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Potential for Fumonisin Production by the Strains of *Gibberella fujikuroi* Species Complex Isolated from Maize Produced in Uganda

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Abstract: The study investigated the fumonisin production potential of 25 isolates of *Gibberella fujikuroi* species complex isolated from freshly harvested maize produced in Uganda. Twenty three out of the 25 isolates tested were able to produce fumonisins. The total fumonisin production varied between 19.4 and 99.8 mg kg⁻¹. Majority of the isolates were high fumonisin producers. The ability to produce fumonisins varied amongst the isolates tested. Strains identified as *Gibberella moniliformis* (GU257904.1) and *Gibberella fujikuroi* (EU979565.1) were generally higher fumonisin producers (39.9-99.8 mg kg⁻¹) compared to those identified as *Gibberella moniliformis* (FJI54074.1) (0-24.9 mg kg⁻¹). The order of fumonisin production was *G. moniliformis* > *G. fujikuroi* > *Fusarium proliferatum*. Seven strains (MRC 9059, MRC 9063, MRC 9054, MRC 9053, MRC 9067, MRC 9055 and MRC 9066) produced higher amounts of total fumonisins than the reference strain (MRC 826). Some unidentified species (MRC 9061, MRC 9051 and MRC 9064) also produced high fumonisins levels of 87.9, 84.3 and 58.4 mg kg⁻¹, respectively. The findings of this study indicated that the *G. fujikuroi* species associated with maize produced in Uganda are high fumonisin producers. These findings emphasize the need to put in place measures to control contamination of maize and maize based products with fumonisins.

Key words: Fumonisin, maize, *Gibberella fujikuroi*, *Fusarium*, *Gibberella moniliformis*

INTRODUCTION

Fumonisin are a large group of secondary toxic metabolites that are produced by several species of the *Gibberella fujikuroi* complex (Menmti *et al.*, 2010; Rocha *et al.*, 2009). They are natural contaminants of many economically important crops worldwide including wheat, sorghum, rice, date fruits and peanuts (Alwakeel and Nasser, 2011; Hinojo *et al.*, 2006; Isakeit *et al.*, 2008) but maize is the most vulnerable of all crops (Gamanya and Sibanda, 2001; Shephard *et al.*, 1996). Fumonisin have also been reported in ready to drink wine (Centeno and Calvo, 2002). Within the *Gibberella fujikuroi* complex, two species namely *Fusarium verticillioides* (Teleomorph, *Gibberella moniliformis* and *G. fujikuroi* mating population A) and *F. proliferatum* (Teleomorph, *Gibberella intermedia*, *G. fujikuroi* mating population D) have been reported as the most prolific producers of fumonisins (Jime'neza *et al.*, 2003; Marasas, 2001). A total of 28 fumonisin analogs have so far been elucidated but fumonisin B₁ (FB₁), B₂ (FB₂) and B₃ (FB₃) are the most abundant in naturally contaminated maize (Rheeder *et al.*, 2002).

Occurrence of fumonisins in food and feed is an increasing health problem worldwide (Reddy *et al.*, 2010)

affecting both animals and humans (Chukwuka *et al.*, 2010; Logrieco *et al.*, 2007). In animals, the effects of fumonisins may vary depending on the physiological, genetic and environmental factors (Akande *et al.*, 2006; Iheshiulor *et al.*, 2011). Dietary exposure to fumonisins causes leukoencephalomalacia in horses (Kellerman *et al.*, 1990), pulmonary oedema in pigs (Smith *et al.*, 1996) and liver cancer in experimental rats (Gelderblom *et al.*, 1991; Voss *et al.*, 1992). In humans, fumonisins are suspected to cause human esophageal cancer (Rheeder *et al.*, 1992; Yoshizawa *et al.*, 1994), liver cancer (Ueno *et al.*, 1997) and neural tube defects (Missmer *et al.*, 2006).

