REAL-TIME PCR AND AGAR PLATING METHOD TO PREDICT *FUSARIUM VERTICILLIOIDES* AND FUMONISIN B₁ CONTENT IN NIGERIAN MAIZE

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Abstract: Eighty maize grain samples collected in Nigeria were investigated for fumonisin B₁ (FB₁) 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. FB₁ 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 = 0.0001 at 95% confidence level), and means of FB₁ and *F. verticillioides* DNA in the yellow and white maize were significantly different. Despite the high consumption of maize in Nigeria, the amount of FB₁ 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 FB₁ being the most prevalent fumonisin (Bankole and Mbekoko 2004; Adejumo et al. 2007a). The concentrations of FB₁ 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 B₁ 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 B₁ 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.
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 immunosassay (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 fumonisins 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 FB1 analysis. Samples were 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 nährstoffarmer 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’-CTTCCGTGCGATGTTCCTC-3’ and VER2 5’-AATTGCGATTTATATATCTA-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.

**Fumonisins B1 analysis**

A HPLC-MS method based on protocols by Royer et al. (2004) was used for FB1 determination. The limit of detection for FB1 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 fumonisins-free maize extract with standard solutions in concentrations corresponding to fumonisins 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 B1 analysis

<table>
<thead>
<tr>
<th>Maize number</th>
<th>Colour</th>
<th>State</th>
<th>Fumonisin B1 [µg/kg]</th>
<th><em>F. verticillioides</em> DNA [µg/kg]</th>
<th><em>F. verticillioides</em> Agar Plate [% inc]</th>
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</table>

* 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 B1

<table>
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<tr>
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<th>FB1 [µg/kg]</th>
<th>DNA [µg/kg]</th>
<th>Agar Plate [% inc]</th>
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<td>39 (48.7%)</td>
<td>45 (56.2%)</td>
</tr>
<tr>
<td>Negative</td>
<td>25 (31.3%)</td>
<td>41 (51.2%)</td>
<td>35 (43.2%)</td>
</tr>
<tr>
<td>Range</td>
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<td>10–126.7</td>
<td>6.7–60</td>
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<tr>
<td>Mean*</td>
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<tr>
<td>Median*</td>
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</tr>
<tr>
<td>Standard Error</td>
<td>18.3</td>
<td>3.4</td>
<td>2.1</td>
</tr>
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</table>

* 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 FB1 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...
Among 55 samples that showed detectable levels of FB₁, 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 FB₁ 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 FB₁ and *F. verticillioides* DNA differed significantly among the white and yellow maize at 95% confidence level. Yellow maize showed more FB₁ (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 DNA and fumonisin B₁.

<table>
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<th>DNA [µg/kg]</th>
<th>Agar Plate [% incidence]</th>
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<td>55 (68.7%)</td>
<td>39 (48.7%)</td>
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<td>Negative</td>
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<tr>
<td>Standard Error</td>
<td>18.3</td>
<td>3.4</td>
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</table>

* mean and median calculation were based on positive samples

There was no significant difference in the mean FB₁ for collection dates (May, June and July) and the 4 states. Although, FB₁ content was highest for July and least in June collections. The FB₁ 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. verticilliodes* 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 obvoid 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 different collection periods, practices and storage conditions (Viquez et al. 1996). 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 B₁ 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 fumonisins 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 B1 WYTWARZANEGO PRZEZ FUSARIUM VERTICILLIOIDES W NIGERYJSKIEJ KUKURYDZY**

Badano 80 prób nasion kukurydzy zebranych w Nigerii na zawartość fumonisinu B1 (FB1) i zasiedlenie ich przez Fusarium verticillioides. Określono zawartość DNA wykorzystując specyficzną dla gatunku metodę real-time PCR i obliczono żywotne elementy rozmnożenia grzyba metodą płytkową. FB1 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 Fusarium verticillioides określona metodą rzeczywistego czasu PCR wynosiła 49,7 µg/kg (zakres: 10–126,7 µg/kg), podczas gdy metoda płytkowa ujawniała obecność F. verticillioides w 45 próbach (średniej występowanie: 21,0%, zakres 6,7–60,0%). Wystąpiły po-wiązania korelacyjne między DNA F. verticillioides ujawnione przez real-time PCR i zasiedleniem w przypadku metody płytkową, (R = 0,71, p = 00001 przy poziomie ufności 95%), a średnie dla FB1 oraz DNA F. verticillioides w żółtej i białej kukurydzy były istotnie różne. Pomimo wysokiej konsumpcji kukurydzy w Nigerii ilość FB1 spożywane przez konsumentów wydaje się niska. Szacunkowe dzienne spożycie fumonisinu wynosiło 0,21 µg/kg w stosunku do wagi ciała.