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Morphological and Molecular Identification of *Aspergillus flavus* Isolated from Rice (*Oryza sativa* L.) Grain Samples in Karnataka and Tamil Nadu in Southern India

T. Dhivya Priya, C. Mahendra, Shamprasad Phadnis* and Anitha Peter

Department of Plant Biotechnology, University of Agricultural Sciences, Bengaluru - 560 065, India.

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Mycotoxin contamination especially aflatoxin is of grave concern in food safety, due to its carcinogenicity. Rice is a staple food for all sections of people in India. The present investigation thus aimed at enumeration of fungal microflora in ten samples of rice collected from Karnataka and Tamil Nadu in South India. The main genera found were *Aspergillus, Cladosporium, Alternaria, Rhizopus and Aspergillus* which was found with highest frequency (90%) and relative density (52%) with *A. ochraceous* and *A. flavus* comprising ninety and sixty percent respectively. The presence of *A. flavus*, a principal aflatoxin producer, was confirmed by polymerase chain reaction (PCR) amplification of the specific internal transcribed spacer (ITS) ITS1-5.8S-ITS2 of the ribosomal DNA region of the fungus and its sequencing in selected isolates. This region is also found in the non-aflatoxigenic *A. oryzae* with 100 % homology. The isolates were hence confirmed as *A. flavus* by amplification of an important gene in aflatoxin biosynthetic pathway, namely *nor*-1 (*afl*D) gene and its expression by reverse transcription PCR (RT-PCR) as this gene is present in both species but expressed only in *A. flavus*. The co-occurrence of *A. ochraceous* in most of the samples also raises concern in consumer health which needs to be addressed during the storage and public distribution of rice. The presence and expression of *nor*-1 gene could also be used as a molecular tool for detection of *A. flavus* in stored rice and other food grains.

Keywords: Rice; *A. flavus*; Aflatoxins; *nor*-1; RT-PCR.

Aflatoxins are a class of mycotoxins produced as secondary metabolites mainly by *Aspergillus flavus* and *Aspergillus parasiticus* and rarely by other species of *Aspergillus* like *A. nominus, A. pseudotamarii, A. bombycis, A. ochraceoroseus* (Varga *et al.,* 2003; Cary *et al*., 2005).The four major aflatoxins are AFB_1, AFB_2 produced by *A. flavus* and AFG_1, by *A. parasiticus*. AFB1 is the most potent human carcinogen (group1) because of its association with hepatocellular carcinoma (IARC 1993; Liu *et al*., 2010).

* To whom all correspondence should be addressed.
E-mail: shamphadnis08@gmail.com

A. flavus and *A. parasiticus* represent the prominent contaminants of cereals such as maize, sorghum, pearl millet, rice, wheat produced in the humid tropics and sub-tropics (Reddy *et al.*, 2010).Because of their adverse effect on human health,they may cause economic problems for international trades and loss to grain producers (Degola *et al*., 2007).

Rice (*Oryza sativa* L.) is one of the most important staple foods in the world. The grains when exposed to heavy rain or high humidity at the time of storage could become susceptible to *Aspergillus* species and aflatoxin contamination (Vasanthi and Bhat, 1998; Reddy *et al*., 2009). Inorder to improve food safety and food security, a number of surveys and monitoring programs have been carried out in several countries (Park *et*

al., 2002; Liu *et al*., 2008). Regulatory authorities in different countries have set maximum tolerance levels for aflatoxin that range from 0 to 50 µg/ kg to control their contamination level in food supply (FAO, 2004). In India, the permissible tolerance limit for aflatoxins in all foods are 30 µg/kg as prescribed under the Food Safety and Standards (Contaminants, Toxins and Residues) Regulations, 2011.

Though conventional procedures of identifying *Aspergillus* species based on microbiological and immunological techniques are reliable, they are generally time consuming, laborious and non-specific and may result in false positives (Degola *et al*., 2007). Hence a precise, responsive and specific PCR reaction based diagnostics has been applied as an alternative assay for detection and identification of the mycotoxin producers in the fungal genera of *Aspergillus* (Niessen, 2007).

PCR amplification of internal transcribed spacer 'ITS1-5.8S-ITS2' of ribosomal DNA (rDNA) combined with sequencing of amplicons is used to identify *Aspergillus* at species level (Chen *et al*., 2002).The results of these methods are generally well correlated with morphological and physiological traits (Rodrigues *et al*., 2011).

