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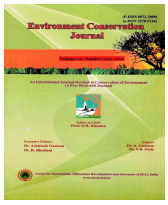

Detection of fumonisin among different strains of *Fusarium* spp. associated with bakanae disease of rice (*Oryza sativa* L.) using molecular markers

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ABSTRACT

Bakanae disease caused by *Fusarium fujikuroi* of basmati rice causes huge economic losses varying with varieties produced, with a frequency of 3.0-95.4%. The *Fusarium* spp. associated with bakanae disease produce fumonisins, a group of structurally similar sphingosine analogue mycotoxins, among which Fumonisin B1 is the most prevalent and active (FB1). The worst harm to both people and animal wellbeing is created by fumonisins, which infect feed and food sources. IARC, a global organization dedicated to cancer research, classified FB1 as a potential causing human cancer (Group 2B). Altogether 26 strains of *Fusarium* spp. from bakanae infected samples of various popular basmati rice varieties collected from Hisar, Jind, Fatehabad, Bhiwani, Sirsa, Panipat, Sonipat, Karnal, Yamunanagar, Kaithal and Kurukshetra (eleven) districts of Haryana state. Two specific primers namely VERTF and polyketide synthase (PKS) (involved in fumonisin biosynthesis) FUM (rp 32 and rp 33) were utilized in this investigation to differentiation between fumonisin-producing and non-producing strains employing PCR technique. Twenty-two strains were significant for the VERTF primer and showed the capacity to generate fumonisin, while four isolates evaluated negative for both primers. The FUM specific primer displayed positive respose only in nine strains and rest were negative. The present study provides a rapid and specific method that helped in accurate differentiation between fumonisin-producing and non-producing strains.

Introduction

In Asian nations where rice is grown, a disease known as bakanae induced by *Fusarium fujikuroi* has gained substantial importance (Asmaul *et al.,* 2021). Hori (1898) was the first to recognise *F. heterosporium* Nees as the disease's cause. Ito and Kimura renamed the imperfect stage *F. moniliforme* and revised Sawada's (1917) description of the fungal sexual stage from *Lisea fujikuroi* to *Gibberella fujikuroi* in 1931 (Sun and Snyder, 1981). In addition to damaging plant tissues, *Fusarium* spp. forms fumonisins, a group of mycotoxins which cause a variety of health problems in animals and humans. Genus *Fusarium*, which includes the major fumonisin producers, is primarily responsible for producing these chemicals (Tyska *et al.,* 2021). Mycotoxins being dangerous natural compounds created by various pathogenic fungus that occur in nature in a variety of products from all over the globe. Food and feed frequently include deadly mycotoxins called fumonisins (FBs), (Li *et al.,* 2022). The potential impacts on the wellbeing of the animals are considered in the

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threat analysis of mycotoxins in animal feed, together with the potential dangers of their compounds transferring to animal - based foods intended for human consumption (Mackay *et al.,* 2022). Fumonisin causes severe species- and organ-specific fatalities, such as neurological problems in horses, pulmonary edoema in pigs, gastrointestinal cancer in people, renal and liver related adverse reactions in rats (Wangia and Kizito, 2020).

In general, the morphological characteristics of fumonisin, which is produced by fungi, are insufficient (Jurado *et al.,* 2010). Using DNA-based molecular methods, precise and specific methodologies for detecting strains that can generate fumonisins have been created. A PCR test for fumonisin detection using the FUM1 primer has been developed in earlier research investigations. According to Moretti *et al.* (2004) the internal transcribed spacer section (IGS) is a non-coding region with a widely varying nucleotide. Two sets of primers (VERTF1/2) were found in the IGS (Patino *et al.,* 2004). Nayak *et al.* (2018) studied genetic variability of fumonisin generating *Fusarium* strains of bakanae employing PCR-RFLP of IGS-rDNA section. Current research intends to provide scientific data for upcoming initiatives to undertake corrective and preventive measures for the management of mycotoxins in the nation. So keeping in view the danger of exposure to carcinogenic compounds due to the large consumption of rice-based diets and a fairly quick precise method that aids in the distinction between fumonisin producing and non-producing strains with accuracy, present study was planned.

