CURRENT BOTANY

Curr. Bot. 2(3): 34-37, 2011

REGULAR ARTICLE

PCR based detection of fumonisin producing strains of *Fusarium verticillioides* and gene related to toxin production

V. Karthikeyan[1], R. Rajarajan[1], S. Patharjan[2], P. Karthikeyan[3] , P. Saravanakumar[4], M. Siva[5], P.S. Aruna Bhavani[5], P. Palani[5]

1 Department of Plant Biology and Plant Biotechnology, D.G.Vaishnav College, Arumbakkam, Chennai 600 106, TN, India

2 PRIST University, Centre for research and Development, Thanjavur, TN, India

3 Department of Genetic engineering, SRM University, Kattankulathur, TN, India

4 Department of Environmental and Herbal Science, Tamil University, Thanjavur, TN, India

5 CAS in Botany University of Madras, Guindy Campus, Chennai, TN, India

KEYWORDS

Fusarium vercillioides, PCR, Fum 5, IGS, Polyketide synthase

CORRESPONDENCE

V. Karthikeyan, Department of Plant Biology and Plant Biotechnology, D.G.Vaishnav College, Arumbakkam, Chennai 600 106, TN, India

E-mail: karthickeyanv@yahoo.com

EDITOR

A. Pandey

CB Volume 2, Year 2011, Pages 34-37

ABSTRACT

Fusarium verticillioides produces Fumonisins are a group of mycotoxins that contaminate food and feed products posses maximum threat to human and animal health. In this work Eighty two strains of Fusarium species collected from infected rice samples were subjected to PCR assay to discriminate fumonisin producing and nonproducing strains with Inter Generic Spacer region (IGS) of rDNA coding units specific primer named as VERTF-1/VERTF-2 were used. 21 isolates of *F. verticillioides* scored positive for VERTF-1/ VERTF-2 pair of primers proves to be potential fumonisin production and 25 isolates were scored negative. Specific primers for polyketide synthase (PKS) gene FUM1-(previously FUM5) were used to all 83 strains resulted in positive signals observed in 21 strains of *F. verticillioides.* This present study proves the efficiency of IGS and gene specific primer also represents well for *Fusarium* strains isolated from rice also. For both primers PCR detection was consistent even at 100 pg/µl concentration of genomic DNA. This quite rapid and specific method helps in accurate discrimination of Fumonisin producing strains.

Introduction

Contamination of rice and rice-based products with fumonisins poses a threat to agriculture and food safety worldwide. As a primary step to reduce the global impact of fumonisins, proper and accurate detection and identification of *Fusarium* species, which are known to produce toxins become necessary. Fumonisins are mycotoxins produced by both rice and maize pathogen *Gibberella fujikuroi* mating population A; anamorph *Fusarium verticillioides* (Sacc.) and several related species. Even though there are some reports available about contamination of grains with fumonisins has been associated with human esophageal cancer in South Africa (Nelson *et al.,* 1993). Fumonisins also cause leucoencephalomalacia in horses, lung edema in swine, and cancer in experimental animals. There was no much importance given to this foolish –disease of rice. In present study we attempted to elucidate the study of fumonisins of rice Bakanae disease from Italy and this part of our work carries information about mycotoxicology.

To develop rapid, sensitive, and accurate detection methods DNA based Molecular techniques are being widely used to identify and discriminate among isolates within a species (Niessen *et al.,* 1998; Gonzalez-Jaen *et al.,* 2004; Jurado *et al.,* 2005; Mule *et al.,* 2005; Nicolaisen *et al.,* 2005). Prevention of toxins entering in food chains is effectively done through early detection and characterization of potential fumonisin-producing strains. The existence of two genetically isolated populations within *F.verticillioides*, which differed in their ability to produce fumonisins were reported previously through its phylogenetic study (Mirete *et al.,* 2004; Moretti *et al.,* 2004). World widely occurring major groups of Fusarium associated with maize and rice whose ability to produce fumonisins was confirmed. The other group did not produce fumonisin and lacked the main genes involved in fumonisin biosynthesis.

