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Resistance to aflatoxin contamination and genotypic colonization of *Aspergillus flavus* and *Aspergillus parasiticus* in groundnuts (*Arachis hypogaea*)

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Groundnut (*Arachis hypogaea*) is an important income-generating crop in sub-Saharan Africa. Biotic factors affecting production include fungal infections caused by *Aspergillus flavus* and *Aspergillus parasiticus* leading to aflatoxin contamination. Resistant cultivars can be a potential cost-effective strategy and a feasible option to small scale farmers. This study aimed to compare the colonizing effect of the *A. flavus* and *A. parasiticus* and to evaluate the genetic resistance of selected groundnut varieties to *A. flavus* and *parasiticus*. The study was conducted at Zambia Agriculture Research Institute, Chilanga, Zambia. Intact, mature, and undamaged kernels of Natal common, Chishango and MGV4 were infected with *A. flavus* and *A. parasiticus*. The experiment followed a layout of 3 (groundnut genotypes) x 2 (*Aspergillus* isolates [*A. flavus (S-strain)* and *A. parasiticus*]) factorial experiment arranged in a completely randomized design, with three replications. Aflatoxin levels ranged from 0.12 to 0.24 µg/ kg in all groundnut genotypes inoculated with *A. flavus* MGV4 was identified as the most resistant genotype exhibiting the lowest levels of aflatoxin content (0.12 µg/kg) (*P*<0.001). On the other hand, *A. parasiticus* was identified as a faster colonizing pathogen than *A. flavus* despite producing negligible amounts of aflatoxins in all evaluated groundnut genotypes.

Key words: Aspergillus species, aflatoxin levels, severity, incidence, seed infection.

INTRODUCTION

Groundnut is an important staple and income-generating crop particularly for women who traditionally manage the crop in sub- Saharan Africa. However, its productivity is affected by both biotic and abiotic factors. The main biotic

constraint to groundnut production is susceptibility to aflatoxin contamination (Boni et al., 2021), thereby posing a threat on food safety and security (Medina et al., 2015). The two fungi are an opportunistic pathogen with a wide host range including, corn, wheat, barley, rice, tree nuts, and cotton seeds (Khan et al., 2021; Elzupir et al., 2015).

In Zambia, groundnuts are grown in almost all agro ecological regions thus the second most widely cultivated crop after maize (Tembo and Sitko, 2013). However, studies in Zambia have shown cases of aflatoxin levels exceeding the acceptable international requirement levels for export greater than 10 µg kg $^{-1}$ aflatoxin levels (Kachapulula et al., 2017a; Mukanga et al., 2019). This has resulted in part being among the lowest exporters of groundnuts in the sub-Saharan region (Sitko et al., 2011) despite having favourable agro-ecological conditions for growing the crop (Mofya-Mukuka and Shipekesa, 2013). Environmental conditions such as high humidity, high temperature, heavy rains, and drought intensity cause mycological dispersion and increase aflatoxin production (Dias et al., 2014; Hamidou et al., 2014).

Aspergillus flavus and *Aspergillus parasiticus* produce aflatoxins as secondary metabolites that cause severe diseases in both animals and humans (Scheidegger and Payne, 2003). The effects of aflatoxin include reduced immune system response in humans, liver cancer in adults as well as stunted growth and cognitive developmental challenges in children (Okello et al., 2010). There are about 13 identified aflatoxin types, among these, AFB_1, AFB_2, AFG_1, and AFG_2 are the most harmful to humans and animals, usually found in feeds and foods (Hamidou et al., 2014). AFB1 has been classified as a Group 1 human carcinogen by the International Agency for Research on Cancer (IARC) (Ostry et al., 2017). The production of conidia and sclerotia in *Aspergillus* species in the soil enables them to survive through harsh weather conditions. These perennation structures that are produced in fungi together with the secondary metabolites are considered as an adaption mechanism to survival and contribute to the organism´s virulence index (Okoth et al., 2016). The direct contact of the groundnut pods with fungal mycelium in the soil provides the main entry for fungal invasion (Torres et al., 2014). While another suggested route of fungal infection is through flowers (Styer et al., 1983). When insects and nematodes damage the pods, this also contributes to seed infection and subsequent aflatoxin contamination (Timper et al., 2004).