Material and Methods

Diseased samples collection

Bakanae infected diseased samples (64) from commonally grown aromatic rice cultivars viz., PB 1121, PB 1401, PB 1718, PB 1509 and Basmati 521, were collected from Hisar, Jind, Fatehabad, Bhiwani, Sirsa, Panipat, Sonipat, Karnal, Yamunanagar, Kaithal and Kurukshetra districts of Haryana during *kharif* season. From these samples, finally twenty six different isolates of *Fusarium* spp. were selected and maintained for this study.

Isolation, purification and maintenance

The infected samples were cut into pieces of three to four mm and surface disinfected using $MgCl_2$ (0.1%). Under completely sterile and aseptic conditions, the cut pieces were distributed evenly over potato dextrose agar media in petri plates. A BOD incubator was used to incubate the inoculated plates at a temperature 25±2°C. Purified and the related fungus from the culture plates, *Fusarium* spp. were identified through microscopic examination (Leslie and Summerell,2006) subcultured using the single spore culture technique (Hansen, 1926) and maintained using potato dextrose agar for future research.

DNA extraction

Twenty six purified strains of *F. moniliforme* were used to isolate DNA using CTAB technique. (Murray and Thompon, 1980). Using a sterile pestle and mortar, the fungus mat was crushed into a powder and then immersed in liquid nitrogen. The powder that resulted was collected in 2 ml centrifuge tubes. The powder was combined with 800 µl of 1% mercaptoethanol-containing CTAB buffer before being placed in the tubes for an hour in a water bath at 65°C. After each 15 minutes, the tubes were carefully turned over to combine the contents, eighty hundred µl of chloroform : isoamyl alcohol (24:1) were supplied after incubating. In order to ensure proper mixing, the materials were chilled to outside temperature and the materials were shaken at seventy rpm for thirty to forty five minutes. After shaken, the samples were placed in a micro-centrifuge at ten thousands rpm for fifteen min. Using a pipette, the liquid was extracted from the tissue waste and placed in new 1.5 ml spin tubes. The supernatant was then given the RNase treatment by having 10 µl of RNase to every tube, which was then keeping them at 37°C for 30 min. Following the addition of 800 µl of cold isopropyl alcohol, moderate inversions were performed. After being kept at four degree celsius for fifteen minutes, materials spun once more for ten minutes at 10,000 rpm. The pellet was rinsed with 70% ethanol after the supernatant was disposed. After being wind drying, the pellet was combined with 50 µl of Tris-EDTA buffer. The isolates' entire DNA was kept in storage at -20°C until usage.

PCR amplification

In this investigation, a PCR test was utilized to differentiation between fumonisin-producing and non-producing strains using two specific primers called VERTF (Vertf 1 and Vertf 2 forward and reverse primers, respectively) and FUM (rp 32 and rp 33 forward and reverse primers), which are both

involved in fumonisin biosynthesis. PCR was conducted in a total amount of 25 µL with every tube holding 12.5 µL of the master mix (Promega corporation, USA) 7.5 µL water, 1.5 µL each primers (Vertf 1-5'-GCGGGAATTCAAAAGTGG CC-3') and (Vertf 2-5'-GAGGGCGCGAAA CGGATCGG-3') as studied by (Patino *et al*., 2004) and FUM (rp 32-5- ACAAGTGTCCTTGGGGTC CAGG-3') and (rp 33-5'-GATGCTCTTGGAAGT GGCCTACG-3') as reported by (Jeon *et al.,* 2013) and 2 µl DNA of each isolates. The primers were synthesized by Integrated DNA Technologies (USA). The PCR conditions used included pre-incubation at 94^0C for 4 min, followed by multiplication for 35 rounds, including denaturation at 94^0C for 1 min, annealing at 60^0C for one min, extension at 72^0C for 1 min and a last extension stage of seven min at 72^0C. Amplicons were observed by electrophoresis on 2% agarose gels utilizing the EtBR dye and documented with a (Bio-Rad, Philadelphia, PA, USA) gel documentation system.

Results and Discussion

A total of twenty six *Fusarium* isolates were analyzed in the present investigation which obtained from common aromatic paddy cultivars. The infected samples were collected from different locations of Haryana state.