Since the intergenic spacer region of the rDNA units (IGS) is a highly variable non-coding sequence, which appears to be a most rapidly evolving spacer region (Moretti *et al.,* 2004) two set of primers (VERTF1/2) were developed by Patino *et al.,* 2004 on the basis of the IGS region to detect *F.verticillioides* and to differentiate fumonisin-producing *F. verticillioides* and their specificity was confirmed by using a combined approach of PCR-RFLP. Furthermore RFLP patterns obtained by Patino *et al.,* 2006 and Gonzalez-Jaen *et al.,* 2004 showed intra-specific variability in the IGS region with isolates of *F. verticillioides* which yielded no amplification with isolates from the fumonisin nonproducing population. These authors also reported that some strains from Nepal, originally reported as fumonisin non producers (Desjardins *et al.,* 2000) were actually low producers and yielded positive amplification with the VERTF-1 and VERTF-2 assay, confirming the efficiency of the PCR-assay to discriminate potential *F. verticillioides* fumonisin producing

strains. Also, Bezuidonhout *et al.,* 1998 developed a multiplex PCR detection method for identification of fumonisin forming fungi using same VERTF1/2 primers. A similar study has been done by Mirete *et al.,* 2004 and Sreenivasa *et al.,* 2008 to evaluate the genetic variability of *F. verticillioides* species by using the primers based on the IGS region and to discriminate the fumonisin producing *F.verticillioides* isolates from Indian Maize kernals. Biochemical analyses indicated that fumonisins are products of a polyketide synthase (PKS) gene called FUM1 (previously FUM5) (Proctor *et al.,* 1999; Proctor *et al.,* 2003; Desjardins *et al.,* 2002).

Despite the agricultural importance of bakanae disease, there has been little information about mycotoxicology available from north western Italy. Though there has been much work done on fumonisin structural elucidation and its wide distribution among species were done, there was no report on discrimination of fumonisin producing strains form rice infecting *G.fujikuroi* and detection of Fum5 genes from the same was not done earlier.

The following objectives were followed for the present study.

In first part of our study is on discrimination of Fusarium strains into fumonisin producing and nonproducing strains for that we used VERTF1/2 and followed by the detection of FUM5 gene responsible for fumonisin production through PCR method and its gene sequencing.

Materials and Methods

Collection Isolation and storage of Fusarium species from rice seeds

Bakanae infected rice samples approximately 10 -15 plants per field were collected during June and July of 2006 and 2007 from the major rice growing areas of North-Western Italy from Piemonte and Lombardic regions which includes Vercelli, Pavia and Novara provinces. Four to five locations at different and opposite directions were chosen as collections sites. Samples were washed well with running tap water. *Fusarium* spp. were isolated from rice seeds by following procedure. Washed the whole seeds in running tap water for 2 h. The palea and lemma were surface treated in the NaOCl solution for 30 s, rinsed twice in SDW, and placed on PDA. Whole seeds, were incubated at 22 to 26°C under cool, white fluorescent lights for 30 min daily to enhance sporulation. Prior to further analysis strains were grown in *Fusarium*-selective agar medium containing pentachloronitrobenzene Komada medium (pH.5.5) (Nelson *et al.,* 1993). Cultures were stored in PDA slants and in glycerol stocks at 4°C for further use. Four representative (ITEM504 (*F.verticillioides*), ITEM231 (*F.verticillioides*), and ITEM1746 (*F.verticillioides*)) strains of the species were obtained from Culture collection of Institute of Sciences of Food Production, Bari, Italy (http://www.ispa.cnr.it/Col-lection).

Modified DNA extraction by CTAB method (Moller et al., 1992)

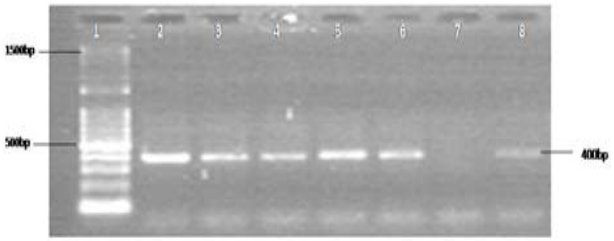
50mg of fungal mycelia was scraped from 10d old PDA cultures, manually ground in 1.5ml of microfuge tubes with micro pestle adding 500ul of pre-warmed (60°C) TES lysis buffer (100mM Tris pH 8.0; 10mM EDTA pH 8.0; 2% SDS).50ug of proteinase K were added to the ground material, incubated in 60°C for 60 min. 140ul of 5M Nacl and 64ul of 10% (w/v) of CTAB were added to the suspension incubated at 65°C for 10 min. DNAs were extracted by adding equal vol. of chloroform:isoamylalcohol (24:1) centrifuged at 14000xg/10min. DNA was precipitated by adding 0.6vol of cold isopropanol and 0.1 vol of 3M sodium acetate pH 5.2 and maintained at -20°C, centrifuged and washed twice with 70% ethanol suspended in 100ul of TE (10mM Tris pH 8.0; 1mM EDTA pH 8.0). RNA was digested by adding 10mg/ml of RNAse A and incubating at 37°C for 45min and stored -20°C for further use.

