Prevalence of fumonisin producing Fusarium verticillioides associated with cereals grown in Karnataka (India)

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Received 10 July 2015; received in revised form 13 June 2016; accepted 5 July 2016

Available online 13 July 2016

Abstract

A total of 135 cereal samples were collected from different districts of Karnataka state, India in which 69 samples were infected with Fusarium species. Among these 51 samples were having Fusarium verticillioides infection and among them 42 samples were positive for fumonisin production. Per cent incidence and frequency were high in maize samples with 33.12% and 47.54%, respectively followed by paddy and sorghum, while pearl millet was free from F. verticillioides infection. Relative density of F. verticillioides association was 59.50% among the screened samples. A total of 326 Fusarium species were isolated by screening 135 cereal samples and among these 194 isolates of F. verticillioides scored positive for VERTF-1 and VERTR species-specific pair of primers. Further amplification with VERTF-1 and VERTF-2 pair of primers recorded 176 isolates of fumonisin producing F. verticillioides. The study revealed high incidence, frequency and relative density of fumonisin producing F. verticillioides and production of fumonisins in cereals. It was amplified using one forward and two reverse primers to discriminate fumonisin producing from fumonisin non-producing F. verticillioides which stresses the need for the development of managemental strategies before they enter into the food chain.

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Keywords: Fumonisin; Fusarium; IGS; Cereal; Maize; Paddy; Sorghum

1. Introduction

Cereals are the basic staple food of India and provide much of the energy and protein for majority of the population. They also have known to contain a range of micronutrients such as vitamin E, some of the B vitamins, sodium, magnesium and zinc. Contamination of cereals due to poor agricultural practices and intermittent rain at the time of harvest by fungal species of Aspergillus, Fusarium and Penicillium are often unavoidable and it is worldwide problem [1,2]. The most common mycotoxins present in cereals are aflatoxins, fumonisins, zearalenone, ochratoxins, T2 toxin and deoxynivalenol [3]. Contamination of cereals and cereal based products with fumonisins poses threat to agriculture and food safety throughout the globe. Food and Agriculture Organization (FAO) estimated that each year 25%–50% of the world’s food crops are contaminated by mycotoxins [4]. Of the fungi involved, the most common are Fusarium species which are associated with cereals all over the world. Totally 70 different Fusarium species were isolated and identified from many substrates throughout the world [2]. Fusarium verticillioides is an important fungal pathogen with a wide range of plant hosts such as maize, paddy, sorghum, etc. [5]. The risk of contamination by fumonisins is related to the association of F. verticillioides species with cereals [6,7]. Fumonisins are considered as agriculturally important environmental toxins produced by F. verticillioides and other Fusarium species in the field or during storage [8]. Fumonisins cause several diseases such as blind staggering and leukoencephalomalacia in horses [9], pulmonary edema in swine [10] and hepatic cancer in rats [11], esophageal cancer, liver cancer [12], skin lesions [13], wound [14], keratitis and polycystic kidney disease in humans. More than ten types of fumonisins have been characterized among
which B1, B2 and B3 are the major types produced [15]. The International Agency for Research on Cancer (IARC) has indicated that FB1 is a possible carcinogen to humans.

Rocha et al. [42] reported high frequency (96%) of \textit{F. verticillioides} in maize grains collected from four different regions of Brazil. \textit{F. verticillioides} and other \textit{Fusarium} species are reported to cause ear rot in maize. \textit{F. proliferatum} was reported along with \textit{F. verticillioides} from Italy [16], Southern Europe [17] and Iran [18]. \textit{F. subglutinans} was the species most frequently recovered from asymptomatic host tissue and was more frequent than \textit{F. verticillioides} [19]. Many instances of asymptomatic infection of \textit{F. verticillioides} in corn have been reported [20,21].

Levic et al., [39] reported dominance and frequency of \textit{Fusarium} species isolated from corn kernels over years; \textit{F. subglutinans} predominated in some years. High prevalence of \textit{F. verticillioides} associated with cereals persisted consistently by molecular based study with species specific primers when compared to conventional methods.

The most reliable method to distinguish between \textit{F. verticillioides} and closely related species is DNA sequence comparison. DNA used included nuclear ribosomal DNA intergenic spacer (IGS), the nuclear ribosomal DNA internal transcribed spacer, genes encoding the translation elongation factor 1 (TEF), \textit{b}-tubulin, calmodulin, cytochrome P450 reductase, and 28S ribosomal RNA [22,23]. One set of species specific primer VERTF-1 [24] and IGS based VERTR primer [25] have been used to differentiate \textit{Fusarium verticillioides} from other \textit{Fusarium} species. The other set of primer included VERTF-1 and VERTF-2 to discriminate fumonisin producing from non fumonisin producing isolates. Aim of the present work was to study the per cent incidence, frequency and relative density of \textit{F. verticillioides} associated with maize, sorghum, paddy and pearl millet using conventional and PCR methods. Further, to know their ability to produce fumonisin by LC MS method.