A. flavus is grouped into two types according to the sclerotia size; S and L morphotypes. The S morphotype has abundant small sclerotia (<400 µm in diameter), with few conidia production and high aflatoxin levels. The L type produces few but large sclerotia (>400 µm in diameter), and abundant conidia with variable aflatoxin levels (Soni et al., 2020). However, because of lesions in the aflatoxin gene cluster, a few L strains do not produce

aflatoxins, hence referred to as atoxigenic (Chang et al., 2005; Adhikari et al., 2016; Khan et al, 2021).

Good management practices have been employed to reduce pre- and post-harvest contamination of aflatoxin but to a limited success (Torres et al., 2014). This is because aflatoxin contamination can occur both in the field and in storage. However, the use of resistant cultivars has being identified as a potential cost-effective strategy in reducing or mitigating aflatoxin contamination in groundnuts and it is feasible to be adapted by small scale farmers (Soni et al., 2020). It is, however, imperative that management action require the understanding of specific individual effect of these pathogens as these may differ according to environments. The specific objectives were therefore *i)* to compare the colonizing effect of the *A. flavus* and *A. parasiticus and ii)* to evaluate the genotypic resistance of selected groundnut varieties to *A. flavus* (S strain) and *A. parasiticus.*

MATERIALS AND METHODS

Study

The study was conducted at the Plant Pathology laboratory at Zambia Agriculture Research Institute, Mount Makulu Central Research Station in Chilanga, Zambia (15.550 °S and 28.183 °E).

Germplasm used for this study

Natal common, Chishango and MGV4 (Table 1) varieties were selected for the experiment based on their high adoption rate by the majority of the small-scale farmers in Zambia (Chirwa et al., 2015; Sally and de Klerk, 2012). Mature, undamaged seeds for each genotype were infected with *A. flavus* and *A. parasiticus*. The experiment was laid following a 3 (groundnut genotypes) x 2 (*Aspergillus* isolates [*A. flavus- S-strain* and *A. parasiticus*]) factorial experiment arranged in a completely randomized design (CRD), with three replications.

Isolation and culturing of *A. flavus* (S-strain) and *A. parasiticus*

Isolates of *A. flavus* (S-strain) and *A. parasiticus* were isolated from a soil sample. The suspension was made by adding 1 g of the soil sample to 10 ml of sterilized distilled water and mixing it thoroughly using a vortex mixer. The suspension was evenly plated on Modified Rose Bengal Agar for fungal isolation (Cotty, 1994) and incubated at 31°C for 3 days in the dark. After 3 days, the spores of colonies were then transferred and cultured on 5/2 growth medium (5% V8 Vegetable Juice; 2% Bacto agar, pH 5.2, 950 ml distilled water) (Cotty, 1989 cited in Kachapulula, 2017b) and incubated using Thermo Forma Series II Water Jacket CO_2 Incubator for another 7 at 30 ± 2 °C days for significant sporulation of isolates (Plate 1A and B). Macroscopic identification of all fungal isolates was done using taxonomic keys and descriptions in the standard manual of fungi (Samson et al., 2010). Afterwards each identified strain was carefully removed or washed off using 0.01% (v/v) Tween$^®$20 (P9416 Sigma-Aldrich) solution while rubbing with a sterilized spreader to make the spores suspension and used for

Table 1. Genotypes used in the study.

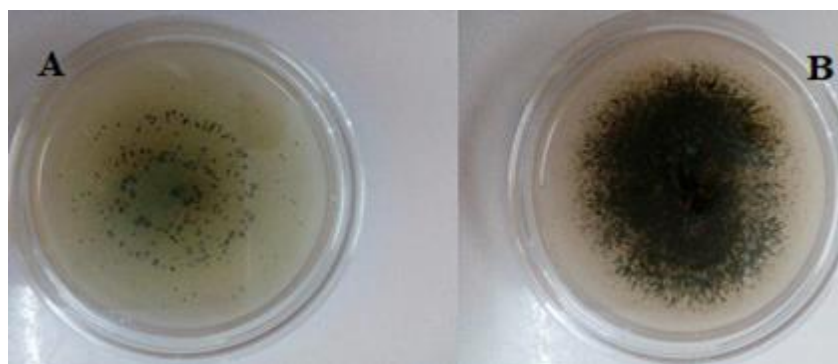| Genotype	Characteristics	Days to maturity	Variety type	Source
Natal common	Small seeded variety with tan kernels	90 - 100	Improved	ZARI*
Chishango	Medium seeded with tan-pink seed colour	120 - 130	Improved	ZARI
MGV4	Medium seeded variety with red kernels	130 - 140	Improved	ZARI

*Zambia Agriculture Research Institute.

