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REAL-TIME PCR AND AGAR PLATING METHOD TO PREDICT *FUSARIUM VERTICILLIOIDES* AND FUMONISIN B_1 CONTENT IN NIGERIAN MAIZE

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Abstract: Eighty maize grain samples collected in Nigeria were investigated for fumonisin B_1 (FB_1) content and *Fusarium verticillioides* colonization. *F. verticillioides* DNA was quantified by species-specific real-time PCR and living propagules of the fungus were counted by agar-plating method. FB_1 was detected in 55 (68.7%) of the total samples (mean: 98.5 μg/kg, range: 10 to 714 μg/kg) at 10 μg/kg detection limit. The mean amount of *F. verticillioides* DNA determined by real-time PCR was 49.7 μg/kg (range: 10–126.7 μg/kg), while agar plate method showed the presence of *F. verticillioides* in 45 samples (mean incidence: 21.0%, range: 6.7–60.0%). There was correlation ties between *F. verticillioides* DNA by real time PCR and fungal colonization by agar plate method (R = 0.71, p = 00001 at 95% confidence level), and means of FB_1 and *F. verticillioides* DNA in the yellow and white maize were significantly different. Despite the high consumption of maize in Nigeria, the amount of FB_1 ingested by consumers appears to be low. The estimated daily intake of fumonisins was 0.21 μg/kg body weight per day.

Key words: maize, fumonisin, mycotoxins, *Fusarium verticillioides,* real-time PCR

INTRODUCTION

The fungal genus *Fusarium* includes many crop pathogens, which reduce grain yield and impair seed quality, causing a potential threat to the global food supply (Strange and Scott 2006; De Venter 2000). They also produce mycotoxins which can accumulate either preharvest or in stored grains (Bottalico 1998) and are responsible for serious chronic and acute diseases in humans and animals leading to feed refusal, vomiting, diarrhoea, dermatitis, hemorrhages and other disease symptoms (Desjardins 2006).

Fusarium mycotoxins most commonly found in grain in Nigeria are fumonisins produced by *F. verticillioides*, with FB_1 being the most prevalent fumonisin (Bankole and Mabekoje 2004; Adejumo *et al.* 2007a). The concentrations of FB_1 in Nigerian samples varied from 10 to 760 μg/kg with a mean level of 117 μg/kg in positive samples (Adejumo *et al.* 2007a). *F. verticillioides* is known worldwide to cause stalk and ear rot of maize (Leslie *et al*. 1990; Logrieco *et al.* 1993; Bottalico 1998; Leslie and Summerell 2006). The contamination of maize with fumonisins is believed to be responsible for a variety of animal diseases, e.g. equine leukoencephalomalacia (ELEM) in horses (Kellerman *et al.* 1990), pulmonary edema in swine (Harrison *et al.* 1990) hepatotoxic, nephrotoxic and carcinogenic effects in rats (Gelderblom *et al.* 1991; Voss *et al.* 1993).

Fumonisin B_1 is suspected to cause esophageal (Rheeder *et al.* 1992; Chu and Li 1994) and liver cancer in humans (Gelderblom *et al.* 1991; Rheeder *et al.* 1992; Voss *et al.* 1993; Chu and Li 1994; Ueno *et al.* 1997).

Apart from fumonisins, mycotoxins zearalenone, deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3-ADON), diacetoxyscirpenol and enniatins A, B and B_1 have been recently detected in Nigerian maize meant for human consumption (Adejumo *et al.* 2007b). *Fusarium* species other than *F. verticillioides* found in maize in Nigeria were *F. acuminatum, F. compactum, F. equiseti, F. graminearum, F. oxysporum, F. semitectum, F. sporotrichioides* and *F. subglutinans* (Adejumo *et al.* 2007a, b). Different *Fusarium* species have different mycotoxins profiles, therefore accurate determination of the species is critical to predict the potential risk of the isolate as well as prevent the toxins from entering the food chain (Jurado *et al*. 2006), but in large samples of grain, one can often find more than one fungal species and each species can or may produce different toxins. Early detection and identification of these pathogens is an integral part of a successful plant disease management.

Conventional diagnostic methods for the detection of fungal pathogens in cereal crops are based on morphological and cultural characteristics which involve visual symptoms of the disease and microscopic observation.

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These methods are multi-stage, slow, time consuming, labor intensive and to a certain degree subjective. *Fusarium* taxonomy requires considerable training because the species are sometimes difficult to distinguish. A further drawback of methods based on pure cultures is that the viability of fungi in seeds after harvest may be reduced (Prange *et al*. 2005). Therefore, there is a need for developing tools that would permit a reliable, rapid, sensitive and specific diagnosis of *Fusarium* species in contaminated samples.