There are 25 aflatoxin biosynthesis genes, of which *nor*-1 (*afl*D) plays an essential role in the early conversion of norsolorinic acid to averantin (Chang *et al*., 1992; Yu *et al*., 2004; David, 2009). Several researchers in recent years have applied molecular tools (genomic PCR) to detect the presence or expression (RT or Real Time PCR) of the aflatoxin biosynthesis genes. However, PCR detection of aflatoxin biosynthesis genes cannot resolute in distinguishing aflatoxigenic from non-aflatoxigenic fungi due to undetected genetic mutation within primer targeted binding site (Levin, 2012).

Reverse Transcription-PCR based screening methods targeting aflatoxin biosynthesis genes (*afl*D, *afl*O, *afl*Q and *afl*P) seems more proficient for distinguishing aflatoxigenic from non- aflatoxigenic strains as they are aimed at gene expression studies (Sweeney *et al*., 2000; Scherm *et al*., 2008; Rodrigues *et al*., 2009).

This preliminary study aimed to throw light on the safety of rice for direct human consumption in the key rice growing states of Southern India, namely Karnataka and Tamil Nadu, with the objectives: (1) Screening of aflatoxigenic *Aspergillus* species in rice (2) Detecting the presence of aflatoxin biosynthesis gene- *afl*D in *Aspergillus* species by genomic PCR and (3) To discriminate aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* using Reverse Transcription PCR (RT-PCR).

MATERIALS AND METHODS

A total of 10 rice grain samples (250 g each) were collected from retail shops and godowns in the states of Karnataka and Tamil Nadu, India (Table 1). The rice samples were sealed in plastic bags, appropriately labeled and stored at 4 ºC for further analysis.

The samples were subjected to direct agar plating to assess the incidence of various *Aspergillus* species. Twenty five to thirty seeds were randomly selected from each sample, surface sterilized with 0.1% sodium hypochlorite for 2 minutes, followed by three washings with sterile distilled water and dried on sterile filter paper. About 8-10 disinfected seeds were aseptically inoculated onto the surface of freshly prepared potato dextrose agar (PDA) (peeled potatoes 200 g L^{-1}; dextrose 20 g L^{-1}; bacteriological agar 15 g L^{-1}) plates and incubated at 25 ºC for 5 days. The PDA plates were supplemented with 0.5mg/ml of chloramphenicol as recommended by Neergard (1973) to inhibit the bacterial growth. Fungal colonies were characterized based on their colony morphology. These colonies were sub cultured and maintained on PDA (Himedia, Mumbai, India) slants for further morphological and microscopic examinations (Barnett and Hunter, 1998). These isolates were coded as indicated in Table 1. The percentage of frequency of occurrence and relative density of fungal species were calculated according to the method of Gonzalez *et al*. (1995) (Table 2).

$$\text{Frequency of occurrence\%} = \frac{\text{No. of samples of occurrence of a genus / species}}{\text{Total no.of samples}} \times 100$$

$$\text{Relative density (\%)} = \frac{\text{No. of isolates of a genus / species}}{\text{Total no. of fungi / genus isolates}} \times 100$$

Screening of *Aspergillus* species on Aspergillus Flavus Parasiticus Agar (AFPA) media

Representative isolates of *Aspergillus* species were transferred onto AFPA media as described by Pitt *et al*. (1983) with minor

modifications (bacteriological peptone 10 g L^{-1}; Yeast Extract 20 g L^{-1}; Ferric Ammonium citrate 0.5 g L^{-1}; chloramphenicol 0.2 g L^{-1}; rose bengal dye 50 mg L^{-1}; bacteriological agar 15 g L^{-1}). The plates were incubated in the dark at 25ºC for 42 to 72 hours to confirm group identification by colony reverse colour (Klich and Pitt, 1988).

