Food Bioscience 13 (2016) 84–88

Contents lists available at ScienceDirect

Food Bioscience

journal homepage: www.elsevier.com/locate/fbio

Multiplex PCR for the early detection of fumonisin producing *Fusarium verticillioides*

Deepa Nagaraj [a], Charith Raj Adkar-Purushothama [b], Sreenivasa Marikunte Yanjarappa [a,*]

[a] Molecular Mycology Laboratory, Department of Studies in Microbiology, Manasagangothri, University of Mysore, Mysore 570006, Karnataka, India
[b] RNA Group/Groupe ARN, Département de Biochimie, Faculté de médecine des sciences de la santé, Pavillon de Recherche Appliquée au Cancer, Université de Sherbrooke, 3201 rue Jean-Mignault, Sherbrooke, Québec, J1E 4K8, Canada

ARTICLE INFO

Article history:
Received 1 July 2015
Received in revised form
7 January 2016
Accepted 22 January 2016
Available online 22 January 2016

Keywords:
Fusarium
Cereals
Maize
Paddy
Sorghum
Spiking
Mycotoxin

ABSTRACT

In the present study, fumonisin producing *Fusarium verticillioides* was specifically detected in pure cultures, cereal samples and plant materials by multiplex PCR using one forward VERTF-1 and two reverse primers VERTR and VERTF-2. A total of 326 *Fusarium* isolates were obtained from maize, sorghum, paddy wheat and pearl millet samples collected from different districts of Karnataka, India. All *Fusarium* species were subjected to single round of PCR with species specific and fumonisin specific primers which recorded 59.50% of *F. verticillioides* and 53.98% of fumonisin producing *F. verticillioides*. Maize samples recorded highest frequency 34.42% of fumonisin producing *F. verticillioides* followed by paddy 28.57% and sorghum 16.66%. Sensitivity of multiplex PCR experiment was conducted by whole grain experiment of the collected cereals, roots and leaves of the cereal samples by diluting the DNA 10 to 100 times in which 1:50, 1:75 and 1:100 diluted samples recorded positive. The developed multiplex PCR assay provided a powerful tool for the accurate detection, identification and discrimination of potential fumonisin producing *F. verticillioides* strains among the population. The present study is the first report of developing the multiplex PCR method for early detection of fumonisin producing *F. verticillioides* from cereal samples, pure cultures and plant parts.

1. Introduction

Mycotoxins are secondary fungal metabolites which regularly occurs in cereals and cereal based products. Food and Agricultural Organization (FAO) has estimated that approximately 25% of crops worldwide are contaminated with mycotoxins every year (Lawlor & Lynch, 2005). Mycotoxis are strictly regulated and most of the countries have set threshold levels for mycotoxin in food and feeds. (Richard, Bennett, Ross & Nelson, 1993). Fumonisins producing *Fusarium verticillioides* infect food grains such as maize, wheat, barley, rice, finger millet, oats and rye (Rashmi et al., 2012). Fumonisins are great threat in foods, feeds and environment since, high levels of fumonisins in foods can have toxic effect ranging from acute to chronic manifestations in humans and animals (ICMSF, 1996). Mycotoxicosis caused by mycotoxins is not pathognomonic; hence determining the cause of specific condition or disease requires confirmation of the presence of toxin in foods and feeds. Earlier the traditional analytical methods such as TLC, HPLC and GC were practised due to their accuracy, precision and specificity to detect mycotoxins but presently many research scholars have developed rapid sensitive molecular methods and immunological assays which have replaced the conventional methods for efficient detection of mycotoxin producing fungi (Niessen, 2007).