The amount of fumonisins produced varies amongst strains (Jurado *et al.*, 2010; Mirete *et al.*, 2003; Rocha *et al.*, 2011). It is categorised strains of *F. verticillioides* into low (traces to 49 µg g⁻¹), intermediate (50-500 µg g⁻¹) and high (>500 µg g⁻¹) FB₁ producers. The ability of a strain to synthesise fumonisins has been associated with the presence of a FUM gene cluster (Butchko *et al.*, 2003; Proctor *et al.*, 2003). However, fumonisin regulation at strain level seems to be a complex process governed by both environmental and genetic factors (Sagaram *et al.*, 2006). Factors like temperature, host, moisture content, pH and nutrients have been reported to influence the expression of FUM

genes and subsequent fumonisin biosynthesis (Picot *et al.*, 2010; Shim and Woloshuk, 2001).

Unlike the other fungal secondary metabolite gene clusters, the FUM gene contains no pathway-specific regulatory gene (Proctor *et al.*, 2003). As such, fumonisin biosynthesis is regulated by other genes such as FCC1, FCK1, PAC1 and GBP1 which are not linked to FUM cluster (Proctor *et al.*, 2003; Sagaram *et al.*, 2006). With the exception of FCC1, FCK1 no definitive epistatic relationship has been demonstrated between the rest of the regulatory genes, an indication that they operate independently (Sagaram *et al.*, 2006). The available information therefore suggests that a multiplicity of factors regulate the biosynthetic route which accounts for the variation of fumonisin levels produced by different strains (Sanchez-Rangel *et al.*, 2005).

In Uganda, ear rots caused by *Fusarium* sp. are a very big problem during maize production (Bigirwa *et al.*, 2006, 2007). The most recent study reported a high incidence of *G. fujikuroi* species in freshly harvested maize (Atukwase *et al.*, 2012). The authors further observed a wide morphological and genetic intraspecific variability amongst the strains isolated from freshly harvested maize. In a related study, all the samples of freshly harvested maize collected from six major maize producing districts in Uganda tested positive for fumonisins with average levels ranging between 0.2 and 10 mg kg⁻¹ (Atukwase *et al.*, 2009). These findings suggested that the *G. fujikuroi* species associated with maize produced in Uganda were potential high fumonisin producers. The major objective of this study was to establish the fumonisin production potential of *Gibberella fujikuroi* strains isolated from maize produced in Uganda. This is the first report concerning the fumonisin production potential of *Fusarium* strains isolated from maize produced in Uganda.

MATERIALS AND METHODS

Fungal isolates: Twenty five strains of *G. fujikuroi* species complex were included in this study. All the strains were isolated from maize obtained from six major maize producing districts in Uganda (Table 1). The isolates are held lyophilised at the culture collection of the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC), Medical Research Council (MRC), South Africa. Molecular analyses were conducted by matching the isolates to 9 strains from National Centre for Biotechnology Information (NCBI) database as follows: *G. moniliformis* (accession No. GU257904.10), 9 isolates; *G. moniliformis* (accession No. FJ154074.1), 4 isolates; *G. fujikuroi* (accession No. EU979565.1),

Table 1: *Gibberella fujikuroi* isolates from different districts tested for fumonisin production potential

Sequence based identity (NCBI best match)	Isolates	District of origin	
<i>G. moniliformis</i> (GU257904.1)	MRC 9048	Kasese	
	MRC 9052	Kapchorwa	
	MRC 9072	Mubende	
	MRC 9063	Masindi	
	MRC 9066	Masindi	
	MRC 9069	Masindi	
	MRC 9067	Masindi	
	MRC 9068	Masindi	
	MRC 9053	Kapchorwa	
	<i>G. moniliformis</i> (FJ154074.1)	MRC 9060	Sironko
		MRC 9070	Mubende
		MRC 9050	Kasese
	<i>G. fujikuroi</i> (EU979565.1)	MRC 9065	Masindi
		MRC 9054	Nakasongola
<i>F. proliferatum</i> (GU066714.1)	MRC 9055	Nakasongola	
	MRC 9059	Nakasongola	
	MRC 9073	Masindi	
	MRC 9062	Sironko	
<i>Fusarium</i> sp. (EF680754.1)	MRC 9071	Masindi	
	MRC 9064	Masindi	
	MRC 9058	Nakasongola	
<i>Fusarium</i> sp. (EF680759.1)	MRC 9049	Kasese	
	MRC 9057	Nakasongola	
<i>Fusarium</i> sp. (GQ141219.1)	MRC 9051	Kasese	
<i>Fusarium</i> sp. (EF680751.1)	MRC 9061	Sironko	