Fungal DNA extraction

DNA was extracted from *Aspergillus* mycelium using Cetyl-trimethyl-ammonium bromide (CTAB) method (Karthikeyan *et al*., 2013). The isolates of *Aspergillus* strains were grown for 3 days in 5 ml of potato dextrose broth supplemented with 0.5 mg/ml chloramphenicol. About 100 mg of mycelium was harvested by filtration, transferred to pre-cooled mortar, frozen in liquid nitrogen and ground to fine powder. The powder was suspended in 1ml CTAB lysis buffer (2% CTAB, 1.4M NaCl, 20mM EDTA, 100mM Tris-HCl, 0.2% β-mercaptoethanol and 1% PVP, pH 8.0) incubated at 65 ºC for 1 hour with gentle inversions. The tubes were spun at 12,000 rpm for 10 min. The clear supernatant was collected and equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and spun at 12,000 rpm for 10 min. The upper aqueous layer containing nucleic acids was precipitated by the addition of equal volume of cold isopropanol and incubated at -80 ºC for 30 min. The DNA pellet recovered was washed with pre-chilled 70% ethanol, air dried, dissolved in 20 µl of TE buffer and stored at -20 ºC for further use. Five microlitre of RNAse (10mg/ml) was added to remove RNA and incubated at 37 ºC for 30 min. The DNA concentration and purity was determined by biospectrometer (Eppendorf).

PCR amplification of ITS region for identification of *Aspergillus flavus*

The ITS1 and ITS4 region primers (White *et al*., 1990) were used for amplification of fungal DNA. All primers were custom-synthesized by Sigma-Aldrich, India (Table 3).

PCR amplification reaction mixture of 40 µl contained 50 ng template DNA, 200 µM dNTPs, 0.5 µM each of ITS1 and ITS4 primers, 1U *Taq* DNA polymerase (GeNei, Bangalore, India) and 10X reaction buffer with 15 mM $MgCl_2$. PCR amplification of 35 cycles was performed in DNA thermocycler (Applied Biosystems) with initial denaturation at 96 ºC for 3 min, denaturation step at 94 ºC for 1 min, annealing at 60 ºC for 30 sec and extension at 72 ºC for 1 min with a final extension at 72 ºC for 10 min. The PCR products were separated on 1.5 % agarose gel containing 10 mg/ml ethidium bromide in 1X TAE buffer and stored at -20 ºC for further use.

Sequence analysis

The partial sequencing of ITS PCR products using ITS1 or ITS4 primers was performed at Chromous Biotech, Bangalore, India. The BLAST analysis of the resultant partial nucleotide sequences was done using NCBI database (Altschul *et al*., 1990) and *Aspergillus flavus* was identified based on E-value (zero).

Molecular detection of *afl*D (*nor*-1) gene in *Aspergillus* species

The PCR reaction was carried out for *nor*-1 (*afl*D) gene using specific primers as reported in literature (Giessen *et al*., 1996) (Table 3). A reaction volume of 20µl was optimized as follows: 10X reaction buffer with 15 mM $MgCl_2$, 200 µM dNTPs, 1U *Taq* DNA Polymerase (GeNei, Bangalore, India), 0.5 µM each of Nor-1 F and Nor-1 R primers, 50 ng template DNA and nuclease free water.

The PCR conditions for 35 cycles of amplification in DNA thermocycler (Applied Biosystems) were 3 min at 94ºC, 1 min at 94ºC, 1 min at 65ºC and 1 min at 72ºC with a final extension at 72ºC for 10 min. The PCR products were then separated and visualized in 1.8 % agarose gel (1X TAE) and the gel was documented using gel documentation unit (Alpha Innotech Corp. USA).

Total RNA extraction and RT- PCR for expression studies of *afl*D (*nor*-1) gene

The Mycelia (200 mg) of *A. flavus* isolates collected from three day old culture grown in 5 ml potato dextrose broth supplemented with 0.1 % peptone (w/v) (Melvin and Alka, 2013) was used for RNA extraction using RNeasy Plant Mini Kit (Qiagen, Germany). Total RNA was extracted as per manufacturer's instructions. The concentration and purity of RNA was checked using Nanodrop spectrophotometer (Eppendorf, Germany). The RNA samples were examined on 0.8 % agarose gel in 1X TAE.