The use of molecular methods in fungal diagnostics to complement morphological identification has become common (Taylor 1999; Donaldson *et al*. 1995). Polymerase chain reaction (PCR) is the most frequently used molecular diagnostic now, replacing enzyme-linked immunoassay (ELISA). PCR detects target DNA sequences in complex mixtures even when the mycelia are no longer viable. Despite this advance, diagnosis based on growing pure cultures of the pathogen still predominates in developing countries due to technical demands and costs associated with molecular techniques.

Various PCR assays have been developed for the identification of mycotoxigenic species of *Fusarium*. The objectives of this study were to (i) apply a rapid and reliable method for species-specific identification and absolute quantification of *F. verticillioides* by real-time PCR to Nigerian maize samples designated for human consumption, (ii) use the PCR method and the traditional agar plating method to predict *F. verticillioides* and fumonisin content in maize.

MATERIALS AND METHODS

Sample collection

Eighty (80) maize samples were collected between May and July of 2005 from farmers, markets and grain shops in south western Nigeria: Ondo, Osun, Ekiti and Oyo states those were 28, 18, 12 and 22 respective samples which were used for the investigation. Five hundred gram seeds per sample were ground with the milling machine (1 mm sieve) and used for FB_1 analysis.

Mycological analysis

Fifteen seeds from each maize sample were surface sterilized with a 1% sodium hypochlorite (NaOCl) solution and 5 seeds were placed per plate into potato dextrose agar (PDA) and incubated at 25°C for 5 days. Fungi were isolated and sub-cultured to obtain pure cultures. Spore suspension of the fungus was prepared and spread onto water agar plates and incubated for 18 h at room temperature. A single germinating conidium of each fungus was then removed and transferred to PDA plates, Spezieller närstoffarmer Agar (SNA) plates and potassium chloride agar (KCLA) and incubated for 7 days at 25°C. After that, SNA plates were placed under UV light for 2 to 4 weeks at 22°C. Fusarium species were identified by morphological characteristics (Leslie and Summerrell 2006).

DNA extraction from maize flour

CTAB methods (Murray and Thompson 1980) with modifications by (Stewart 1993) and (Brandfass and Karlovsky 2006) were used for DNA extraction, while the quality and concentration was assessed by agarose electrophoresis (Sambrook *et al*. 1989).

Determination of *F. verticillioides* DNA by real-time PCR

Real-time PCR protocol was developed based on primers specific for *F. verticillioides* described by Mule *et al*. 2004 and S. Nutz, C. Brandfass and P. Karlovsky, in preparation. The primer pairs specific to *F. verticillioides*: VER1 5′-CTTCCTGCGATGTTTCTCC-3′ and VER2 5′-AATTGGCCATTGGTATTATATATCTA-3′ were used to amplify the calmodulin gene region

with PCR product of 578 bp. Thirty-two samples were tested in parallel by performing 40 cycles of amplification in iCycler System. The cycling protocol for primer set consisted of denaturation at 94°C for 50 s, 62°C for 50 s, 72°C for 1 min with a final extension step of 72°C for 7 min. The detection of fluorescence was carried out in the annealing step of each cycle. Following the amplification, melting curves were acquired by heating the samples to 95°C for 1 min, cooling to 55°C for 1 min, followed by 60 cycles with 10 s and increasing the temperature after cycle 2 by 0.5°C (Schnerr *et al*. 2001). The fluorescence of SYBR GREEN I was measured after each PCR step on iCycler (BioRad, Hercules, CA, USA). Calibration was carried out with purified *F. verticillioides* DNA in 0.5 pg, 5.0 pg, 50 pg and 500 pg added to maize flour matrix. A standard curve of PCR product ratio of the DNA concentration standards against the cycle threshold gave a linear regression of $Y = -3.721 + 15.538$ ($r^2 = 0.99$). PCR product ratios were converted to DNA concentrations (picograms of total DNA), then to microgram per kilogram (μg/kg) flour.

Fumonisin B_1 analysis

A HPLC-MS method based on protocols by Royer *et al*. (2004) was used for FB_1 determination. The limit of detection for FB_1 determined at a signal to noise ratio of 3 : 1 was 10 μg/kg maize flour, the limit of quantification was determined at signal to noise ratio of 10 : 1. The calibration curve was prepared by spiking fumonisin-free maize extract with standard solutions in concentrations corresponding to fumonisin contents from 6.25 to 2 000 μg/kg flour.