2 isolates; *F. proliferatum* (accession No. GU066714.1), 4 isolates; *Fusarium* sp. (accession No. EF680751.1), 4 isolates; *Fusarium* sp. (EF680759.1), 1 isolate; *Fusarium* sp. (accession No. GQ141219.1), 1 isolate and *Fusarium* sp. (accession No. EF680751.1), 1 isolate. A strain of *Fusarium pseudonygamai* which was isolated from Nakasongola and was closely related to some *G. moniliformis* species was also tested. In addition, we included *F. verticillioides* (MRC 826), a well known fumonisin producing strain isolated from maize produced in Transkei region, South Africa to act a reference (Fandohan *et al.*, 2005).

Fungal substrate (maize grains): Freshly harvested maize cobs (20 kg) of Longe IV variety was purchased from a farmer in Masindi district. The maize cobs were transported in perforated polythene bags to the Microbiology Laboratory at the Department of Food Technology and Nutrition, Makerere University and kept at -18°C until the time it was sterilised.

Establishment of toxin production potential of the isolates

Preparation of substrate (maize grains): Maize grains in lots of 250 g were cleaned, wrapped in three layers of aluminium foil and autoclaved at 121°C for 15 min. The sterile maize was rehydrated by direct addition of 40% sterile water to obtain water activity suitable for mould growth and fumonisin production (Llorens *et al.*, 2004). The grains were aseptically sealed in stomacher bags and

kept at 4°C for 2 days. The bags containing the maize were turned 6 times everyday to allow uniform absorption of moisture.

Preparation of inoculum: All the strains used in the study were sub-cultured by transferring a small portion of the sporulating mycelia to the centre of a petri plate (90 cm) containing Potato Dextrose Agar (PDA) (Difco, Detroit MI). The inoculated petri plates were incubated at 25°C for 7 days (Samapundo *et al.*, 2007).

Inoculation of maize with isolates: Approximately 25 g of rehydrated maize was aseptically weighed into sterile petri plates (90 cm) and properly spread to form a single layer. Using a sterile cork-borer, a 5 mm diameter agar disk was cut from the margin of the seven day old mycelia growing on PDA and transferred to the centre of the plate containing maize. The inoculated plates (triplicates) were incubated at 25°C for 7 days. Triplicate plates containing sterile maize but not inoculated with the fungal isolates were also incubated to serve as a control for determining the original levels of fumonisins in maize. A typical *F. verticillioides* (MRC 826) known to be a high fumonisin producer (Rheeder *et al.*, 2002) was incubated under similar conditions to act as a reference.

Total fumonisin quantification: Total fumonisin concentration of both inoculated and non-inoculated maize samples was determined using the Fluorometer method with slight modification to cater for high levels of fumonisins expected on maize cultures. The modification involved adjusting the amount of sample used for total fumonisins extraction. Instead of the 50 g of sample used in a typical Vicam method, this study used 5 g. This applied for both the cultured maize and the control. The contents (maize and mould) from two randomly selected plates for each strain were finely ground using a warring blender (HGBTWTS3, Torrington, USA). Five grams (5 g) of the ground sample was mixed with 5 g of sodium chloride and 100 mL of methanol/water (80: 20) solution. The mixture was blended at high speed for 1 min and filtered through a 24 cm fluted filter paper (VICAM, Watertown, USA). The extract (10 mL) was diluted with 40 mL of Phosphate Buffered Saline (PBS)/0.1% Tween-20 wash buffer and filtered through a 1.0 micro fibre filter paper and passed through an immunoaffinity column (VICAM, Watertown, USA). The column was washed with 10 mL of PBS/0.1% Tween-20 wash buffer followed by 10 mL of PBS. Fumonisins were eluted from the column using 1 mL HPLC grade methanol and collected in a cuvette. A mixture (1 mL) of developer A and developer B were added to the elute in a cuvette and placed in the Fluorometer (VICAM, Watertown, USA)

for fumonisin quantification in parts per million (ppm). All the solvents and reagents used were purchased from Microsep (Pty) Ltd. (Bramley, South Africa). Amount of total fumonisins produced by the isolate growing on maize cultures was calculated as follows:

$$\text{Total fumonisins (mg kg}^{-1}\text{)} = (\text{B}-\text{A})\times\text{C}$$

Where:

B: Total fumonisin in inoculated maize

A: Total fumonisins in non-inoculated maize (control)

C: Extraction sample reduction factor (10)

Statistical analyses: The fumonisin production data was analysed using SPSS for windows version 15 (SPSS, Chicago IL). One sample t-test ($p < 0.05$) was used to compare the mean total fumonisin production between the test strains and the reference strain (MRC 826).

