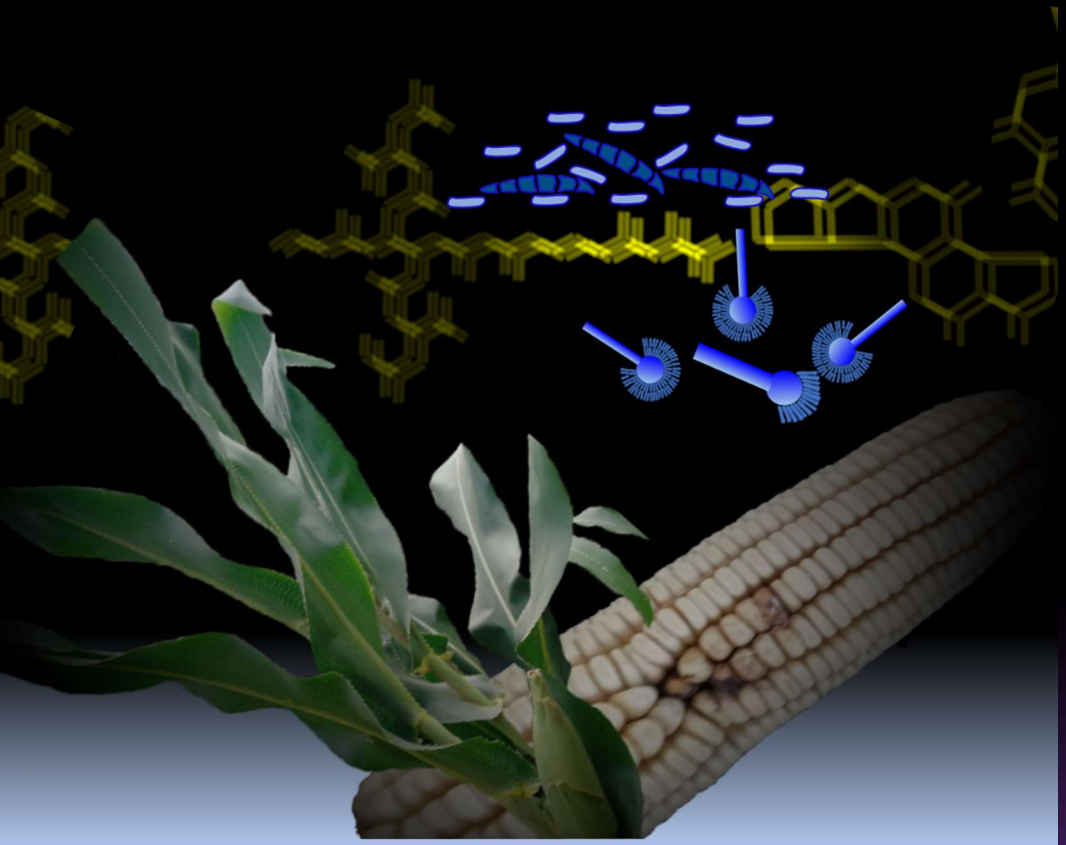
