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Variation in *in vitro* Fumonisin B_1 Production by Different *Fusarium verticillioides* Isolates in Kenya

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Abstract: Several *Fusarium verticillioides* isolates from different maize growing regions in Kenya were isolated and evaluated for their ability to produce fumonisin B_1 (FB_1). The toxin was quantified using a directly competitive ELISA method. There were differences in the ability of the various isolates to produce FB_1. Six isolates of *F. verticillioides* from every region produced varying amounts of FB_1 *in vitro*. The overall mean FB_1 level in positive isolates was 1513.3 μg kg^{-1}. Of all the isolates used in the study only 26% did not produce detectable levels of FB_1, whereas 74% produced varying amounts of FB_1 between 69 to >5000 μg kg^{-1}. Within every given region there was variation in the ability *F. verticillioides* isolates to produce FB_1. Isolates from Malava, Tongaren and Kakamega showed great variation among themselves. All isolates from Kitale, Tongaren, Kakamega and Embu produced detectable levels of FB_1. This data puts to rest the speculation that *F. verticilliodes* isolates from Kenya may be low FB_1 producers. Kenya and most countries that allow free movement of maize need to reconsider their free domestic movement policy in order to avoid introduction of prolific isolates in otherwise 'pest free' areas.

Key words: *Fusarium verticillioides* · Ear rot · Maize · Mycotoxins · ELISA · Kenya

INTRODUCTION

Maize (*Zea mays* L.) is a staple food crop in Kenya. The crop is grown on about 1.4 million hectares that yield an estimated 28 tonnes annually [1]. Despite increasing maize demand in Kenya, its production is on the decline due to drought, low soil fertility, pests and diseases [1-4]. One of the main concerns is maize ear rot disease which not only causes rotting of maize but also contaminate it with mycotoxins. The major and dominant ear rot fungi in Kenyan maize is *Fusarium verticillioides* [2,5,6]. The fungi is the most important producer of fumonisins, a group of mycotoxins that have the ability to cause equine leukoencephalomalacia, pulmonary edema, human esophageal cancer and rat liver cancer [7-9]. In Kenya, mycotoxicoses recurs in certain areas indicating some existence of a conserved factor (s) to such areas. Given that most maize varietes are widely planted and their seed are supplied by the same companies we thought that the role of Agro-ecological zone on the ability of isolates to produce FB_1 should be investigated. The purpose of this study was therefore to determine if difference (s) exists in the FB_1 production ability of various *F. verticillioides* isolates from different agro-ecological zones in Kenya.

MATERIALS AND METHODS

Sample Collection and Preparation: Maize samples were collected from Kakamega, Shikoti, Tongareni, Kitale, Embu, Kitui and Malava. A half a kilogram of maize sub-samples was collected from ten farmers from each site and constituted to form a sample. They were then transported in cotton bags to prevent moisture migration and heating. The moisture content was determined using a moisture meter and where it fell above 13%, the samples were dried till moisture content reduced to between 11-13%. Moisture content below 13% prevents growth of saprophytic fungi and hence maintains the integrity of the sample. The sample from each region was divided into three portions equally using a Pascal's Cascade Rotary Divider (Model 1) with a medium cone cap. A third of the sample was kept at KARI-Kakamega laboratory and another third was kept at Kenyatta University as a reference sample the third was used for isolation of the different *F. verticillioides* isolates *at* Kenyatta University research laboratory.

