Chapter

Fungal Biomass Load and *Aspergillus flavus* in a Controlled Environment

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Abstract

Fungal biomass quantification is critical in understanding the interactions between the pathogen and susceptibility or resistance of the host plant as well as identifying competition between individual fungal spp. in disease progression. In the present chapter, two maize lines grown in different climatic regions of Kenya were infected with an aflatoxigenic *A. flavus* isolate (KSM014) and fungal colonization of the maize plant tissues was monitored by measuring fungal biomass load after 14 days in a controlled environment. The objective of the study was to determine whether the maize line colonized was a factor in increasing or limiting the growth of an aflatoxigenic strain of *Aspergillus flavus*.

Keywords: fungi, *Aspergillus flavus*, aflatoxins, maize lines

1. Introduction

Fungal biomass quantification is critical in understanding the interactions between the pathogen and susceptibility or resistance of the host plant in identification and competition in individual species of fungi in diseases progression [1].

The quantification and detection of biomass of fungi in plant host tissues have been conducted using polymerase chain reaction methods [2, 3]. Some authors, Sanzani et al. [2] showed that, the sensitivity of quantitative polymerase chain reaction (qPCR) can be applied to measure infections at very low volumes, thus, corresponding to the quantity of the pathogen that might be present during the latent or time of and or at asymptomatic infections. qPCR also enables the evaluation of stages of infection in plant tissues and the quantification of a fungal pathogen throughout the entire disease cycle [2].

Coninck et al. [1] developed a qPCR assay for detection and quantification of *Cercospora beticola* fungi in leaves of sugar beet. Moreover, Waalwijk and co-workers [4], using a gene involved in fumonisin biosynthesis developed a qPCR assay to quantify and detect fumonisin producing *Fusarium verticillioides* strains from maize obtained from South African subsistence farmers. These results were then compared to the fungal DNA content and with the fumonisin levels of the respective *F. verticillioides* strain. A qPCR assay was also developed by Nicolaisen et al. [5] using Ef1α for quantification and detection of 11 *Fusarium* spp. isolated from field materials associated with wheat and maize. Similarly, Korsman and co-workers [6], demonstrated the use of qPCR assay for detection and quantification of Gray leaf spot disease in maize leaves using cytochrome P450 reductase gene. These studies demonstrated

the potential use of qPCR for detection and quantification of fungal pathogens and for probable selection of resistant plant cultivars in breeding lines. This also helps in understanding the processes involved in infection in a host-pathogen system and providing information on the bioecology [7, 8].

Mayer et al. [9] and Jurado et al. [10] used single copy mycotoxin biosynthetic genes to develop PCR assays for detecting mycotoxigenic fungi. Assay sensitivity increased when ITS1 and ITS2 spacer regions were included as, these regions have sufficient variability to enable discrimination of closely related species in the genus *Aspergillus* [11]. Subsequently, these regions have been successfully used for detection and identification of aflatoxigenic *Aspergillus* spp. [12, 13].

The objective of this study was to develop a sensitive, specific qPCR assay for quantifying *A. flavus* biomass in infected maize tissues. The assay was used to measure, the sensitivity of two dry land African maize lines grown in Kenya KDV1 and GAF4, when infected with an aflatoxigenic isolate (KSM014). Similar studies have been done with other fungal species, but this study is the first where the biomass of *A. flavus* from infected maize was detected and quantified with qPCR. This approach also could be used to discriminate between inbred maize lines that are sensitive or resistant to specific *A. flavus* strains and to help understand the mechanism of the maize defense response to *A. flavus*.

2. Materials and methods

2.1 Cultures of fungi

The aflatoxigenic *A. flavus* KSM014 isolate was cultivated and maintained as described previously [14] and thereafter stored as spore suspension in 15% glycerol for short term storage at −20°C or for long term storage at −80°C prior to DNA/RNA extraction.