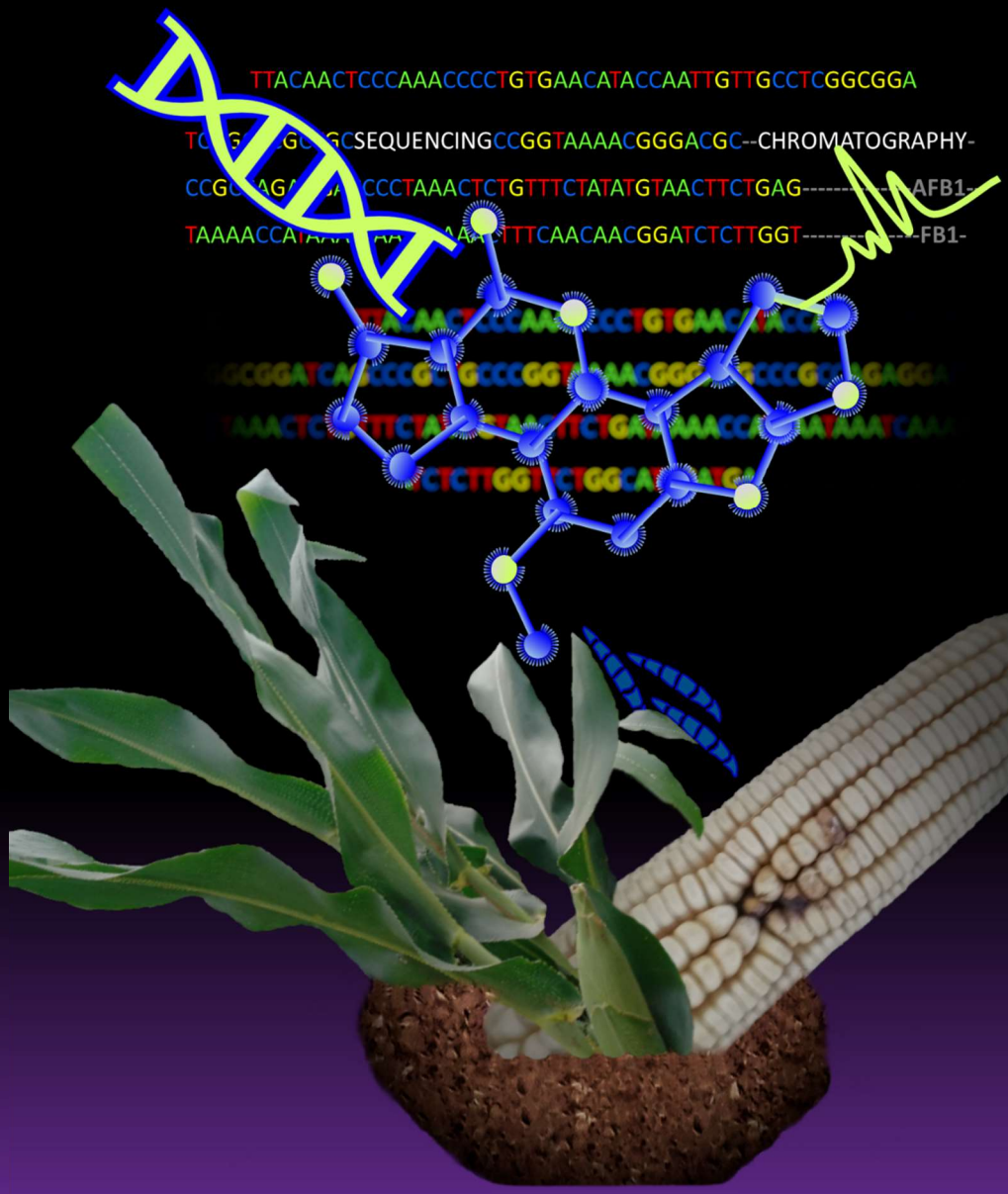


