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Modulation of Aflatoxin Production by Interaction of *Aspergillus* Species from Eastern Kenya

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Abstract: *Aflatoxin contamination of grain has continued to pose a significant threat to sustainable food security and trade worldwide. In the field, there are incidences of varying contamination levels in grain within the same niche. We hypothesize that the variation could be due to fungal species interaction at the kernel level. Seventeen isolates (14 Aspergillus flavus and 3 Aspergillus parasiticus) from Eastern Kenya were selected and confirmed for toxigenicity using Dichlorvos-Ammonia method, then cultured based on their isolation frequencies and co-existence in nature. The fungi were co-cultured using maize kernels as growth substrate, which was then used to estimate aflatoxin produced in a competitive ELISA. A one-sample two-tailed t-test was carried out to determine the degree of significance in aflatoxin production. Eight isolates were non-toxigenic, while nine were toxigenic. When co-cultured with some non-toxigenic isolates such as A. parasiticus (2EM0601), the most toxigenic A. flavus isolate (1EM1901) significantly increased aflatoxin production, while it reduced with others. These observations warrant investigation on the interaction of Aspergillus species in culture especially given their diverse toxigenic potential. We concluded that colony-mediated aflatoxin production could explain the variations of toxin levels observed in freshly collected field samples.*

Keywords: *Toxigenicity; aflatoxins; fungal interactions; Aspergillus; Dichlorvos-Ammonia*

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I. Introduction

Aflatoxins are potent hepatotoxic and carcinogenic metabolites produced by fungi of the genus *Aspergillus.* The important members of this group include *A. flavus* and *A. parasiticus,* which contaminate various oil-rich seeds including maize (*Zea mays*), groundnuts (*Arachis hypogaea*) and cotton (*Gossypium hirsutum*) [1]. The consumption of food with high levels of aflatoxins is detrimental to both human and animal health leading to acute and chronic aflatoxicosis. Kenya is now ranked high among countries with recurrent and severe episodes of outbreaks of aflatoxicosis. The most severe case was reported in the Eastern part of the country, where it resulted in 125 fatalities in 2004 [2]. Subsequent surveys over three years found that aflatoxin levels were lethal and as high as 38,000 ppb [3].

The perennial high levels of aflatoxins recorded in Kenya have aroused research efforts towards their management [4]. Biological control using non-toxigenic *Aspergillus flavus* to compete against toxigenic species has shown great potential in cotton [5]. The non-toxigenic *A. flavus* has subsequently been formulated into different commercial products AflaSafe™ and Aflaguard® for use in peanut and maize production in Africa [6, 7]. The current efforts are geared towards the development of biological control agents for the Kenyan environment. However, and an important prerequisite is to understand the nature of interactions among different strains of *A. flavus* and *A. parasiticus*. It has been recorded that aflatoxigenesis is dependent on various factors including temperature, moisture, pressure, and competition by colonies [8, 9].

The current class of biocontrol agents being deployed work based on 'competitive exclusion' where one strain out-competes another for nutrient acquisition, reproduction, and space [10]. We hypothesize that the interaction between strains in section *Flavi* is mediated by contact between hyphae of the interacting species and possibly other unknown mechanisms. Such interactions influence aflatoxin synthesis either positively or negatively. In the selection of candidate strains for biocontrol, it is imperative to decipher the dynamics of species interaction and its effect on toxigenicity. The current study sought to establish the impact of interactions between the congeneric species of *Aspergillus* (*A. flavus* and *A. parasiticus*) previously isolated from a common niche [11].

II. Materials and Methods

2.1. Fungi Isolates Culture

The fungi used in this study were previously isolated from maize from Eastern Kenya. The samples were isolated from the same maize kernel samples, and this information was used to determine their co-existence [11]. Seventeen isolates were retrieved from storage and sub-cultured on Czapek Dox Agar medium (HiMedia Laboratories Pvt. Ltd).

