

Research Paper

Incidence and multiplex PCR based detection of trichothecene chemotypes of *Fusarium culmorum* isolates collected from freshly harvested Maize kernels in Southern India

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Abstract

Hundred *Fusarium culmorum* strains, isolated from freshly harvested maize grain samples from Southern parts of India, were incubated in czapek-dox medium and analyzed for trichothecene (DON/NIV) production. The mPCR assay was standardized targeting trichothecene metabolic pathway genes viz., *Tri6*, *Tri7*, *Tri13* for detection of trichothecene (DON/NIV) chemotypes and *rDNA* gene for specific detection of *F. culmorum* species. Primers for targeted genes were designed and used to predict whether these isolates could produce deoxynivalenol/nivalenol, 94 isolates were able to produce DON/NIV by mPCR assay. Chemical analysis of DON/NIV was carried out for mPCR positive isolates by high performance-thin layer chromatography (HPTLC). To check the practical usefulness of developed mPCR assay, 150 field samples of maize were evaluated and results were compared with conventional HPTLC method. Out of 150 samples, 34% samples stayed as a positive for NIV contamination whereas 44% were found to have deoxynivalenol contamination. Moreover, mPCR results are equivocally matched with the HPTLC chemical analysis for field samples. Chemotyping of *F. culmorum* isolates were reported for the first time from India, and highlights the important potential of *F. culmorum* to contaminate maize with DON/NIV.

Key words: deoxynivalenol, nivalenol, multiplex PCR assay, HPTLC.

Introduction

The genus *Fusarium* is a common fungal contaminant of many economically important field crops and food products, which causes a major contamination in human and animal nutrition. *Fusarium* sp. infects many important food grains, such as maize, wheat, barley, rice, millet, oat and rye, and produce highly toxic secondary metabolites known as mycotoxins. The major classes of *Fusarium* mycotoxins are trichothecenes and fumonisins. According to the type of trichothecene production, some *Fusarium* species, like *F. culmorum* and other species have been divided into two chemotypes: (i) the nivalenol chemotype, which includes isolates producing nivalenol and fusarenone X, and (ii) the deoxynivalenol chemotype, which includes isolates producing deoxynivalenol and acetyldeoxynivalenol (Langseth *et al.*, 1999; Muthomi *et al.*, 2000). Trichothecenes can cause wide range of acute and chronic effects

in humans and animals through ingestion of food and feed prepared from cereal crops contaminated with the toxins (Mello *et al.*, 1999). The effects include skin inflammation, digestive disorders, tachycardia, oedema and haemorrhages in several internal organs, haemolytic disorders, impairment of immune disorders and nervous disorders (IARC, 1993).

Contamination of maize grains with trichothecenes is a common problem throughout the World, especially in temperate regions like India (Janardhana *et al.*, 1999; Venkataramana *et al.*, 2011). In Southern India, maize is grown under different ecological conditions and is harvested with fairly high trichothecene contaminations (Janardhana *et al.*, 1999). Recent reports indicate that maize is prone to fungal infection during the pre and post harvest period (Bilgrami *et al.*, 1980; Sinha, 1990). Vasanthkumar (Vasanthkumar, 1986) studied the infection of maize by field and storage

moulds during pre and post harvest practices in relation to seed-borne fungal diseases of maize. Thimmappaiah *et al.* (1987) reported the natural occurrence of *Fusarium* toxins such as T-2 toxin, deacetoxyscirpenol and zearalenone in peanut, sorghum and maize from Mysore districts of Southern India. Pre-harvest succession of fungi in ripening maize grains has been reported by Banerjee *et al.* (Beyer *et al.*, 2005).