Plate 1. A *A. flavus* (S-strain) colony exhibiting a yellowish green colour; **B:** *A. parasiticus,* exhibiting *a dark green colour.* Both isolates of *A. parasiticus* and *A. flavus* where cultured on 5/2 growth medium.

inoculation as done by Singh et al. (2016).

Constitute of modified Rose Bengal Agar (MRB)

Modified Rose Bengal (MRB) medium had the following ingredients per 1000 ml: 3.0 g sucrose, 3.0 g $NaNO_3$, 0.3 g KH_2PO_4, 0.7 g K_2HPO_4, 0.5 g $MgSO_4$ $7H_2O$, 0.5 g KCl, 10.0 g NaCl, 20 g Bacto agar, 50 mg Chloramphenicol, micronutrients, and the antibiotics (dichloran, streptomycin and rose bengal). We combined sucrose, inorganic salts, and micronutrients with 5 ml raised Bengal stock in an appropriate volume of deionized water and adjusted the pH to 6.5. The mixture was then dispensed into media bottles and agar was added and the mixture brought to a boil. At this point the solution was stirred until the agar was melted and the Chloramphenicol was added. After autoclaving for 15 min at 120 °C, the medium was cooled on a stir plate to between 50 and 60 °C. Then, dichloran (10 mg L^{-1}) and streptomycin (50mg L^{-1}) were added and after stirring for 5 to 10 min the medium was poured (15-20 ml per 100 mm plate).

The micronutrients of Adye and Mateles (A&M) stock solution contained the following ingredients per liter: 0.7 mg $Na_2B_4O_7$ $10H_2O$, 0.5 mg $(NH_4)_6MO_7O_{24}$ $4H_2O$, 10g Fe_2 $(SO_4)_3$ $6H_2O$, 0.3 mg $CuSO_4$ $5H_2O$, 0.11 mg $MnSO_4$ H_2O, 17.5 mg $ZnSO_4$ $7H_2O$. One ml of this stock solution was added to each litre of medium before autoclaving. Concentrated micronutrients were solubilized by acidifying the stock with hydrochloric acid (HCl) to about pH 2.0. Dichloran stock solution consisted of 250 mg technical dichloran (96.5%) dissolved in 20 ml acetone and brought to volume in a 250 ml volumetric flask with 95% acetone. Rose Bengal Stock consisted of 500 mg Rose Bengal moistened with 30 ml 95% ethanol and brought up to volume in a 100 ml volumetric flask with distilled water. Streptomycin stock consisted of 1.0 g streptomycin sulfate in 100 ml distilled water; this stock was filter sterilized before use. Chloramphenicol stock consisted of 2.5 g Chloramphenicol dissolved in 95% ethanol and brought up to volume with 95% ethanol in a 100 ml volumetric flask. MRB was chosen as a medium for isolation due to its increased inhibition of fungi outside the Aspergillus group (Cotty, 1994).

Estimation of conidial suspension

To have a uniform and equal number of spores and to avoid biasness on the rate of colonization, the conidial suspension was estimated. The number of spores per milliliter in the suspension of each isolate grown for 7 days at 30 ± 2^oC was described as by (Khan et al., 2021). After gently washing off the isolates from the growth media with 0.01 % Tween 20 solution, 600 µl of absolute ethanol was transferred into vials followed by another 600µl of spore suspension, and 10.8 µl of 50% ethanol. The mixture was homogenized by slowly inverting the vials for 3 times. The spores were then measured using a turbidimeter (Oberco Hellige TB200) and estimated using the formulas below:

1. NTU initial = xNTU x 20   8058138806
2. No. of spores/ml = NTU x 49,937

where, *NTU* = Nephelometric Turbidity Units and x = is the average mean of the three readings taken on the turbidimeter. The final spore count and concentration of 10^5 spores/ml was then adjusted using method described by Dania et al. (2014):

$C_1V_1 = C_2V_2$

Table 2. The rating scale used to score for severity of *A. flavus* and *parasiticus* on kernels.