On the fungal contamination dynamics in maize: towards competitive exclusion to control mycotoxins

Bwalya Katati

2023

On the fungal contamination dynamics in maize: towards competitive exclusion to control mycotoxins



Bwalya Katati

Propositions

1. Non-pathogenic *Sarocladium* is the silver bullet to vanquish *Fusarium*'s fumonisin in maize.
   (this thesis)
2. Sacrificing fitness for mycotoxin production does not confer an advantage for toxigenic *Flavi* over atoxigenic counterparts.
   (this thesis)
3. The word 'and' should never appear in a hypothesis.
4. Randomly Amplified Polymorphic DNA PCR (RAPD) is best placed in the museum of lost scientific glories.
5. Research quality assessment must factor in the researcher's resource envelope.
6. The dykes are undutchable.
7. Social media has reduced freedom of public photography.

Propositions belonging to the thesis, entitled
"On the fungal contamination dynamics in maize: towards competitive exclusion to control mycotoxins"

Bwalya Katati
Wageningen, 2nd June 2023.

On the fungal contamination dynamics in maize: towards competitive exclusion to control mycotoxins

Bwalya Katati

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This research was conducted under the auspices of the C.T. De Wit Graduate School for Production Ecology and Resource Conservation

On the fungal contamination dynamics in maize: towards competitive exclusion to control mycotoxins

Bwalya Katati

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A. P. J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 2 June 2023
at 4 p.m. in the Omnia Auditorium.

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Contents

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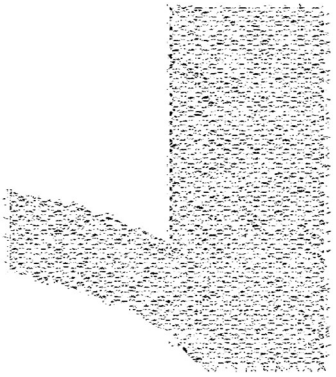


" …it came to pass
that the food
they had kept
got contaminated…" –

from Exodus 16:20
(The Bible)

Chapter I

General introduction

1.0 Overview

Fungi infecting crops are ubiquitous. In this thesis, I explore the contamination dynamics of fungi in preharvest maize with respect to abiotic and biotic stimuli. *Aspergillus* is of special interest due to its highly carcinogenic aflatoxin (AF), particularly AF-B1. In addition, *Fusarium* is of second interest due to the fumonisin (FB) also implicated in carcinogenicity, particularly FB1. *Aspergillus* section *Flavi* (*Flavi,* for short) are a diverse group of fungal species in the genus *Aspergillus* most of which are known for the production of B and G AFs. Examples of the *Flavi* include *A. flavus, A. parasiticus, A. nomius, A. pseudotamarii, A. bombycis, A. toxicarius, A. parvisclerotigenus* and *A. minisclerotigenes*. The main concept of study is how niche partitioning in fungi in response to specific exogenous abiotic stimuli may affect contamination and control of the mycotoxins FB and AF. I particularly look at rainfall as an abiotic stimulus influencing the fungal microbiome composition, AF and FB, as well as the influence of antioxidant on AF production in *Flavi*. My thesis further explores fungal correlations (Figure 1), as a biotic factor, in terms of how abundances change among fungi in response to the abiotic stimulus rainfall.

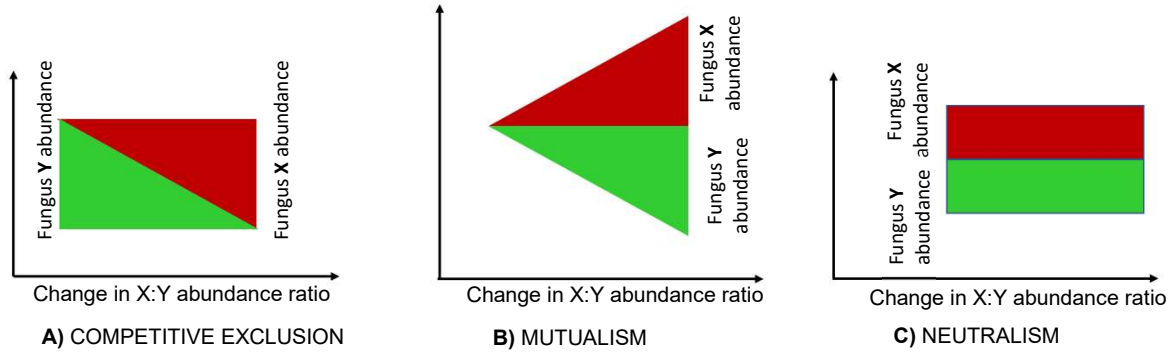


Figure 1. Idealistic correlations between two fungal genera with: (A) competitive exclusion (negative correlation); (B) mutualism; (C) neutralism (lack of correlation).

Identification of fungi in the mycobiome with negative correlation on *Fusarium* or *Aspergillus* suggests competitive exclusion, an indication of the prospective utilisation of such fungi in the control of *Fusarium* and *Aspergillus* proliferation on maize and subsequently their mycotoxins, FB and AF. In addition, identification of abiotic conditions that could have a diminutive effect on abundance of toxigenic *Flavi* compared to atoxigenic counterparts, or indeed able to suppress AF production by *Flavi*, could be useful in the control of the AF in maize as well.

