

## DETECTION OF FUMONISIN PRODUCING *FUSARIUM VERTICILLIOIDES* IN PADDY (*ORYZA SATIVA* L.) USING POLYMERASE CHAIN REACTION (PCR)

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### ABSTRACT

The study reports the occurrence of fumonisin producing *Fusarium verticillioides* in 90 samples of stored paddy (*Oryza sativa* L.) collected from different geographical regions of Karnataka, India. Fumonisin producing *F. verticillioides* was identified based on micromorphological characteristics and PCR using two sets of primers. One set of primers was *F. verticillioides* species specific, which selectively amplified the intergenic space region of rDNA. The other set of primers was specific to fumonisin producing *F. verticillioides*. Eight paddy samples were positive for *F. verticillioides*. Eleven isolates obtained from these samples were capable of producing fumonisin.

**Key words:** *Fusarium verticillioides*, fumonisin, paddy, PCR

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### INTRODUCTION

Mycotoxigenic fungi are natural contaminants of cereals worldwide (9). Mycotoxins are secondary metabolites of fungi, which may contaminate animal and human foods. Grain spoilage reduces the nutritional value of cereals, resulting in 5% losses of foodstuff in world. Although there are many species of toxigenic moulds, only a few mycotoxins, particularly those affecting cereals, are considered to be significant for humans (26).

Fumonisin is one of the most recently discovered cytotoxic and carcinogenic mycotoxins, mainly produced by *Fusarium verticillioides* (Sacc.) Nirenberg (previously known as *Fusarium moniliforme*, Sheldon) (7). To date, 28 structurally related fumonisin analogues have been identified, only three of them fumonisin B<sub>1</sub> (FB<sub>1</sub>), B<sub>2</sub> and B<sub>3</sub> occur abundantly (13). Among different fumonisins isolated and characterized, fumonisin B<sub>1</sub> is known to cause a unique neurotoxic syndrome called equine leukoencephalomalacia (ELEM) in horse (23). It has been shown that fumonisin B<sub>1</sub> is hepato carcinogenic to rats and is also associated with pulmonary edema in swine (19). Ingestion of moldy corn, infected by *F. verticillioides* is linked with high

incidence of human liver cancer and esophageal cancer, in the regions of South Africa and China (14). The International Agency for Research on Cancer (IARC) evaluated FB<sub>1</sub>, as possible carcinogen to humans (Group 2B) (10).

In India, paddy (rice) is the staple food for 65% of the population and is a high calorific cereal grain (2). Despite the elementary importance of paddy, the unfavorable conditions during harvesting and processing, paddy crop is susceptible to fungal infection and subsequent deterioration during storage. Earlier studies revealed the occurrence of many mycotoxigenic fungi including species of *Fusarium* on paddy (17). Therefore the detection and control of *Fusarium* species is crucial to prevent toxins entering the food chain.

PCR protocols based on rDNA sequences have been extensively used for the accurate detection of *Fusarium* species. Ribosomal DNA sequences have been considered stable and occur in multiple copies, possess characteristics that are suitable for the detection of pathogens up to the species level (11). Many *Fusarium* species including *F. verticillioides* are mainly seed-borne in paddy (12). However, these moulds are less studied and it is not known whether the isolates on paddy have the potential to produce fumonisins. Hence, the present

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investigation was conducted to know the fumonisin producing *F. verticillioides* occurring on paddy using PCR.

## MATERIALS AND METHODS

### Collection of paddy samples

For isolation of mycotoxigenic fungi 90 different paddy samples intended for human consumption were collected from farmers of various districts of Karnataka, India, during the months of June-2006 to September-2006. Samples (0.5kg) were packed in sterile polythene bags, appropriately labeled. The grain sample was stored in the laboratory at 5°C and mycological analysis was started within a week (18).

### Media for isolation of *Fusarium* species

Isolation of *Fusarium* species from paddy was done both by standard blotter as well as agar plating methods. *Fusarium* species were isolated from paddy, by plating on selective medium such as modified malachite green agar (MGA) medium containing 2.5 mg/lit. malachite green oxalate (6) and maintained on modified Czepek dox agar (CZA) and Spezieller Nährstoffarmer agar media (SNA) (16).

