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TOOLS FOR *FUSARIUM* MYCOTOXIN REDUCTION IN FOOD AND FEED CHAINS
RESEARCH PAPERS

Identification and quantification of fumonisin-producing *Fusarium* species in grain and soil samples from Egypt and the Philippines

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Summary. Fumonisins are considered among the important mycotoxins associated with human esophageal cancer and livestock diseases. These mycotoxins are mainly produced by *Fusarium verticillioides* in tropical and subtropical regions such as the Philippines and Egypt and humid temperate regions of the world. The classical taxonomy of fumonisin-producing fungi is challenging, and species-specific PCR reactions are commonly used to clearly identify species within these complexes. The aim of this study was to isolate, identify and quantify fumonisin-producing species in maize, wheat and soil samples from Egypt and the Philippines, and to test Eppendorf-Agar as a long term preservation method. We isolated 44 single spore isolates (39 from Egypt and five from the Philippines) from the collected samples (25 isolates from maize, five from wheat and 14 from soil). In addition, we quantified the content of fumonisin-producing fungi DNA from 15 maize samples and six wheat samples from Egypt, and from six maize samples from the Philippines. morphological and microscopic identification indicated that 21 isolates from Egypt and five from the Philippines were *F. verticillioides*, one isolate was *F. proliferatum* and two isolates were *F. nygamai*. Molecular identification indicated that all these isolates belonged to *F. verticillioides*. Most were from maize, four were from soil and only one was from wheat. Other *Fusarium* species isolated included *F. oxysporum* and *F. solani*. No *F. graminearum* isolates were found. The quantitative PCR (qPCR) results obtained using the Taqfum-2f, Vpgen-3R primer pair and the FUMp probe for quantification of fumonisin-producing *Fusarium* species showed that fumonisin-producing *Fusarium* isolates were present in four maize samples from the Philippines and eight maize samples from Egypt. The *Fusarium* DNA levels from fumonisin-producing isolates were in the range of 13×10^{-3} to 61×10^{-1} ng ng^{-1} total DNA in positive samples, except in one maize sample from the Philippines with high concentration of >0.5 ng ng^{-1} total DNA. This indicates that >50 % of all DNA was *Fusarium* DNA. No fumonisin-producing *Fusarium* DNA was detected in the wheat samples and in the remaining maize samples. These results showed that PCR-techniques based on qPCR can be used to identify fumonisin-producing *Fusarium* species and quantify risks of mycotoxin contaminated grains.

Key words: preservation, maize, wheat, mycotoxins, qPCR.

Introduction

Maize is one of the most important world food crops, and is very susceptible to fungal contamination which can occur during pre- and post-harvest. The identification of the *Fusarium fujikuroi* species complex based on morphological characteristics is challenging, even for experts (Leslie and Summerell, 2006; Rossi *et al*., 2009). Species-specific PCR is commonly used to clearly identify species inside fungus complexes such as *Fusarium verticillioides* and *F. proliferatum* (Rahjoo *et al*., 2008, Waalwijk *et al*., 2008). These are the main species producing fumonisins (FBs), a group of mycotoxins related to several human and animal diseases (Desjardins, 2006).

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Many types of FBs, including FB1, FB2 and FB3, are known to contaminate maize (Sydenham *et al.*, 1991), wheat and barley (Aziz *et al.*, 2004). Fumonisin B1 is a highly toxic FB and it is also teratogenic (Marasas *et al.*, 2004) and carcinogenic (Lemmer *et al.*, 1999; Gelderblom *et al.*, 2001). The International Agency for Research on Cancer (IARC) classified FB1 in Group 2B (IARC, 2002). Recent surveys have raised concerns about the extent of FB1 contamination and its implications for animal health and productivity (Rodrigues and Naehrer, 2012; Boutigny *et al.*, 2014; Cendoya *et al.*, 2014; Abd-El Fatah *et al.*, 2015). Aziz *et al.* (2004) reported that FB1 was detected in Egyptian wheat, maize and barley seeds. The early detection and quantification of grain contamination by fumonisin-producing *Fusarium* species is necessary to reduce food-borne illnesses. Consumption of fumonisin-contaminated maize leads to disruption of sphingolipid metabolism, associated with human esophageal cancer and several toxicoses in livestock animals, and increases risk for neural tube defects in children (Marasas, 1995; Marasas *et al.*, 2004). The regulatory limit for fumonisins in maize and by-products established by European Union and Food and Drug Authority to prevent exposure of individuals to these fungal toxins is 200 to 4,000 μg kg^{-1} (van Egmond *et al.*, 2007).