The samples were then incubated at 28°C for five days. Using the morphological and cultural characteristics [12], the identity of the isolates was confirmed as *A. flavus* (1EM1901, 1EM1201, 1EM2606, 1EM2901, 1EM4501, 1EM4501, 1EM4503, 2EM0501, 2EM0502, 2EM0602, 2EM1201, 2EM3503, 2EM3506, and 2EM6103) and *A. parasiticus* (1EM2902, 2EM0402, and 2EM0601) as described by Salano et al. [11]. The identification code was retained as they had been first isolated [11]. Subsequently, the fungal strains were sub-cultured on Yeast Extract Sucrose medium (mixed with 50mg/L streptomycin sulfate and penicillin, Zhonghuo Pharmaceuticals, China) and maintained for seven days at 28°C. This was done by inoculating the isolates at two points (same isolate) of the Petri dishes (90 × 15mm (Aptaca™, Italy)) to form dual colonies (Fig. 1).

2.2 Dichlorvos-Ammonia Method for Testing Toxigenicity

For the DV-AM method, the fully grown isolates initially cultured on the Czapek Dox Agar medium were sub-cultured on aflatoxin-inducing Yeast Extract Sucrose (YES) medium (amended with 50mg/L streptomycin sulfate and penicillin, Zhonghuo Pharmaceuticals, China) and maintained for seven days at 28°C. The culturing and testing for toxigenicity of the isolates on the aflatoxin-inducing YES media was sequentially done following the protocols for the DV-AM method, as described by Kushiro et al. [13]. The method briefly involved: (i) Dichlorvos (Amiran Kenya Ltd) was diluted with methanol in 250 fold ratio premixed with the media prior to solidification, (ii) the isolates were incubated in darkness at 28°C for 5 days, (iii) 0.2 mL of ammonium hydroxide solution was poured onto the inside of the lid of the petri dish plate used to set up the DV-AM method, (iv) the isolates were categorized as either toxigenic or non-toxigenic depending on their ability to produce aflatoxins (shown by a characteristic red coloration at the underside of the plates), and (v) the plate images of the toxigenic isolates were further analyzed with ImageJ 1.x software for enhanced detection [14] (Fig. 3)

2.3 Competition Studies

One *A. flavus* toxigenic isolate (1EM1901) was considered for combinatory studies with its randomly selected congeneric isolates based on their coexistence (occurrence in the field). The isolates combined with 1EM1901 were: 1EM1201, 1EM2606, 1EM2901, 1EM1EM4501, 1EM4501, 1EM4503, 2EM0402 2EM0501, 2EM0502, 2EM0601, 2EM0602, 2EM1201, 2EM3503, 2EM3506, and 2EM6103. Aside from the isolates used in combinations, three other non-toxigenic species; *A. terreus, A. flavus,* and *A. niger* also used to study the mechanisms of interactions between species of *Aspergillus* (Fig. 3C and 4). The three isolates were, however, excluded in combinatory studies with the toxigenic 1EM1901 isolate. The reason for was that the three isolates were intended to show the mechanisms of interaction of *Aspergillus* isolates but not the ability to modulate aflatoxin production of the aggressive fungi.

Maize kernels were used as a medium for fungal growth to simulate the natural infestation of *Aspergillus* species in the field. Maize kernels were surface sterilized with 25 % (v/v) sodium hypochlorite (NaOCl) for 15 minutes, then rinsed with sterile distilled water. Subsequently, the maize kernels were treated with ammonium hydroxide (2.0% NH_4OH) treatment for 15 minutes and rinsed with sterile distilled water. The maize kernels were then left overnight inside the laminar flow with the ultraviolet light on. The maize kernels were then loaded into sterile 25 mL sample bottles (Rudolph Research Analytical). Each of the non-toxigenic isolates was combined with the toxigenic species (1EM1901) at a concentration of 1×10^6 conidia/mL (Fig. 2). Sixteen isolates combinations were done, and each was replicated three times and cultured in independent sample bottles. Ammonia-treated maize samples with 1000µL of sterile water were used as a negative control in the study. All the inoculated maize samples and controls were incubated and maintained at 28°C for five days.

Incubation of the fungi using the bottles packed with maize as substrate offered a good simulation of the actual niche (temperature of 33 °C and relative humidity above 15%) for aflatoxin production [15]. These standard growth conditions minimized chances of variation in aflatoxin production as affected by macro and micro-climatic conditions [16].