Statistical analysis

Statistix 8.1 Analytical Software, 2003 was used for statistical analyses. The data were arcsine transformed. Analysis of variance (ANOVA) was done and Tukey HSD All-Pairwise Comparisons Test at 5% significance level was used to compare the means. Spearman Rank Correlation coefficient was used to evaluate the intensity of the relationships between the agar plate methods and the results of the real time PCR.

RESULTS

F. verticillioides was detected by species-specific PCR amplification in 39 (48.7%) of the maize samples, while agar plate method enabled the identification of *F. verticillioides* in 45 (56.2%) maize samples (Tables 1, 2).

Table 1. Detection of *F. verticillioides* in maize by seed agar plate method and real time PCR and Fumonisin B_1 analysis

Maize number	Colour	State	Fumonisin B_1 [µg/kg]	*F. verticillioides* DNA [µg/kg]	*F. verticillioides* Agar Plate *[% inc]
1	2	3	4	5	6
36	yellow	Ondo	16	26.7	0
42	white	Ondo	112.8	50	33.3
43	yellow	Ondo	303.6	73.3	40
44	yellow	Ondo	58.4	56.7	26.7
58	yellow	Ondo	13	43.3	13.3
91	yellow	Ekiti	22.4	26.7	20
114	yellow	Ekiti	231.3	46.7	0
140	white	Oyo	11.7	32.3	6.7
146	yellow	Oyo	17.7	23.3	26.7
152	white	Oyo	47.1	43.3	33.3
200	white	Ondo	103.8	50	6.7
245	white	Osun	112.9	60	40
264	white	Osun	40	36.7	13.3
274	yellow	Oyo	53.3	90	40
287	yellow	Osun	61.8	26.7	26.7
289	yellow	Osun	2.5	33.3	6.7
294	yellow	Oyo	713.8	56.7	46.7
296	white	Osun	100.9	70	13.3
309	yellow	Osun	212	70	13.3
332	white	Oyo	42.8	43.3	33.3
336	yellow	Oyo	31.7	43.3	33.3
339	yellow	Oyo	210.5	126.7	13.3
345	white	Oyo	195.8	36.7	6.7
350	white	Oyo	38.1	20	20
355	yellow	Oyo	694.2	70	40
359	white	Oyo	125.1	36.7	6.7
367	yellow	Oyo	10.9	60	6.7
378	yellow	Oyo	36.9	30	40
382	white	Oyo	52.2	43.3	26.7
391	white	Oyo	55.9	36.7	20
399	white	Oyo	103.2	120	60
38	yellow	Ondo	0	20	0
69	yellow	Ekiti	0	70	46.7
102	white	Ondo	0	50	13.3
211	white	Ondo	0	23.3	0
267	yellow	Osun	0	66.7	0
307	yellow	Osun	0	23.3	20
342	white	Oyo	0	63.3	13.3
361	white	Oyo	0	40	26.7
10	white	Ekiti	17.8	0	13.3
21	yellow	Ondo	0.7	0	6.7
28	yellow	Ondo	132.5	0	0
29	yellow	Ondo	132.3	0	0
30	white	Ondo	9.8	0	20
40	yellow	Ondo	9.3	0	0
50	yellow	Ondo	9.4	0	6.7
52	yellow	Ondo	63	0	0
54	yellow	Ondo	13.2	0	0
55	yellow	Ondo	48.1	0	0
62	yellow	Ondo	81.4	0	0
73	yellow	Ekiti	16.9	0	6.7
96	white	Ekiti	99.6	0	6.7
126	yellow	Ondo	40.3	0	0
143	white	Oyo	23.9	0	0
189	yellow	Ondo	32.9	0	13.3
190	yellow	Ondo	221	0	0
209	white	Ondo	90.6	0	13.3
216	yellow	Ondo	91.4	0	0
252	white	Osun	0.3	0	13.3
258	white	Osun	2	0	0
259	white	Osun	44.5	0	0
260	white	Osun	25.2	0	0
262	white	Osun	125.8	0	0
266	yellow	Osun	28.7	0	0
291	yellow	Osun	82.2	0	0
292	yellow	Osun	46.5	0	6.7
311	yellow	Osun	16.1	0	0
348	white	Oyo	10.5	0	0
386	white	Oyo	95.7	0	6.7
1	white	Ondo	0	0	0
4	white	Ekiti	0	0	0
6	yellow	Ekiti	0	0	0
15	white	Ekiti	0	0	0
23	yellow	Ondo	0	0	0
89	white	Ekiti	0	0	0
94	white	Ekiti	0	0	0
131	white	Ondo	0	0	0
205	yellow	Ondo	0	0	0
263	white	Osun	0	0	0
346	white	Ekiti	0	0	0