First strand cDNA was synthesized from total RNA in a 20 µl reaction using AMV-RT enzyme (GeNei, Bangalore, India). RT reaction was set up by adding 8 µl of RNA sample (3 µg) and 1µl of *Nor*-1 reverse primer, in a 0.2 ml polypropylene tube. This mixture was heated to 70 °C for 5 min.

and immediately chilled for 5 min. cDNA synthesis was performed in PCR machine (Bio-Rad MyCycler) with the cycle parameters programmed for one cycle of 42°C for 1 h.The reaction was stopped by heating the mixture at 75°C for 20 min. Two microlitre of the above synthesized cDNA was directly amplified using same Nor-1 primers and PCR conditions as used for genomic DNA amplification. RT-PCR products were separated by agarose gel electrophoresis as described above. The expression of the housekeeping gene *tub1*, coding β-tubulin was used as a control.

RESULTS AND DISCUSSION

Morphological and microscopic characteristics were studied for the identification of seed borne isolates using manual keys. Among 149 isolates, the fungal genera *Aspergillus* was in copious numbers and represented 52% of the isolates followed by the population of *Cladosporium* (34%), *Alternaria* (12%) and *Rhizopus* (2%). Makun *et al.* (2007) also described similar mycoflora associated with Nigerian rice. However, in this study only the genera *Aspergillus* was further analyzed for identification of the various species as many of the fungi are mycotoxigenic.

Five different species of *Aspergillus* were recovered in our present study. They were ranked according to their isolation frequency and relative density. The *Aspergillus* sp. identified were *A. ochraceous* which was the predominant species and recovered with highest percentage frequency (90%) followed by *A. flavus /A.oryzae* (60%), *A. quadrilineatus* (60%), *A. niger* (30%) and *Emericellanidulans* (30%). Similarly, relative density was found higher in *A. ochraceous* (23%) followed by *A. flavus/A. oryzae* (11%), *A. quadrilineatus* (9%), *Emericella nidulans*(6%) and *A. niger* (3%) (Table 2). These results are in agreement with several reports on incidence of mycotoxigenic *Aspergillus sp*. of rice in India. When moisture content was higher than the desired level in rice grains, it resulted in the invasion of field and storage fungi leading to grain discoloration/loss in viability, quality and toxin contamination (Reddy *et al*. 2008). *Aspergillus*

Table 1. Rice samples collected from Karnataka and Tamil Nadu

S. No	Place of rice sample collection	Coding of *Aspergillus* isolates
1	Gadag, Karnataka, India	-
2	Bangalore, Karnataka, India	BF1,BF2
3	Mysore, Karnataka, India	MYF1,MYF2,MYF3,MYF4,MYF5
4	Madikeri, Karnataka, India	CF1,CF2,CF3,CF4
5	Haveri, Karnataka, India	HF1,HF2
6	Mandya, Karnataka, India	-
7	Devenahalli, Karnataka, India	DEF1,DEF2
8	Madurai, TamilNadu, India	-
9	Shivagangai, Tamil Nadu, India	SHF1,SHF2
10	Salem, Tamil Nadu, India	-

Table 2. Frequency and relative density percent of the fungal species isolated from rice grains

Isolated fungi	No. of isolates	No. of positive samples	Frequency	Relative density
A. ochraceous	34	9	90	23
A. flavus	17	6	60	11
A. quadrilineatus	14	6	60	9
Emericellanidulans	9	3	30	6
A. niger	4	3	30	3
Cladosporium	50	9	90	34
Alternaria	18	3	30	12
Rhizopus	3	1	10	2

flavus and *Aspergillus parasiticus* are the most frequent aflatoxin producers found in agricultural commodities among 22 closely related species of *Aspergillus* in section *Flavi* (Godet and Monoaut, 2010). These species could be distinguished based on macro and microscopic characteristics.

The seed borne *A. flavus* colonies obtained on PDA at 25 ºC (Fig.1a) initially displayed creamy white to white color mycelia, forming yellow-green conidia and later was found with exudates on their surface. Wheat brown colored sclerotia were also present in large numbers (Fig. 1b). The microscopy predominantly showed septate hyphae, thick walled conidiophores, coarsely rough elongated vesicles, with conidia size around 2-3 µm with biserrate conidial heads confirming the identity of the fungus (Fig.2).

The isolates of *A. flavus* were further confirmed and distinguished from other closely related species *A. nomius* and *A. oryzae* on AFPA media. After 48 hours of culture conditions, *A. flavus* produced a bright orange color on the reverse side of the plate due to the production of aspergillic acid whereas, *A. nomius* and *A. oryzae* produced a yellow /brown and cream reverse color respectively as reported by Doster *et al*. (2009) (Fig.3).