Scale	Disease reaction	Description (growth on the kernels)
1	Highly resistant	invisible mycelial growth
2	Resistant	1-20% surface coverage of mycelial growth
3	Moderately resistant	21-50% surface coverage of mycelial growth
4	Susceptible	51-70 % surface coverage of mycelial growth
5	Highly susceptible	71-100% surface coverage of mycelial growth

where, C_1 = Initial inoculum concentration, V_1 = Initial volume of water used in streaking the culture plate C_2 = Final inoculum concentration desired, and V_2 = Final volume of water to be added to obtain desired concentration.

Fungal Inoculation of groundnuts

Before inoculating the seeds with the two strains, the seeds were sterilized using tyndallisation method (Jung et al., 2009). This was done by placing the seeds in vials and heating them to 121°C for 30 min in an Autoclave SX Series TOMY Digital machine for three successive intervals to destroy the endospores as well as any surface spores. Thereafter, 20 ml containing approximately 10^5 spores/ml concentration for each strain was used to inoculate the sterilized seeds in the vials. The immersed seeds were left for 30 min and later transferred onto the 24-well crystal-clear plates for optimal visibility using forceps. No strain was added to the controls except sterilized distilled water. To increase humidity, the middle wells of the plates were filled with sterilized water and sealed with parafilm and incubated at 28°C for 10 days.

Scoring the rate of colonization

To determine the rate or effect of colonization for the two *Aspergillus* spp. severity and incidence scores were performed for each treatment. Percent-severity kernel infection (PSKI): scores on each kernel on a scale of 1-5 (Table 2) (kwemoi et al, 2010).

Determination of aflatoxin levels by High-Performance Liquid Chromatography (HPLC)

Sample extraction

Twenty-four kernels from each replication were ground using a Waring Lab Blender after which 25 g of the sample was accurately weighed, mixed with 5 g NaCl and transferred into a clean beaker. Prior to homogenizing, 125 mL of methanol: water (70:30) was added and blended for 2 min. The mixture was filtered using No. 1 Whatmann filter paper (Sigma-Aldrich, WHA1001090). The extraction and clean up was done as by Vicam (2021): AflaTest[TM] [WB] SR[+] instruction manual No. 715007173 Rev B.

Data analysis

The data collected was subjected to analysis of variance (ANOVA) using GenStat 13th edition software to determine differences in aflatoxin contamination levels of the genotypes. The means were separated using the least significant difference (LSD) at 95% Confidence Interval (CI).

RESULTS

Aflatoxin levels in the groundnuts

The results revealed that only the interaction effect (Genotype × Inoculation treatments) was significantly different (*P*<0.001). The contamination level in the three groundnut genotypes were however below the regulatory limits (10 µg kg^{-1}) and toxin levels ranged from 0.12 to 0.24 µg kg^{-1} in groundnut inoculated with *A. flavus* in all the genotypes (Table 3). Toxins were however not detected in *A. parasiticus* colonized genotypes. Chishango showed the highest levels of aflatoxin contamination (0.24 µg kg^{-1}), followed by Natal common (0.20 µg kg^{-1}) and lastly MGV4 (0.12 µg kg^{-1}).

Colonization of groundnuts by *A. flavus* (S-strain) and *A. parasiticus*

The results obtained showed significant difference (*P*<0.001) in inoculation treatment main effect. While genotypic and the interaction effects were not significant (*P*>0.05). Further analysis showed that clear mean differences across genotypes were observed between the control and inoculation treatments [*A. parasticus* and *A. flavus*] (Figures 1 to 3) with *A. parasticus* exhibiting highest values at day 3 and 7.

DISCUSSION

Aflatoxin contamination levels by *A. flavus* (S-strain) and *A. parasiticus*

The significant differences in aflatoxin contamination across genotypes observed in all inoculations with *A. flavus* could be due to differences in genetic make-up of groundnut genotypes employed in the study. Resistance to *A. flavus* was identified to be conditioned by additive gene action, associated by multiple genes, and highly influenced by the environment (Jayaprakashi et al., 2019). In our study, MGV4 was identified as the most resistant genotype to aflatoxin accumulation, implying that MGV could possess the highest accumulation of

Table 3. Concentration of aflatoxin in groundnut kernels analysed by HPLC 10 days after inoculation with *A. parasiticus* and *A. flavus* (S-strain)

