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Screening of fusarium species in maize using molecular techniques

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ABSTRACT

Contamination by mycotoxins in human food and animal feed is a worldwide problem because mycotoxins are naturally occurring toxic substances. Human ingestion of mycotoxins mainly occurs from the consumption of mycotoxins in residues and metabolites in animalderived foods such as milk or meat products. Present results describe the detection of mycotoxins in samples of maize seed collected from different localities Out of one thirty samples nine samples tested, 4 samples were found to be contaminated with fusarium species. FUM1 key genes that are involved in the production of the fumonisn, The fragments obtained were resolved on the DNA for generating data of fragment sizes. Use of the PCR combined with the Bioanalyzer offered significant benefits over traditional agarose gel electrophoresis and staining methods. Amplification of the gene fragments was also achieved using PCR with genes.

Key words : *Maize, PCR, Gene specific primers*

Introduction

Fumonisins are another group of mycotoxins produced by *fusarium* species, chiefly those associated with pink ear rot of maize. Consumption of fumonisin contaminated maize has been linked to high rates of esophageal cancer10 (*Parry et al.,* 1995). Fumonisins consist of a linear carbon backbone substituted at various positions with hydroxyl, methyl and tricarboxylic acid groups. The forms generally present in naturally contaminated maize are members of the B series, FB1, FB2, FB3 and FB411; Three species are most commonly associated with pink ear rot, *F. proliferatum, F. verticillioides* and *F. subglutinans.* Only *F. verticillioides* and *F. proliferatum* are able to produce fumonisins while *F. subglutinans* and *F. proliferatum* can produce moniliformin and beauvericin (Bottalico, 1998).

Fusarium verticillioides produces the mycotoxins fumonisns that are known to be potentially carcinogenic to humans and toxic to animals. A cluster of 15 genes are known to be involved in the biosynthesis of these mycotoxins, among which, FUM1 is the largest, encoding a polyketide synthase, which catalyzes the synthesis of a polyketide that forms a large portion of the fumonisn structure (Da Silva *et al.,* 2007).

The present study therefore utilizes the genes described above, namely, FUM1 for fumonisn detection.

Materials and Methods

Primer and probe designing for amplification of gene fragments

The following genes have been identified based on their functionality in producing the toxins in the pathways.

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– *Fusarium verticilloides* FUM1gene, for measuring Fumonisin production.

Gene sequences from at least 10 different strains of the each of the bacterial species described above, were obtained from GENBANK and saved in EDITSEQUENCE software of Lasergene 8.0 version. Subsequently, using megalign software of the same version, these ten sequences were aligned using clustal W. Homologous regions marked in red were identified and two different primers for forward and reverse amplification were chosen for each gene from these regions, to amplify fragments of different sizes for the four different genes. Primer 3.0 software was used for designing primers, which were selected in such a manner so as to amplify 300 bp region from FUM1 fene. Sequences of forward and reverse primers used for amplification of these gene fragments are given in Table 1.

Fungal strains procurement

The four fungal strains, *Aspergillus flavus, Aspergillus parasiticus, Aspergillus ochraceus* and *Penicillium verrucosum* were obtained from National Chemical Laboratories, Pune, India. Similarly, another four fungal strains, *Aspergillus parasitiicus, Aspergillus ochraceus, Fusarium culmorum, and Fusarium verticillioides* were obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India.

Sub-culturing of fungi

The NCL cultures were inoculated onto PDA (potato dextrose agar) plates and sub-cultured. After 8 days of growth, the fungal mycelia were scraped from the surface of the plates and DNA was extracted. These samples were used as positive controls for PCR.

F.verticillioides isolates were cultured in 250 mL Erlenmeyer flasks containing 50 mL of the following fumonisin-inducing liquid medium (Jimenez *et al.*, 2003) : malt extract (0.5 g/l), yeast extract (1 g/l), peptone (1 g/l), KH_2PO_4 (1 g/l), $MgSO_4 \cdot 7H_2O$ (0.3 g/l), KCl (0.3 g/l), $ZnSO_4 \cdot 7H_2O$ (0.05 g/l), $CuSO_4 \cdot 5H_2O$ (0.01 g/l) and fructose (20 g/l). After 8 days of growth, the fungal mycelia were scraped from the surface of the plates and DNA was extracted. These samples were also used as positive controls for PCR

DNA isolation

Fungal DNA was isolated using Stratagene DNA extraction kit, with a few modifications, as per the method described (Brown *et al.*, 2001). The procedure involves digestion of cellular proteins using solution II provided in the kit, which is the extraction buffer. 1% beta-mercaptoethanol was added to solution II to obtain better digestion of cellular proteins. Subsequent removal of the proteins was achieved by "salting out" using standard 5M sodium chloride, followed by precipitation of the DNA with ethanol and resuspension in the buffer of choice.