The most significant observation in this study was the detection of *A. ochraceous* in many of the samples. According to Varga *et al*. (1993), *A. ochraceous* and *A. niger* are mycotoxigenic in producing ochratoxin (OTA), which has been shown to have nephrotoxic, immunotoxic, genotoxic and teratogenic effects and classified as a possible human carcinogen (group 2B) by IARC, 1993.

Molecular methods have been extremely useful for the identification of *Aspergillus* species and their systematic studies (Rodrigues *et al*., 2007). A single isolate representing a particular *Aspergillus* species from every rice sample was randomly selected for amplification and partial sequencing of ITS regions. Genetic identification of the selected isolates MYF1 and CF1 was accomplished by amplification of the ITS1-5.8 S-ITS2 regions of rDNA using universal primers ITS1 and ITS4 (White *et al*., 1990). The amplified PCR products of 600 bp (Fig.4) is in accordance with the findings of Henry *et al*. (2000), who have reported the ITS amplicons for *A. flavus* ranging in size from 565-613 bp.

The BLAST analysis of partial nucleotide ITS sequences (MYF1 and CF1) using NCBI database with the already deposited sequences was done to confirm the identity of *A.flavus* (Fig.5). In addition, the isolates MYF1 and CF1 showed 100% identity with *A. oryzae* which supported the findings of Kurtzman *et al*., (1986) who found that *A. flavus* and *A. oryzae* have 100% DNA complementarity. Nikkuni *et al*., (1998) showed that this methodology did not allow the discrimination of species of *A. flavus* and *A. oryzae* and hence should be confirmed by gene expression studies using reverse transcription (RT) PCR.

It has been reported that atleast 25 genes are identified for aflatoxin biosynthesis and clustered within a 70 kb DNA region in the chromosome (Yu *et al*., 2004). One of them is *nor*-1 (*afl*D) gene. This is the first gene in the biosynthetic pathway which encodes a reductase enzyme that catalyzes the conversion of norsolorinic acid (the first stable pathway intermediate) to averantin (Chang *et al*., 1992; Trail *et al*., 1994). This gives rise to AFB_1 and AFG_1, or AFB_2 and AFG_2, depending on the existing branch point. The presence or absence of this gene in the fungi is thus an indication as to whether the fungal isolate is aflatoxigenic or non-aflatoxigenic.

In the present study, 7 out of 17 *Aspergillus* isolates (BF1, MYF1, CF1, HAF1,

Table 3. PCR primers for identification and expression of *nor*-1 gene of *Aspergillus flavus*

Target genomic region	Primer	Primer Sequence (5'-3')	PCR amplicon size (bp)	RT-PCR amplicon size (bp)
ITS1	ITS1-F	TCCGTAGGTGAACCTGCGG	600	-
ITS2	ITS4-R	TCCTCCGCTTATTGATATGC		
*afl*D(*nor*-1)	Nor-1F	ACCGCTACGCCGGCACTCTCGGCAC	400	400
	Nor-1R	TTGGCCGCCAGCTTCGACACTCCG		
*tub*1	Tub1-F	GCTTTCTGGCAAACCATCTC	1498	1108

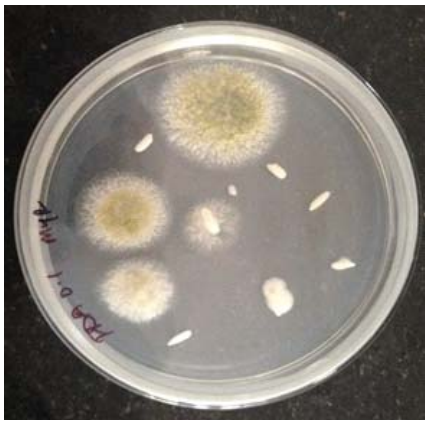


Fig. 1(a). Three day- old seed borne colonies of *A. flavus* on PDA at 25°C

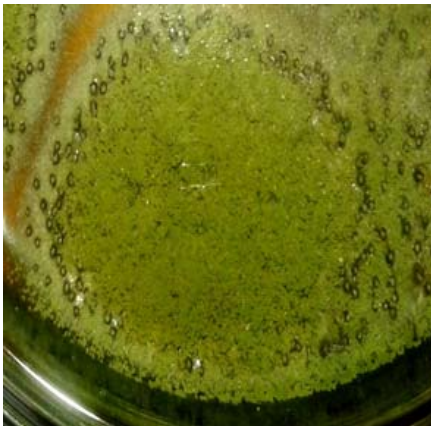


Fig. 1(b). Six day- old colonies of *A. flavus* with formation of sclerotia on PDA at 25°C