The usual methods for chemotyping of *Fusarium* isolates are high performance liquid chromatography (HPLC) or gas chromatography/ mass spectroscopy (GS/MS) analysis of extracts from substrates such as maize artificially inoculated with *Fusarium* (Sugiura *et al.*, 1990; Muthomi *et al.*, 2000). These methods are commonly applied due to their high sensitivity and specificity. However, these methods are rather labour-intensive and require sophisticated instrumentation and skilled operators. Recent studies are focusing genotypic identification of toxigenic fungi through molecular methods. DNA based methods that rely on the amplification of the genes involved in the biosynthesis of trichothecenes also are available. Specific PCR primers have been developed to the specific genes (*Tri5*, *Tri6*, *Tri7* and *Tri13*) which are involved in trichothecene metabolism (Venkataramana *et al.*, 2011; Lee *et al.*, 2001). Some of the genes have been sequenced and found to be functional in NIV-producing isolates and nonfunctional in DON-producing isolates (Lee *et al.*, 2002, 2001; Chandler *et al.*, 2003).

The distribution of each chemotype/genotype varies by geographic region. Thus, strains of *F. culmorum* with DON and NIV chemotype/ genotype are known from several countries, including UK (Jennings *et al.*, 2004), Germany (Muthomi *et al.*, 2000), the Netherlands and Norway (Langseth *et al.*, 1999), Italy (Gang *et al.*, 1998), France (Bakan *et al.*, 2001), USA (Mirocha *et al.*, 1994), Canada (Abramson *et al.*, 2001). However in India, we are first time reporting incidence of trichothecene chemotypes of *F. culmorum* on maize kernels by multiplex PCR method.

In the present study a novel mPCR method was developed for detection of trichothecene chemotypes of *F. culmorum*. Developed method was successfully evaluated in terms of sensitivity, specificity and reliability on to artificially contaminated samples as well as contaminated field samples of maize.

Materials and Methods

Sampling area

A total of 150 freshly harvested maize kernel samples were randomly collected from Karnataka and Andhra Pradesh, two districts from each state viz., Kusalnagara and Mysore districts of Karnataka; Srikakulam and Vizianagaram districts of Andhra Pradesh, India. Collected samples were stored at 4 °C for further analysis of mycoflora and toxin detection.

Isolation and identification of moulds from infected maize grains

Isolation of mycoflora

F. culmorum and other fungal genera were isolated from the infected maize grains using Potato Dextrose Agar (PDA) media. The morphological identification of *Fusarium* species were followed by the previous reports of Nelson *et al.* (1983). The pure cultures of *Fusarium* was maintained on SNA plates and stored at 4 °C for analysis of toxins.

DNA extraction

DNA was extracted from pure cultures of *Fusarium* sp., using commercially available DNA extraction kits (QUIAZEN, Gambh).

Isolation of DNA from contaminated food grains

Twenty grams of contaminated food sample was ground in coffee grinder for 90 s and then 0.2 g ground grain was mixed in 1 mL lysis buffer (100 mM Tris-HCl, 50 mM EDTA, 150 mM NaCl and 1% SDS) and homogenized by gentle mixing and kept it for water bath at 60 °C for 10 min. Samples were centrifuged at 12000 x g for 5 min and 500 µL of supernatant was mixed with 150 µL of 5 M potassium acetate and incubated on ice for 10 min. After centrifugation a 400 µL of supernatant was mixed with 300 µL of ice cold isopropanol to precipitate the DNA, the resulting pellet was washed with 70% ethanol, dried under air and dissolved in 50 µL of Tris-EDTA (pH 8.0).

Development of primers and PCR analysis

Designing of primers

DNA sequences were analysed and aligned by Clustal method. Primers were designed using the aligned gene bank database sequences viz., *Tri6*, *Tri7*, *Tri13* and *rDNA* genes for the specific detection of nivalenol and deoxynivalenol producing *F. culmorum*. Total of four primer pairs were designed using Gene runner software (<http://www.generunner.com>). Primer sequences are listed in Table 1. Before standardizing mPCR protocol, all designed primers were evaluated on to array of fungal species to check the specificity and sensitivity.

Multiplex PCR assay

Multiplex PCR was carried out for DON and NIV producing *F. culmorum* in an Eppendorf master cycler gradient (Hamburg, Germany) with a reaction volume of 30 µL. The amplification mixture consisted of template DNA (1.0 µL), MgCl₂ (2.0 mM), 1X PCR buffer (Sigma), dNTP mix (200 µM, MBI, Fermentas), Taq polymerase (1 unit, Sigma) and primer pairs specific to the targeted genes *Tri6*, *Tri7*, *Tri13* and *rDNA* were added at a concentration of 100 nM, 150 nM, 200 nM and 50 nM, respectively. The PCR cycling conditions were carried out with an initial de-

Table 1 - Primers used in this study.