RESULTS

The results of the fumonisin production potential of the isolates tested in the study are presented in Fig. 1. Twenty three out of the 25 isolates tested were able to produce fumonisins on maize cultures. The total fumonisin production varied between 19.4 and 99.8 mg kg⁻¹. In comparison with the reference strain (MRC 826), majority (64%) of the strains tested were found to be high fumonisin producers. A one sample t-test indicated that of the 23 strains that produced fumonisins, only 9 produced significantly lower ($p < 0.05$) fumonisins than the reference strain (MRC 826). Under the culture conditions used in this study, strain MRC 9066 produced the highest amount of total fumonisins (99.8 mg kg⁻¹) whereas MRC 9057 produced the lowest (19.4 mg kg⁻¹). Although, not significantly different ($p > 0.05$), seven strains (MRC 9059, MRC 9063, MRC 9054, MRC 9053, MRC 9067, MRC 9055 and MRC 9066) produced higher amounts of total fumonisins (93.20-99.8 mg kg⁻¹) than the reference strain (90.4 mg kg⁻¹).

In terms of intraspecific variability, the results in Fig. 1 indicate that isolates identified as *G. moniliformis* (GU257904.1) were higher fumonisin producers than those identified as *G. moniliformis* (FJI54074.1). Amongst the *G. moniliformis* (GU257904.1) isolates, the lowest fumonisin producer was MRC 9048 (39.9 mg kg⁻¹) whereas, the highest was MRC 9066 (99.8 mg kg⁻¹). In contrast, the highest fumonisin producer amongst *G. moniliformis* (FJI54074.1) was MRC 9060 (24.9 mg kg⁻¹) whereas, MRC 9050 and MRC 9065 did not produce any detectable amounts of fumonisins. Both isolates of

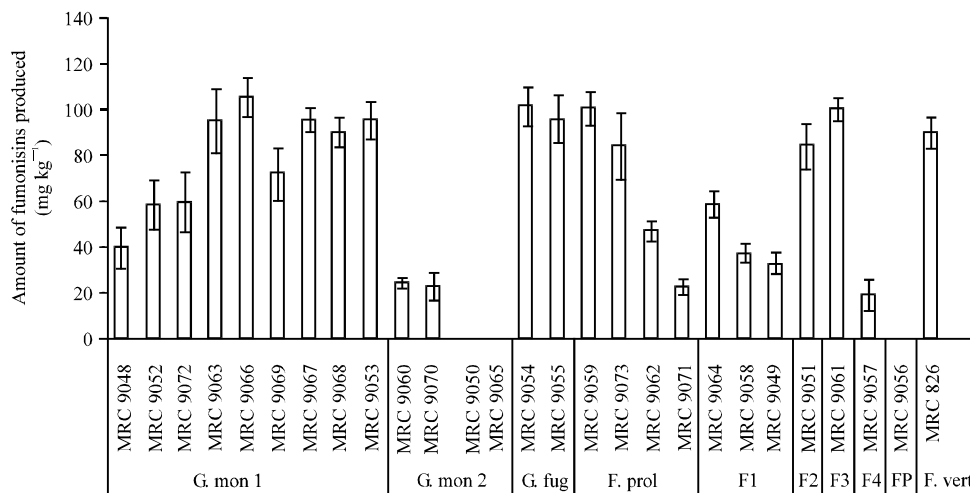


Fig. 1: Fumonisin production potential of *G. fujikuroi* strains isolated from maize produced in Uganda. G. mon1: *Gibberella moniliformis* (GU257904.1), G. mon2: *Gibberella moniliformis* (FJ154074.1), G. fug: *Gibberella fujikuroi* (EU9779565.1), F. prol: *Fusarium proliferatum* (GU066714.1), F1: *Fusarium* sp. (EF680754.1), F2: *Fusarium* sp. (GQ141219.1), F3: *Fusarium* sp. (EF680751.1), F4: *Fusarium* sp. (EF680759.1), FP: *Fusarium pseudonygamai*, F. vert: *F. verticillioides* (Reference strain)