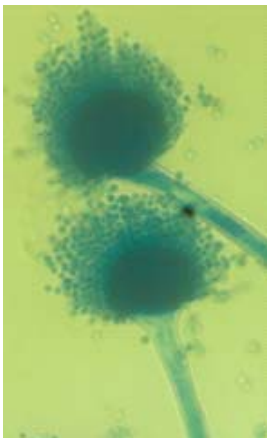


Fig. 2. Microscopic observation of *A. flavus* under 40 × magnification

DEF1, SHF1 and SHF2), each representing the rice samples positive for *A. flavus* contamination were screened for the presence of *nor*-1 gene of aflatoxin biosynthesis gene cluster.

PCR amplification of *nor*-1 gene yielded a single DNA fragment of size 400 bpin all the seven isolates (Fig. 6). These results indicated that *nor*-1 gene was present in all strains either aflatoxigenic or non-aflatoxigenic. However, the successful amplification of the target aflatoxin biosynthesis gene cannot be a proof for aflatoxin production (Levin, 2012). Since the genes may be cryptic and not expressed due to an undetected mutation external to the amplicon sequence, *A. oryzae* and *A. sojae* which are non-aflatoxigenic may also contain some genes (*nor*-1, *ver*-1, *omt*-A and *afl*R)

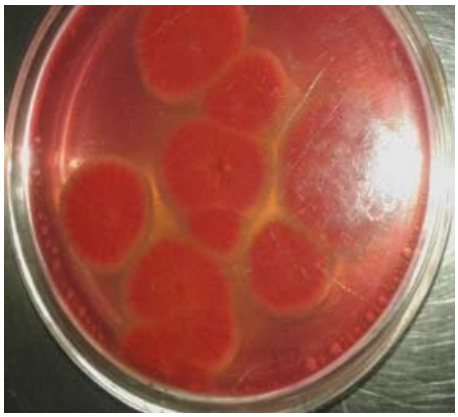
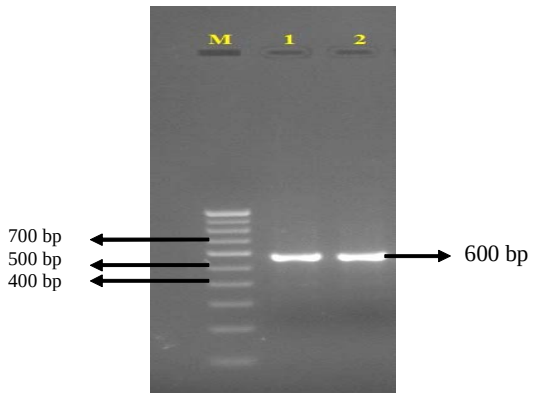


Fig. 3. Five day- old colonies of *A. flavus* on AFPA at 25 °C producing bright orange reverse side



Lanes 1-3: M-100 bp ladder; 1-MYF1; 2-CF1

Fig. 4. Agarose gel electrophoresis of internal transcribed spacer (ITS) region of PCR products