2. Material and methods

2.1. Collection of samples

A total of 135 cereal samples (61 maize, 42 paddy, 24 sorghum and 8 pearl millet) were collected from different districts of Karnataka state during November 2012–May 2014. All the collected samples (0.5 kg) were packed in sterile polythene bags, labeled appropriately and maintained at 4 °C. They were subjected to mycological analysis.

2.2. Isolation of \textit{Fusarium} species

Sampling was done by hand halving method according to International Seed Testing Association (ISTA 2003). The incidence of \textit{Fusarium} species was analyzed using both standard blotter and agar plating methods [26]. Two hundred grains from each sample were placed on moist blotting material as well as on agar media. Melachite Green Agar 2.5 (MGA-2.5) was used as the selective isolation medium [11]. The plates were incubated with alternating periods of 12 h darkness/light at 25 ± 2 °C for seven days. After incubation, plates were visualized for \textit{Fusarium} species by micro-morphological studies. \textit{Fusarium} species were transferred onto Potato Dextrose Agar (PDA), (Himedia, India) to identify at the species level using fungal taxonomic keys [2,27]. All fusarium isolates were maintained on Czapek Dox Agar slants at 4 °C for further studies.

Percent incidence, frequency and relative density were calculated according to the following formula;

\[
\text{Percent incidence} = \frac{\text{No. of grains infected with} \text{Fusarium sp.}}{\text{Total no. of grains plated}} \times 100
\]

\[
\text{Frequency} = \frac{\text{No. of samples with} \text{Fusarium sp.}}{\text{Total no. of samples analyzed}} \times 100
\]

\[
\text{Relative density} = \frac{\text{No. of} \text{Fusarium sp. isolated}}{\text{Total no. of Fusarium plated}} \times 100
\]

2.3. DNA Isolation from \textit{Fusarium} species

Based on the morphological characters, a total of 372 \textit{Fusarium} isolates were inoculated to 500 μL of potato dextrose broth in 2 mL microrefuge tubes and incubated with alternating periods of 12 h darkness/light at 25 ± 2 °C for 4 days. From the resulting mycelium DNA was extracted [28]. The mycelial mat was pelleted by centrifugation at 5000 r per minute for 5 min. The pellet was ground in microrefuge tubes with blunt ends of sterile disposable pipette tips in 500 μL of lysis buffer (20% SDS, PVP, 0.1 mol/L EDTA, 2 mol/L Tris–HCl, lithium chloride, pH 8.0) and incubated at 65 °C for 15 min. During incubation, the mixture was briefly vortexed 2–3 times. The samples were then treated with 500 μL of phenol: chloroform (1:1, v/v) and vortexed for 1 min and the supernatant was collected after centrifugation at 3000 r per minute for 5 min at 4 °C. DNA was precipitated with an equal volume of ice-cold isopropanol, and incubated at −20 °C for 60 min and centrifuged at 8000 r per minute for 8 min at 4 °C. The pellet obtained was rinsed with 70% ethanol, air-dried, resuspended in 50 μL of nuclease free water and for PCR.

2.4. Primers for PCR

Isolates were confirmed as \textit{Fusarium verticillioides} with the use of forward primer VERTF-1 (5′-CGC GGA ATT CAA AAG TGG CC-3′) designed by Patino et al. [24] and the reverse primer VERT-R (5′-CGA CTC ACG GCC AGG AAA CC-3′) designed by Sreenivasa et al. [29]. These were used to identify \textit{F. verticillioides} strains at the species level. The isolates were tested using the PCR specific assay for fumonisin-producing \textit{F. verticillioides} with primers VERTF-1 (5′-CGC GGA ATT CAA AAG TGG CC-3′) and VERTF-2 (5′-GAG GCC GCG AAA CCG ATC GG-3′) as described by Patino et al. [24]. The expected PCR amplicon sizes were 1016-bp and 400-bp for the primers VERTF-1/VERT-R and VERTF-1/VERTF-2, respectively.
2.5. PCR amplification