### Isolation of *Fusarium* species

For mycological studies, samples were subjected to sampling by hand halving method according to International Seed Testing Association (ISTA) (18). 400 paddy grains were surface sterilized, by 1% sodium hypochlorite for 1 min. and rinsed twice in sterile distilled water for 30 seconds (21). 200 surface sterilized grains were plated (10 per plate) on MGA2.5 medium containing chloramphenicol (50 mg/l) and the remaining 200 grains were plated (25 per plate) equidistantly on moist blotters. The plates were incubated at 26°C for 5 - 6 days. The developing fungal colonies were counted directly after incubation and species of *Fusarium* were sub cultured and identified on potato dextrose agar (PDA) medium (5,16).

### DNA extraction for PCR

The genomic DNA was extracted from all the isolates of *F. verticillioides* (n=27), *F. proliferatum* (n=4), *F. anthophilum* (n=6), *F. graminearum* (n=4) and *Alternaria solani* (n=1) (negative control) respectively. Each fungal species was inoculated to 500 µl of sterile potato dextrose broth in 2 ml microfuge tubes, aseptically. The microfuge tubes were then incubated for 5 days at 26°C. Each fungal isolate was subjected to modified DNA extraction method of Zhang *et al.* (28). Briefly, microfuge tube containing mycelial growth was centrifuged at 5000 rpm (REMI C24 Cooling Centrifuge) for 8 min. and the broth was discarded. The mycelium was resuspended in the cell lysis buffer (2% CTAB, 1.4M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, pre-heated at 65°C). The microfuge tubes were heated at 65°C for 20 min. in a water bath. To these tubes, equal

volume of phenol: chloroform (1:1) was added and centrifuged at 3000 rpm for 5 min. The supernatant was transferred to new microfuge tubes and an equal volume of isopropyl alcohol was added. The microfuge tubes were kept at -20°C for 2 hours for precipitation and then centrifuged at 8000 rpm for 8 min. The DNA pellet was dried and resuspended in 20 µl of nuclease free water and used directly for PCR analysis.

### Primers for PCR

VERT-1 and VERT-2 set of primers (5'- GTCAGAA TCCATGCCAGAACG -3' and 5'-CACCCGCAGCAAT CCATCAG-3'), was used specifically to detect *F. verticillioides*. Another set of primers, VERTF-1 (5' - GCGGGAATTC AAAAGTGGCC -3') and VERTF-2 (5' - GAGGGCGCGAAA CGGATCGG -3') as described by Patino *et al.* (22), was used to detect fumonisin producing ability of *F. verticillioides*. The expected amplicons sizes were 800 bp and 400 bp respectively. The primers were purchased from Bangalore Genei, Bangalore (India).

### Polymerase chain reaction (PCR)

The total genomic DNA from *F. verticillioides* (n=27), *F. proliferatum* (n=4), *F. anthophilum* (n=6), *F. graminearum* (n=4) and *Alternaria solani* (n=1) (negative control) was subjected to PCR analysis using an Advanced Primus 25 Thermocycler (Peqlab, Germany). The PCR mixture included 2 µl of genomic DNA, 1 µl of each primer (20 pmol), 0.5 µl of Taq DNA polymerase (3U/µl), 2.5 µl of 10X PCR buffer, 2.5 µl of MgCl<sub>2</sub> and 1 µl of 2 mM dNTPs and the final volume was made up to 25 µl with nuclease free water. The reagents were purchased from Bangalore Genei, Bangalore (India).

The PCR conditions were maintained at 94°C for 4 min. for initial denaturation, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, primer extension at 72°C for 1 min. and final extension at 72°C for 5 min. Amplified products were analyzed on 1.5% agarose gel in 1X TAE buffer (40 mM Tris acetate and 1.0 mM EDTA) and documented with a gel documentation system (UTP-Bio Doc, USA).