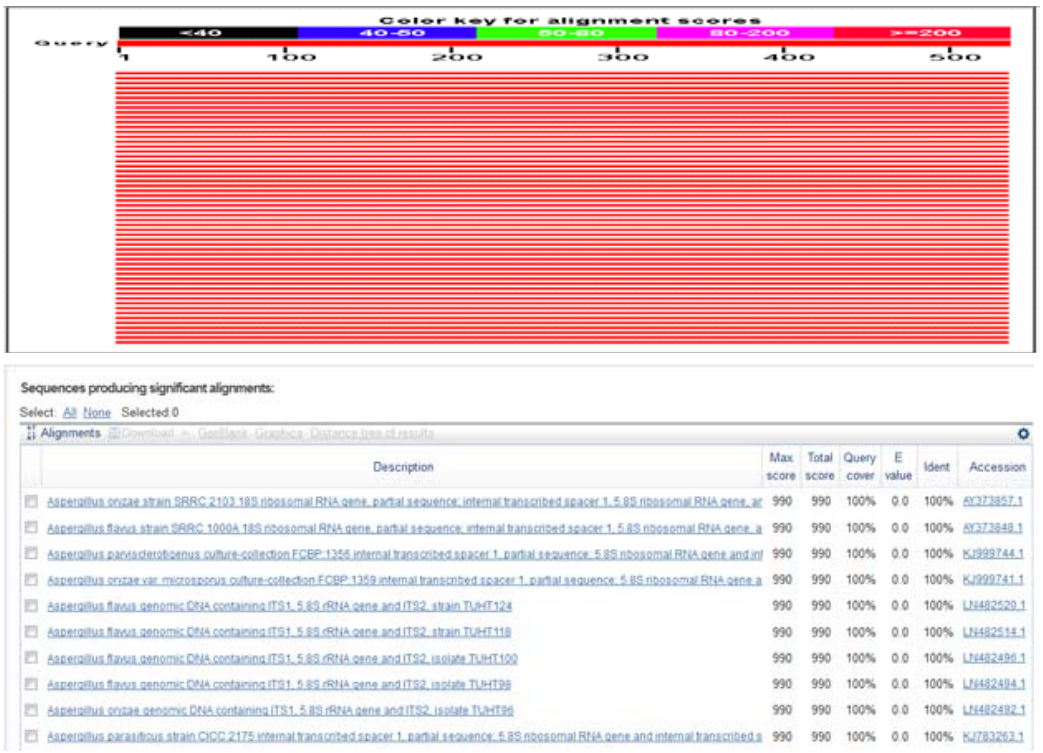
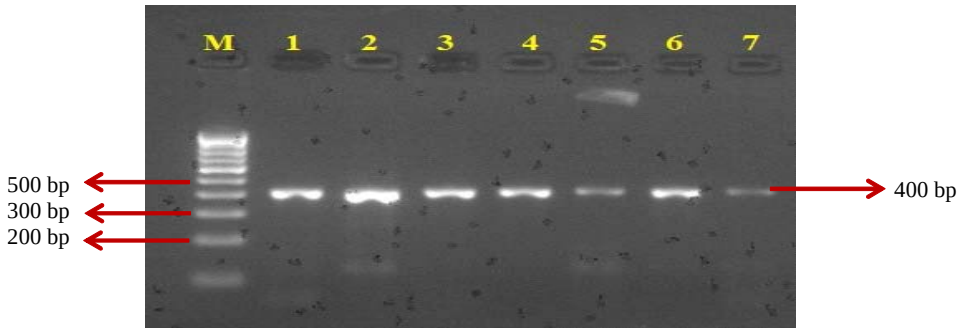


Fig 5(a). BLAST analysis of partial sequence of ITS regions of MYF1 isolate of *A. flavus* using NCBI database

Fig. 5(b). BLAST analysis of partial sequence of ITS regions of CF1 isolate of *A. flavus* using NCBI database

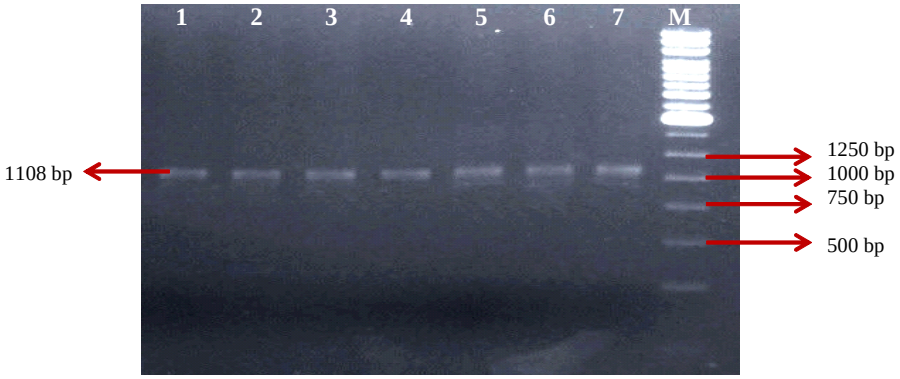
needed for aflatoxin biosynthesis but are not expressed (Woloshuk *et al*., 1994; Klich *et al*., 1995, 1997; Kusumaoto *et al*., 1998; Watson *et al*., 1999). This holds true even in the case of non-aflatoxigenic isolates of *A. flavus* (Criseo *et al*., 2001).