The isolated 372 DNA samples were subjected to PCR (Sure cycler 8000 Agilent technologies) using VERTF-1 and VERT-R set of primers with the PCR conditions 95 °C for 2 min of initial denaturation, 94 °C for 30 s of denaturation, 61 °C for 30 s of annealing, primer extension at 72 °C for 1 min and final extension at 72 °C for 13 min. VERTF-1 and VERTF-2 set of primers were used to discriminate fumonisin producing and non producing F. verticillioides with the PCR conditions 95 °C for 2 min of initial denaturation, 94 °C for 30 s of denaturation, 60 °C for 30 s of annealing, primer extension 72 °C for 45 s and final extension at 72 °C for 13 min. PCR reaction included 12 μL of master mix (Genei PCR Master mix), 1 μL DNA, 0.6 μL of each primer and 10.8 μL of water in a total volume of 25 μL reaction mixture. PCR products were analyzed in 1.5% agarose gel (50× TAE-Tris base, glacial acetic acid, 0.5 mol/L EDTA, pH 8) and image was documented with a gel documentation system (Vilber Lourmat-Lab India, Hyderabad) after staining with ethidium bromide.

2.6. Sequencing and construction of phylogenetic tree

Randomly selected six PCR amplified products (four F. verticillioides isolates confirmed up to species level with VERTF-1 and VERT-R primer and two F. verticillioides isolates amplified with VERTF-1 and VERTF-2 primer for the presence of gene coding for the production of fumonisin) were sequenced (Amnion Sequencing, Bangalore, India). The sequences were deposited at NCBI, GenBank, USA. Phylogenetic tree was constructed by online software MEGA 5.1 using neighbor joining method for the sequenced isolates.

2.7. Analysis of fumonisin-producing ability of Fusarium isolates by LC/MS

Randomly selected nine isolates of F. verticillioides which were identified up to species level as well as fumonisin producing F. verticillioides confirmed by PCR (three isolates each from maize, paddy and sorghum) were tested for their ability to synthesize fumonisin B1. Each isolate was artificially inoculated with the concentration of 1 mL of 10⁶ spores/mL into 5 g autoclaved each cereal in culture tubes. The tubes after incubation for 27 days were finely ground using liquid nitrogen, with sterile pestle and mortar and used for fumonisin extraction. Ground sample weighing 0.4 g was taken in a sterile glass vial and suspended with 2 mL acetoniitrile/water (1:1, v/v) extraction solvent and allowed for equilibration overnight in a gel rocker at 28 ± 2 °C. The extracts were syringe filtered using 0.45 μm nylon membrane filters and liquid chromatography/mass spectrometry (LC/MS Waters Acquity Synapt G2, United States) was performed for the sample extracts. A column C18 was used at 50 °C with sample temperature being 24 °C for a run time of 8 min and mobile phase was water/acetonitrile. MassLynx SCN781 software was used to validate the LC/MS results. All sample extractions were carried out in triplicate.

### Table 1

<table>
<thead>
<tr>
<th>Sl. no.</th>
<th>Cereal samples</th>
<th>Number of samples</th>
<th>Place of collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Maize</td>
<td>61</td>
<td>Mandy, Madikeri, Mysore, Tumkur, Shimoga, Hospet, Chitradurga, Bagalkote, Dharwad, Hubli, Bellary, Chamarajanagar, Bangalore</td>
</tr>
<tr>
<td>2</td>
<td>Paddy</td>
<td>42</td>
<td>Mysore, Mandy, Hassan, Shimoga, Tumkur</td>
</tr>
<tr>
<td>3</td>
<td>Sorghum</td>
<td>24</td>
<td>Mysore, Mandy, Chamarajanagar, Dharwad, Tumkur, Davangere, Chitradurga, Hubli, Bellary, Hassan</td>
</tr>
<tr>
<td>4</td>
<td>Pearl millet</td>
<td>08</td>
<td>Chamarajanagar, Tumkur, Bellary, Hospet, Bagalkote</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>135</td>
<td></td>
</tr>
</tbody>
</table>

### 3. Results

Mycological analysis using standard blotter and agar plate methods showed that, among 135 cereal samples (maize-61, paddy-42, sorghum-24, pearl millet-8) collected (Table 1), 69 samples (maize-37, paddy-22, sorghum-9, pearl millet-1) were positive for Fusarium infection and 51 samples were having F. verticillioides contamination. Among these 42 samples were fumonisin producing F. verticillioides (Table 2).