* percentage incidence [% inc] was based on 15 surface-disinfected seeds

Table 2. Detection of *F. verticillioides* in maize by agar plate method and real time PCR, analysis of and fumonisin B_1

	F. verticillioides		
	FB_1 [µg/kg]	DNA [µg/kg]	Agar Plate [% incidence]
Positive samples	55 (68.7%)	39 (48.7%)	45 (56.2%)
Negative samples	25 (31.3%)	41 (51.2%)	35 (43.7%)
Range	10–714	10–126.7	6.7–60
Mean*	98.5	49.7	21.0
Median*	53.3	43.3	13.3
Standard Error	18.3	3.4	2.1

* mean and median calculation were based on positive samples

There was a significant correlation between *F. verticillioides* DNA content and the amount of the fungus detected by the agar plate method (R = 0.71, p = 0.0001 at 95% Confidence Level). Comparing the PCR analysis and agar plate method showed that only 71% of maize samples positive for *F. verticillioides* by agar plating were confirmed by species-specific PCR. *F. verticillioides* was not detected by agar plate method in 5 samples that were positive in PCR analysis, while 11 samples that showed positive for *F. verticillioides* by agar plate method were negative in PCR analysis.

Fifty five samples (68.7%) contained detectable FB_1 levels, mean 98.5 µg/kg and range 10–714 µg/kg (Table 2). Out of these, thirty samples contained detectable amounts of *F. verticillioides* DNA, while 36 samples showed the presence of this *Fusarium* species by agar plate method. Interestingly, the real time PCR detected *F. verticillioides* in

9 samples with FB_1 levels under the quantification limit of HPLC-MS. The agar plate method confirmed the PCR result for 6 samples.

Among 55 samples that showed detectable levels of FB_1, 25 samples showed negative PCR results for *F. verticillioides*, while 19 samples showed negative results by agar plate method. However, among the 25 samples positive for FB_1 but negative by PCR, the results of agar plate method showed the presence of *F. verticillioides* and *F. proliferatum* in 8 and 17 maize samples, respectively.

Results in table 3 show that means of FB_1 and *F. verticillioides* DNA differed significantly among the white and yellow maize at 95% confidence level. Yellow maize showed more FB_1 (97 µg/kg and 41 µg/kg in yellow and white maize, respectively) and *F. verticillioides* DNA (32 µg/kg 19 µg/kg, respectively).

Table 2. Detection of *F. verticillioides* in maize by agar plate method and real time PCR, analysis of and fumonisin B_1

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Standard Error	18.3	3.4	2.1

* mean and median calculation were based on positive samples

There was no significant difference in the mean FB_1 for collection dates (May, June and July) and the 4 states. Although, FB_1 content was highest for July and least in June collections. The FB_1 content was also highest in maize samples from Oyo state, followed by Ondo, Osun and least in Ekiti state.

DISCUSSION

One of the reasons for the differences in *F. verticillioides* DNA content and the amount of fungus detected by the agar plate method is that *F. species* in the Liseola section (especially *F. thapsinum, F. proliferatum* and *F. verticillioides*) exhibit similar morphological features: dark to violet colour on PDA plates, absence of chlamydospores on KCL agar (swollen cells may look like chlamydospores for *F. proliferatum*), narrow/straight shaped macroconidia, oval to obovoid microconidia in long chains and on false heads (Leslie and Summerell 2006). Accurate morphological identification of *Fusarium* species is therefore difficult. It may be the partitioning of fungal material to samples at low levels of fungal content in grain. Sampling technique was found to exert a major influence on analytical results of *Fusarium* contamination studies (Mulfinger *et al*. 2000). In this study, seed plating was done on 15 kernels (3.72 g), while a 0.1 g sub-sample from 50 g maize flour was used for DNA extraction and PCR analyses. This could be due to absence of infected kernels in the ground maize or the amount of fungal material that was below the detection limit of the PCR. The plate method may be inflating by the incidence of *F. verticillioides*, given that other species are misidentified with plate assay but do not show up in PCR assay.