A PCR product of size approximately 400bp was observed for primers (Verf 1 and Vertf 2) in Fig. (1a) lane 2 M 100 bp lanes (3-14) isolates numbers as FM 3, FM 7, FM 10, FM 12, FM 16, FM 18, FM 20, FM 25, FM 28, FM 31, FM 34 and FM 36 (Table 1) and in Fig. (1b) lane 1 M 100bp lanes (2-15) isolates FM 37, FM 40, FM 44, FM 50, FM 51, FM 52, FM 53, FM 56, FM 59, FM 60, FM 62, FM 63, FM 64 and FM 66 (Table 1) in sequence. Twenty two isolates were showing fumonisin producing ability and four isolates (FM 52, FM 56, FM 59 and FM 62) were negative.

Similarly in Fig. (1c) lane 2 M 100 bp lanes (3-14) isolates viz., FM 3, FM 7, FM 10, FM 12, FM 16, FM 18, FM 20, FM 25, FM 28, FM 31, FM 34 and FM 36 (Table 1) and in Fig. (1d) lane 1 M 100bp lanes (2-15) isolates numbers as FM 37, FM 40, FM 44, FM 50, FM 51, FM 52, FM 53, FM 56, FM 59, FM 60, FM 62, FM 63, FM 64 and FM 66 (Table 1) in sequence for FUM gene a product of size approximately 680bp was observed. Nine isolates viz., FM 3, FM 7, FM 10, FM 12, FM 16, FM 18, FM 40, FM 52 and FM 56showed amplification of FUM (rp 32 and rp 33 primers) therefore regarded as producer of fumonisin while the remaining strains were non-fumonisin producers.

This study further demonstrates that PCR analysis is an effective and fast way to detect fumonisin producing *Fusarium* strains. Similar to our study, as we detect almost 85% fumonisin producing strains, Nayak *et al.* (2014) detected 85% fumonisin producers among 28 *Fusarium* isolates from Indian rice cultivars with a rapid molecular method using primer Fum5 F and Fum6 R.

Elsharnouby *et al.* (2015) studied twelve *Fusarium* isolates for PCR assay to distinguish between isolates that produce fumonisin and those that do not. Single strain (*F. verticillioides*) from damaged maize, 10 samples (*F. moniliforme*) from infested paddy with bakanae disease, and one strain (*F. solani*) from damaged wheat. In that investigation, two distinct primers by the names of VERTF-1 and FUM1 were employed. The polyketide synthase (PKS) gene FUM1 and the intergenic spacer region (IGS) of rDNA. Only sample (*Fusarium solani*) was evaluated negatively for both primers while 11 strains were positive for the VERTF-1 primer and had the capability to generate fumonisin. Five strains of *Fusarium moniliforme* while one of *Fusarium verticillioides*, respectively, responded negatively to the primer FUM1. In this study, Vertf primer also showed good results as in our investigation in comparison to primer FUM. *Fusarium* spp. isolates infecting wheat and maize were also used in this investigation while we studied only rice isolates.

Deepa *et al.* (2015) collected a total of 135 cereal samples from different districts of Karnataka, 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. Similar to our study, Vertf primer also showed good results for fumonisin detection among different strains of *Fusarium* species.

Hinojo *et al.* (2006) showed deviation in methods as used in present study for detection of fumonisins among the isolates, optimized analytical method for

Table 1: Details of *Fusarium* isolates collected from different locations of Haryana for fumonisin producing and non-producing ability