2.2 Maize cultivars

GAF4 maize lines and KDV1 varieties were obtained from Kenya Agricultural and Livestock Research Organization (KALRO), Kenya. The selection of the varieties are focused mainly on their drought tolerance and the agro-ecological zones in which they were grown. Striga tolerant variety (GAF4) is produced by KALRO Kibos, Kisumu County. GAF4 is cultivated in Homa Bay, Kisumu and Busia counties [15]. The Kenya Dryland Varieties 1 is an open pollinated hybrid recommended for medium to low altitude areas. KDV1 is drought tolerant, matures early and produce flowers after germination between 45 and 52 days. It is mainly cultivated in Homa Bay and Makueni regions (http://drylandseed.com).

2.3 Media preparation and reagents

Phytagel, Nicotinic acid, Glycine, Thiamine hydrochloride, Murashige and Skoog medium (MS), Potassium hydroxide, Pyridoxine hydrochloride and Myo-inositol were from Sigma-Aldrich (USA). MS vitamins; 5 g myo-inositol, 500 mg Thiamine-HCl, 500 mg pyridoxine-HCl, 250 mg nicotinic acid and 100 mg glycine were filter sterilized after preparation in distilled water and thereafter stored at −20°C according to the instructions of the manufacturer's (Sigma-Aldrich, USA). The modified MS media was briefly prepared, 2.15 g MS salt was dissolved in sterile H_2O, thereafter, 10 ml MS vit. added and pH 5.7 adjusted using 1 M KOH and volume further adjusted to 1 l using sterile H_2O. 5 g of phytagel was added to MS media

and heated in microwave to dissolve the salts. Fifty milliliters of the media was dispensed into tissue culture bottles, autoclaved and thereafter, cooled in the level 2 biosafety cabinet for approx. 1 h prior to inoculations as previously described [14].

2.4 Seed sterilization and *Aspergillus flavus* infection

The seeds were sterilized in a biosafety cabinet, level 2 [Contained Air Solutions (CAS) BioMAT2, UK]. Twenty milliliters of 95–100% ethanol was used for sterilization of viable seeds for 1 min and briefly shaken for 15 s. The alcohol was replaced with 20 ml of sodium hypochlorite (2.5%). After 15 min of reaction at room temperature, the mixture was shaken for 30 s and thereafter, the liquid discarded. 30 ml of sterile H_2O was used 5× to wash the seeds with intermittent shaking after every wash. 50 ml of sterile H_2O was added and left to stand for 1 hr at rmt. The H_2O was replaced with 20 ml of 2% Tween 20 and shaken for 30 s. Conidia suspensions adjusted to 1×10^6 conidia ml^{-1} using a hemocytometer was used to inoculate the seeds. The seeds in the tubes were para filmed after sealing and kept for 30 min in a shaking incubator at 30°C. Controls were treated with sterile H_2O instead of spores of conidia and thereafter, incubated following the same conditions. The seeds were left to dry in Petri dishes after inoculations overlaid with filter paper overnight (Whatman No. 1). The seeds were germinated in a plant growth chamber, Conviron (Winnipeg, Manitoba, Canada) set at 28°C after subsequent inoculations onto tissue culture bottles. The germination and growth were observed for a 14-day period, tissues of the plant (roots and shoots) were separately harvested, flash frozen in liquid nitrogen prior to DNA/RNA extraction and stored at −80°C.