G. fujikuroi (EU9779565.1) produced higher levels of fumonisins (above 95 mg kg⁻¹) than the reference strain. Similar intraspecific variability was observed amongst *F. proliferatum* (GU066714.1) isolates. MRC 9059 produced the highest amount (93.20 mg kg⁻¹) of total fumonisins and MRC 9072 produced the least amount (22.9 mg kg⁻¹).

Fumonisin production capacity also seemed to vary within the species. Generally, isolates of *F. verticillioides* (*G. moniliformis*) were higher fumonisin producers than those of *F. proliferatum*. Important to note is that some unidentified species, MRC 9061, MRC 9051 and MRC 9064 produced relatively high fumonisin levels of 87.9, 84.3 and 58.4 mg kg⁻¹ respectively. *F. pseudonygamai* did not produce detectable amount of fumonisins.

DISCUSSION

Results of this study confirmed the presence of high fumonisin producing strains in maize produced in Uganda. Under the culture conditions used in this study, the fumonisin production profile of majority of the isolates was similar to that of *F. verticillioides* (MRC 826) which is one of the most prolific fumonisin producing strains (Fandohan *et al.*, 2005; Rheeder *et al.*, 2002). The findings are in agreement with results of the previous study (Atukwase *et al.*, 2009) which reported that fumonisin levels in maize produced Uganda were above the 4 mg kg⁻¹ recommended by the Food and Drug

Administration (FDA) for unprocessed maize destined for human consumption (FDA, 2001).

The study observed wide intraspecific and interspecific variability in the fumonisin production capacity of the isolates. Whereas, majority of the isolates produced fumonisins, the levels produced varied from strain to strain and from species to species. Similar findings have been reported by other researchers in Brazil (Rocha *et al.*, 2011), Mexico (Sanchez-Rangel *et al.*, 2005) and Benin (Fandohan *et al.*, 2005). The variation in fumonisin production potential has been attributed to genetic differences within the strains (Sagaram *et al.*, 2006). A number of studies have reported a positive relationship between fumonisin production and the expression of FUM1 genes (Jurado *et al.*, 2010; Lopez-Erassquin *et al.*, 2007; Rocha *et al.*, 2011). In contrast, other studies have reported isolates which contained FUM1 genes but did not produce fumonisins *in-vitro* (Sanchez-Rangel *et al.*, 2005). To date, it has been established that there are a number of fumonisin regulating genes that do not reside in the FUM cluster (Sagaram *et al.*, 2006). Such genes include FCC1 which codes for a cyclin-like protein that regulates fumonisin production and fungal conidiation (Shim and Woloshuk, 2001); PAC1, which appears to be a transcriptional repressor of fumonisin biosynthetic genes (Flaherty *et al.*, 2003) and ZFR1, which has been characterized as a regulators of secondary metabolism in other fungi (Flaherty and Woloshuk, 2004). Thus, the

presence/absence of these genes and their effect on the expression of the FUM genes could be responsible for the variation in fumonisin production potential of an individual strain.

Results from this study indicated that the fumonisin production potential of some *F. proliferatum* isolates was not significantly different from that of the reference strain. Isolates of *F. proliferatum* capable of producing high amounts of fumonisins have also been reported by Rocha *et al.* (2011) and Jurado *et al.* (2010). The findings of this study present a challenge with regard to the safety of maize produced in Uganda given the fact that both *F. verticillioides* and *F. proliferatum* were the most frequently isolated species in freshly harvested maize (Atukwase *et al.*, 2012).

CONCLUSION

Strains of *Gibberella fujikuroi* complex associated with maize produced in Uganda are high fumonisin producers. The current findings together with previous reports on fumonisin contamination in freshly harvested maize indicate that fumonisins are important mycotoxins in Uganda. This emphasizes the need to put in place measures to control contamination of maize and maize based products with fumonisins.

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