SN	Isolates ID	Locations (Districts)	Isolates positive/ negative (Vertf)	Isolates positive/ negative (FUM)	Variety grown
1	FM 3	Dhad (Hisar)	+	++	PB 1121
2	FM 7	Gurana (Hisar)	+	++	PB 1121
3	FM 10	Kheri Jalab (Hisar)	+	++	PB 1121
4	FM 12	Intal Khurd (Jind)	+	++	PB 1121
5	FM 16	Ikkas (Jind)	+	++	PB 1121
6	FM 18	Saniana 1 (Fatehabad)	+	++	PB 1121
7	FM 20	Saniana 2 (Fatehabad)	+	--	PB 1401
8	FM 25	Pirthala1 (Fatehabad)	+	--	PB 1121
9	FM 28	Pirthala 2 (Fatehabad)	+	--	PB 1121
10	FM 31	Kungar (Bhiwani)	+	--	PB 1509
11	FM 34	Alakhpura (Bhiwani)	+	--	PB 1121
12	FM 36	Barsi (Bhiwani)	+	--	PB 1121
13	FM 37	Patli Dabar (Sirsa)	+	--	PB 1401
14	FM 40	Mochiwali (Sirsa)	+	++	PB 1121
15	FM 44	Bajekan (Sirsa)	+	--	PB 1121
16	FM 50	Naiwala(Sirsa)	+	--	PB 1718
17	FM 51	Bapoli (Panipat)	+	--	PB 1509
18	FM 52	Panipat 1	-	++	PB 1718
19	FM 53	Panipat 2	+	--	PB 1718
20	FM 56	Sonipat	-	++	PB 1121
21	FM 59	Taraori (Karnal)	-	--	PB 1121
22	FM 60	Sikri (Karnal)	+	--	PB 1718
23	FM 62	Kartarpur (Yamunanagar)	-	--	PB 1509
24	FM 63	Sandhala (Yamunanagar)	+	--	PB 1121
25	FM 64	Kaithal	+	--	Basmati 521
26	FM 66	Babain (Kurukshetra)	+	--	PB 1509

Note: Isolates positive for primer Vertf shown by symbol (+) and negative (-) and isolates positive for primer (FUM) shown by symbol (++) and negative (--)

determination of fumonisins in rice was applied to the study of FB_1 and FB_2 production by four isolates of the *G. fujikuroi* species complex in rice cultures carried out at different temperatures and water activities to establish the influence of strain and environmental conditions on fumonisin production in this cereal. In general, fumonisin production was the highest at 20°C and lowest at 37°C. Four of the five assayed water activity (a_w) values (0.97, 0.98, 0.99, and 1.0) did not affect significantly fumonisin accumulation but fumonisins were not detected in cultures when a_w was 0.96.Similar to our study, Maheshwar *et al.* (2009) studied 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 micro-morphological 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. Less no. of isolates were taken for the detection of fumonisins as comparisons to our investigation.

Sreenivasa *et al*. (2008) reported that out 83 *Fusarium verticillioides* strains 64 were positive for Fumonisin production. Choi *et al.* (2018) assessed the genetic potential for fumonisin production among different isolates using a PCR assay designed to detect the presence of the FUM1 gene.The results of this assay showed that about 98% of the FFSC isolates tested were positive for FUM1

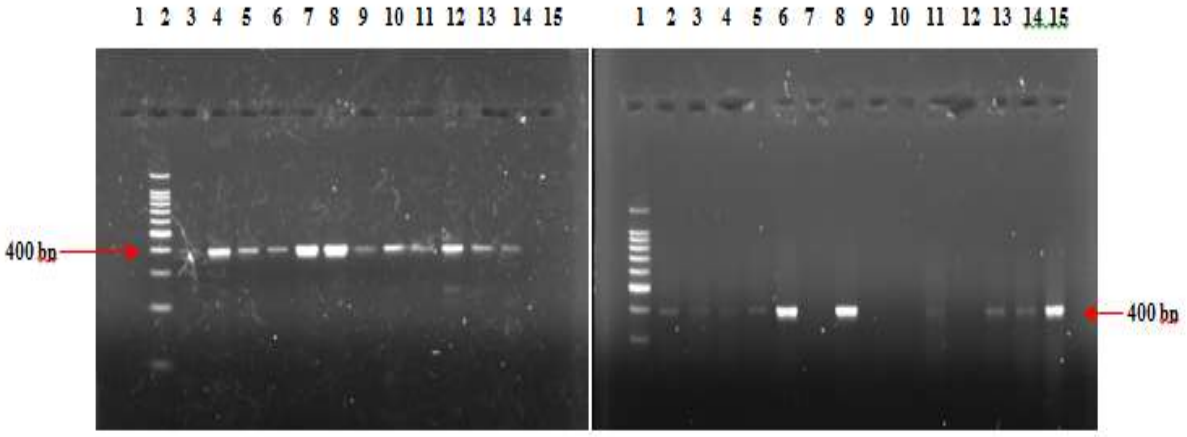

Fig. (1a) lane 2 M 100 bp lanes (3-14) isolates FM 3 FM 7, FM 10, FM 12, FM 16, FM 18, FM 20, FM 25, FM 28, FM 31, FM 34 and FM 36 size 400 bp