2.4 Determination of Aflatoxin Levels

After the 5-day incubation of the maize samples and controls, the samples were pulverized using manual hand-grinder (Whatman® Inc.). Approximately 20g of the pulverized maize samples were then mixed with 100mL methanol (70% v/v) to extract aflatoxins. The mixtures were then filtered, and 10mL of the filtrate was aliquoted into 14-mL BD Falcon™ test tubes (BD Biosciences, Bedford, USA) and labeled accordingly.

Total aflatoxin ELISA analysis was then performed using the total competitive ELISA kit (Helica Biosystems Inc®, Santa Ana-USA) according to the manufacturer's instructions. The absorbance of aflatoxin was read at 450nm using a microplate reader (Mindray® Inc. Nanshan, Shenzhen, China) (Fig. 5).

2.5 Data Analyses

One sample two-tailed *t*-test was performed to determine whether both reduction and increase in aflatoxin levels were not due to chance using Statistical Package for Social Science (SPSS) version 20.0 (IBM Corp., Armonk, NY). The changes in aflatoxin levels were expressed as percentages with regards to the combination of non-toxigenic species with the toxigenic congeneric isolate (1EM1901).

III. Results

Of the 17 isolates tested, eight isolates *A. flavus* (1EM1201, 1EM2901, 2EM3503, 2EM0501, 2EM1201, 1EM2606) and *A. parasiticus* (2EM0601 and 1EM2902) were confirmed to be non-toxigenic strains by DV-AM method. Another eight isolates were toxigenic *A. flavus* (2EM0502, 1EM4503, 2EM0602, 1EM4501, 2EM6103, 2EM3506, 1EM4502) and one *A. parasiticus* (2EM0402) (Table 1.).

Table 1. Aflatoxin level changes for the toxigenic isolate 1EM1901 when co-cultured with non-toxigenic isolates

	Isolates	DV-AM Toxigenicity	Aflatoxin Concentrations (ppb)	Percentage Change in Aflatoxin Levels
1	1EM1901/2EM0402		3.08 ± 0.65	86.66***
	2EM0402 (*A. parasiticus*)	+	6.03 ± 0.56	48.92
2	1EM1901/1EM1201		20.50±2.02	11.35
	1EM1201 (*A. flavus*)	-	16.48±0.09	19.61
3	1EM1901/1EM2901		13.73 ± 0.81	40.63
	1EM2901 (*A. flavus*)	-	3.24 ± 0.49	76.40
4	1EM1901/2EM0601		24.61±0.26	-*6.4
	2EM0601 (*A. parasiticus*)	-	15.36±0.06	37.59
5	1EM1901/2EM0502		21.93±1.27	5.19
	2EM0502 (*A. flavus*)	+	10.35±0.48	52.80
6	1EM1901/2EM3503		21.61±1.04	6.57
	2EM3503 (*A. flavus*)	-	7.30±0.56	66.22
7	1EM1901/2EM0501		21.63±1.07	6.48
	2EM0501 (*A. flavus*)	-	7.27±0.56	66.39
8	1EM1901/1EM4503		19.60±0.67	15.23
	1EM4503 (*A. flavus*)	+	12.71±0.33	35.15
9	1EM1901/2EM0602		23.91±0.08	-*3.39
	2EM0602 (*A. flavus*)	+	5.49±0.55	77.04
10	1EM1901/2EM1201		17.91±0.31	22.54
	2EM1201 (*A. flavus*)	-	4.46±0.53	75.10
11	1EM1901/1EM2606		21.70±1.08	6.14
	1EM2606 (*A. flavus*)	-	7.42±0.56	66.81
12	1EM1901/1EM4501		7.44±0.81	67.91***
	1EM4501 (*A. flavus*)	+	4.97±0.05	33.20
13	1EM1901/2EM6103		23.90±0.06	-*3.35
	2EM6103 (*A. flavus*)	+	7.32±0.56	69.37
14	1EM1901/2EM3506		17.87±0.39	22.73
	2EM3506 (*A. flavus*)	+	5.92±0.56	66.87
15	1EM1901/1EM2902		16.65±0.11	27.99
	1EM2902 (*A. parasiticus*)	-	6.02±0.56	63.84
16	1EM1901/1EM4502		21.64±1.08	6.44
	1EM4502 (*A.flavus*)	+	10.35±0.48	52.17
17	Maize (Ammonia Untreated)		5.67±0.29	
18	Maize (Ammonia treated)		2.66±0.27	
19	1EM1901 (*A. flavus*)	+	23.12 ± 0.21**	Positive Control

The signs + and – indicate whether the species is toxigenic or non-toxigenic respectively based on DV-AM method.