Recently, monitoring the expression of aflatoxin genes using RT-PCR has been applied for the detection and differentiation of aflatoxigenic strains of *Aspergillus* sp. (Rodrigues *et al*., 2007).



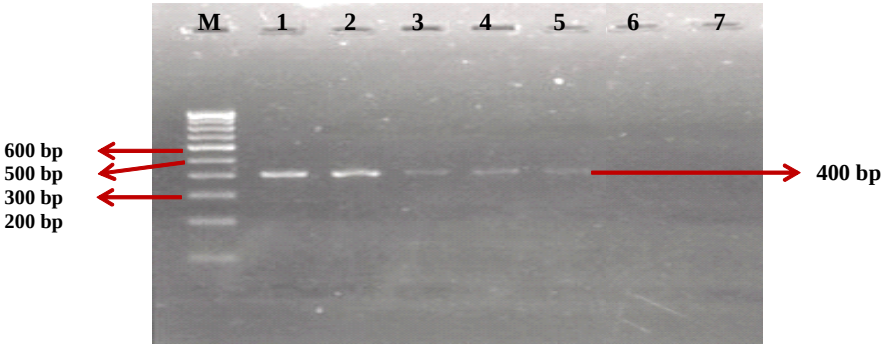
Lanes 1-8: M-100 bp ladder; 1-BF1; 2-MYF1; 3-CF1; 4-HF1; 5-DEF1; 6-SHF1; 7-SHF2

Fig. 6. Agarose gel showing the pattern of PCR products detecting *afl*D gene



Lane 1-8: M-250 bp Ladder; 1-MYF1; 2-CF1; 3-DEF1; 4-SHF1; 5-SHF2; 6-BF1;7-HF1

Fig. 7(a). Representative electrophoretic band pattern of RT-PCR products indicating expression of β-*tub*



Lane 1-8: M-100 bp Ladder; 1-MYF1; 2-CF1; 3-DEF1; 4-SHF1; 5-SHF2; 6-BF1;7-HF1

Fig 7(b). Representative electrophoretic band pattern of RT-PCR products indicating expression of *afl*D

In our study, the isolates which were positive for genomic PCR for *nor*-1 (*afl*D) were subjected to gene expression analysis by RT-PCR, targeting the biosynthetic gene. A house keeping gene, *tub1* was used as positive control for RT-PCR. Total RNA was extracted from 48h old mycelium grown under aflatoxin inducing conditions on potato dextrose broth supplemented with 1% peptone (Melvin and Alka, 2013).

The isolates MYF1, CF1, DEF1, SHF1, SHF2 (5/7) which showed positive for RT-PCR in terms of expression of *nor*-1 (*afl*D) gene, displayed a characteristic 400 bp size amplicon as expected (Fig. 7). It was found that the *nor*-1 gene expressed in these isolates, were also detected by conventional monomeric PCR (Fig .6). Thus a good correlation between gene detection and gene expression was observed for all these isolates.

Two isolates BF1, HF1 (2/7) failed to generate a positive transcription signal for the target gene (Fig .7) though the gene was detected in these isolates by genomic PCR. All of these strains were considered as aflatoxin non-producers which is probably due to point mutation in aflatoxin gene cluster (Chang *et al*., 2005). According to Tominaga (2006), *A. oryzae* and *A. sojae* contained aflatoxin biosynthesis genes (*nor*-1, *ver*-1, *omt*-A and *afl*R) but, were not expressed. This may explain the lack of aflatoxin production in these traditional Asian food fermenters.

The current study suggests that the *nor*-1 gene is present and expressed in several isolates. This however, has to be demonstrated by quantification of aflatoxins and other molecular methods such as multiplex RT-PCR which, correlate the expression of several genes in aflatoxin biosynthesis and its production.

The results of this preliminary study concluded the contamination of toxigenic *Aspergillus flavus* in rice grains under storage conditions. The high levels of *Aspergillus ochraceous* strains found in most of the samples is alarming as the co-occurrence of AFs and OTA in these natural combination could be synergistic, additive or antagonistic in human health (Miller, 1995). This warrants appropriate crop and commodity management practices to reduce the exposure of humans and animals from mycotoxins produced by *Aspergillus* species.

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