Aspergillus flavus Biomass in Maize and Use of a Biocontrol Strategy to Limit Aflatoxin Production.

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1. Introduction

- The quantification of fungal biomass is critical in understanding the interactions between host plant susceptibility or resistance to a fungal pathogen as well as identifying competition between individual fungal species during disease progression [1-2].
- RT-qPCR has been used to detect and quantify the fungal biomass in various plant host tissues [1–2].
- High level of sensitivity of qPCR enables the measurement of very low infection titres which could correspond to the amount of pathogen present at the time of infection or during latent, non-symptomatic infections.
- The use of atoxigenic *A. flavus* strains for biocontrol is directly connected with methods for detecting aflatoxins as it confirms that the biocontrol agent reduces aflatoxin contamination.
- The levels of aflatoxin in foods and feeds are strictly regulated [3,4]. This requires rapid, sensitive, quantitative and relatively easy techniques for aflatoxin detection at various stages in the food chain [4,5].
- Bio-analytical techniques TLC, HPLC & LC-MS/MS is widely used in aflatoxin detection and is considered the gold standard for aflatoxin detection [4,5] .

Maize inspection by Researcher and fluorescence

Fungal growth on maize kernels on PDA media

Bread

Fruits

Fig. 1: Mycotoxin infected food & feeds

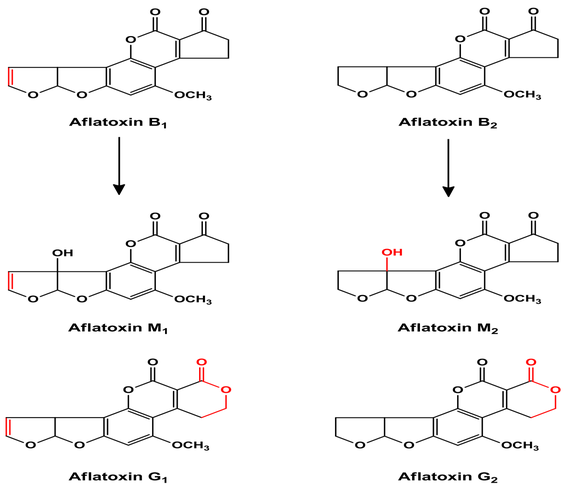
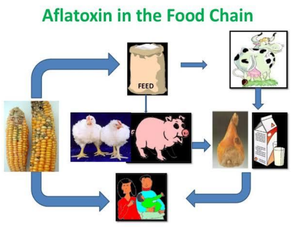


Fig. 2: Chemical structure of aflatoxins.



AFM_1, AFM_2 are derivatives of AFB_1 & AFB_2 respectively

Mycotoxin infected maize burnt-Kenya

2. Objectives

i. To test and develop a qPCR assay to quantify the amount of *A. flavus* biomass in infected maize tissues.
ii. To use the qPCR assay to investigate whether the respective maize lines KDV1 and GAF4, grown in different regions of Kenya, contribute to increasing or limiting the biomass of KSM014, an aflatoxigenic strain.
iii. To evaluate the selected method's ability to differentiate atoxigenic and aflatoxigenic *A. flavus*.
iv. To investigate the *in-vivo* biocontrol assay of maize kernels colonized by atoxigenic *A. flavus* strain KSM012, (NCBI_accession MG385137) as a biocontrol agent to minimize aflatoxin contamination by the toxigenic *A. flavus* strain KSM014, (NCBI_accession MG385138).