## RESULTS AND DISCUSSION

Mycological analysis using standard blotter and agar plate methods yielded 27 isolates of *F. verticillioides*, in 8 out of 90 paddy samples screened. The isolates of *F. verticillioides* showed cultural and micro morphological characters typical for *F. verticillioides* as described by Booth (5), Leslie and Summerell (16).

This finding is in good agreement with the earlier studies, which reported the *F. verticillioides* from paddy. Taligoola *et al.* (25) reported *F. verticillioides* at a relatively higher frequency (12.5%) on the paddy seeds. *Fusarium* infection and fumonisin contamination is a major problem in Pakistan and 20% of the

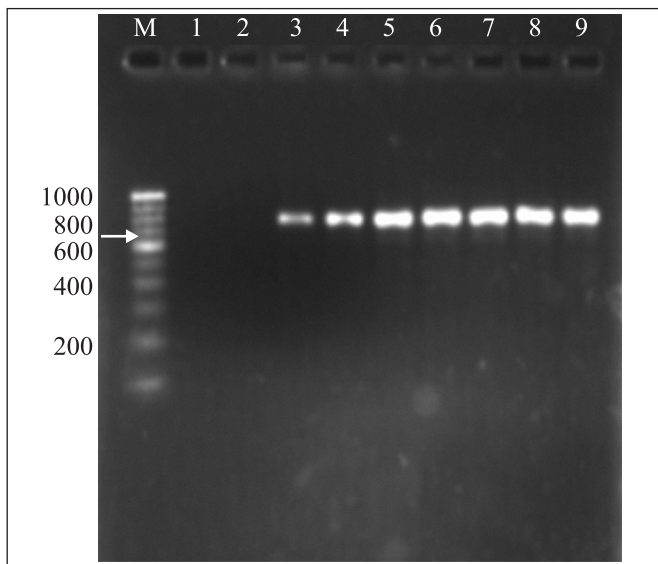
seeds were found naturally infected by *F. verticillioides* (4). Desjardins *et al.* (2000) have reported 2980µg/g of fumonisin production from rice culture. This indicates that, even 20% of *F. verticillioides* infection can be a serious problem, as paddy is the staple food for people of Asia.

*F. verticillioides* was the most common fungi and found in 89.3% of maize samples from south-western Nigeria (3). In Asia, the incidence of *Fusarium* and fumonisin contamination is highly prevalent. *F. verticillioides* contamination has been reported from Nepal, where both local and improved varieties of paddy are affected by *F. verticillioides* (8).

When genomic DNA of all the 27 isolates of *F. verticillioides* subjected to PCR, the expected 800bp amplified product specific to VERT-1 and VERT-2 was amplified. Such amplified product was not detected in other *Fusarium* species tested namely, *F. proliferatum* (n=4) and *Alternaria solani* (n=1) (Fig. 1) (Table 1).

Further, 11 out of 27 *F. verticillioides* isolates scored positive for the VERTF-1 and VERTF-2 set of primers and the expected 400 bp amplicon was detected, indicating their potential fumonisin producing ability. No such amplification was detected in other *Fusarium* species (Fig. 2) (Table 1). Similar work was done in maize by Pamphile and Azevedo, (20) and reported the endophytic *F. verticillioides* by PCR analysis.

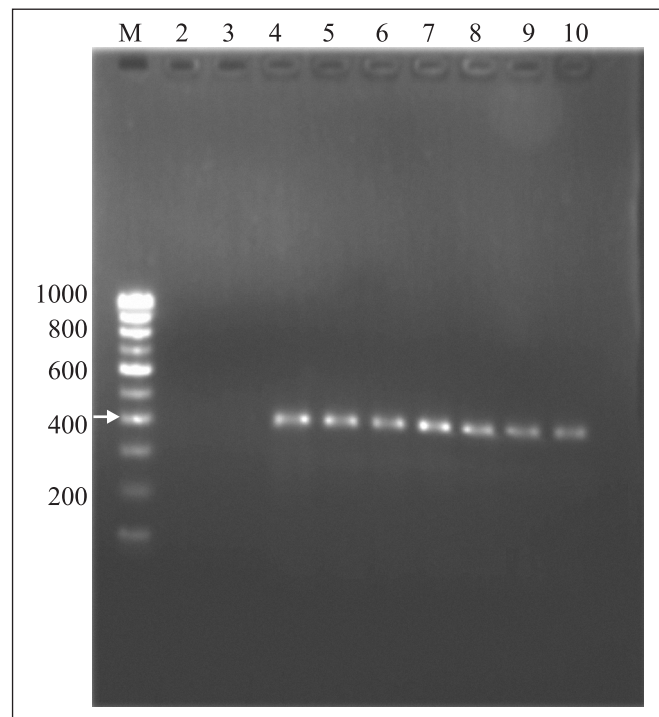
Paddy is the most important food crop with more than 90% of global production occurring in tropical and semi-tropical Asia. In several Asian countries, it provides 50 - 70% of the energy