Genotype	Inoculation treatment	Total aflatoxin (µg/kg)
Natal common	*Parasiticus*	0
	flavus	0.20[b]
	Control	0
MGV4	*Parasiticus*	0
	flavus	0.12[c]
	Control	0
Chishango	*Parasiticus*	0
	flavus	0.24[a]
	Control	0
LSD		*0.03*
P value		*<0.001*
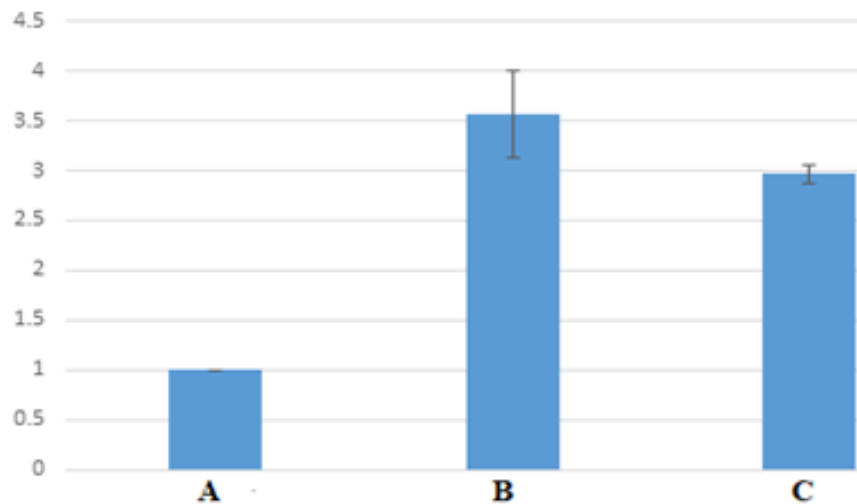
Figure 1. Mean severity (colonization) score across genotypes of fungal pathogens at the first (1) day after inoculation. A- Control, B- *Aspergillus parasticus*, C- *A. flavus.*

desirable allele's associated with resistance to aspergillus infection. Thus, MGV4 can be crossed with a susceptible genotype to create a mapping population for use in identifying linked molecular markers for resistance to aflatoxin accumulation (Ndeke and Tembo, 2019; Mbwando et al., 2016). A previous study identified both major and minor QTL associated with resitance to aflatoxin flavus accumulation (Yu et al., 2019). Thus a mapping population developed by using MGV4 as a resistant parent could aid in identfing stable QTLs. Chishango and Natal common may not be the best susceptible candidate as their observed contamination levels were below the acceptable limit (10 µg kg $^{-1}$), implying that their genetic response is in the resistant blanket.