2.5 The extraction of gDNA from maize tissues and *Aspergillus flavus*

A 100 mg of each of the following samples: control healthy maize tissues, infected and *A. flavus* KSM014 mycelia was used to extract DNA following the method of Möller et al. [16] with some modification. Briefly, 100 mM Tris pH 8.0, 2% SDS, modified TES buffer, 2% (w/v) polyvinylpyrrolidone (PVP) and 10 mM EDTA was prepared. 5 μl RNase (10 mg/ml) and 450 μl of TES buffer was added to microtube (2 ml) containing the tissues and thereafter, homogenized by vortexing for 15 min or with a microtube pestle. Twenty microlitres of Proteinase K (1 μg/μl) was added and vortexed for 1 min, thereafter, incubated for 1 h at 60°C. Seventy microliters of 10% CTAB (0.1 vol.) and 160 μl of 5 M NaCl (0.3 vol. was added and incubated at 65°C for 10 min). Seven hundred and fifty microliters of chloroform/isoamyl-alcohol (24:1) was added, vortexed for 5 min, incubated on ice for 30 min and centrifuged at 14,000 rpm for 10 min. The aqueous phase was transferred cautiously onto a new microtube (2 ml) and 300–350 μl isopropanol (0.55 vol.) added and left to stand for 30 min at RT after mixing gently for 30 s. The mixture was centrifuged for 10 min at 14,000 rpm. Supernatant was cast-off, the pellets rinsed with chilled 700 μl of 70% ethanol twice and centrifuged again at 14,000 rpm for 2 min after mixing gently. The pellets were air dried and dissolved in 40 μl TE buffer (10 mM Tris-Cl pH 8, 1 mM EDTA pH 8) or nuclease free water after discarding the ethanol. The integrity of DNA was assessed on a 1% agarose/EtBr gel and the concentration quantified on a Nano-Drop™ 1000 spectrophotometer (Nano Drop Technologies, USA). DNA was diluted to 10 ng/μl for further analysis.

2.6 Designing of primers

Sets of 3 primers (**Table 1**); elongation factor 1 alpha (*Ef1a*), *β*-tubulin, and membrane protein (*MEP*) were used in the current research. *MEP* and *Ef1a* were

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. [17]
Elongation Factor 1 alpha (*EF1α*)	CGTTTCTGCCCTCTCCCA	TGCTTGACACGTGACGATGA	102	62 ^{0} C	Nicolaisen et al. [5]
β-TubM	TCTTCATGGTTGGCTTCGCT	CTTGGGTCGAACATCTGCT	118	62 ^{0} C	Mitema et al. [18]

Table 1.
Specific primers used in the current study.

obtained from Dr. Shane Murray (pers. comm) whereas, *β*-tubulin was designed in Primer3 ver. 4.0 programme [19]. Secondary structure formation was evaluated in DNAMAN software ver. 6.0 (Lynnon LLC., USA) and further verified in OligoAnalyzer Tool (Integrated DNA Technologies). The melt curve and PCR analysis were used to identify both non-specific and specific amplification.

2.7 PCR amplification

Conventional polymerase chain reaction amplification was carried out in volumes of 25 μl and consisted of 0.5 μl of 10 μM dNTPs (Bioline), 10× reaction buffer with $MgCl_2$, 1 μl of 10 μM forward and reverse primers, 0.2 μl Kapa Taq, 1 μl of 10 ng DNA template, and sterile H_20. Protocol performed and followed for cycling conditions were: 1 cycle for 5 min at 94°C followed by 35× (for 30 s at 94°C, for 45 s at 60°C, for 90 s at 72°C). Elongation step was achieved at 72°C for 7 min and finally at 4°C for 1 min. The products of PCR were assessed on 2% agarose/EtBr gel in TAE1 X buffer (Tris–acetate 40 mM and EDTA 1.0 mM). Fermentas (100 bp DNA ladder) was used as a molecular size marker.

2.8 Standard curves and fungal quantification

Ten-fold serial dilutions of pooled 10 ng gDNA extracts from *A. flavus* and control plants were used to create standard curves. The threshold cycle (Ct) values for each dilution were plotted against the logarithm of the starting quantity of the template. The amplification efficiencies were created from the std. curve slopes according to the methods [13, 20]. Additionally, linear regression curves were drawn, and the qPCR efficiency was calculated as: $E = 10\left(\frac{-1}{Slope}\right)$.

The quantity of targeted DNA in an unknown sample was inferred from the respective std. curves.