3. Materials & Methodology

i. **Cultures of fungi:** Obtained from [4]. Five *A. flavus* isolates: KSM012, KSM014, HB021, HB026 and HB027 were grown on both aflatoxin inducing (YES) & non-inducing medium (YEP), 7 d, 30 ° C [4].
ii. **Maize cultivars:** KDV1 and GAF4 were purchased from KALRO, Nairobi, Kenya
iii. **Seed sterilization and *A. flavus* infection:** 20 seeds were sterilized in 95-100% EtOH, NaOCl & dH_20 and were inoculated (in triplicates) with 20 ml conidia suspensions (1 x 10^6 conidia ml^{-1})
iv. **DNA extraction from *A. flavus* and maize tissues:** 100 mg mycelia (infected and control healthy maize tissues following the method of [4] with modifications)
v. **Primer design:** Three sets of primers (Table 4); *β-tubulin*, *Elongation factor 1 alpha* (*Ef1α*) and Membrane protein (MEP) were used in this study (Primer3 ver. 4.0 programme [4]) Table 4. Potential secondary structure formation was assessed in DNAMAN software ver. 6.0 (Lynnon LLC., USA) and further verified in OligoAnalyzer Tool (Integrated DNA Technologies). The PCR and melt curve analysis were used to identify both specific and non-specific amplification.
vi. **PCR amplification:** Cycling conditions : 1 cycle at 94 ° C for 5 min, by 35 x (at 94 ° C for 30 s, at 60 ° C for 45 s, at 72 ° C for 90 s). Elongation step was for 7 min at 72 ° C & held at 4 ° C. The PCR assed, 2 % agarose/EtBr gel & Fermentas 100 bp DNA ladder.
vii. **Aflatoxin standards, standard curves and fungal quantification:** 200 µg l^{-1} AFB1, 50 µg l^{-1} AFB2, 200 µg l^{-1} AFG1 and 50 µg l^{-1} AFG2 were used. The threshold cycle (Ct) values were plotted against the logarithm of the starting quantity of the template. Efficiencies of amplification were generated from the slopes of the standard curves' slopes [2]. Linear regression curves were drawn, and the qPCR efficiency was calculated as: $E = 10(\frac{-1}{Slope})$.

The amount of target DNA in an unknown sample was extrapolated from the respective standard curves.

viii. **Metabolite extraction:** TLC, HPLC and optimization
ix. ***In vitro* co-infection of maize lines and Biocontrol strategy**
x. **Statistical analysis:** GraphPad Prism, ANOVA, TMCT & Post-test for linear trend analysis

$$1 - \frac{\boldsymbol{TACM\ co-inoculted\ with\ both\ atox\ and\ aflatox\ isolates\ of\ A.\ flavus}}{\boldsymbol{TACM\ inoculated\ with\ the\ aflatoxigenic\ isolate\ alone}} \times 100$$

Standard deviations of mean differences in aflatoxin levels were calculated as a measure of variability in efficacy. The efficiency (E) of each isolate was calculated as: $\boldsymbol{E = \frac{R}{\frac{A}{A+T}}}$

Table 1 Specific primers used for total fungal and strain quantification

Primer name	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product size (bp)	Ta	Reference
Membrane Protein (MEP)	TGTACTCGGCAATGCTCTTG	TTTGATGCTCCAGGCTTACC	203	64 ^{0} C	Manoli et al., 2012
Elongation Factor 1 alpha (*EF1α*)	CGTTTCTGCCCTCTCCCA	TGCTTGACACGTGACGATGA	102	62 ^{0} C	Nicolaisen et al., 2009
β-TubulinM	TCTTCATGGTTGGCTTCGCT	CTTGGGTCGAACATCTGCT	118	62 ^{0} C	Mitema et al., 2018

4. Results & Discussion

Colonization by *A. flavus*

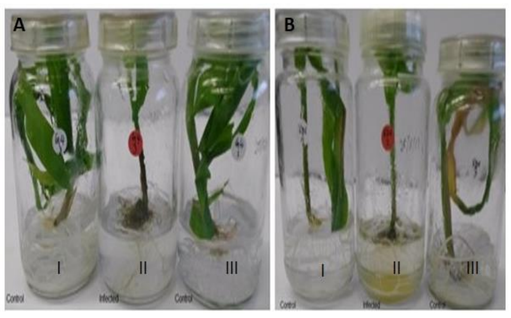


Fig. 3. The respective GAF4 (**A**) and KDV1 (**B**) maize lines with and without *A. flavus* KSM014 infection. The uninfected maize control plants (I & III) and the infected plants (II) are shown 14 days after germination