Polymerase chain reaction

A standard polymerase chain reaction (PCR) protocol is used to amplify the IGS gene region. VERTF-1 (forward primer; 5-GCG GGA ATT CAA AAG TGG CC-3) and VERTF-2 (reverse primer;5-GAG GGC GCG AAA CGG ATC GG -3 (Patino *et al.,,* 2004) primers are used in a PCR reaction, with Reactions were performed in volumes of 50 µl and contained 20ng of fungal DNA for each reaction. The reaction mixture consisted of a 10X buffer (Taq Polymerase Kit – Quagen, Italy) solution consisting of 50 mM KCl and 10 mM Tris-HCl (pH 9), 0.2 mM of each dATP, dCTP, dGTP, and dTTP, 0.5 mM of each forward and reverse primer pairs, and 1.5 mM of MgCl2. Amplification was performed using the Biomerta gradient thermo cycler. The PCR program included the following temperature regime: 95°C for 3 min, The remaining steps in the program were repeated 32 times and consisted of 95°C for 1 min, 60°C for 1 min, 72°C for 3 min, and the final extension was at 72ºC for 5 min. The PCR products (15 µl) were separated by gel electrophoresis in 2.0 % agarose at 1 X TAE gel and stained with Cyber safe (Invitrogen-Italy) for photography. An ~700 bp product is amplified. The PCR conditions and reaction mixture for Fum5 (5-GTC CTA CGC GAT ACA TCC CAC AAT -3) and Fum 6 (5-GAT CAA GCT CGG GGC CGT CGT TCA TAG-3 (Baird *et al.,* 2008) gene is same except the annealing temperature 60°C.

Results and Discussion

All 83 *Fusarium* isolates were tested with VERTF-1/ VERTF-2 set of primers to analyze for their ability to produce fumonisin. 21 isolates of *F.verticillioides* DNA out of 83 Fusarium isolates showed the expected 400-bp amplicons. As expected no amplification was observed in negative control DNAs (*F. proliferatum)* (Fig. 1).



Lane 1 - 100bp DNA ladder;
Lane 2,3,4,5,6, and 8 shows the amplification of 400bp of IGS primers used on *F. verticillioides*

Fig. 1: PCR amplification of *F.verticillioides* by VERTF-1 and VERTF-2 primers

In this study, the use of this set of primers detected the occurrence of 25 isolates of *G.fujikuroi* that did not showed amplification and, therefore, should not be potential fumonisin producers. The VERTF-1/2 set of primers were used to analyze 83 *F.verticillioides* used for their potential to produce fumonisin. The excepted 400bp amplicon was detected only with the DNA from 64 of the 83 isolates of *F.verticillioides* similarly no amplification was seen with DNA from *F. proliferatum* that was used as negative control.

Another interesting finding of this work is the IGS primer is only efficient against *F.verticilliodes* but finding of Fum 5 gene with Fum 5&6 primer is efficient over *F.proliferatum*, *F.graminarium*, *F.napiforme* and *G.fujikuroi* also. Four gene sequences were deposited to NCBI gene bank (sequences furnished below). Compared to IGS primer the fum5 gene detection is more accurate and reliable method. Sensitivity of the PCR reactions was evaluated furring the investigation. Decreasing amounts of the genomic DNA of *F.verticillioides* were used in order to determine the minimum amount of input DNA required to produce detectable product with primers. Using 10 replicate samples per DNA concentration, amplification products of the primers were visible on gels from 30ng/µl, 20 ng/µl, 10 ng/µl, 1 ng/µl and 100pg/ µl. Among the 82 strains twenty six selected strains which showed positive signal amplification to VERTF1/2 primers were subjected to FUM5 primers. Among twenty six eighteen strains showed clear amplification of FUM5 gene of 419bp size fragments (Fig.2).