Ten nanograms of DNA isolated from infected and healthy maize roots and shoots respectively were used to assess primer specificity. For the exclusion of false negative results, template DNA samples from fungi were assessed for polymerase chain reaction amplification with primer pairs *EF1a* and *β*-Tub. DNA extracted from pure fungal cultures (*A. flavus*) and control plant tissues were pooled, diluted to 10 ng/μl and used to evaluate the quantity of fungal DNA template in the infected plant tissue. The final fungal DNA template concentrations were 1, 5×10^{-1}, 2.5×10^{-1}, 1.25×10^{-1}, 6.25×10^{-2}, 3.125×10^{-2} ng/μl. These dilutions were used to estimate the detection limits of the *EF1a* and β-Tub primer pair in the infected plant tissues. Serial dilutions of extracted DNA from healthy maize tissue were prepared to gauge the detection limits of the *MEP*. For normalization and quantification of gene between different samples, the amount of fungal DNA as calculated by the *Ct* value for *β*-Tub and/or *EF1a* was divided by the amount of maize DNA as calculated by the *Ct* values for MEP. Rotor Gene 6000 2 plex HRM (Corbett Life Science Research, Australia) was used to assess the profiles of gene expression. Kapa *SYBR* Fast Kit, Master mix (Kapa BioSystems, South Africa) containing DNA polymerase, reaction buffers, dNTPs and 3 mM $MgCl_2$ were used for each polymerase chain reaction. Final concentrations of 10 μM gene specific primers (0.4 μl reverse and 0.2 μl forward), 1× Kapa SYBR green and 1 μl gDNA template were prepared to 20 μl total volume using nuclease free H_2O. Primer sets of specific genes (**Table 1**) were used in separate reactions which were performed in triplicate.

The quality and integrity of the isolated DNA, samples from infected and control tissues of the maize, and saprophytic fungi were subjected to polymerase chain reaction analysis with the reference genes under the following amplification

conditions: for 10 min at 95°C; 35 cycles for 3 s at 95°C, for 20 s at 64°C, for 1 s at 72°C for *MEP* and at annealing temperature 62°C for both *Ef1a* and β-tubulin.

2.9 Statistical analysis

The statistical analysis was performed as previously described [14].

3. Results and discussion

3.1 Gene specificity and qPCR assays

To our knowledge, a qPCR assay for the detection and quantification of *A. flavus* biomass using extracted fungal DNA from control or infected maize tissues has not been previously reported. Since this is the first report, our discussion will be in comparison with reports for *Fusarium* spp. and related fungi where this assay is more commonly used.

In this study, the qPCR assay was developed to specifically detect and quantify *A. flavus* gDNA in maize tissues. Primers were designed, and their specificity was confirmed by testing against control and infected tissues (**Figure 1**). The fungal biomass in the co-infected shoots differed from the fungal biomass in the roots according to 1-way ANOVA analysis and TMCT test ($P < 0.05$).

Amplification of the MEP gene (203 bp) was used to detect maize DNA, while amplification of *β*-tubulin (118 bp) and *Ef1a* (102 bp) were used to detect *A. flavus* DNA (**Table 1**; **Figure 1**). The specificity of the primer pairs was determined by conventional PCR (**Figure 1**) after *A. flavus* KSM014 infection of GAF4 and KDV1 maize lines. *A. flavus* DNA extracted from infected maize plant tissues, for both lines, gave an amplification product for both *β*-tubulin (118 bp) and *Ef1a* (102 bp) (**Figure 1**). However, there was amplification product for *Ef1a* than there was for to *β*-tubulin (**Figure 1**), especially in the roots. The *MEP* gene (203 bp) was amplified in both control and infected maize plants for both lines (**Figure 1**). *MEP* amplification was plant specific and *β*-Tub and *Ef1a* were fungal specific. Based on these results, *β*-Tub is a better marker for detecting *A. flavus* in infected maize tissues than was *Ef1a* (**Figure 1**) and was used for fungal biomass determination.