F. verticillioides can be an endophyte in maize, the production of fumonisin depends not only on the presence of the fungus, but also on the correct environmental conditions for the strains present to be able to make the toxin. Fumonisin non-producing strains from field samples are known (Desjardins *et al*. 2000), but in general the proportion of these strains to the total population is relatively small. Other explanation for samples with the fungus but no toxin may be because the fungus appeared late and did not have time to produce the toxin or that it was misidentified.

The results on FB_1 and *F. verticillioides* DNA among the white and yellow maize confirmed an earlier report (Rava *et al*. 1996), but they contradicted another (El-Sayed *et al.* 2003). The endosperm of the latter is reported to have a soft and floury texture, whereas that of the former have a hard texture (Williams *et al.* 1992) with an enriched content of vitamin E which acts as an antioxidant during storage (Katta *et al.* 1997). Apart from these differences, fungal contamination and FB_1 content may be affected by different collection periods, practices and storage conditions (Viquez *et al.* 1996).

Whole seed plating appears superior to qualitative PCR-based testing when assessing the risk of mycotoxin contamination because it provides an estimate of the degree of fungal infection in the sample, while a qualitative PCR-based method can only indicate the presence or absence of the target species (Demeke *et al.* 2005). The agar method is also more suitable in detecting viability of the pathogens after thermal treatment (Clear *et al.* 2002) or spraying with a fungicide. However, PCR-based tests are preferable to conventional methods for screening for quarantine pathogens, to estimate the risk of mycotoxin contamination by toxigenic *Fusarium* species and for the assessment of fungal mass (Waalwijk *et al.* 2004). A major limitation is the lack of discrimination between living and dead material.

The low levels of fumonisins reported here could be due to a number of other species than *F. verticillioides*, many of these species are not usually considered significant fumonisin producers, even though they might be able to produce amounts such as those reported here. As many of these strain might formerly have been identified as "*F. moniliforme*" the current identification as *F. verticillioides* might also be incorrect. The contamination of Nigerian maize with fumonisin B_1 in the four states studied was below the legal limits of 2 000 µg/kg currently adopted in North America and European Community. Taking into account a relatively high average consumption of maize in Nigeria (180 g/person/day), the estimated daily intake of fumonisins was 0.21 µg/kg body weight per day. This is below the recommended tolerable daily intake (TDI) of 2 µg/kg established by the Scientific Committee on Food (SCF) (European Commission 2003). From the above it may be concluded that the consumption of maize in the four studied states does not pose health risks, but this is different in rural villages where daily consumption is

higher and grain quality is much lower than it would be in the city. There will be variation in mycotoxin content across agroecological zones and seasons and our data reflects only the situation in one season. Extension of the toxicological data sets to assess human health risk from fumonisin by sampling more growing seasons, and ideally by monitoring the fumonisin contamination continuously, is desirable.

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POLISH SUMMARY

REAL-TIME PCR I METODA PŁYTKOWA DO PRZEWIDYWANIA ZAWARTOŚCI FUMONISINU B_1 WYTWARZANEGO PRZEZ *FUSARIUM VERTICILLIOIDES* W NIGERYJSKIEJ KUKURYDZY

Badano 80 prób nasion kukurydzy zebranych w Nigerii na zawartość fumonisinu B_1 (FB_1) i zasiedlenie ich przez *Fusarium verticillioides*. Określono zawartość DNA wykorzystując specyficzną dla gatunku metodę real-time PCR i obliczono żywotne elementy rozmnożeniowe grzyba metodą płytkową. FB_1 wykryto w 55 (68,7%) przypadkach w ogólnej liczbie prób (średnia: 98,5 µg/kg, zakres: 10 do 714 µg/kg), przy limicie wykrywalności 10 µg/kg. Średnia ilość DNA *F. verticillioides* określona metodą rzeczywistego czasu PCR wynosiła 49,7 µg/kg (zakres: 10–126,7 µg/kg), podczas gdy metoda płytkowa ujawniła obecność *F. verticillioides* w 45 próbach (średnie występowanie: 21,0%, zakres 6,7–60,0%). Wystąpiły powiązania korelacyjne między DNA *F. verticillioides* ujawnione przez real-time PCR i zasiedleniem w przypadku metody płytek agarowych, (R = 0,71, p = 00001 przy poziomie ufności 95%), a średnie dla FB_1 oraz DNA *F. verticillioides* w żółtej i białej kukurydzy były istotnie różne. Pomimo wysokiej konsumpcji kukurydzy w Nigerii ilość FB_1 spożywanego przez konsumentów wydaje się niska. Szacunkowe dzienne spożycie fumonisinu wynosiło 0,21 µg/kg w stosunku do wagi ciała.