Primer	Sequence (5'-3')	Product size	Targeted gene	Accession No	Annealing temperature
Fcu F	GATGCCAGACCAAGACGAAG	302 bp	<i>rDNA</i> 1-302	AY880844.1	58 °C
Fcu R	GGTTAGAATCATGCCGACC				
tri6 F	GATCTAAACGACTATGAATCACC	541 bp	<i>TRI6</i> 99-640	AY134893.1	58 °C
tri6 R	GCCTATAGTGATCTCGCATGT				
tri7F	ATAGGTACCGGATCGCAGG	794 bp	<i>TRI7</i> 1-794	FJ152469.1	58 °C
tri7R	CCGAAAGCCTCTAATAGTGT				
tri13 F	GTTGCAGTTCGCTTGATTTTCG	1000 bp	<i>TRI13</i> 60-1060	FJ152465.1	58 °C
tri13 R	GTTGCAGTTCGCTTGATTCAG				

naturalization at 94 °C for 4 min, followed by 30 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1.5 min, with a final extension of 72 °C for 8min.

Specificity and sensitivity of mPCR on artificially contaminated maize grains

The specificity of the mPCR primers was determined against different organisms shown in (Table 2). Sterile maize grains (5 g) were experimentally spiked with *F. culmorum* spore suspensions at different concentrations (1×10^5 , 1×10^4 , 1×10^3 and 1×10^2 cfu g⁻¹). Negative controls were kept without inoculation of spores. All the samples were enriched for two days, further DNA was isolated and analysed by mPCR assay.

Application of mPCR on field samples

Analysis of field samples

Maize samples were collected from various fields of Andhra Pradesh and Karnataka, India and processed as described earlier and all of these samples were subjected to mPCR assay and toxins are analyzed by HPTLC.

Chemical analysis of DON and NIV

Extraction and cleanup

Briefly, 50 g of the well ground sample was extracted with 250 mL of acetonitrile-water (60:40, v/v) using high speed blending for 2 min. The extract was filtered through Whatman No. 4 filter paper and an equal volume of ethyl acetate was added to the filtrate and separated the lower chloroform fraction and evaporated to dryness. Dried compound was diluted with PBS and passed through immunoaffinity columns (VICAM, USA) for clean-up. After washing with water, DON /NIV were eluted with methanol. These extracts further used for HPTLC analysis.

HPTLC detection

High performance thin layer chromatography (HPTLC) technique was used to detect the specific chemotypes of (DON /NIV) *F. culmorum*. Chromatography was carried out on 10 x 10 cm precoated silica gel HPTLC plates (Merck). Test samples and standards were applied

Table 2 - Standard cultures used in this study.

SNO	Name	Source
1	<i>Fusarium culmorum</i> ITCC146	IMTECH
2	<i>Fusarium culmorum</i> ITCC149	ITCC
3	<i>Fusarium culmorum</i> ITCC148	ITCC
4	<i>Fusarium graminearum</i> MTCC 1893	MTCC
5	<i>Fusarium graminearum</i> ITCC 1805	ITCC
6	<i>Fusarium moniliforme</i> ITCC 3362	ITCC
7	<i>Fusarium moniliforme</i> ATCC 14164	IMTECH
8	<i>Fusarium oxysporum</i> NCIM 1072	NCIM
9	<i>Fusarium oxysporum</i> IMTECH 2480	IMTECH
10	<i>Fusarium solani</i> ITCC3359	IMTECH

*IMTECH- Institute of Microbial Technology; ITCC- Indian Type Culture Collection; ATCC-American Type Culture Collection; NCIM - National Centre for Industrially important Microorganisms.

with automatic TLC sampler (ATS III) from CAMAG (Muttentz, Switzerland), with a 50 mm run length using: chloroform + methanol + water (9+1+0.2, v/v/v.) as mobile phase. Fluorescence detection was carried out by post-chromatographic derivatization with 10% aluminium chloride (Sigma) in methanol-water mixture. The plate was immersed in the derivatization solution using a dipping device III (CAMAG) and heated for 20 min at 110 °C. The plate was then scanned at 366/ > 400 nm with a densitometer TLC Scanner 3 (CAMAG) using a slit dimension of 5.0 x 0.5 mm and scanning speed of 40 mm s⁻¹ (18). DON and NIV standards (Sigma) at a concentration of 0.1 mg/mL in methanol: water (1:1) was used as positive controls for HPTLC detection.