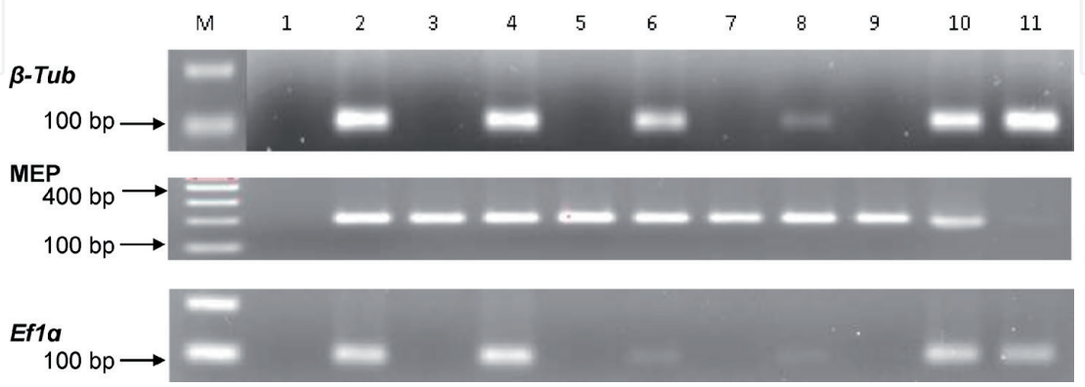

Figure 1.
*Gel images of the quantitative polymerase chain reaction amplicon sizes for maize maker gene (*MEP*) and* A. flavus *maker genes (*Ef1α, β-*tub) assessed on 2% agarose/EtBr gel run at 80 v for 45 min. M. 100 bp ladder; 1. NTC; 2. Pooled samples (maize gDNA and pure fungal gDNA); 3. GAF4 (control roots); 4. GAF4 (infected roots) 5. GAF4 (control shoots); 6. GAF4 (infected shoots); 7. KDV1 (control shoots); 8. KDV1 (infected shoots); 9. KDV1 (control roots); 10. KDV1 (infected roots); 11. KSM014 (positive control).*

3.2 Colonization of plant tissues by *Aspergillus flavus*

Aspergillus flavus KSM014 infection of both maize lines resulted in changes in maize phenotype with the KDV1 showing more severe symptoms that GAF4 (**Figure 2**). After 3–14 days post infection, the infected kernels for both maize lines showed stunted growth compared to control kernels (**Figure 2**). Additionally, the shoots and roots exhibited minimal growth with the *A. flavus* fungi colonizing the kernels and this could possibly explain the reason for stunted growth or germination. The phenotypic observations suggest that KDV1 maize line grown in Makeuni is more susceptible to fungal infection (*A. flavus*), whereas GAF4, grown in Kisumu and Homa bay appeared more resistant to the infection (**Figure 2**).

The observed phenotypic characteristics were further supported by the detection and quantification of fungal biomass load in gDNA extracted from infected and control plant tissues as revealed by the qPCR assay (**Figure 3**).

Insignificant difference was observed in biomass of fungi between infected plant tissues for the GAF4 and the control maize line (**Figure 3a**). In contrast, significant differences in biomass of fungi for the KDV1 maize line was exhibited upon infection ($p < 0.05$) for both the shoot and root tissue (**Figure 3b**). Fungal gDNA level was observed to be lower in the infected GAF4 maize line tissues compared to KDV1 suggesting that GAF4 was more resistant to *A. flavus* KSM014 infection than KDV1 (**Figure 3**).

The fungal biomass of *Alternaria dauci* was observed to be equivalent in two carrot cultivars between 1 and 15 days of post-inoculation, whereas it was found to be four-fold higher in the more susceptible cultivar between 21 and 25 days post-inoculation [21]. This suggests that fungal pathogens may colonize both susceptible and resistant cultivars in a similar manner during the first stages of the interaction, but fungal development is subsequently restricted in the partially resistant cultivar due to putative plant defense mechanisms [21].