Quantification by nano drop

Quantification of DNA in the final volume was done using nanodrop (Thermo Scientific).

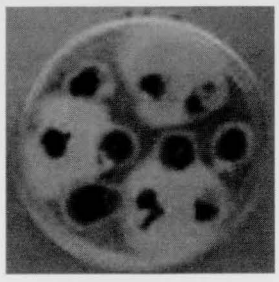
Polymerase Chain reaction

Initially, amplification of the gene fragments was achieved in normal PCR using ABI Veriti ® Thermal Cycler supplied by Applied Biosystems International Incorporated, USA. A reaction mixture of 20 ml lml was prepared containing 1 mL each of forward and reverse primers (10ml M); dNTPs, 2mM, 2ml l;10X TAQ buffer, 2m l, TAQ DNA polymerase (Stratagene), 0.2 ml l; 2 ml l of template DNA (100ng/ml l); sterile distilled water, 11.8 ml l, and added to 8-strip PCR tubes. Cycling conditions used were denaturation at 94°C for 4 min, followed by 35 cycles of denaturation, annealing and extension at 94°C for 45 sec, 58°C for 45 sec, 72°C for 45sec, and final extension for 8 min at 72°C. Agarose gel electrophoresis in a 2% agarose gel was used for analyzing the PCR products.

Results and Discussion

Sub-culturing of fungus

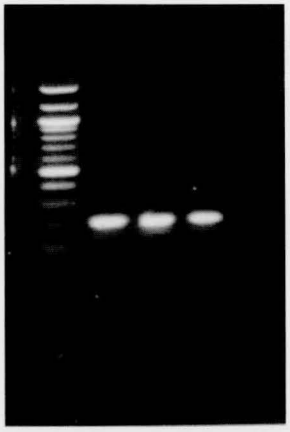
Fungal strains procured from NCL, Pune and MTCC, Chandigarh, were sub-cultured and DNA was isolated for PCR. Fig.1 shows the fungal strains obtained after sub-culturing for eight days for four strains obtained from NCL, Pune.



D-*P.verrucosum*

DNA extraction

DNA extraction yielded DNA of good quality and quantity as observed in the nanodrop and depicted in Fig. 2. DNA quantity ranged from 110 to 322 ng/mL and 260/280 ration ranged from 1.8 to 2.0.



Lane 1: 100 bp DNA ladder
Lanes 1-3: FUM gene fragments 200 bp;

PCR amplification

PCR amplification of the four genes using the primers designed was done from fungal DNA samples initially using ABI Veriti ® Thermal Cycler supplied by Applied Biosystems International Incorporated, USA, to ascertain if the gene fragments are being optimally amplified. All four gene fragments that were amplified were of the desired size which was confirmed by using a 100 bp DNA ladder (Sanger *et al.*, 1977).

Scientists (D'Mello *et al.*, 1999) have developed a specific real-time reverse transcription-PCR (RT-PCR) assay to quantify the level of expression of two genes of the fumonisin biosynthetic cluster in *F. verticillioides*: fum1 (that encodes a polyketide synthase enzyme) and the ABC transporter encoding gene fum19. The level of expression of both genes was compared with the amount of fumonisin B1 (FB1), measured by HPLC, produced by several strains of F. verticillioides in liquid culture. The results indicated a good correspondence between the levels of fum1 and fum19 expression and the production of fumonisin B1. The analysis described provides a good approach for the rapid and specific detection and characterization of the potential ability of *F. verticillioides* strains to produce fumonisins. The present study utilized a set of primers to successfully amplify a fragment of the FUM1 gene, which was 300 bp long, from DNA of F. verticilloides (Russell and Paterson, 2006).

Conclusion

This study focuses on an application oriented collaborative approach dealing with the development of rapid and easy PCR based assay for detection of mycotoxins, namely, aflatoxin, deoxynivalenol (DON), fumonisin and ochratoxin (OTA) in selected food grains. Our investigation addresses the area of mycotoxin producer detection or detection of the relevamt genes involved in mycotoxin production, using biotechnological approaches. This will give a clear indication of the potential for toxin production by the contaminating fungi, prior to actual determination of the toxin content by chromatography. For this approach, the biochemistry has to be determined sufficiently to enable the development of gene probes of the pathway. Based on the biochemical pathways of mycotoxin biosynthesis already described, the genes for the four toxins have been selected for primer designing. Fragments of the appropriate size for which the primers have been designed to amplify, have been obtained, both by PCR analysis, namely, gene 300 bp for FUM1 gene. Use of this method on actual field samples of food grains and food products, would be useful in gene detection at critical control points in food production. If this method is coupled with mycotoxin quantitation by chromatographic methods in the final food product, it would be very significant useful information in prevention and control of mycotoxin contamination, thereby reducing morbidity that may due to their consumption.

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