From a Zambian perspective, the full community structure of fungi contaminating maize and how rainfall patterns may influence its composition remains a knowledge gap. This is despite maize being a cereal of such a high national importance. Furthermore, the *Flavi* community at species level still remains underexplored. This is despite the importance of *Flavi* community in defining the risk for maize AF contamination considering that within *Flavi*, certain species may have a higher propensity for aflatoxigenic virulence compared to others. For example, the higher AF virulence of *A. minisclerotigenes* in comparison with *A. oryzae*. The proliferation and dominance of *A. minisclerotigenes* in this case would pose a higher risk of AF contamination of the maize than *A. oryzae*.

This chapter will introduce the research in my thesis by presenting the background, the research problem, aims, objectives and research questions, and how these all cumulate to the rationale for the research.

1.1 Background

1.1.1 Maize mycobiome and mycotoxins in relation to the abiotic environment

A widely propagated grass transcending global grain, maize (Z*ea mays*) (Shahbandeh, 2021) is a widely consumed cereal in sub-Saharan Africa, Latin America and Asian countries, providing essential calories for both human and livestock. Unfortunately, maize is also a perfect substrate for a spectrum of fungi. The importance of maize fungal infection is that besides being drivers of disease themselves, some species of the various fungi produce harmful metabolites termed mycotoxins (D. Barug *et al.*, 2006; Munkvold *et al.*, 2019), with which they contaminate the maize. Common members of fungi thriving on preharvest maize include *Fusarium*, *Aspergillus*, and *Penicillium* (Ekwomadu *et al.*, 2018; Simpasa *et al.*, 2018).

The contamination of different crops with different fungi is fostered by abiotic and biotic factors. Rainfall, temperature and crop nutrient are known to be the important abiotic ones (Bernáldez *et al.*, 2017; Daou *et al.*, 2021; Roucou *et al.*, 2021). Such abiotic factors shape fungal specific niches and shape the dynamics of an ecological system (Arroyo *et al.*, 2008; Giorni *et al.*, 2009; Medina *et al.*, 2017; Mellon *et al.*, 2000; Mellon *et al.*, 2005; Sejakhosi Mohale *et al.*, 2013; Perrone *et al.*, 2020; Samsudin *et al.*, 2016). The fungal niche partitioning can result in different fungi having specific adaptations to specific crops or particular abiotic stresses. For example, while *Trichoderma* is a wheat

endophyte, *Fusarium* is both a maize and wheat endophyte, while fungi such as *Sarocladium* are largely regarded rice pathogens (Sakthivel *et al.*, 2002). Moderately xerophilic fungi such as *Aspergillus* and *Penicillium* are likely to proliferate in maize under dry conditions. For example, low rainfall areas are known to be prone to *Aspergillus* section *Flavi* species (*Flavi*) and AF contamination (Jaime-Garcia & Cotty, 2004; Kachapulula *et al.*, 2017b), which may indicate the better adaptation of *Flavi* to such abiotic conditions. Although the *Flavi* are a diverse group under the genus *Aspergillus*, key reported members are *A. flavus* and *A. parasiticus*. (Akello *et al.*, 2021; Dooso Oloo *et al.*, 2019; Horn, 2003; Kachapulula *et al.*, 2017b). Through niche partitioning, several fungi may thrive on the same host crop but utilise different niche resources. In case of niche overlaps, fungi may compete for the same niche resource, especially when the niche partitioning is not strictly delineated (Chesson, 2000): by competitive exclusion, propagules of one fungus may become more prominent than another. In quantifiable terms this could be seen as a negative correlation in abundance of one fungus against the other. An example of the competitive exclusion of one fungus by another is the case of *Fusarium* and *Trichoderma*, which has been deployed for the competitive exclusion of *Fusarium* in wheat (Filizola *et al.*, 2019; Juan Palazzini *et al.*, 2018). A similar approach is the deployment of non-aflatoxin producing *A. flavus* for the competitive exclusion of toxigenic *Flavi* (Alaniz Zanon *et al.*, 2018; Bandyopadhyay *et al.*, 2016; Cotty & Bhatnagar, 1994; Senghor *et al.*, 2020).