Isolation and Identification of *F. verticillioides*: The *F. verticilliodes* isolation was on PCNB agar medium that

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is a selective medium for isolation of *Fusarium* species. An isolation method by Castella *et al*. [10] was used. It involves dipping seeds in 70% ethanol then surface sterilizing in 1% sodium hypochloride for two minutes before rinsing twice in sterile distilled water and drying between sterile filter papers. Five seeds were then plated on PCNB media in triplicates and incubated at 25°C for five days. The colonies of observed fungal growth were sub-cultured on agar agar media till a pure culture of suspected *F. verticillioides* isolates were obtained. It is important to carry out sub culturing on agar than other rich media to avoid loss of FB_1 producing abilities of the isolates [11]. The *F. verticillioides* suspected colonies were then sub-cultured on Sucrose Nutrient agar placed under Non Ultra Violet light of alternating 12 hours of light and darkness for seven days. Pink coloured cultures were then selected. The cultures were viewed by cutting 1 cm^2 of SNA with the fungal colony and mounting directly on the slide with a drop of water and cover slip (SNA is transparent) then confirmed according to Booth, [12] and Nelson *et al.* [13]. After confirmation single conidial isolation was done onto slants for every *F. verticillioides* isolates followed by storage at 20°C to await FB_1 production evaluation.

Evaluation of FB_1 Production Ability by Different *F. verticillioides* Isolates: Each *F. verticilliodes* isolate was cultured on 2 plates of Potato Dextrose agar 25°C for 10 days. Conidia of different *F. verticilliodes* isolates were suspended in sterile water and 50 ml of 10^7 spores/ml inoculated on moistened corn (200 g of kernels and 200 ml of sterile water) in half litre conical glass jars previously autoclaved at 121°C for one hour on each of the two consecutive days to ensure they were sterile and could not harbor ear rot fungi. Cultures were then incubated in the dark with shaking in process for 28 days at 25°C. The cultures were then dried at 45°C for 72 hours. The dry sample was ground fine using a coffee blender with ethanol cleaning between samples and stored at 0°C until analysis. Each one of the maize cultures was then assayed for FB_1 by Enzyme Linked Immunosorbent Assay (ELISA) on microtiter plates. The FB_1 standard toxins were purchased from Sigma Chemicals USA. Preparation of toxin-Horseradish Peroxidase conjugate was by periodate method. Summarily the immunogen was coupled to keyhole limpet hemocyanin via glutaoaraldehyde reaction and coated to micro titer plates where toxin levels were determined based on absorbance at 450 nm as described by Usleber, *et al.* [14] and Gathumbi *et al.* [15]. Each sample was analysed in duplicate and the average calculated. The detection limits for the toxin was 10 ug kg^{-1} to 5000 ug kg^{-1}.

RESULTS

Variation in the ability of *F. verticillioides* isolates to produce FB_1 was quite high and is summarized in Table 1. The six isolates from every region produced varying amounts of FB_1 *in vitro*. For Kitale FB_1 level ranged from 89 μg kg^{-1} to 878.5 μg kg^{-1} with a mean of 406 μg kg^{-1}. One of the isolates from Tongaren did not produce detectable levels of FB_1 whereas the positive samples produced FB_1 in the range of 140.5 μg kg^{-1} and 4423 μg kg^{-1}. The mean level of FB_1 was 1154.7 μg kg^{-1}. All isolates from Kakamega produced detectable levels of FB_1 ranging from 116.5 μ kg^{-1} to 3606 μg kg^{-1}. The mean FB_1 level was 962.8 μg kg^{-1}. Three isolates from Kitui did not produce detectable FB_1 amounts. Those that produced ranged between 69.0 μg kg^{-1} and 754.5 μg kg^{-1}. The mean FB_1 levels for Kitui isolates was 357.5 μg kg^{-1}. Two Malava isolates did not produce detectable levels of $FB_1$where as the lowest FB_1 level was 147.0 μg kg^{-1} and the highest was greater than 5000 μg kg^{-1}. The mean FB_1 level in positive isolates was 1513.3 μg kg^{-1}. All Embu isolates were positive with a range of 116.5 to 1014.5 μg kg^{-1}. Only one isolate from Shikoti produced FB_1 of 124.5 μg kg^{-1}. Of all the isolates used in the study, only 26% did not produce detectable levels of FB_1 whereas 74% produced varying amounts of FB_1. Within a given region variation in the ability of FB_1 was evident especially for Malava, Tongaren and Kakamega isolates (Table 1). All isolates from Kitale, Tongaren, Kakamega and Embu produced detectable levels of FB_1.