Fumonisin biosynthetic (*FUM*) gene clusters have been reported in *F. verticillioides*, *F. proliferatum*, and a single strain of *F. oxysporum* (Proctor *et al.*, 2003; 2008; Waalwijk *et al.*, 2004). The gene *FUM1* encodes a polyketide synthase necessary for the production of fumonisins, which catalyses the initial steps in fumonisin biosynthesis (Bojja *et al.*, 2004). This gene can be used to detect FB1 production by *F. verticillioides* (López-Errasquín *et al.*, 2007). In developing countries such as Egypt and the Philippines, it is advantageous to find simple, easy and inexpensive methods to detect, quantify and preserve the toxigenic fungi. The objectives of the present study were to isolate, identify and quantify fumonisin-producing species in maize, wheat and soil samples from Egypt and the Philippines, and to test PDA-Eppendorf as a long term preservation method for *F. verticillioides* isolates.

Materials and methods

Fungal isolation, and phenotypic identification

Isolations were performed onto potato dextrose agar (PDA) or *Aspergillus flavus* and *parasiticus* agar (AFPA). For individual grain samples, grains were surface disinfected with 5% sodium hypochlorite (NaOCl) for 1 min followed by rinsing three times with sterile water. The disinfected grains (five of maize and 10 of wheat grains per Petri plate, and three plates per sample) were placed onto PDA or AFPA and then incubated for 3–5 d at 25°C. For soil samples, 2 g of each sample was suspended in 6 mL of sterile distilled water in sterile polystyrene tubes and mixed on a rolling mixer for 20 min. (Donner *et al.*, 2009). Resulting supernatant was inoculated onto Petri plates (100 μL on each plate) containing PDA or AFPA. After incubation, *Fusarium* colonies were transferred to PDA plates and single spore isolates were obtained through serial dilution of a spore suspension of each isolate, and spreading subsamples onto water agar in Petri plates, followed by incubation for 12 h. Three single spore microcolonies were then transferred onto PDA medium and incubated for 24 h. These colonies were used as sources for Eppendorf-agar storage (see below).

All isolates were identified based on macroscopic and microscopic characteristics according to Barnett and Hunter (19720 and Nelson *et al.*, 1983), at the Laboratory of Mycology and Phytopathology, All-Russian Institute of Plant Protection (VIZR), St. Petersburg-Pushkin, Russia.

Eppendorf-agar long term preservation of isolates

The single spore *F. verticillioides* isolates were each cultured in an Eppendorf tube containing 0.5 mL PDA for 1 week at 25°C, and then moved to cold storage at 4°C. The viability of the isolates was tested by subculturing them onto PDA in Petri dishes at 3 month intervals for 27 months.

DNA extraction and PCR

DNA extraction

The *Fusarium* isolates were cultured on malt extract medium (30 g L^{-1} malt extract, 5 g L^{-1} peptone) at 25°C for 3 d. A mycelial disk of each isolate was then transferred into an Eppendorf tube. DNA was extracted by the octanol/ isopropanol method, as described by Paavanen-Huhtala *et al.* (1999). The quality of DNA was confirmed by using ITS (Table 1) primers amplifying *Fusarium* DNA (Yli-Mattila *et al.*, 2004).

Fumonisin- and species-specific primer PCRs

The primer pair Verprof/VERTI-R (Table 1) was used for identification of fumonisin-producing *F. verticillioides* isolates, while the primer pair Taqfum-f 2/VPgen- R3 (Table 1) was used for identification of *F. verticillioides*, *F. proliferatum, F. globosum* and *F. nygamai* isolates. These fungi are the most important fumonisin-producing species (Waalwijk *et al*., 2008; Table 1). The primer pair Fg11/Fg11r was used for detection of *F. graminearum* DNA in the maize samples, as described by Doohan *et al.* (1998).

A PTC-200 DNA DNA Engine Thermal cycler (MJ Research, Inc.) was used for PCR amplification. Aliquots (5 μL) of each PCR product were analyzed by electrophoresis in TBE buffer in 1.0% agarose gels and visualized using the ChemiDoc MP Imaging System (Bio-Rad).

Quantitative PCR

The total amounts of *F. verticillioides*, *F. proliferatum, F. globosum* or *F. nygamai* DNA were quantified by qPCR from 15 maize samples and six wheat samples from Egypt, and from six maize samples from the Philippines, as described by Waalwijk *et al.* (2008). This used the Taqfum-2f, Vpgen-3R primer pair and the FUMp probe (CAATGCCATCTTCTTG). A Bio-Rad IQTM5 Real-Time PCR Detection System (Bio-Rad) was used for running qPCR samples. *Fusarium* species DNA amounts in grain samples was determined as total DNA, quantified using a Qubit fluorometer (Invitrogen), as described by Yli-Mattila *et al.* (2011).