Among the samples, maize showed maximum incidence of F. verticillioides with 8.5% incidence in Mysore, 3.5% in Madikeri and 3.6% in Bellary samples. Further 2% incidence was in paddy from Hassan and 1.4% incidence was in sorghum from Mysore samples (Table 3). Pearl millet was free from infection. Frequency of F. verticillioides among screened samples was high (47.54%) with maize followed by paddy (42.85%) and least in sorghum (16.66%). Among the screened samples maize (40.98%), paddy (33.33%) and sorghum (12.5%) samples were found contaminated with fumonisin producing F. verticillioides (Table 2). Of the 326 Fusarium isolates obtained from 69 infected cereal samples, 194 were F. verticillioides which were isolated from 51 cereal samples. The relative density of the isolated F. verticillioides species was 59.50% whereas other Fusarium species screened had relative density of 40.5% (Fig. 1).

### Table 2

Frequency of cereal samples infected with Fusarium and its species with fumonisin production.

<table>
<thead>
<tr>
<th>Cereal samples screened</th>
<th>Infected with Fusarium sp. %</th>
<th>Infected with F. verticillioides %</th>
<th>Infected with Fumonisin producing F. verticillioides %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>60.65</td>
<td>47.54</td>
<td>40.98</td>
</tr>
<tr>
<td>Paddy</td>
<td>52.38</td>
<td>42.85</td>
<td>33.33</td>
</tr>
<tr>
<td>Sorghum</td>
<td>37.5</td>
<td>16.66</td>
<td>12.5</td>
</tr>
<tr>
<td>Pearl millet</td>
<td>12.5</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not detected.
Table 3
Percent incidence (%) of Fusarium verticillioides in cereal samples.

<table>
<thead>
<tr>
<th>Cereal samples</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Maize</td>
<td>8.5</td>
</tr>
<tr>
<td>Paddy</td>
<td>0.75</td>
</tr>
<tr>
<td>Sorghum</td>
<td>1.4</td>
</tr>
<tr>
<td>Pearl millet</td>
<td>–</td>
</tr>
</tbody>
</table>

Note: 1, Mysore; 2, Mandya; 3, Hassan; 4, Shimoga; 5, Tumkur; 6, Madikeri; 7, Chitradurga; 8, Hospet; 9, Bagalkote; 10, Dharwad; 11, Hubli; 12, Bellary; 13, Chamrajnagara; 14, Bangalore, 15, Davangere; –, no samples collected.

Fig. 1. Relative density of the F. verticillioides isolated from cereal samples.

When all the 326 Fusarium species isolates were subjected to PCR amplification with VERT primers, 194 isolates scored positive with species specific VERTF-1 and VERTR (1016 bp) primers, and 176 isolates scored positive for VERTF-1 and VERTF-2 (400 bp) primers (Fig. 2). Hence, among the 194 isolates, 176 isolates were fumonisin producing F. verticillioides and 18 isolates were non-fumonisin producing. F. verticillioides (MTCC 156) strain was used as positive control and F. gramineorum (MTCC 1893) was used as negative control (Fig. 3).

For reconfirmation of PCR results the randomly selected PCR amplified products of VERTF-1/VERTR and VERTF-1/VERTF-2 were subjected to sequencing. Sequencing reads of PCR analyzed products were tested using nucleotide megablast. The sequences were 99% similar to Giberella moniliformis strains. The same sequences were deposited at NCBI and obtained the accession numbers KJ410046, KJ410047, KR061317, KR061318 with VERTF-1/VERTR primers and KJ410767, KJ410768 with VERTF-1/VERTF-2. The sequencing result provides as reconfirmation for PCR carried out for the F. verticillioides strains isolated from the cereals. The sequenced isolates are represented by phylogenetic tree analysis (Fig. 4).

The fumonisin producing ability was reconfirmed through LCMS results and three randomly selected representative isolates from maize, paddy and sorghum were compared with toxin standard of fumonisin B1 with retention time of 1.76 min and molecular weight of 722.535 g/mol. Six isolates (two each from maize, paddy, sorghum) recorded as fumonisin producing and three isolates were non fumonisin producers (one each from maize, paddy and sorghum) when compared to standard graph (Fig. 5).

