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., 2016). 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).

Germlasm 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 (Chiriwa 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 CO₂ 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.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Characteristics</th>
<th>Days to maturity</th>
<th>Variety type</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natal common</td>
<td>Small seeded variety with tan kernels</td>
<td>90 - 100</td>
<td>Improved</td>
<td>ZARI*</td>
</tr>
<tr>
<td>Chishango</td>
<td>Medium seeded with tan-pink seed colour</td>
<td>120 - 130</td>
<td>Improved</td>
<td>ZARI</td>
</tr>
<tr>
<td>MGV4</td>
<td>Medium seeded variety with red kernels</td>
<td>130 - 140</td>
<td>Improved</td>
<td>ZARI</td>
</tr>
</tbody>
</table>

*Zambia Agriculture Research Institute.

Plate 1. 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* were cultured on 5/2 growth medium.

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 NaNO₃, 0.3 g KH₂PO₄, 0.7 g K₂HPO₄, 0.5 g MgSO₄ 7H₂O, 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 (50 mg 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 Na₂B₄O₇ 10H₂O, 0.5 mg (NH₄)₂MoO₄ 2H₂O, 10g Fe₃ (SO₄)₂ 6H₂O, 0.3 mg CuSO₄ 5H₂O, 0.11 mg MnSO₄ H₂O, 17.5 mg ZnSO₄ 7H₂O. 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 (95.5%) 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°C 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. \( NTU_{initial} = xNTU \times 208058138806 \)
2. \( \text{No. of spores/ml} = NTU \times 49.937 \)

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.

<table>
<thead>
<tr>
<th>Scale</th>
<th>Disease reaction</th>
<th>Description (growth on the kernels)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Highly resistant</td>
<td>invisible mycelial growth</td>
</tr>
<tr>
<td>2</td>
<td>Resistant</td>
<td>1-20% surface coverage of mycelial growth</td>
</tr>
<tr>
<td>3</td>
<td>Moderately resistant</td>
<td>21-50% surface coverage of mycelial growth</td>
</tr>
<tr>
<td>4</td>
<td>Susceptible</td>
<td>51-70 % surface coverage of mycelial growth</td>
</tr>
<tr>
<td>5</td>
<td>Highly susceptible</td>
<td>71-100% surface coverage of mycelial growth</td>
</tr>
</tbody>
</table>

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 $121^\circ$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 $28^\circ$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) (Iwemoi 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): AfalTest™ with 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 ($10 \mu g \ kg^{-1}$) and toxin levels ranged from 0.12 to 0.24 $\mu g \ kg^{-1}$ 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 ($0.24 \mu g \ kg^{-1}$), followed by Natal common ($0.20 \mu g \ kg^{-1}$) and lastly MGV4 ($0.12 \mu g \ kg^{-1}$).

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 treatment [A. parasiticus and A. flavus] (Figures 1 to 3) with A. parasiticus 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)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Inoculation treatment</th>
<th>Total aflatoxin (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natal common</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parasiticus</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>flavus</td>
<td>0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MGV4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parasiticus</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>flavus</td>
<td>0.12&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chishango</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parasiticus</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>flavus</td>
<td>0.24&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>LSD</td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>P value</td>
<td></td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Figure 1. Mean severity (colonization) score across genotypes of fungal pathogens at the first (1) day after inoculation. A- Control, B- *Aspergillus parasiticus*, 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 resistance to aflatoxin flavus accumulation (Yu et al., 2019). Thus a mapping population developed by using MGV4 as a resistant parent could aid in identifying 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<sup>-1</sup>), 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...
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. parasiticus. In that vein A. flavus (S-strain), observed with detectable aflatoxin may be regarded as a relatively more virulent pathogen compared to A. parasiticus. 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 Aspergillus colonisation and aflatoxin contamination do not correlate implying that the two mechanisms may be influenced by different genes and environmental conditions.
(Jayaprakash et al., 2019; Wang et al., 2017)

Conclusion

The results showed that *Aspergillus parasiticus* 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.

REFERENCES