Flavi are important to maize due to the carcinogenic AF production of some species (IARC, 2012). While rainfall is a key abiotic factor in maize fungal infection, factors exacerbating the actual grain infection by *Flavi* include damage of kernels by insects (Barry *et al.*, 1985; Marsh & Payne, 1984; Widstrom, 1979; Widstrom *et al.*, 1975; Windham *et al.*, 1999), breed type of seed and husk condition (Barry *et al.*, 1986). In addition, the grain condition also dictates the contamination levels of grain with AF (Xu *et al.*, 2022). With soil being the generally agreed reservoir for *Flavi*, the diversity of the soil *Flavi* is considered to have a bearing on the risk factor for crops *Flavi* and AF contamination (Donner *et al.*, 2009; Kachapulula *et al.*, 2017b; Njoroge *et al.*, 2016).

AFs and FBs are among the important and widely studied mycotoxins on the global food safety landscape. AFs are mainly produced by *Aspergillus flavus* and *A. parasiticus* species, while FBs are largely produced by *Fusarium* species. Some secondary metabolites produced by some fungi are beneficial to humankind such as

penicillin produced by *Penicillium chrysogenum* (García-Estrada *et al.*, 2020) or *P. nalgiovense (Moavro et al., 2019)*. Unfortunately a large number of the mycotoxins either have no known benefit to human but rather harmful effects on human health and on livestock productivity (Peng *et al.*, 2018; Robens & L., 1992). For example, FB1 has been found to be the most toxic (Wentzel *et al.*, 1992; Yu *et al.*, 2020) of the Fumonisin-B variants (FB1, FB2, FB3, FB4). Of the unmasked *Fusarium* mycotoxins (Broekaert *et al.*, 2015), FB1 is on average the larger proportion (70-80%) of the total FBs in maize grain (Gil-Serna *et al.*, 2014). More importantly perhaps is aflatoxin-B1 (B1) due to its high carcinogenicity (IARC, 2012; McCullough & Lloyd, 2019; Rushing & Selim, 2019; Wogan, 1992) and lethal potency (Azziz-Baumgartner *et al.*, 2005; Kamala *et al.*, 2018; Njapau & Lewis, 2006).

1.1.2 Oxidative stress and aflatoxin (AF) production in *Flavi*.

The role of AF in *Flavi* continues to be explored. Amongst the roles include a response to biotic interactions among fungi, with microbials, or even with insects interacting with the fungi. Hence, it has been assigned a defensive role against microbials including fungivores (Arai *et al.*, 1967; Drott *et al.*, 2017; Trienens *et al.*, 2010). In addition, it has been reported to be a signature response to stress such as drought stress which can lead to an increase in expression of aflatoxin biosynthetic pathway genes (Angel Medina *et al.*, 2015) to enable the fungus continue to grow. Furthermore, it has also been demonstrated to be a response to oxidative stress (J. C. Fountain *et al.*, 2016; Fountain *et al.*, 2014; Jayashree & Subramanyam, 2000; Narasaiah *et al.*, 2006). The implication of the link of AF production to oxidative stress is that this could potentially lead to elevated levels of AF in crops that have low antioxidant levels, such as maize. Turning this around, increasing the antioxidative capacity of a host plant tissue is suggested to reduce AF production by *Flavi* (J. C. Fountain *et al.*, 2016), which would subsequently reduce the AF contamination, for example in the maize grain. This would also imply that crops such as maize, which are naturally deficient in elements such as selenium (Se) (Bocchini *et al.*, 2018; Schiavon *et al.*, 2020), may be more prone to AF contamination. Se is a known antioxidant and helps break down oxidative elements in the cells, which could otherwise damage living tissue and DNA (Chiang *et al.*, 2017; Vali *et al.*, 2018). Due to deficiency of maize in micronutrients such as iron, vitamin-A, Zinc (Goredema-Matongera *et al.*, 2021) as well as Se (Bocchini *et al.*, 2018; Schiavon *et al.*, 2020), coupled with the global deficiency of Se in world soils (dos Reis *et al.*,

2017; Haug *et al.*, 2007), it is not uncommon for the cereal to be biofortified to improve the nutrients availability in the crop. This can be through conventional breeding, genetic modification, or foliar application of the target mineral. This alters the abiotic environment of the seed, and this could have a bearing on the *Flavi* contamination dynamics and AFs.

