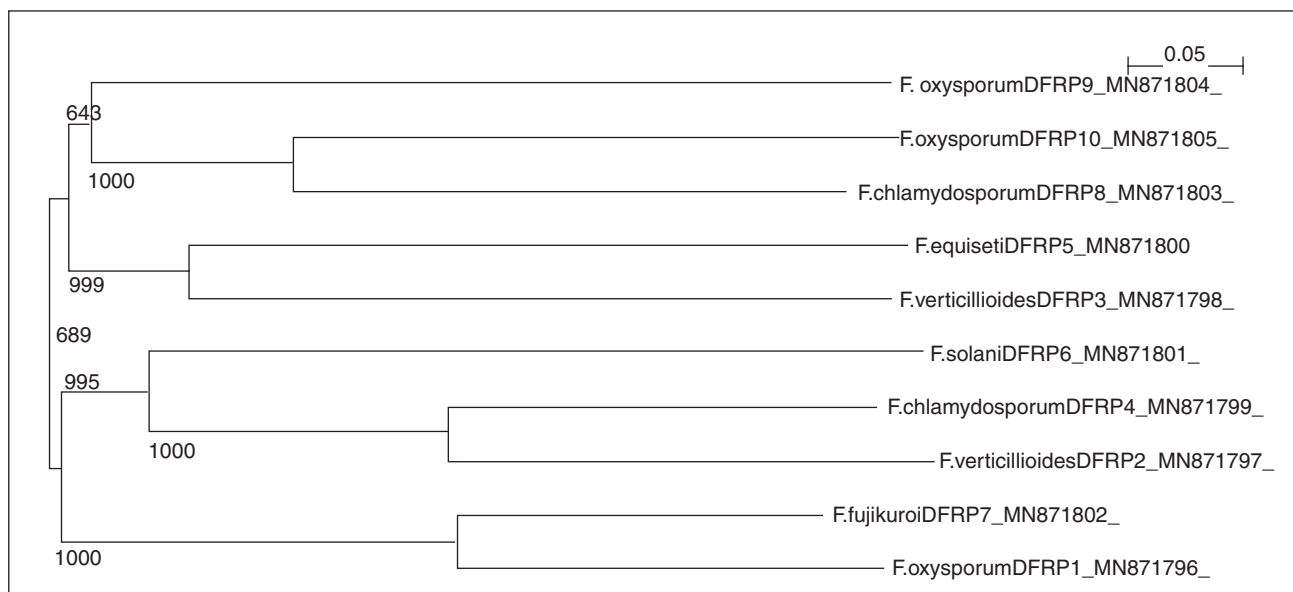


Fig 2 Phylogenetic tree deduced from the ITS gene sequences. Numbers at nodes are percentage bootstrap values based on 1000 replications.

MN871803 for *F. chlamydosporum* DFRP8, MN871804 for *F. oxysporum* DFRP9 and MN871805 for *F. oxysporum* DFRP10 (Table 1). Out of the 10 *Fusarium* isolates, nine were obtained from paddy, whereas only one isolate *F. oxysporum* DFRP10 was obtained from sorghum. Fungal species identification by using phenotypic characteristics is misleading; this is particularly intricate in case of genus *Fusarium* because of the existence of several conflicting taxonomy. Molecular detection methods have been used to detect contamination in cereals by *Fusarium* species (Sreenivasa *et al.* 2008, Abd-Elsalam *et al.* 2003). In this regard, the present investigation sequencing technique after PCR was used to detect *Fusarium* species associated with the cereal crops which serve as secondary confirmation to the microscopy – based identification.

Phylogenetic analysis

Combined analysis of 10 *Fusarium* species was done for the similarity clusters with external nodes indicated with NCBI accession numbers (Fig 2), for individual *Fusarium* species and branches reflected the nodes from the major clade. As in the physiological characteristics, it was clear that the isolated strains were a member of the family Necteriaceae and the genus *Fusarium*. Consequently, the isolated strains were named as *Fusarium oxysporum* DFRP1, *F. verticillioideis* DFRP2, *F. verticillioideis* DFRP3, *F. chlamydosporum* DFRP4, *F. equiseti* DFRP5, *F. solani* DFRP6, *F. fujikuroi* DFRP7, *F. chlamydosporum* DFRP8, *F. oxysporum* DFRP9, *F. oxysporum* DFRP10 for further studies based on their similarity index in the NCBI Database. The sequence readings obtained for all the *Fusarium* species were also used for the construction of phylogenetic tree to know the evolutionary relationships among the *Fusarium* species based on their genetic closeness. *Fusarium* species