- indicate the percentage increase by 1EM1901 when combined with non-toxigenic isolate and reduction in the toxin potential by non-toxigenic within the toxigenicity threshold respectively.*

****shows two toxigenic fungi that elicited significant aflatoxin reduction.*

*** shows the aggressive toxigenic fungus that was combined with its congeneric isolates.*

All the isolates used in combinatory studies were able to cause a change in aflatoxin production by the aggressive isolate 1EM1901 (*A. flavus*). The changes were either an increase or decrease in aflatoxin production. Precisely, thirteen of the isolates (1EM1201, 1EM2606, 1EM2901, 1EM2902, 1EM4501,

1EM4502, 1EM4503, 2EM0402 2EM0501, 2EM0502, 2EM1201, 2EM3503 and 2EM3506) were able to cause a reduction in aflatoxin levels below FDA allowable limits (20ppb for human consumption) against the aggressive toxigenic isolate (1EM1901). When cultured alone, the isolate 1EM1901 showed aflatoxin level of 23.12 ± 0.21 ppb. The changes observed was either an increase or decrease in aflatoxin production from the initial concentration of the isolate 1EM1901 (23.12 ± 0.21 ppb). However, only two toxigenic fungi *A. parasiticus* (2EM0402) and *A. flavus* (1EM4501) elicited a significant reduction in toxin production by the toxigenic isolate to levels below 10ppb; 3.08 ± 0.65 ppb and 7.44±0.81 respectively. Despite this reduction in aflatoxin level, it was observed that no non-toxigenic isolate was able to cause a reduction of aflatoxin production by the aggressive fungi below 10 ppb. Three isolates *A. parasicitus* 2EM0601 (non-toxigenic), *A. flavus* 2EM0602 (toxigenic) and *A. flavus* 2EM6103 (toxigenic) led to an increase in aflatoxin levels by the aggressive fungi (1EM1901). Of the eight toxigenic isolates, three caused an increase in toxin production when co-cultured with the toxigenic isolate 1EM1901, whereas five toxigenic isolates (1EM4502 and 2EM3506) caused a reduction in toxin production.

The maize samples used as culture substrate were treated with ammonia. Ammonia treatment led to aflatoxin reduction in maize from 5.67±0.29 ppb to 2.66±0.27 ppb. The initial aflatoxin concentration for maize before fungal inoculation was, therefore, 2.66±0.27 ppb. For the negative control, aflatoxin levels remained at 2.66±0.27 ppb after the incubation period.

One sample *t*-test was done to check whether there was a significant difference between the aflatoxin concentrations means for the 16 combined sets as compared to the aggressive fungi. Since the sample sizes are equal (N=16 for each group), and that the *p*-value of the Levene's Test for Equality of variances is greater than 0.05, the hypothesis was therefore accepted hence concluding that the variances of the population were equal. Equal variances assumed were determined using the SPSS.

The hypothesis that the means of the combined sets and non-toxigenic were not different failed to be accepted at α=0.05 (t_{15}= -3.011, p=0.009< 0.05). This means that there is a change in aflatoxin production when non-toxigenic isolates are combined with the toxigenic isolate (1EM1901). That is, the reduction in aflatoxin concentration is not just by chance but through the effects of non-toxigenic isolates on toxigenic isolate.