Results

Forty-four single spore isolates (39 from Egypt and five from the Philippines) were obtained from the collected samples. Twenty-five isolates were from maize grains, 14 were from soil and five were from wheat grains.

All *F. verticillioides* isolates in addition to several *Aspergillus flavus* and *A. parasiticus* isolates, one isolate of *Bacillus subtilis*, two isolates of *B. amyloliquefaciens* and one *Streptomyces* isolate showed significant viability during the 27 month preservation using the Eppendorf-agar method.

Morphological identification (using microscopic characters) indicated that 21 isolates from Egypt and five from the Philippines belonged to *F. verticillioides*, one isolate was *F. proliferatum* and two were *nygamai* (Table 2). Other *Fusarium* species included two *F. oxysporum* isolates and five of *F. solani*.

Molecular identification showed that all fumonisin-producing *Fusarium* isolates, including the *F. proliferatum* isolate, two *F. nygamai* isolates and one *F. oxysporum* isolate identified by morphological characters, belonged to *F. verticillioides* (Table 2). Most of the *F. verticillioides* isolates were from maize. Two *F. verticillioides* isolates, from Egypt and from Philippines, the only *F. oxysporum* isolate and all *F. solani* isolates were collected from soil. Only one *F. verticil-*

Table 1. Primer sequences used in PCR: ITS1/ITS4 for confirming the quality of DNA, Taqfum-2F/Vpgen-3R for detecting four *Fusarium* species producing fumonisins, Verpro-F/VERTI-R for detecting fumonisin-producing *F. verticillioides*,, and Fg11/Fg11r primer pair used for detection of *F. graminearum*.

Primer	5′ > 3′ sequence	Target sequences and species	Amplicon size(bp)	Author
ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	Fungal ribosomal DNA	650-700	Yli-Mattila *et al*. (2004)
Taqfum-2F Vpgen-3R	ATGCAAGAGGCGAGGCAA GGCTCTCA/ GGAGCTTGGCAT[1]	All fumonisin producers *F. verticillioides, F. proliferatum, F. globosum* and *F. nygamai*	180	Waalwijk *et al*. (2008)
Verpro-F VERTI-R	GCCATGCGTCACGGCCAC GGAGTAGACAGGGTATTTGC	*F. verticillioides* fumonisin producers	235	Waalwijk *et al.* (2008)
Fg11 Fg11r	CTCCGGATATGTTGCGTCAA GGTAGGTATCCGACATGGCAA	*F. graminearum*	400	Doohan *et al*. (2008)

Table 2. Morphological and molecular identification of the isolated fungi, and collection sites.

Isolate code[1]	Morphological identification[2]	Molecular detection and identification of fumonisin producing fungi[1]			Collection site	Sample code
		Taqfum-2F Vpgen-3R PCR	Verpro-F VERTI-R PCR	Fungus species[2]		
1 E	F. v	+	+	F. v	Maize sample from Awsiem Tuesday market (Balady*)	M11 b
2 E	F. v	+	+	F. v	Maize sample from Awsiem Tuesday market (Hokoma Monofia)	M7 b
3 E	F. v	+	+	F. v	Maize sample from Awsiem Tuesday market (Balady Bahary*)	M10 b
4 E	F. v	+	+	F. v	Maize sample from Awsiem Tuesday market (Hegry Elmanashy*)	M9 b
5 E	F. v	+	+	F. v	Maize sample from Awsiem	M1 b
6 E	A. f	-	-	-	Maize sample from El-Beharia)	M14 b
7 E	F. v	+	+	F. v	Maize sample from el- Fayiom	M16 b
8 E	F. v	+	+	F. v	Maize sample from El-Beharia (Balady*)	M4 b
9 E	A. s	-	-	Not identified	Wheat sample from el-Monofia	
10 E	Un known	-	-	Not identified	Wheat sample from Awsiem Tuesday market (Balady*)	W8
11 E	F. p	+	+	F. v	Maize sample from Awsiem	M12 b
12 E	F. o	+	+	F. v	Soil sample from el-mansoria	S5 b
13 E	F. v	+	+	F. v	Maize sample from Awsiem Tuesday market (Hokoma Monofia*)	M7 b
14 E	F. v	+	+	F. v	Maize sample from Awsiem Tuesday market (Balady Bahary*)	M10 b
15 E	F. v	+	+	F. v	Maize sample from Awsiem Tuesday market (Hegry Elmanashy*)	M9 b
16 E	F. v	+	+	F. v	Maize sample from Monofia	M2 b
17 E	F. s	-	-	Not identified	Soil sample from Ekashef area in Awsiem	S4 b
18 E	F. v	+	+	F. v	Maize sample from Awsiem Tuesday market yellow maize Awsiem)	M15 b
19 E	F. v	+	+	F. v	Maize sample from Awsiem Tuesday market (yellow maize argantiny*)	M13 b
20 E	F. v	+	+	F. v	Wheat sample from Awsiem Tuesday market (Balady*)	W4 b
21 E	F. v	+	+	F. v	Maize sample from Awsiem Tuesday market yellow maize El-Beharia)	M14 b
22 E	F. v	+	+	F. v	Maize sample from Awsiem Tuesday market yellow maize El-Beharia)	M14 b
23 E	F. v	+	+	F. v	Maize sample from Awsiem Tuesday market (Hokoma El-Beharia*)	M8 b