Genotypic colonization of *A. flavus* (*S-strain*) and *A. parasiticus*

The results showed *A. parasiticus* was a fast colonizing pathogen (Figure 1 and 2) though no detectable mycotoxins were observed in the associated genotype, as compared to genotypes associated to inoculation with
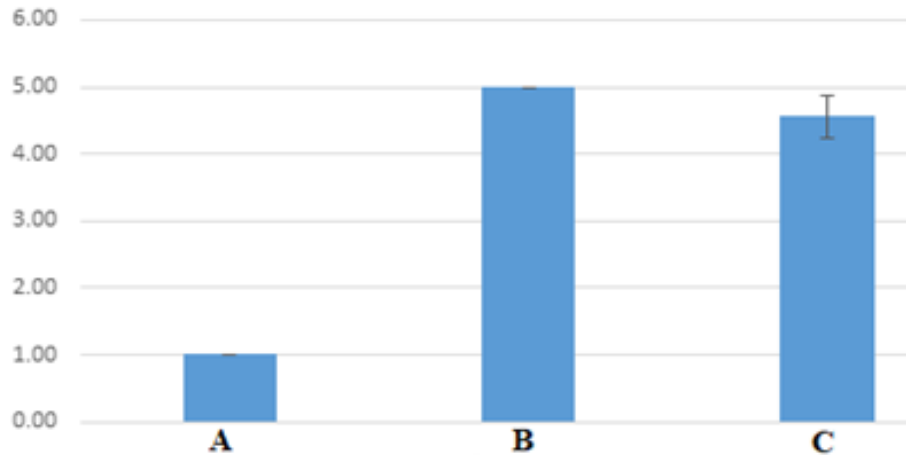
Figure 2. Mean severity (colonization) score across genotypes of fungal pathogens at the seventh (7th) day after inoculation. A- Control, B- *Aspergillus parasticus*, C- *A. flavus.*
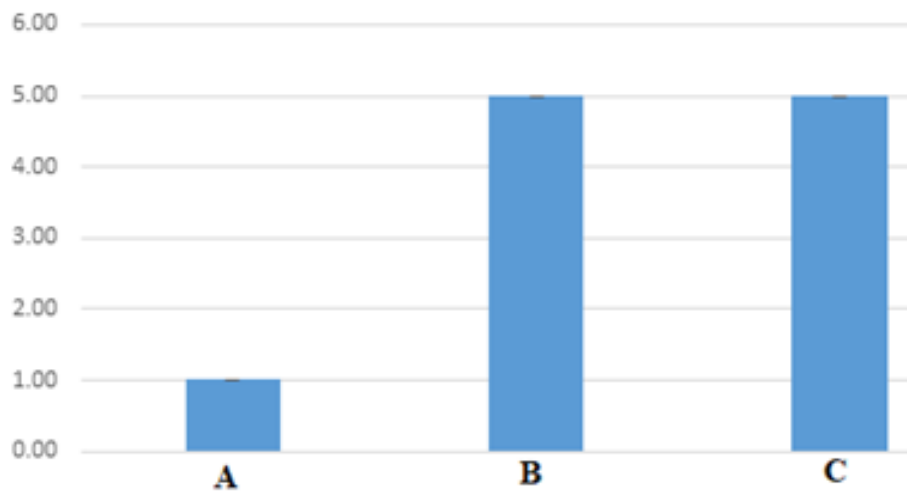

Figure 3. Mean severity (colonization) score across genotypes of fungal pathogens at the tenth day after inoculation. A- Control, B- *Aspergillus parasticus*, C- *A. flavus.*

A. flavus (S- strain). An indication that *A. flavus* (*S- strain*) is probably a more disastrous pathogen and requires a relatively more urgent attention than *A. parasticus.*In that vein *A. flavus* (S- strain), observed with dectatable aflatoxin may be regarded as a relatively more virulent pathogen compared to *A. parasticus*. This study stands in contrast to Horn (2005) who reported that *A. flavus* is a significantly more aggressive colonizer than *A. parasiticus* on groundnut.

Variations in perceived virulence levels could also be attributed to differences in sclerotia production. In this experiment *A. flavus* produced more sclerotia than *A. parasiticus,* which is an indication that there may be a correlation between the production of sclerotia and aflatoxin levels. However, other findings suggest that the abundance or indeed presence of sclerotia may not be used as a measure of strain toxicity. In their findings, they suggest that isolates without sclerotia had a higher production of toxins and vice versa (Okoth et al., 2016). Conceivably, the non-detection of toxin production in *A. parasiticus* may be that the strain was atoxigenic although it is a rare case (Horn et al., 1996).

Furthermore, it was deduced that *Aspergilus* colonisation and aflatoxin contamination do not correlate implying that the two mechanisms may be influenced by different genes and enviromemental conditions

(Jayaprakash et al., 2019; Wang et al., 2017)

Conclusion

The results showed that *Aspergillus parasticus* was the most colonizing pathogen. Conversely, the inability to produce detectable aflatoxin in similar inoculated genotypes compared to *A. flavus* (S-strain) qualifies *A. flavus* as the more virulent pathogen. This study revealed that MGV4 was the most resistant genotype to aflatoxin contamination. However, the other genotypes were also in the resistant blanket as there aflatoxin levels were below the acceptable limit (10 µg.kg^{-1}). Further studies can be exploited to assess the performance of other cultivated varieties for resistance in the quest to identify, susceptible genotypes that could aid in molecular mapping studies. With such an understanding of the resistance or susceptibility to aflatoxin contamination, this will guide plant breeders to consider this trait in groundnut breeding. In addition, we argue that the variations observed in aflatoxin contamination (infection, colonisation and subsequent aflatoxin production) in our study could depend on the genetic makeup of the genotype and toxigenicity of the pathogen itself. In conclusion, small holder farmers may benefit greatly from the breeding of host plant resistant genotypes as the technology can easily be disseminated and is more cost-effective in its application.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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