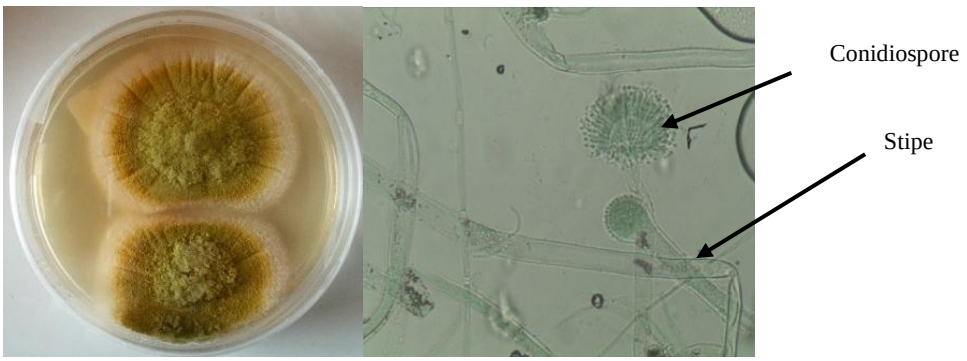

Fig. 1. Dual colonies of *A. flavus* on Czapek Dox Agar medium and the microscopic image of the conidiophores

Fig. 2. Untreated (A); and ammonia-treated maize (B); non-toxigenic isolate (2EM6103) with toxigenic isolate (1EM1901) (C)

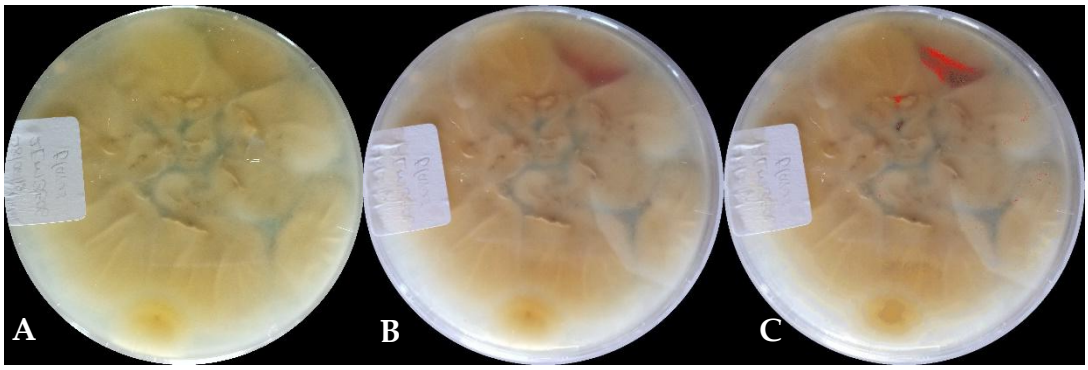

Fig. 3. (A) Dichlrovos treated culture before ammonia treatment (B) Dichlorvos fungal isolates after ammonia treatment (C) ImajeJ 1.x analyzed the image for the DV-AM positive fungal isolate

The three other fungi isolates (*A. flavus, A. terreus* and *A.niger,* (not included in the combination study) had been used to explain the possible mechanisms of fungal interactions in culture. A conspicuous inhibition of *A. flavus* by *A. terreus* was observed on culture plates hence explaining the possible mechanism for aflatoxin reduction (Fig. 4 A). A positive interaction was, however established when *A.flavus* and *A.niger* were grown together on PDA medium (Fig. 4 B).

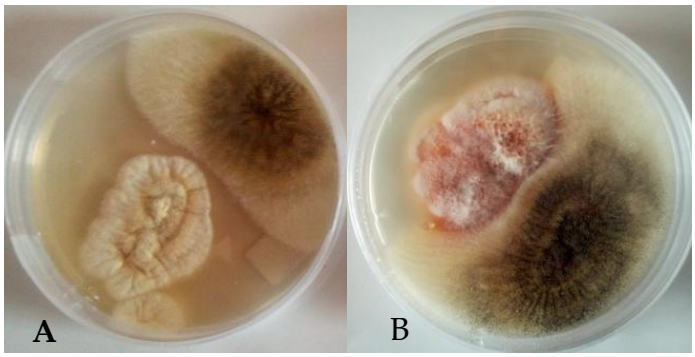

Fig. 4. (A) Inhibitory mechanism shown by *A. terreus* and *A. flavus*, and (B) positive interaction of *A.flavus* and *A. niger* (grown on PDA medium)