The main objective of the present work was to standardize a multiplex PCR assay for fumonisin producing *F. verticillioides* screened from cereal samples collected from different parts of Karnataka. Multiplex PCR method exhibits great flexibility in experimental design and overcomes limiting primer kinetics and fragment competition. Recently number of uniplex PCR techniques have been adapted to multiplex amplification for diagnosis of genetic and infectious disease for identification of person, population, pathogens and helps in revealing organization of human genome (Edwards & Gibbs, 1994). In the present study, the standardized multiplex PCR assay was evaluated with pure cultures and sensitivity of the assay was tested with spiked cereal samples (maize, paddy, sorghum) and plant parts (root, leaf) and the assay results were compared with LCMS.

* Corresponding author.
E-mail address: sreenivasamy@gmail.com (S. Marikunte Yanjarappa).

http://dx.doi.org/10.1016/j.fbio.2016.01.001
2212-4292/

Table 1
Percent incidence of *Fusarium* sp. and fumonisin producing *F. verticillioides* based on m-PCR and percent of infection in each cereal samples.

Cereal samples	*Fusarium* sp. (%)		*F. verticillioides* (%)		Fumonisin producing *F. verticillioides* (%)	
	cereals	m-PCR	cereals	m-PCR	cereals	m-PCR
Maize	60.65	48.40	47.54	33.12	40.98	31.59
Paddy	52.38	42.02	42.85	24.53	33.33	20.85
Sorghum	37.50	8.8	16.66	1.84	12.50	1.53
Pearl millet	12.50	0.6	ND	ND	ND	ND
Wheat	ND	ND	ND	ND	ND	ND

ND-not detected.

2. Materials and methods

2.1. Mycological analysis

A total of 137 cereal samples including maize (61 samples), paddy (42 samples), sorghum (24 samples), pearl millet (8 samples) and wheat (2 samples) were collected from different districts of Karnataka state from November 2012 to May 2014 and were subjected to mycological analysis. Isolation of *Fusarium* species was carried out by using both standard blotter method and agar plating method (Mathur & Kongsdal, 2003). Melachite Green Agar (MGA-2.5) was used as the selective isolation medium (Bragulat, Martinez, Castella & Cabanes, 2004). The plates were incubated with alternating periods of 12 h darkness and 12 h light at $25 \pm 2°$ C for seven days. After incubation, the plates were observed for studying micro-morphological characteristics of *Fusarium* species. *Fusarium* species were transferred onto potato dextrose agar (PDA) plates to identify them at the species level using fungal taxonomic keys (Booth, 1977; Leslie & Summerell (2006)). All the fungal isolates were maintained on czapek dox agar slants at 4 °C for further studies.

2.2. DNA isolation from Fusarium species

Based on the morphological characters, 298 *Fusarium* isolates were inoculated to 500 µL of potato dextrose broth in 2 mL microfuge tubes and incubated with alternating periods of 12 h darkness and 12 h light at 25 ± 2 °C for four days. The resulting mycelium was used for DNA extraction as per the protocol described by Sreenivasa, Dass, Charith raj and Janardhana (2006).

2.3. Primers for PCR

Isolates were confirmed as *Fusarium verticillioides* by the use of forward primer VERTF-1 (5′- GCG GGA ATT CAA AAG TGG CC -3′) designed by Patino, Mirete, Gonzalez-Jaen, Mule, Rodriguez and Vazquez (2004) and the reverse primer VERT-R (5′- CGA CTC ACG GCC AGG AAA CC-3′) designed by Sreenivasa , Jaen, Dass, Charith Raj and Janardhna (2008) that was used to identify *F. verticillioides* strains at the species level. The isolates were tested using the PCR specific assay for fumonisin-producing *F. verticillioides* with primers VERTF-1 (5′- GCG GGA ATT CAA AAG TGG CC −3′) and VERTF-2 (5′- GAG GGC GCG AAA CGG ATC GG −3′) as described by Patino *et al.* (2004) and the expected PCR amplicon sizes were 1016-bp and 400-bp, respectively.