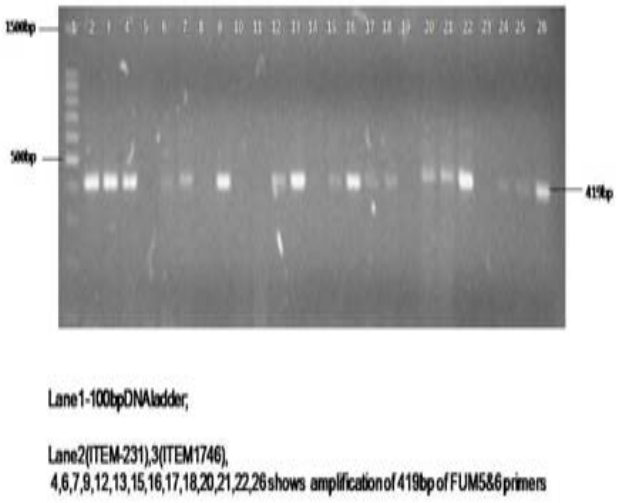


Fig. 2: PCR amplification of FUM5 gene of *F.verticillioides*

Primer specificity

The efficiency of PCR depends on designing good specific primers and developing appropriate conditions for DNA amplification. In order to check the specificity of the IGS primer and gene specific primer these two primers were used with the DNAs of other *Fusarium* species and which includes *F. culmorum*, *F. subglutinas*, and *F. graminearum* as a negative control for IGS and for Fum5 *Aspergillus sps*. Which also posses PKS domine gene cluster used as a negative control, which fails to amplify the specific band size. The annealing temperature 53.2^{0}C for VERTF and 60°C for Fum 5 was used during PCR reaction to ensure specific binding of the primers. No cross-reaction were observed with the other species tested. In the present investigation, we found that the *G.fujikuroi* isolated from different regions of the North Western Italy have shown variability with reference to potential ability to produce fumonisins as analyzed by PCR.

In conclusion, primer Fum 5 consistently produced a product of bp 419 that was effective for identification of *G.fujikuroi* associated with rice tissues. No PCR products were produced within the closely and distantly related taxa. future research can now be conducted to determine the level of fumonisin production by HPLC method. The main objective of this study is to Descrimination of fumonisin producing and non producing Fusarium strains through IGS primer and PCR based detection of polyketide synthase gene fum 5 from different Fusarium species were achieved methodologically.

G. fujikuroi was clearly the dominant species and would probably be the main source of the fumonisin production in cereal samples (Kedera *et al.,* 1998; Grim *et al.,* 1998). Previously, sequences from internal transcribed spacer (ITS) regions ITS1 and ITS2 were successfully used for design of specific primers to identify select *Fusarium* spp. (Grim *et al.,* 1998; Bluhm *et al.,* 2002; Bluhm *et al.,* 2004; O'Donnell *et al.,* 1995). However, none of the PCR assays were capable of differentiating fumonisin producing from non-producing strains. But in our present study, the use of this set of primers detected the occurrence of certain isolates that did not showed amplification and, therefore, should not be potential fumonisin producers, similar study has been done by Mirete *et al.,* 2004. Though these IGS primers were tested against only *F.verticillioides* in our present study we used the same to *G.fujikuroi* since the *F.verticillioides* also belongs to the matting population of *G. fujikuroi.*

Previous workers Baird *et al.,* 2008 and Srinivasa *et al.,* 2008 challenged this primers only with maize infecting *Fusarium* tissues this is the first effort on rice represented same banding size for both VERTF and FUM5 primers. Since the *Fusarium* posses number of species in its complex its quite tricky and some way it may leads to the misconception among the species but the present molecular biology field reveals a better strategy to confirm the species in a very specific manner that PCR techniques allows a efficient discrimination of *F.verticillioides* from other toxigenic *Fusarium* species. The detection limits ranges between 5 to 10 ng of DNA template per reaction is adequate for the specific amplification. There is a urgent need to develop a species specific identification of *Fusarium* species because each of them posses a specific toxigenic profile. The usage of these kind of specific primers will enhance the rapid detection of species and provide tolls to prevent health risks caused by mycotoxin contamination in foods and feeds. Early and specific detection of this pathogenic fungus leads to the better understanding of the pathogen, which pave the way of effective disease control. The PCR assay described in this work provides a useful tool for rapid and sensitive detection and differentiation of potential fumonisin-producing *F.verticillioides* species.