1.1.3 Mitigating aflatoxin (AF) and fumonisin (FB) in maize

Due to harmfulness of AF (Cao *et al.*, 2022; Ivanovics *et al.*, 2021) and FB (Chen *et al.*, 2018; Kimanya *et al.*, 2010; Yu *et al.*, 2021) on both human and livestock, a number of interventions have been sought. Traditional methods are the more widely applied Good Agronomic Practices (GAP) as described by the World Health Organization (WHO, 2018b). These include sorting out of poor looking grain as this may more likely have higher mycotoxin contamination (Matumba *et al.*, 2021; Xu *et al.*, 2022); avoiding loose husk cover ears as these may harbour higher fungal loads, further risking contamination of tight husk cover ears and good quality grain with *Flavi* (Barry *et al.*, 1986; Xu *et al.*, 2022) and subsequently mycotoxin contamination. Beyond GAP measures, novel technological approaches such as breeding for germplasm naturally resistant to AF/FB contamination (WHO, 2018b) and the use of competitive exclusion in fungi have been pursued. Competitive exclusion has largely relied on identification of fungal species or genera as the Biological Control Agent (BCA) able to outcompete mycotoxigenic fungus (Bandyopadhyay *et al.*, 2016; Cotty & Bhatnagar, 1994; K. C. Ehrlich *et al.*, 2015; Filizola *et al.*, 2019; Moral *et al.*, 2020; Juan Palazzini *et al.*, 2018; Senghor *et al.*, 2020). One of the critical attributes of the BCA is that it should have the ecological adaptation to naturally thrive in the environment in which the pathogen thrives.

1.2 Research Problem

AF and FB continue to debilitate the global food safety landscape and are the key fungal metabolites that punctuate the mycotoxin contamination diction in Sub-Saharan Africa. Although some genera have been identified, the full spectrum of fungi contaminating maize, and how abiotic factors like rainfall can shape its composition has been underexplored. Although competitive exclusion of *Fusarium* by *Trichoderma* has been tried with success in crops such as wheat (Filizola *et al.*, 2019; Juan Palazzini *et al.*, 2018), identification of other potential fungi that can serve as Biological Control

Agents (BCA) to counteract *Fusarium* and FBs has not been exhaustive. Finding additional potential BCA is especially important as so far, the competitive exclusion of *Fusarium* in maize has not been successful at field level (Kagot *et al.*, 2019). With respect to *Aspergillus*, it is plausible that certain fungal genera would thrive within the same natural environment in which the genus *Aspergillus* thrives, but having a negative correlation with it (meaning one outcompetes the other within a given niche).

The importance of identifying a genus outside *Aspergillus* as a prospective BCA is that this would abate fears associated with the risk for genetic recombination between atoxigenic and toxigenic strains of the *Aspergilli* (Alberts *et al.*, 2017). Currently, the biocontrol of AF relies on the co-mingling between toxigenic and atoxigenic *Flavi* . This occurs through thigmoregulation, involving contact in hyphae between toxigenic and atoxigenic *Flavi* such that the atoxigenic interfere with growth of the toxigenic counterpart (Huang *et al.*, 2011; Xing *et al.*, 2017). In addition, it also relies on the degradation of B1 by atoxigenic *Flavi* initially produced by the toxigenic *Flavi* (Maxwell *et al.*, 2021). It is suggested by some studies that co-mingling between toxigenic and atoxigenic *Flavi* may potentially lead to genetic recombination between the two *Flavi* variants (Horn *et al.*, 2009; Olarte *et al.*, 2012; Varga *et al.*, 2014) hence the fears. Therefore, as an alternative to the current competitive exclusion of *Aspergilli*, abiotic conditions that can alter the population dynamics in favour of atoxigenic *Flavi* over toxigenic counterparts, or simply deter the production of AF in *Flavi,* would have to be explored. This is on basis that the success of a fungus to colonise the crop is based on niche adaptation which is dictated by the abiotic environment.