2.4. Multiplex PCR Amplification

All the isolated 326 DNA samples were subjected to multiplex PCR using a set of one forward VERTF-1 and two reverse primers

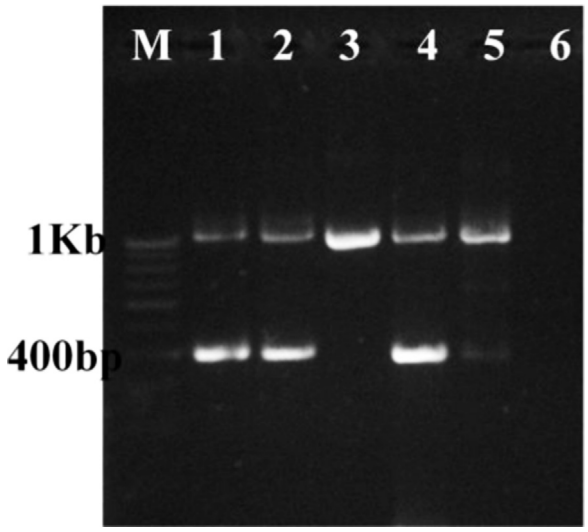

Fig. 1. Multiplex PCR image showing amplification of pure cultures with three primers VERTF-1, VERTR and VERTF-2. Lane **M**-100bp marker, Lane **1**-Positive control (MTCC 156), Lane **2,4 and 5**- Positive isolates of fumonisin producing *F. verticillioides* with 1016bp and 400 bp, Lane **3**-Negative isolate of fumonisin producing *F. verticillioide* with 400 bp, Lane **6**-Negative control (MTCC 1893).

VERTF-2/VERTR with the following PCR conditions: 95 °C for 2 min of initial denaturation, 94 °C for 30 sec of denaturation, 61 °C for 30 sec of annealing, primer extension at 72 °C for 1.5 min and final extension at 72 °C for 13 min. The PCR reaction included 2 µL of dntps, 2.5 µL of Taq buffer, 2.5 µL of $MgCl_2$, 0.125 µL of Taq polymerase, 1 µL DNA, 0.8 µL of each primer (20 pico moles) and 14.475 µL of water in a total volume of 25 µL reaction. PCR was performed by using Sure cycler 8000 (Agilent technologies). Later PCR products were analyzed in 1.5% agarose gel (50x TAE-Tris base, Glacial acetic acid, 0.5 M EDTA, pH-8) and gel was documented using a gel documentation system (Vilber Lourmat-Lab India, Hyderabad) after staining with ethidium bromide.

2.5. Sensitivity of the multiplex PCR assay in spiked cereal samples and plant materials

To test the sensitivity of the multiplex PCR assay cereal samples such as maize, paddy and sorghum were spiked with *F. verticillioides* and half the portion of grains was sown for observing the growth of plants after incubation for five days, the other half portion of grains was used for DNA isolation by crushing the infected grains in 2 mL microfuge tubes with 500 µL of lysis buffer and further the procedure of DNA isolation of *Fusarium* species was followed from the grown plants. Each root and leaf (1 g) was ground using ice-cold pestle and mortar with 500 µL of CTAB buffer, was transferred to microfuge tubes and incubated in water bath for 15 min at 55 °C and centrifuged for 5 min at 12000 rpm. Supernatent was transferred to clean microfuge tubes to which 250 µL of chloroform and isoamyl alcohol in 24:1 were added and centrifuged at 1300 rpm for 1 min. Upper aqueous layer was transferred to clean fresh microfuge tubes and to which 50 µL of 75 M ammonium acetate were added followed by 500 µL ice cold absolute alcohol. Tubes were inverted several times and incubated for 1 hr at −20 °C after addition of ethanol to precipitate DNA. Tubes were centrifuged at 1300 rpm for 1 min for the formation of pellets. Supernatent was removed and pellets were washed twice with 70% alcohol and pellets were dried completely. Pellets were resuspended in 50 µL of DNAse free water and stored at −20 °C.

Isolated DNA from spiked plant material was subjected to multiplex PCR assay by following same conditions and PCR mixture as explained earlier. DNA isolated from spiked cereal samples