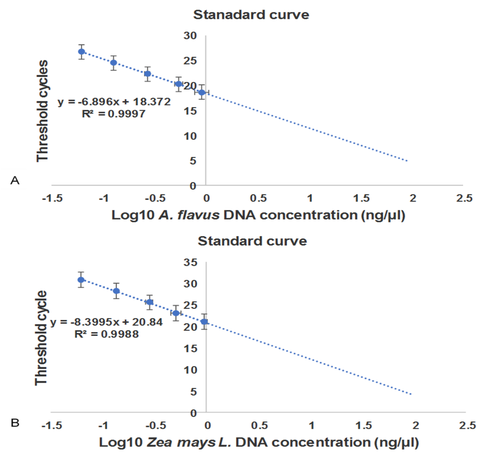


Fig. 5. RT-qPCR SYBR green assay. The curves illustrate the linear regression, efficiency and sensitivity of qPCR for early fungal detection employing marker genes, *β-Tub* and *MEP* to a total of 10 ng genomic DNA. (**A**) standard curve for *β-Tub* amplification using *A. flavus* DNA diluted in maize carrier DNA (**B**). standard curve for MEP amplification from using serial dilutions of maize genomic DNA. (Error bars shows the standard deviations of the mean triplicate gDNA concentrations)

Gene specificity and RT-qPCR assays

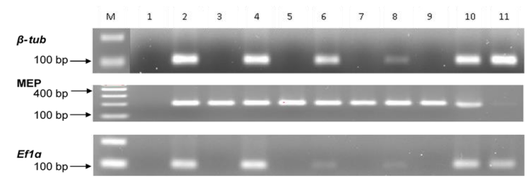


Fig. 4. Gel electrophoresis of qPCR amplicons for *A. flavus* maker genes *β-tub*, *Ef1α* & maize marker gene (MEP), on 2 % agar/EtBr. M. 100 bp ladder; 1. NTC; 2. Pooled samples (Pure FgDNA and MgDNA); 3. GAF4 (CRts); 4. GAF4 (IRts) 5. GAF4 (CSts); 6. GAF4 (ISts); 7. KDV1 (CSts); 8. KDV1 (ISts); 9. KDV1 (CRts); 10. KDV1 (IRts); 11. KSM014 (+ve *A. flavus* cntrl).

Table 2. Phenotypic characteristic measurements of control and infected tissues

Maize line	Phenotypic characteristic: Control roots Exp 1	Exp 2	Exp 3	Average (mm)	Control shoots Exp 1	Exp 2	Exp 3	Average (mm)	Infected roots Exp 1	Exp 2	Exp 3	Average (mm)	Infected shoots Exp 1	Exp 2	Exp 3	Average (mm)
GAF4	285	260	278	274.33	352	322	312	328.67	134	113	98	115	142	185	111	146
KDV1	272	252	232	252	344	300	323	322.33	78	83	84	81.67	82	87	91	86.67

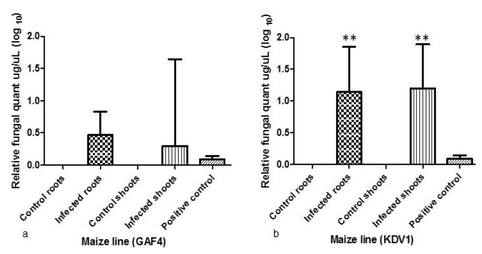


Fig. 6. qPCR analysis showing fungal load of *A. flavus* KSM014 in the root and shoot tissue of GAF4 and KDV1 maize lines respectively. Fungal biomass was measured in infected and non-infected (control) GAF4 (**a**) and KDV1 (**b**) maize lines within 14 days where the *A. flavus β-tub* gene was used for fungal quantification against the maize MEP gene. A one-way ANOVA and Tukey's Multiple Comparison Test revealed ($P < 0.05$). Asterisks indicate significance and the error bars shows standard mean deviation ($n = 3$)