**Figure 1.** Agarose gel (1.5%) showing 800bp amplified products of VERT-1 and VERT-2 regions of *F. verticillioides* DNA. Lane M: 1000 bp DNA marker; Lane 1: *F. proliferatum*; Lane 2: *A. solani*; Lane 3 - 9: *F. verticillioides*.

**Table 1.** Total number of *Fusarium* isolates tested and number of isolates positive for fumonisin production as analyzed by PCR.

| <i>Fusarium</i> species            | No. of <i>Fusarium</i> isolates tested | No. of <i>Fusarium</i> isolates positive for VERT-1 and VERT-2 set of primers | No. of <i>Fusarium</i> isolates positive for VERTF-1 and VERTF-2 for set of primers |
|------------------------------------|--|---|---|
| <i>F. verticillioides</i>          | 27                                     | 27  | 11  |
| <i>F. proliferatum</i>             | 04                                     | 00  | 00  |
| <i>F. anthophilum</i>              | 06                                     | 00  | 00  |
| <i>F. graminearum</i>              | 04                                     | 00  | 00  |
| <i>Alternaria solani</i> (control) | 01                                     | 00  | 00  |



**Figure 2.** Agarose gel (1.5%) showing 400bp amplified products of VERTF-1 and VERTF-2 fumonisin producing regions of *F. verticillioides* DNA. Lane M: 1000 bp DNA marker; Lane 2: *F. anthophilum*; Lane 3: *F. graminearum*; Lane 4 - 10: *F. verticillioides*.

and protein dietary requirements (2). Therefore, studies on the incidence and prevention of mycotoxigenic fungi on paddy are very important. Systematic investigation on contaminating fungal species and their accurate identification is of paramount importance to take the appropriate preventive measures.

The PCR based method used in the present study was found to be quick and more sensitive in identification of *F. verticillioides* isolates, in comparison with agar plating method, which takes a minimum of 5-6 days. The PCR based method was also able to distinguish morphologically similar but toxigenically different *F. verticillioides* isolates.

The study revealed the prevalence of *F. verticillioides* in paddy and confirmed the association of fumonisin producing *F. verticillioides* isolates in paddy, collected from different regions of Karnataka state. The study, suggests the need for sensitive techniques for the quantitative analysis of fumonisins in paddy intended for human consumption. By such investigations it is possible to prevent the exposure of humans and animal life to toxic substances such as fumonisins.

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#### RESUMO

##### **Deteção de *Fusarium verticillioides* produtor de fumonisin em arroz empregando Reação de Polimerase em Cadeia**

O estudo relata a ocorrência de *Fusarium verticillioides* produtor de fumonisin em 90 amostras de arroz armazenado, coletado de diferentes regiões geográficas de Karnataka, Índia. *F. verticillioides* produtor de fumonisin foi identificado baseado em características micromorfológicas e PCR empregando dois sets de primers. Um dos sets era *F. verticillioides* espécie-específico, que amplificava seletivamente a região do espaço intergênico do rDNA. O outro set de primers era específico para *F. verticillioides* produtor de fumonisin. Oito amostras de arroz foram positivas para *F. verticillioides*. Onze isolados obtidos dessas amostras foram produtores de fumonisin.

**Palavras-chave:** *Fusarium verticillioides*, fumonisin, arroz, PCR

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