4. Discussion

During the last decade, fumonisin producing F. verticillioides and related species have received worldwide attention. The non-scientific methods of agricultural practices, poor storage facilities and unfavorable environmental conditions from the time of harvest to storage/marketing have led to colonization of fumonisin producing fungi [30]. Moulds, besides depleting the nutrients, also produce toxic substances that have potential health hazards to animals and in turn to humans [31,32]. In this study 29 maize samples exhibited the association of F. verticillioides out of 37 samples of maize screened for Fusarium species and this was followed by paddy and sorghum but pearl millet was devoid of F. verticillioides infection (Table 2). F. verticillioides was considered as predominant species on maize with signifi-
cant levels of fumonisin [33] and G. fujikuroi was the dominant species that would probably be the main source for fumonisin production in cereals [34,35]. In the present study 194 F. verticillioides were isolated (maize-108, paddy-80, sorghum-6) from 326 Fusarium species (maize-158, paddy-137, sorghum-29, pearl millet-2) screened from 135 cereal samples (Fig. 2).

Even after certain measures taken for the control of fumonisin production, the association of fumonisin producing F. verticilloides is increasing day by day. One hundred and seventy six fumonisin producing F. verticillioides (maize-103, paddy-68, sorghum-5) were isolated from screened 326 Fusarium species in the present work (Fig. 2). Among the 45 maize samples collected from south Karnataka, 25 samples were found to be highly infected with FB1 producing F. verticillioides [36]. Among the 22 fumonisin producing isolates screened, 18 were F. verticillioides in which 17 (94.4%) isolates produced fumonisins (FB1 + FB2) at concentration range of 0.07–121.45 μg/g [37]. An epidemiological survey conducted in Karnataka and Andhra Pradesh during 1997 revealed that consumption of mouldy grains affected 1424 persons in 27 villages [38]. Greater attention is bestowed to investigate Fusarium species worldwide as they reduce the value of cereals used as food and feed. Cereals contaminated with toxigenic species cause acute and chronic poisoning and allergic symptoms to animals and human. Fungi causing deterioration of cereals especially in maize, paddy and sorghum are a major problem because they produce mycotoxins [40].

In the present study, the PCR assay used for the identification of F. verticillioides isolates was quick, accurate and more sensitive, as compared to conventional methods. It was shown that 194 out of 245 isolates were morphologically identified as F. verticillioides and this was further confirmed by VERTF1/VERT-R species-specific primers (Table 4). Among the remaining 51 isolates of Fusarium, 33 were identified as F. proliferatum and 18 as Fusarium species. F. proliferatum and F. verticillioides are having similar morphological characters. Fabrico Lanza et al. [41] did molecular identification of Fusarium isolates by PCR and species-specific primer pairs. Initially they identified five species as F. proliferatum based on morphological criteria but based on PCR test they were identified as F. verticillioides. Molecular analysis using species-specific PCR primers makes possible the precise identification of Fusarium species in such cases where morphological identification is not possible. PCR method also differentiates morphologically similar but toxigenically different F. verticillioides isolates. Present study revealed the association of 53.98% of fumonisin producing F. verticillioides with cereal samples collected from different regions of Karnataka state.

In conclusion, data on the per cent incidence, frequency and relative density of F. verticillioides would be of great sig-

![Fig. 4. Represenation of phylogenetic tree for sequenced isolates of F. verticillioides.](image)

![Fig. 5. Isolate representing production of fumonisin toxin with molecular weight of 722.548 g/mol when compared to standard graph.](image)

<table>
<thead>
<tr>
<th>Cereal samples screened</th>
<th>No. of F. verticillioides screened</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conventional</td>
</tr>
<tr>
<td>Maize</td>
<td>135</td>
</tr>
<tr>
<td>Paddy</td>
<td>101</td>
</tr>
<tr>
<td>Sorghum</td>
<td>9</td>
</tr>
<tr>
<td>Pearl millet</td>
<td>ND</td>
</tr>
<tr>
<td>Total</td>
<td>245</td>
</tr>
</tbody>
</table>

ND, not detected.
nificance for predicting the extent of post-harvest infection, colonization and subsequent deterioration of cereals. Further, it also helps us to know the dry matter loss, nutritional changes and the extent of fumonisin levels during storage. Such a data is of immense value for assessing the possible health hazards in humans and animals upon consumption of such contaminated food grains. The high incidence of mycotoxigenic *F. verticillioides* is of primary concern for policy makers and food experts in this region to reduce the economic losses caused by these fungi and also to minimize the exposure of human and animal life to the potential risks of mycotoxins.

**Conflict of interest**

The authors declare that there are no conflicts of interest

**Acknowledgements**

We thank the Department of Science and Technology (DST-SERB) India, for providing grants through Young Scientist FAST TRAK research project (No. SR/FT/LS-176/2009; 30.04.2012) to Dr. M Y Sreenivasa, Principal Investigator. We thank the Institute of Excellence, University of Mysore, for their valuable support.

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