Aflatoxin Analyses after Biocontrol Strategy

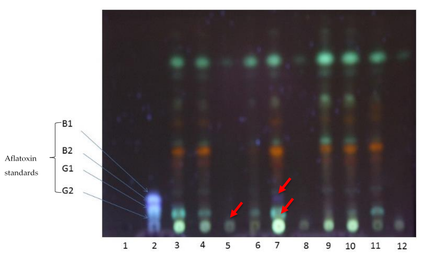


Fig. 8. TLC plates showing the presence or absence of mycotoxins from the isolates in comparison with the aflatoxin standards at long wavelength, 365 nm. Lanes: (**1**) Blank; (**2**) Standard; atoxigenic KSM012 and aflatoxigenic KSM014 ratios for the GAF4 line (**3**) G100/0; (**4**) G75/25; (**5**) G50/50; (**6**) G25/75; (**7**) G0/100; and (8) K100/0; (**9**) K75/25; (**10**) K50/50; (**11**) K25/75; (**12**) K0/100 for the KDV1 line (K). (**B1**: aflatoxin AFB1; **B2**: aflatoxin AFB2; **G1**: aflatoxin AFG1; **G2**: aflatoxin AFG2)

***In-vitro* biocontrol strategies in aflatoxin management and *Aspergillus flavus*.**

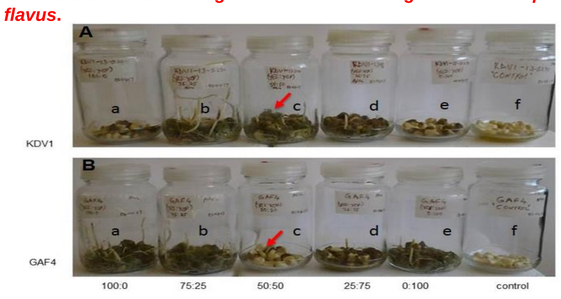


Fig. 7. Biocontrol approach with atoxigenic KSM012 and aflatoxigenic KSM014 strains of *A. flavus* to mitigate aflatoxin production. The kernels for sensitive (**A**; KDV1) and resistant (**B**; GAF4) maize lines were co-infected at different ratios (0:100; 25:75; 50:50; 75:25; 100:0) with atoxigenic and aflatoxigenic strains

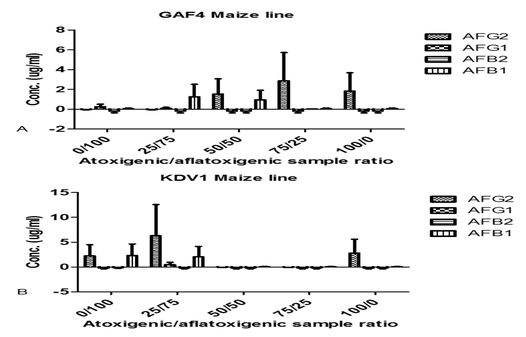


Fig. 9. HPLC analysis highlighting biocontrol of aflatoxigenic (KSM014) by atoxigenic (KSM012) *Aspergillus flavus* when co-infected at different ratios in maize lines GAF4 (A) and KDV1 (B) respectively. (**AFB1**: aflatoxin B1; **AFB2**: aflatoxin B2; **AFG1**: aflatoxin G1; **AFG2**: aflatoxin G2)