(Continued)

Table 2. (Continued).

Isolate code[1]	Morphological identification[2]	Molecular detection and identification of fumonisin producing fungi[1]			Collection site	Sample code
		Taqfum-2F Vpgen-3R PCR	Verpro-F VERTI-R PCR	Fungus species[2]		
24 E	F. v	+	+	F. v	Maize sample from Monofia	M2 b
25 E	F. v	+	+	F. v	Maize sample from Awsiem Tuesday market (Balady*)	M11 b
26 E	F. v	+	+	F. v	Maize sample from Awsiem Tuesday market (Balady*)	M11 b
27 E	F. v	+	+	F. v	Maize sample from Awsiem Tuesday market (yellow maize argantiny*)	M13 b
28 E	F. n	+	+	F. v	Soil sample from Eltarbeaa area in Awsiem	S8 b
29 E	F. s	-	-	Not identified	Soil sample from Eltarbeaa area in Awsiem	S8 b
30 E	F. s	-	-	Not identified	Soil sample from Eltarbeaa area in Awsiem	S8 b
31 E	F. s	-	-	Not identified	Soil sample from Eltarbeaa area in Awsiem	S8 b
32 E	Unknown	-	-	Not identified	Wheat sample from Awsiem	W1
33 E	P. spp.	-	-	Not identified	Wheat sample from Tanta	W3
34 E	P. spp.	-	-	Not identified	Soil sample from Tanta	S6 b
35 E	F. o	-	-	Not identified	Soil sample from Eltarbeaa area in Awsiem	S8 b
36 E	F. n	+	+	F. v	Soil sample from Elhaswa area in Awsiem	S7 b
37 E	F. s	-	-	Not identified	Soil sample from Tanta	S6 b
38 E	F. s	-	-	Not identified	Soil sample from Elhaswa area in Awsiem	S7b
39E	*Alternaria-Stemphylium*	-	-	Not identified	Wheat sample from Awsiem Tuesday market (Monofia)	W2
1 P	F. v	+	+	F. v	Soil sample from Visayas	S3
2 P	F. v	+	+	F. v	Soil sample from Mindanao	S18
-	Not detected	-	-	-	Maize sample from Mindanao	M1
3 P	F. v	+	+	F. v	Maize sample from Visayas	M2
4 P	F. v	+	+	F. v	Maize sample from Mindanao	M3
-	Not detected	-	-	-	Maize sample from Mindanao	M4
-	Not detected	-	-	-	Maize sample from Visayas	M5
5 P	F. v	+	+	F. v	Maize sample from Visayas	M6

[1] E, Egypt; P, Philippines.
[2] F. v, *Fusarium verticillioides;* A. f, *Aspergillus fumigatus*; A. s, *Alternaria-Stemphylium;* F. p, *F. proliferatum;* F. o, *F.oxysporum;* F. s, *F. solani;* F. n, *F. nygamai; P.* spp., *Penicillium* spp.
* Commercial name of the grain.

Table 3. The concentrations of DNA of fumonisin-producing fungi maize (M) and wheat (W) grain samples from the Philippines and Egypt.