Fig. (1b) lane 1 M 100 bp lanes (2-15) isolates FM 37, FM 40, FM 44, FM 50, FM 51, FM 52, FM 53, FM 56, FM 59, FM 60, FM 62, FM 63, FM 64 and FM 66 size 400 bp

Figure (1a) and (1b): Details of *Fusarium* isolates for fumonisin producing and non-producing ability by using primers (Verf 1 and Vertf 2)

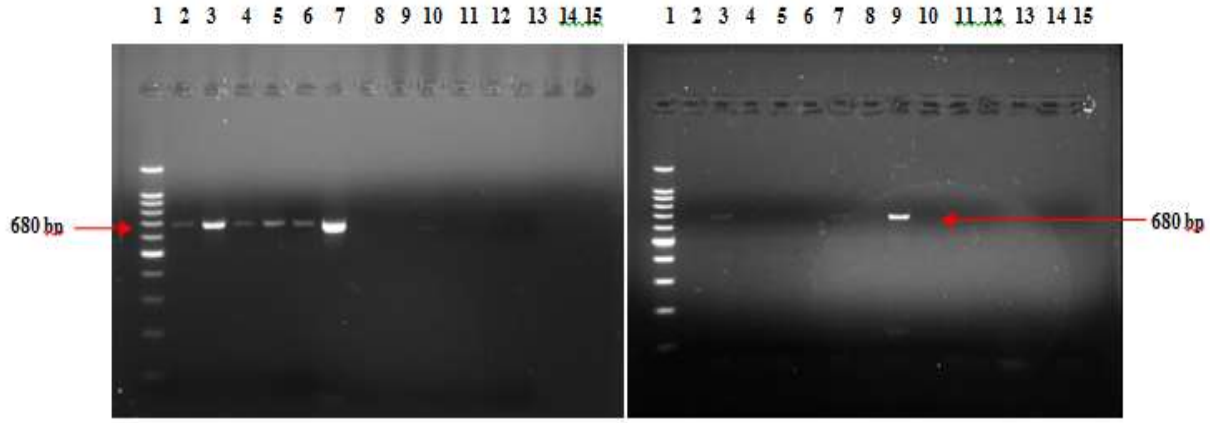

Fig. (1c) lane 1 M 100 bp lanes (2-13) isolates FM 3 FM 7, FM 10, FM 12, FM 16, FM 18, FM 20, FM 25, FM 28, FM 31, FM 34 and FM 36 size 680 bp

Fig. (1d) lane 1 M 100 bp lanes (2-15) isolates FM 37, FM 40, FM 44, FM 50, FM 51, FM 52, FM 53, FM 56, FM 59, FM 60, FM 62, FM 63, FM 64 and FM 66 size 680 bp

Figure (1c) and (1d): Details of *Fusarium* isolates for fumonisin producing and non-producing ability by using primers FUM (rp 32 and rp 33)

including all of the *F. fujikuroi*, *F. proliferatum*, *F. verticillioides* and *F. thapsinum* isolates. Actual production ability was assessed in rice medium and 76.0% of *F. fujikuroi*, 96.3% of *F. proliferatum*, and 94.1% of *F. verticillioides* isolates produced both FB1 and FB2. There was a precise detection of fumonisins producing strains using rapid method similar to our investigation. Similar to our study, as we detects the fumonisins among *Fusarium* spp. which causing bakanae, Jeon *et al*. (2013) reported that three isolates of *F. fujikuroi*, *F. proliferatum* and *F. verticillioides* were found to have FUM1 (the fumonisin biosynthetic gene); however, FUM1 was not found in isolates of *F. concentricum*.

Conclusion

Four strains were evaluated negatively for both primers, while 22 strains tested positive for VERTF primer and showed the capability to generate fumonisin. The primer FUM (rp32 and rp 33) showed positive signal in nine strains and rest of all were negative. The approach presented in this investigation enables quick and precise differentiation between fumonisin-producing and non-producing strains. According to the study, sensitive methods are required for the quantification of fumonisins in rice meant for human consumption. Through this research, it will be feasible to protect both humans and animals from hazardous compounds like fumonisins.

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Conflict of interest

The authors declare that they have no conflict of interest.

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