- The KDV1 maize line grown in Makueni county is more susceptible to *A. flavus* infection (Figs. 3-9).
- The GAF4 maize line grown in Kisumu and Homa bay counties appeared more resistant to the infection (Figs. 3-9).
- The qPCR assay: The *β-Tub* gene was a better marker compared to *Ef1α* (Figs. 4-6: Tables 1, 2.). *β-tub* gene quantified *A. flavus* biomass in both shoot and root tissue of different maize lines.
- We measured fungal biomass fourteen days after infection when symptoms of the infection were phenotypically visible. Debode *et al.* 2009, detected the presence of *Colletotrichum acutatum* by qPCR in strawberry leaves two hours post-inoculation even though the first disease symptoms appeared only after 96 hours. Similarly, Divon & Razzaghian, 2012, could measure *Fusarium langsethiae* DNA in oats independent of the disease symptoms. Both findings demonstrated that fungal presence can be detected earlier, enabling the selection of resistant plants even when samples are indistinguishable based on visual assessment (Fig. 6).
- KDV1's increased susceptibility to *A. flavus* KSM014 infection could be a contributing factor to the previously reported frequent aflatoxicosis outbreaks and high levels of aflatoxin contamination for Makueni and the neighbouring regions [2,4,5]. These results are also consistent with our previous study which showed that the majority of *A. flavus* isolates from Makueni produced high amounts of aflatoxin AFB1, AFB2 [2].
- The detection limits and sensitivity, including linearity of our results were similar and compared favourably to those of Gallo *et al.* 2010 and Malachová *et al.* 2014, who obtained LOD range of 0.6-1.9 µg/kg and a LOQ range of 0.02-0.05 mg/kg for aflatoxins extracted from highly contaminated animal feedstuff (Fig.5).
- Biocontrol strategy showed that aflatoxin production by the aflatoxigenic strain, KSM014, was inhibited by the atoxigenic strain, KSM012 at specific ratios (Figs. 7-9). These observations suggest that upon colonization of kernels by the aflatoxigenic isolate, the atoxigenic strain has the potential to limit colonization of the aflatoxigenic isolate leading to inhibition or reduced aflatoxin levels. Competitive exclusion by atoxigenic isolates results in reduced amount of aflatoxin during co-infection [2], a process aided by primary host contact. Huang *et al.* 2014 showed that both the down-regulation of aflatoxin biosynthesis and variance in ability among fungal isolates to utilize nutrient resources could limit the amount of aflatoxin produced.
- Biocontrol of aflatoxins by atoxigenic isolates is a cost-effective method for managing aflatoxins and could provide a long-term solution to aflatoxin contamination in developing countries, including sub-Sahara Africa [2] (Fig.7-9).
- Based on our study, KSM012 might be a candidate for aflatoxin and *A. flavus* mitigation in Kenya and thus further study on this strain is warranted.

5. Conclusion

- The *β-tub* gene is a potential marker for quantification of the *A. flavus* biomass load in maize plants compared to the *Ef1α* gene and both were specific for *A. flavus*.
- The MEP primers were specific for maize and had no cross contamination with *A. flavus* DNA.
- The specificity of the qPCR assay for *A. flavus* biomass quantification makes it potentially useful tool for screening of *A. flavus* maize lines for resistance to *A. flavus* and associated breeding strategies, identifying potential asymptomatic infections and help understand the mechanism of maize defense response to *A. flavus* infection.
- We found KDV1 maize line, which is cultivated in Makueni region and its environs to be more susceptible to *A. flavus* infection than GAF4 maize line grown in other regions of Kenya. This, susceptibility may be one of the possible reasons for the frequent cases of aflatoxicosis in Makueni than in Nandi, Kisumu and Homa Bay.
- We showed that a biocontrol strategy using the atoxigenic *A. flavus* isolate KSM012, was able to inhibit aflatoxin production by the aflatoxigenic *A. flavus* isolate KSM014 after co-infection of maize kernels at a 50:50 ratio.
- These findings are promising and might be suitable for future development of a biocontrol system appropriate for aflatoxin mitigation against aflatoxigenic *A. flavus* isolates in Kenya.

References

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