Sample code	Total DNA (ng ng^{-1})
M1	Not detected
M2	0.0027
M3	Not detected
M4	0.0014
M5	>0.5
M6	0.0052
M1b	Not detected
M2b	<0.001
M3b	0.006
M4b	0.021
M5b	<0.001
M6b	<0.001
M7b	0.0122
M8b	0.018
M9b	Not detected
M10b	Not detected
M11b	Not detected
M12b	Not detected
M13b	Not detected
M14b	Not detected
M15b	0.061
W1	Not detected
W2	Not detected
W3	Not detected
W4	Not detected
W5	Not detected
W6	Not detected
W7	Not detected
W8	Not detected
W9	Not detected

lioides isolate was obtained from wheat grains, and no other *Fusarium* spp. were isolated.

The qPCR results obtained using the Taqfum-2f, Vpgen-3R primer pair and FUMp probe for quantification of the fumonisin-producing *Fusarium* isolates (Table 3) showed that these fungi were present in four maize grain samples from the Philippines and eight samples from Egypt. The *Fusarium* DNA levels were in the range of 13×10^{-3} to 61×10^{-1} ng ng^{-1} total DNA in positive samples, except in one maize sample from the Philippines with a concentration > 0.5 ng ng^{-1} total DNA. This indicates that more than 50% of all DNA was *Fusarium* DNA. No fumonisin-producing *Fusarium* DNA was detected in the wheat samples and in the remaining maize samples, although in four of these maize samples and in one wheat sample *F. verticillioides* isolates were found. No *F. graminearum* DNA was detected in the maize samples from the Philippines. On the other hand, DNA of fumonisin-producing *Fusarium* was detected in two maize samples (M4 and M5), and but there are no fungi isolated from these samples.

Discussion

This study isolated fumonisin-producing *Fusarium* strains from maize, wheat and soil samples from Egypt and the Philippines. This is in accordance with several reports, which have indicated the presence of these species in Egyptian maize and feed samples (El-Habbaa, *et al*., 2003; Abo El Yazeed, *et al*., 2011 and Abd-El Fatah *et al*., 2015) and also in the Philippines samples (Cumagun *et al*., 2009, Magculia and Cumagun, 2011).

The molecular detection of fumonisin production based on the Verprof, VERTI-R primer pair for *F. verticillioides* and the Taqfum-f2, VPgen-R3 primer pair for all fumonisin-producing species (Waalwijk *et al*., 2008) showed that all *Fusarium* isolates were positive for FBs production. Similar results were reported by Abd-El Fatah *et al.* (2015). The fungus identifications based on morphological characteristics were mainly in agreement with the PCR results using species-specific primers, except for five isolates (one was identified as *F. proliferatum*, another as *F. oxysporum* and two as *F. nygamai*). This highlights the difficulty in identification of *Fusarium* species using traditional methods (Leslie and Summerell, 2006; Rossi *et al*., 2009). Our results showed that use of PCR-based identification methods as a fast and cheap way for detection of contamination of grain samples with fumonisin-producing fungi.

The Eppendorf-agar method is suitable for preserving *F. verticillioides* for more than 2 years. This method was also effective for long-term storage of several *Aspergillus flavus* and *A. parasiticus* isolates, *Bacillus subtilis, B. amyloliquefaciens* and *Streptomyces* isolates. This method is easy to perform and reduces the time, cost and the risk of contamination. Several methods have been used for microorganisms, including repeated subculturing on agar slants (Onions, 1971), preservation under mineral or paraffin oil (Perrin, 1979), freeze-drying (lyophilization) (Tan *et al*., 2007) and cryopreservation (Homolka and Lisa, 2008). All of these methods are more time consuming and expensive than the Eppendorf-agar method described here. However, further research should be carried out to assess effects of this storage storage method on the genetic stability of preserved microbes and suitability for different microbial species.

The qPCR results showed that DNA of fumonisin-producing fungi was detected only in maize grain samples. This was in agreement with number of *Fusarium* isolated from maize, indicating that maize grain is very susceptible to *F. verticillioides* and *F. proliferatum.* In addition, no *F. graminearum* was isolated from the samples, and DNA of this species was not detected in any sample. This is probably due to the unfavourable weather conditions that occur in Egypt and the Philippines for growth of this species. The presence of DNA offumonisin-producing *Fusarium* in samples M4 and M5, although no fungi were isolated from these samples, demonstrated the stability of DNA for detection and quantification of potential mycotoxin contamination, even after death of mycotoxin-producing fungi.

In conclusion, the Eppendorf-agar method is suitable for long-term preservation of *F. verticillioides*. PCR-based techniques could be used to identify fumonisin-producing *Fusarium* species and quantify the risk of contaminated cereal grains, especially in the developing countries.

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