In this study, the polyketide synthase gene FUM5 region was selected since it was thought to have unique primer binding sites for distinguishing fumonisin forming fungi and development of primers could target toxin production. Several factors can affect *in vitro* production of fumonisin including water activity, limited nitrogen and pH of 5.9 levels (Miller, 2001) presence of mycotoxin gene only not 100% enough to designate that the pathogen is completely potential to produce fumonisin it lies in their expression in association with plant system. But this gene identification leads to further studies like gene expression and level of fumonisin production at different stages of fungus. Detection of fumonisin producing *Fusrium* species based on the molecular methods are rapid, as there is no need to culture organisms prior to their identification. The molecular method results are specific, since identification of species is made on the basis of genotypic differences and are highly sensitive, detecting the target DNA molecules in complex mixtures, even when the mycelia are no longer viable.

Acknowledgements

The work was supported by the Italian Ministry for Environment Territory and Sea (Frame work programme "Sustainable Agriculture"). Under mutual collaboration agreement between University of Madras, India and Italian Ministry for University and Research and by the Compagnia di San Paolo, Turin. The authors would like to thank Agrinnova, ENSE, Ente Nazionale delle Sementi Elette and ISPA –Bari for providing rice seeds and reference strains. And BMR genomics University of Padova Italy for providing gene sequencing facility.

References

Baird, R., Abbas, H. K., Windham, G., Williams, P., Baird, S., Ma, P., Kelley, R., Hawkins, L. and Scruggs, M. 2008. Identification of Select Fumonisin Forming *Fusarium* Species Using PCR applications of the Polyketide Synthase Gene and its Relationship to Fumonisin Production *in vitro*. *Int. J. Mol. Sci.,* **9**: 554-570.

Bezuidonhout, S. C., Gelderblom, W.C., Gorst-Allman, C. P., Horak, R. M. and Marasas, W.F.O. 1988. Structure elucidation of fumonisins, mycotoxins from *Fusarium moniliforme*. *J. Chem. Soc., Chem. Commun.,* **52**: 743-745.

Bluhm, B. M., Cousin, M. A. and Woloshuk, C. P. 2004. Multiplex real-time PCR detection of fumonisin producing and trichothecene-producing groups of *Fusarium* species. *J. Food Protect.,* **67**: 536-543.

Bluhm, B. M., Flaherty, J. E. and Cousin, M. A. 2002. Woloshuk CP. Multiplex polymerase chain reaction assay for the differential detection of trichothecene and fumonisin-producing species of *Fusarium* in cornmeal. *J. Food Protect.*, **65**: 1955-1961.

Desjardins, A. E., Munkvold, G. P., Plattner R. D. and Proctor. R. H. 2002. Fum1: a gene required for fumonisin biosynthesis but not for maize ear rot and ear infection by *Gibberella moniliforme* in field tests. *Mol. Plant Microbe Inter.* **15**: 1157-1164.

Desjardins, A. E., and Plattener, R. D. 2000. Fumonisin B1-nonproducing strains of *Fusarium verticillioides* cause maize (*Zea mays*) ear infection and ear rot. *J. Agri. Food chem.* **48**: 5773–5780.

Gonzalez-Jaen, M. T., Mirete, S., Patino, B., Lopez-Errasquin, E. and Vazquez, C. 2004. Genetic markers for the analysis of variability and for production of specific diagnostic sequences in fumonisin-producing strains of *Fusarium verticillioides*. *Eur.J. Plant Pathol.* **110**: 525–532.

Grim, C. and Geisen, R. 1998. A PCR-ELISA for the detection of potential fumonisin producing *Fusarium* species. *Lett. Appl. Microbiol.* **26**: 456–462.