IV. Discussion

As has been reported, some non-toxigenic *Aspergillus* species can be stimulated to produce toxins when in contact with other species [17, 18]. There are earlier assertions that aflatoxin production by toxigenic isolates is only possible when non-toxigenic isolates outcompete their toxigenic congenerics [19-21]. In contrast, the current findings shed new insights into the inhibition of aflatoxin production. Two toxigenic isolates *A. parasiticus* (2EM0402) and *A. flavus* (1EM4501) caused a significant reduction in aflatoxin production when cultured with the aggressive toxigenic isolate *A. flavus* (1EM1901). The mechanisms behind this modulation are dependent on myriad factors that have not been entirely explained, and thus the reduction of aflatoxin production by toxigenic isolates is confounding. Nevertheless, there are attempts to explain the factors behind such observation among section *Flavi,* but opinion differs.

Huang et al. [8], demonstrated that aflatoxin production is thigmo-regulated between the colonies of different *Aspergillus* species. This observation is consistent with our study where the conidia of the isolates were premix and thus possible interaction of the hyphae during fungal growth. The combination of non-toxigenic isolates with the toxigenic ones caused an insignificant change in aflatoxin production with some increasing while others are decreasing. This could imply the involvement of other factors influencing aflatoxin production or even unique individual species factors. The current study confirmed that both positive and negative feedback interactions play a significant role in aflatoxin production. This is demonstrated by the interaction between *A. flavus* and non-toxigenic *A. niger* and *A. terreus* in the isolates confirmed the two modes of fungal interactions. Besides fungi-fungi interactions, the genetic variability of the species especially with the native strains collected in the Eastern part of Kenya might influence aflatoxin production [22]. This was recently demonstrated in variation in metabolic profiles among *A. flavus* isolates from Eastern Kenya. This variation was attributable to the unique genetic makeup of the species and the growth media [23]. In the current study, it is possible that the alteration of aflatoxin production could be due to chemotrophic interactions leading to a fusion between hyphae of mature colonies [8, 17]. The inoculation of the isolates conidia on maize as a growth substrate simulates

fungal growth in the field conditions. We hence affirm that interactions occurring between *Aspergillus* species in the field conditions play a part in inferring aflatoxin-producing capabilities to toxigenic fungi. Modulation of aflatoxin production is, however, a myriad of complex events that range from genetic properties of the isolates to their phenotypic characteristics that warrant investigation.

In terms of species-specific interaction and its effect on aflatoxin production by either *A. parasiticus* or *A. flavus*, there was no observable inhibition by either species. The two non-toxigenic species of section *Flavi* have equal potential in the modulation of aflatoxin production by toxigenic species. However, *A. flavus* species could be more effective at reducing the aflatoxin levels and hence effective biocontrol candidate [24]. In the current study, the species that were able to reduce the aflatoxin production significantly could be good biocontrol candidates, especially in the Kenyan environment. However, since not all of them were non-toxigenic and this observation warrants more attention to ascertain the behavior of toxigenic isolates under diverse environmental conditions. Given the ongoing climatic changes affecting the aflatoxin structural *aflD* and regulatory *aflR* genes, aflatoxin production by *A. flavus* is likely to be increased through elevated expression of the two genes [25, 26]. The current study offers intriguing insights into the possible effects of climatic conditions on the aflatoxin-producing capabilities when species interact in the field. The implication of this study thus presents a new dimension in search of environmentally compatible and genetically stable biocontrol candidates. Further studies need to be done to establish the genetic interactions of both toxigenic and non-toxigenic *Aspergillus* species in regards to modulation of aflatoxin production.

V. Conclusion

The premise of the current study is that the observed variations in aflatoxin production levels by fungi when cultured in the same niche. Their interaction affects aflatoxins production by individual fungi. This occurs through some unknown mechanisms attributed to colony-mediated aflatoxin production. These findings underscore the need for a broad survey before the introduction of biological control products.

Conflict of Interest

The authors declare that they have no competing interests.

Authors' Contributions

MKL: Performed the experiments, generated data, and drafted the manuscript; MAO and FJT: Designed the research topic, sourced funding, supervised the project and reviewed the manuscript; HMM: Provided fungal molecular expertise, supervised the project, analyzed the data and reviewed the manuscript. All authors approved the final draft.

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