It must be noted that we measured fungal biomass 14 days after infection when symptoms of the infection was phenotypically visible. However, other fungal biomass studies have shown that specific fungi could be identified even before the development of the symptoms. The presence of *Colletotrichum acutatum* by qPCR in

Figure 2.
The GAF4 and KDV1 maize lines after 14 days of growth with and without Aspergillus flavus *KSM014 infection. The red sticker shows infected maize plants while the white stickers are the control, uninfected maize plants.*

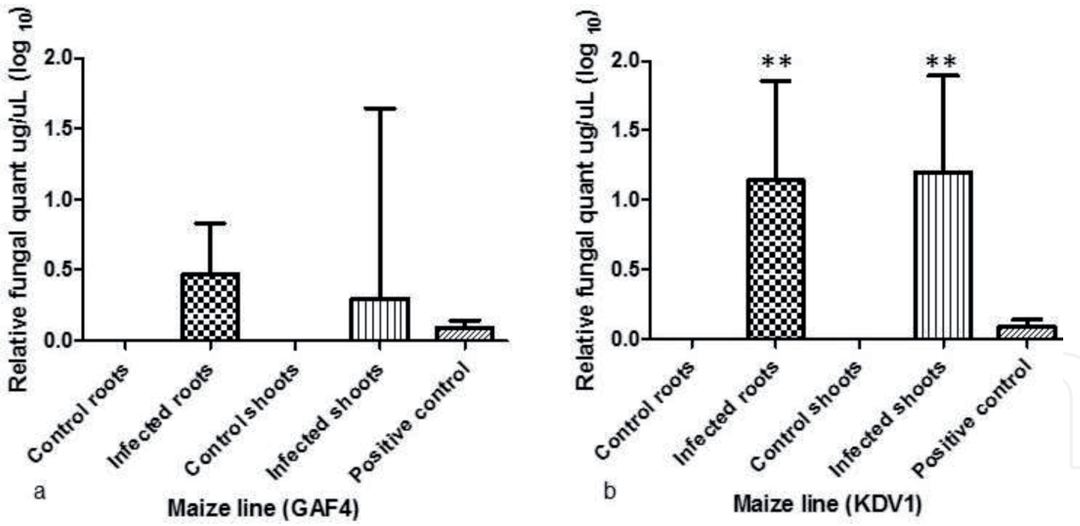

Figure 3.
Quantitative polymerase chain reaction analysis indicating fungal load of A. flavus *KSM014 in the shoot and root tissues of KDV1 and GAF4 maize* lines respectively. Biomass of fungi was measured in infected and non-infected *(control) GAF4 (a) and KDV1 (b) maize lines after 14 days where the A. flavus β-tub gene was used for quantification of fungi against the maize* MEP *gene. Tukey's multiple comparison test and one-way ANOVA ($P < 0.05$), was done where the asterisks indicate significance and the error bars shows standard mean deviation.*

strawberry leaves was detected by Debode et al. [22] 2 h post-inoculation whereas the initial symptoms of the disease appeared only after 96 h. Similarly, *Fusarium langsethiae* gDNA was accurately measured by Divon and Razzaghian [23] in oats independently from symptoms of the disease. These findings show the specificity and efficiency of the qPCR assay for the detection and quantification of fungal pathogens upon infection at early stages, before symptomatic appearances.

GAF4 is a *Striga* spp. resistant maize line cultivated in Kisumu, Kibos, Homa Bay and some parts of Nandi, while KDV1 is an open pollinated maize variety cultivated in Makueni and the neighboring counties. The observation that KDV1 maize line as more susceptible to aflatoxigenic *A. flavus* (KSM014) infection could be one of the contributing factors to why Makueni and the neighboring regions are more prone to frequent aflatoxicosis outbreak and high levels of aflatoxin contamination of the maize used for consumption.

The current study relates to the previous findings on Makueni maize samples [18] where they screened the strains of *A. flavus* isolated from maize kernels obtained from Makueni region on CAM media and found that there was significant variation in production of blue (toxigenic) and green (atoxigenic) fluorescence by most isolates. Seventy eight percent of the isolates from Makueni were observed to produce high amounts of aflatoxin AFB1, AFB2, the most potent carcinogen compared to other regions under study [18]. Additionally, studies conducted by Probst et al. [24] in eastern Kenya, revealed a similar result where they performed culture-based methods to monitor and describe the population structures of aflatoxigenic fungi and its closely associated strains on maize kernels. Moreover, a related study by Lewis et al. [25] and Klich [26] observed that in sub-Saharan Africa, products from subsistence farmers may reach the final consumer without the appropriated monitoring, resulting in critical risks for human health.