Jurado, M, Vazquez, C., Patino, B. and Gonzalez-Jaen, M. T. 2005. PCR detection assays for the trichothecene-producing species *Fusarium graminearum, Fusariumculmorum, Fusarium poae, Fusarium equiseti* and *Fusarium sporotrichioides*. *Syst. Appl. Microbiol.* **28**: 562–568.

Kedera, C. J, Plattner, R. D. and Desjardins, A. E. 1999. Incidence of *Fusarium* species and levels of fumonisin B1 in maize in western Kenya. *Appl. Environ. Microbiol.* **65**: 41–44.

Miller, S. D. 2001. Factors that affect the occurrence of fumonisin. *Environ. Health Persp.***109**: (Suppl.-2), 321-324.

Mirete, S., Vazquez, C., Mule, G., Juradob, M. and Gonzalez-Jaen, M. T. 2004. Differentiation of *Fusarium verticillioides* from banana fruits by IGS and EF-1α sequence analyses. *Eur. J. Pl. Pathol.* **110**: 515–523.

Moller, E. M., Bahnweg, G., Sandermann, H. and Geiger, H. H. 1992. A simple and efficient protocol for isolation of high mole cular weight DNA from filamentous fungi, fruit bodies and infected plant tissue. *Nucleic Acid Res.* **20**: 6115-616.

Moretti, A. , Mule, G., Susca, A., Gonzalez-Jaen, M. T. and Logrieco, A. 2004. Toxin profile, fertility and AFLP analysis of *Fusarium verticillioides* from banana. *Eur. J. Pl Pathol.* **110**: 601–609.

Mule, G., Gonzalez-Jaen, M. T., Hornok, L., Nicholson, P. and Waalwijk, C. 2005. Advances in molecular diagnosis of toxigenic *Fusarium species*: a review. *Food Addit. Contam.* **22**: 316–323.

Nelson, P. E., Desjardins, A. E. and Plattner, R. D. 1993. Fumonisin, mycotoxins produced by fusarium species: biology, chemistry and significance. *Ann.Rev.Phytopathol.* **31**: 233-252.

Nicolaisen, M., Justesen, A. F., Thrane, U., Skouboe, P. and Holmstrom, K. 2005. An oligonucleotide microarray for the identification and differentiation of trichothecene producing and nonproducing *Fusarium* species occurring on cereal grain. *J.Microbiol. Meth.* **62**: 57–69.

Niessen, L. and Vogel, R. F. 1998. Group-specific PCR-detection of potential trichothecene-producing *Fusarium species* in pure cultures and cereal samples. *Syst. Appl.Microbiol.* **21**: 618–631.

O'Donnell, K. and Gray, L. E. 1995. Phylogenetic relationships of the soybean sudden death syndrome pathogen *Fusarium solani* f. sp. *solani* inferred from rDNA sequence data and PCR primers for its identification. *Mol. Plant Microbe Inter.* **5**: 709-716.

O'Donnell, K. 1992. Ribosomal DNA internal transcribed spacers are highly divergent in the phytopathogenic ascomycete *Fusarium sambusinum* (*Gibberella pulicaris*). *Curr. Genet.* **22**: 213-220.

Patino, B., Mirete, S., Gonzalez-Jaen, T., Mule, G., Rodriguez, T. M. and Vazquez, C. 2004. PCR detection assay of fumonisin-producing *Fusarium verticillioides* strains. *J. Food Prot.* **6**: 1278–1283.

Patino, B., Mirete, S., Vazquez, C., Jimenez, M., Rodriguez, T. M. and Gonzalez-Jaen, T. 2006. Characterization of *Fusarium verticillioides* strains by PCR-RFLP analysis of the intergenic spacer region of the rDNA. *J. Sci. Food Agri.* **86**: 429–435.

Proctor, R. H., Brown, D. W., Plattner, R. D. and Desjardins, A. E. 2003. Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in *Gibberella moniliformis*. *Fungal Genet. Biol.* **38**: 237-249.

Proctor, R. H., Desjardins, A. E. , Plattner, R. D. and Hohn, T. M. A. 1999. polyketide synthase gene 1999;

Sreenivasa, M. Y., González Jaen, M. T., Sharmila Dass, R., Charith Raj, A. P. Janardhana, G. R. 2008. *Fusarium verticillioides* Isolated from Indian Maize Kernels: *Food Biotech.***22:**160–170.