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

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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 stratergies before they enter into the food chain.

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]. Contaminantion 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 staggers 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

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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 *F. verticillioides* in maize grains collected from four different regions of Brazil. *F. verticillioides* and other *Fusarium* species are reported to cause ear rot in maize. *F. proliferatum* was reported along with *F. verticillioides* from Italy [16], Southern Europe [17] and Iran [18]. *F. subglutinans* was the species most frequently recovered from asymptomatic host tissue and was more frequent than *F. verticillioides* [19]. Many instances of asymptomatic infection of *F. verticillioides* in corn have been reported [20,21]. Levic et al., [39] reported dominance and frequency of *Fusarium* species isolated from corn kernels over years; *F. subglutinans* predominated in some years. High prevalence of *F. verticilliodes* associated with cereals consistently proved by molecular based study with species specific primers when compared to conventional methods.

The most reliable method to distinguish between *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 1α (TEF), 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 differeniate *Fusarium verticillioides* from other *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 *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 Fusarium species

Sampling was done by hand halving method according to International Seed Testing Association (ISTA 2003). The incidence of *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 *Fusarium* species by micro-morphological studies. *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;

$$\text{Percent incidence (\%)} = \frac{\text{No. of grains infected with } \textit{Fusarium} \text{ sp.}}{\text{Total no. of grains plated}} \times 100$$

$$\text{Frequency (\%)} = \frac{\text{No. of samples with } \textit{Fusarium} \text{ sp.}}{\text{Total no. of samples analyzed}} \times 100$$

$$\text{Relative density (\%)} = \frac{\text{No. of } \textit{Fusarium} \text{ sp. isolated}}{\text{Total no. of } \textit{Fusarium} \text{ plated}} \times 100$$

2.3. DNA Isolation from Fusarium species

Based on the morphological characters, a total of 372 *Fusarium* isolates were inoculated to 500 μL of potato dextrose broth in 2 mL microfuge 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 microfuge 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 *Fusarium verticillioides* with the use of forward primer VERTF-1 (5′-GCG 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 *F. verticillioides* strains at the species level. The isolates were tested using the PCR specific assay for fumonisin-producing *F. verticillioides* with primers VERTF-1 (5′-GCG GGA ATT CAA AAG TGG CC-3′) and VERTF-2 (5′-GAG GGC GCG AAA CGG 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.