Results

Incidence of mycotoxigenic *Fusarium culmorum* and other fungi

A total of 150 *Fusarium* isolates were identified from the maize samples of the present study. Out of 150 fungal isolates, 100 isolates were identified with *F. culmorum* morphologically (45 isolates from Karnataka and 55 iso-

lates from Andhra Pradesh) and remaining 50 isolates from other species including *F. sporotrichioides*, *F. verticillioides* and *F. proliferatum* (Table 3).

Multiplex PCR assay application and chemical analysis of pure cultures of fungi

Primer concentrations (100 nM of Tri6, 150 nM of Tri7, 200 nM of Tri13 and 50 nM of rDNA) and annealing temperature (58 °C) were standardized to get a uniform amplification of all the genes targeted for mPCR assay (Figure 1). Out of 150 *Fusarium* isolates, 100 were showed positive signal for *rDNA* gene specific to *F. culmorum* (94 toxigenic to DON/NIV and remaining were non toxigenic by mPCR). However, 54 and 34 strains of them were positive for DON and NIV respectively, and rest of the 7 strains were stayed as negative for the chemical analysis by HPTLC.

Contamination studies

The detection limit for deoxynivalinol and nivalenol producing *F. culmorum* strains from spiked samples by mPCR was 1×10^3 cfu g⁻¹ maize grains. Samples tested with initial fungal load of 1×10^3 cfu g⁻¹ and above concentrations following 48 h enrichment at 30 °C were positive for both toxins.

mPCR and chemical analysis of field samples

Out of 150 maize samples collected for mPCR studies, 78% samples were showed positive for toxigenic *Fusarium* species. However, only 34% of the total analysed samples were positive for NIV and 44% positive for DON chemotypes. Toxin analysis by HPTLC providing equivocal results with mPCR for both the groups of toxins.

Discussion

It would be more meaningful if analytical systems are made available for low cost, simple to use qualitative and

for quantitative assessments of the mycotoxigenic fungi and mycotoxins present in the different food matrices. Conventional methods for the detection of *Fusarium* based on sporodochia with abundant macroconidia on the chaff surface is time consuming and laborious, however, PCR assays have proven to be very useful and sensitive where sporodochia are absent or in poorly developed state (Nicholson *et al.*, 1998). Multiplex PCR assays have been applied for the detection of air samples and for diseased plant, animal and human tissues for the presence of bacteria, parasites, viruses and fungi (Yenny *et al.*, 2009; Mohd *et al.*, 2010). In this study, we attempted a mPCR assay with an objective to obtain simultaneous detection of trichothecene chemotypes of *F. culmorum* spp. that are commonly associated with *Fusarium* disease.

A total of 150 *Fusarium* isolates originated from Andhra Pradesh and Karnataka, India were assayed by mPCR/HPTLC for their mycotoxin chemotypes. Results were showed that 100 strains have the *rDNA* gene specific to *F. culmorum*. Additionally mPCR amplification of *Tri6*, *Tri7* and *Tri13* alleles suggests that 94% of the *F. culmorum* isolates are able to produce DON/NIV. Some other researchers observed that, the same NIV and DON accumulation by *F. culmorum* in wheat grains (Kammoun *et al.*, 2009; Lobna *et al.*, 2010). However, to our knowledge in India, we are reporting first time DON/NIV chemotypes in maize kernels. In the present study, DON chemotypes were more aggressive when compared to NIV chemotypes of *F. culmorum*. The higher incidence of DON chemotypes may be due to intra specific variation of gene clusters of *F. culmorum* strains (Bakan *et al.*, 2001). The findings of Jennings *et al.* (2004) were confirmed our results, that DON chemotypes dominance in *F. culmorum* strains. Whereas, Lauren *et al.* (1992) and Lee *et al.* (2002) reported contradictory results that NIV dominants in *F. culmorum*.