reported in the present study such as *F. verticillioideis*, *F. proliferatum* are designated the potential producer of fumonisins (Omori *et al.* 2018). Desjardins *et al.* (2000) reported that the predominant *Fusarium* species in surface-disinfested seeds with husks were species of the *Gibberella fujikuroi* complex, *G. fujikuroi* mating population C and *G. fujikuroi* mating population. He also reported that the rice culture produced 2980 µg/g of fumonisin. Nagaraja *et al.* (2016) reported the highest percentage of incidence in paddy was *F. verticillioideis* (3.21 %), followed by *F. graminearum* (1.97 %) and *F. proliferatum* (1.11 %) and least was *F. avenaceum* (0.09 %). The growth also causes discoloration of grain, heating, mustiness, dry matter loss, and production of several secondary metabolites such as mycotoxins, which are potentially dangerous to humans and animals (Bhattacharya and Raha 2002). *Fusarium* occurred at much lower relative frequency in samples, 18.18% in wheat and 50% in barley as compared to *Aspergillus*, samples collected at harvest time. The conventional methods of fungal isolation performed at late stages, i.e. during harvest time and, particularly in the case of stored seeds might strongly under represent those species having their favourable environmental conditions at earlier times. The main *Fusarium* species isolated from wheat and barley was *F. oxysporum* and *F. chlamydosporum*. This profile is compatible with conditions during maize growth in field even approaching harvest time. Direct species specific PCR assays on samples collected at late stages of cereal growth and stored samples might provide a more sensitive detection method for early colonizers like *Fusarium* (Gil-Serna *et al.* 2013). This indicates that *Fusarium* infection can be a serious problem, as paddy is the staple food for Asian countries. Therefore, studies on the prevalence and incidence of mycotoxigenic fungi in paddy is very important.

In one report, a high percentage of isolates of mycotoxigenic *Fusarium* were isolated from finger millet of Andhra region contaminated with mycotoxins such as Zearalenone (ZEA), T-2 toxin, nivalenol (NIV) and Deoxynivalenol (DON) and Deoxyscripenol (DAS) (Penugonda *et al.* 2011). Seventy *Fusarium* species incident on different hosts with different geographical locations was recorded by Leslie and Summerell (2006). Differences in the climatic and the environmental conditions in field among locations and years might be responsible for differences in *Fusarium* diversity and incidence in the studies compared. In particular, some reports point out that *F. verticillioides*, *F. proliferatum* and *F. equiseti* might tolerate better low water potential conditions than *F. graminearum* (Marin *et al.* 2012, Marin *et al.* 2015). Therefore, it would be useful to determine the occurrence and prevalence of certain *Fusarium* species at these early critical stages in order to predict which toxin or toxins could be expected and the control measures to be taken. Additionally, species specific toxigenic fungal detection could be helpful at harvest time and/or in subsequent postharvest stages.

Conclusion

The study revealed the prevalence of *Fusarium* in paddy and sorghum collected from two districts of Karnataka state. The study suggested the need for sensitive techniques for the quantitative analysis of mycotoxin in paddy and sorghum intended for human consumption. *Fusarium* wilt caused by these species are responsible for serious economic losses, the disease in the field conditions appears late in the crop growth and controlling becomes difficult. So, early detection of disease is required to control the pathogenesis. The genus reported is a prolific producer of toxins and that it has a tremendous impact on humans and cattles who eat grains that contain these fungi. The study indicated the extent of contamination and seed damage with respect to physiological and biochemical quality parameters of cereals. Growth of *Fusarium* species on cereals reduce the germination besides the loss of carbohydrate, protein and oil content increases the moisture content, free fatty acid and thus reduces the dry matter content. The data on the diversity of *Fusarium* species on paddy would be of great value for this region for predicting the extent of post-harvest infection, colonization and subsequent deterioration of cereal grains.

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