DISCUSSION

Mycotoxins are fungal metabolites capable of having acute toxic,carcinogenic, mutagenic, teratogenic, immuno-toxic and oestrogenic effects to man and animals [10]. Common mycotoxins in maize include aflatoxins, fumonisins, moniliformin, deoxynivalenol and zearalenone. *Fusarium verticillioides* is the leading fungal species in producing fumonisins that have been shown to cause equine leukoencephalomalacia, pulmonary edema, human esophageal cancer and rat liver cancer [16]. The data on FB_1 production by *Fusarium verticilliodes* isolates from Kenyan maize revealed wide spread producibility of the toxin by this domestic isolates. This was comparable to those reported by Castelia *et al.* [10], Leslie *et al*.[17] and Platter *et al.*[18] but deviated by being higher than those recorded by Lee *et al.*[14]. Results from this study refute earlier claims that *Fusarium verticilliodes* isolates from Kenya may be low FB_1 producers Kedera *et al.* [20]. In some cases large differences in FB_1 production was observed among isolates recovered from the same region

Table 1: Fumonisin B_1 levels produced by different *F. verticillioides* isolates

	Source of maize used to isolate *F. verticillioides* and amount of FB_1 produced						
Isolate No.	Kitale	Tongaren	Kakamega	Kitui	Malava	Embu	Shikoti
C001	754.5	336.0	3606.0	754.5	ND	ND	ND
C002	143.0	ND	761.5	ND	ND	ND	ND
C003	301.0	140.5	1014.5	248.5	>5000	>5000	124.5
C004	272.5	165.0	116.5	69.0	147.0	147.0	ND
C005	878.5	4423.0	154.5	ND	709.0	709.0	ND
C006	89.0	709.0	123.5	ND	197.0	197.0	ND
Mean	406.4	1154.7	962.8	357.3	1513.3	1513.3	124.5

[1]Numbers are in μg kg^{-1}. [2]Mean is average for FB_1 regions positive isolates. [3]ND means FB1 absent or level below 10 μg kg^{-1}

showing that studies aiming to test a single *Fusarium verticilliodes* isolate per maize sample or any other unit may be misleading because the isolate that may be implicated to produce the FB_1 level in the sample or responsible for a disease due to consumption of a maize sample may not be the one isolated. The study has also established that different *Fusarium verticilliodes* isolates are capable of producing significant quantities of FB_1 and this isolates are not limited/confined to a given region of the country however the ability of these isolates to produce FB_1 in the field need to be investigated further with emphasis on the diversity of the climates in the maize growing areas of Kenya with the aim of putting in place measures that can prevent introduction of prolific strains to areas that don't have them. Based on this FB_1 levels in *in vitro* it is obvious that regardless of the mechanism of managing ear rot applied, *Fusarium* resistance in maize gotten through resistance breeding seems to be a worth option in reducing FB_1 hazard in Kenya. Alternatively, genetic engineering of maize with antifungal genes targeting ear rot fungi may be promising.

In sub Saharan Africa the diet is mainly maize based with a 400 g average daily intake per person. In Europe the daily intake is 10 g per person [12]. Though difficult to estimate we think the toxigenic isolates can be able to produce FB1 beyond levels acceptable by regulatory bodies worldwide. In Asia maximum acceptable limit for FB1 range between 5 to 35 μg kg^{-1}, Latin America ranges between 2 to 35 μg kg^{-1} where as North America ranges between 0 and 5 μg kg^{-1} [16]. The detection limitations of our protocol could not establish if the 11 that tested negative isolates produced any amount of the toxin (between 0 and 10 μg kg^{-1}) were above the threshold set for feed and food by most regulatory organizations. Mycotoxin screening methods like ELISA used in this study make it possible to screen large samples compared to other methods like HPLC and TLC which need heavy investment in reagents, equipment and training of staff.

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