Moreover, the current study developed a qPCR assay using *A. flavus* gDNA and the *β*-tubulin gene for the quantification of *A. flavus* in maize tissue. Due to its high sensitivity and specificity, qPCR has been incorporated in official protocols of the European Plant Protection Organization (http://archives.eppo.org/index.htm) for the production, certification and assessment of healthy plant

materials [27, 28]. This could therefore, in future, provide a screening strategy for finding African maize cultivars that are resistant to *A. flavus* infection or as an assessment of healthy maize plants. Zhao et al. [29] developed a qPCR assay for the detection of *Magnaporthe poae* resistant *Poa pratensis* (Kentucky bluegrass turf), which typically needed 3 weeks to detect using conventional culture-based methods. Further, Montes-Borrego et al. [30] demonstrated that fungal presence can be detected earlier, enabling the selection of resistant plants even when samples are indistinguishable based on visual assessment. Lastly, the early detection of latent infections of rust on leaves of cereals was used to estimate infection levels before the appearance of the disease [2].

The genomic DNA extracted from the co-infected shoots of both maize lines showed varied concentrations of fungal biomass load compared to the roots according to analysis using 1-way ANOVA and TMCT test ($p < 0.05$). The quantification of *Verticillium dahliae* gDNA in different tomato cultivars also revealed the concentration of pathogen DNA in plant tissues increased and decreased in susceptible and resistant cultivars, respectively [31]. Similarly, significant differences were found in the amount of *F. oxysporum* DNA in roots of different chickpea cultivars [32], while the detection of *Phomopsis sclerotioides* in pumpkin, melon, cucumber and watermelon showed that infection and rate of disease development of this polyphagous pathogen may vary according to the host [33]. In general, Vandemark and Barker [34], concluded that low levels of pathogen DNA in resistant plants is indicative of a mechanism that inhibits pathogen growth, whereas, the presence of a relatively high amount of pathogen DNA in asymptomatic plants indicates a resistance mechanism based on tolerance rather than on true resistance.

4. Conclusion

The study demonstrated that KDV1 maize line was more susceptible to *A. flavus* infection when compared to GAF4. This also implies that a possible reason for the frequent cases of aflatoxicosis in Makeuni county is the fact that the KDV1 maize line is grown in that region is more susceptible to *A. flavus* infection.

The *β*-tubulin gene is a potential marker for quantification of the *A. flavus* biomass load in maize plants compared to *Ef1α*. The *MEP* gene for maize gDNA was also found to be plant specific by the absence of cross-reaction with fungal gDNA. The specificity of the qPCR assay for *A. flavus* biomass quantification makes it a useful tool in other areas such as screening of *A. flavus* resistant maize lines for breeding, determining possible asymptomatic infection and in plant-pathogen interaction studies.

The next chapter will focus on in vitro biocontrol approach in aflatoxin mitigation and bio-analytical approaches to detect and quantify aflatoxins. The aim is to determine whether biocontrol can minimize aflatoxin production and to find important metabolites that are produced by specific *A. flavus* isolates.

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Authors contribution

Data curation, Alfred Mitema; Formal analysis, Alfred Mitema and Naser Aliye Feto; Funding acquisition, Nasesr Aliye Feto; Investigation, Alfred Mitema; Methodology, Alfred Mitema and Naser Aliye Feto; Resources, Alfred Mitema and Naser Aliye Feto; Validation, Alfred Mitema; Writing—original draft, Alfred Mitema; Writing—review & editing, Alfred Mitema and Naser Aliye Feto.

Conflicts of interest

The authors declare no conflict of interest. The authors are responsible for the content and writing of the paper.

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