The four DNA amplicons scored (Figure 1) were serve as a diagnostic tool for the early detection of trichothecene chemotypes of *F. culmorum*. The 300 bp amplicon is signifies the *rDNA* gene specific to *F. culmorum*, 546 bp region of *Tri6* gene is specific to all trichothecene producing *Fusarium* species (both type A and type B) whereas, 900 bp *Tri7* gene region specific to NIV chemotype and 1000 Bp region of *Tri13* gene is specific to DON chemotypes of *F. culmorum* was used in this study.

Results of the molecular assays (mPCR) were confirmed by chemical analysis by HPTLC. Thus HPTLC conducted on 94 mPCR positive *F. culmorum* isolates revealed that 54 isolates were positive for DON and 33 were positive for NIV toxin, whereas, 7 strains were negative for chemical analysis. The variation in mPCR and HPTLC analysis is not unexpected since Quarta *et al.* (2005) and Ramana *et al.*, (2011) compared the molecular analysis with chemical analysis for toxigenic *Fusarium* species and made the similar kind of findings. Thus while a positive trichothecene

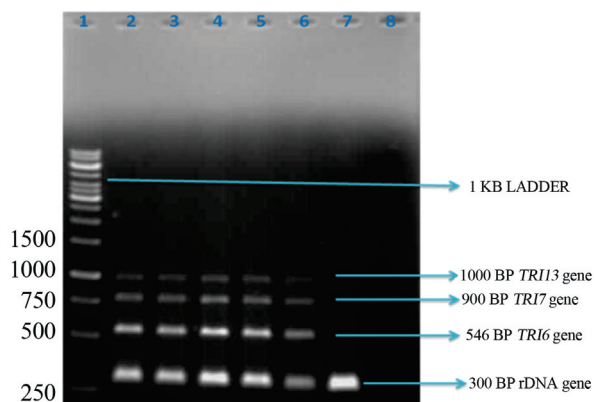


Figure 1 - Multiplex PCR photograph for DON and NIV producing *F. culmorum* Lane M: 1 kb DNA Ladder (MBI Fermentas, Mumbai, India); lanes 1 and 2: *F. culmorum* standard cultures; lanes 3, 4 and 5: *F. culmorum* isolates; lane 6 non toxigenic *F. culmorum* isolate; lane 7: negative control.

genotype indicates the potential for trichothecene biosynthesis, only a test for the toxin itself can be used to determine if and how much toxin a strain has produced. In vitro, DON/NIV may be produced by utilizing very different culture conditions such as whole grain, solid substrate fermentation, or liquid cultures using a defined minimal medium. Our results corroborate that growth conditions greatly influence the amount of mycotoxin produced. trichothecene biosynthesis may be regulated by temperature (Ramirez *et al.*, 2006), relative humidity (Beyer *et al.*, 2005), and substrate composition (O'Neill *et al.*, 1993). Results lead to the conclusion that in vitro assays could not appropriate to predict production of DON in the field as suggested by Gang *et al.* (1998). The physiology of plant-hosts and pathogenesis of the strain itself may further influence mycotoxin accumulation under field conditions. However, in the case of field samples mPCR results are equivocally matched with the chemical analysis. So, the newly developed mPCR assay is an alternative for the time consuming and laborious conventional culture methods for early assessment of trichothecene chemotypes of *F. culmorum* from field samples. The present research has demonstrated that, the occurrence of *F. culmorum* in Southern India (Andhra Pradesh and Karnataka) is dominant; it may due to cool climatic conditions and high moisture and rainfalls. Global variation in DON/NIV production by isolates of *F. culmorum* and distribution of these isolates geographically and by host are important in Plant pathology and food safety and security.

Conclusion

High levels of toxigenic *F. culmorum* incidence in maize samples demonstrates the need for better surveillance and monitoring by policy makers or food toxicologists to reduce the exposure of human and animal life to toxic compounds produced by fungi.

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