Although the soil is the natural reservoir for *Flavi*, it remains unclear how the *Flavi* diversity on soil may resonate with its diversity on maize. If the diversity in soil mirrors that on maize, it would become possible to predict the risk for AF contamination on the grain based on *Flavi* presence in soil. For example, some studies have predicted risk of *Flavi* and AF contamination in maize on basis of the soil *Flavi* toxigenicity (Donner *et al.*, 2009; Kachapulula *et al.*, 2017b; Njoroge *et al.*, 2016). However, it is probable that the diversity of *Flavi* on maize and soil would differ considering that the two are different ecological niches. Therefore, the extrapolation of the risk of infection of maize with *Aspergilli* on the basis of the soil population may require a further level of validation by better understanding the correlation in *Flavi* community structure between soil and maize. This would provide better intelligence in evaluating the risk of contamination of

maize with *Flavi* and AF based on the influence of the diversity of soil *Flavi* on maize *Flavi*, and subsequently the influence of maize *Flavi* diversity on AF. In Zambia, in addition to the soil-maize community structure of *Flavi*, the genetic diversity of *Flavi* naturally contaminating the maize remains unexplored. This creates the AF contamination risk of unidentified species such as *A. aflatoxiformans* or *A. minisclerotigenes*, the latter which is highly virulent and implicated in high contamination levels of AF in maize in eastern Kenya (Dooso Oloo *et al.*, 2019). This warrants the need to further catalogue the *Flavi* community in Zambia.

Considering that the production of AF has been linked to mitigation of oxidative stress in *Flavi* (Jake C. Fountain *et al.*, 2016; Fountain *et al.*, 2014; Jayashree & Subramanyam, 2000; Narasaiah *et al.*, 2006), it remains unclear whether the role of AF in toxigenic and atoxigenic *Flavi* is the same, considering that atoxigenic strains do not produce the AF. If the roles are different, it may imply that the response of the atoxigenic and toxigenic *Flavi* to an exogenous antioxidant would be different, for example because they differentially affect Darwinian fitness. If the two species variants respond differently to antioxidant, increasing the antioxidative capacity of the maize grain would imply either of the two would be fitter than the other upon infection of such grain due to difference in utilisation of the antioxidant. Toxigenic strains expend energy producing the AF through an intricate anabolic nexus of enzymatic conversion of precursor molecules to AFs. Under the same abiotic conditions, preventing the AF production through an antioxidant such as selenium (Se) would imply that the toxigenic strains would be fitter than the atoxigenic counterparts on assumption that the energy expended on AF production is liberated for other purposes such as growth or spore production. From a practical perspective, this would be viewed as the potential for biofortification of crop with such antioxidant to lead to the promotion of toxigenic isolates over atoxigenic counterparts, particularly in natural ecosystem where competition for niches occurs. It is noted that maize is naturally deficient in micronutrients such as Se. Hence the crop can at times be biofortified with the element. Considering that fitness response between toxigenic and atoxigenic *Flavi* may likely be different due to presence of such an antioxidant in their environment, it warrants the need to investigate the effect of Se on fitness of the two wildtype *Flavi* variants. This warrants the need to investigate and establish what sort of effect an antioxidant would have on fitness of toxigenic and atoxigenic strains. In addition, it would be useful to

validate the effect of the antioxidant on AF production in toxigenic *Flavi* as well as effect on degradation of B1 by the atoxigenic *Flavi*.

1.3 Aims and Objectives

The aim of my thesis is to describe the contamination dynamics of maize with fungi, FB and AF in relation to biotic and abiotic perturbation with Zambia as a relevant study area (Figure 2). The specific objectives and associated specific research questions of this study are outlined in Table 1 below.

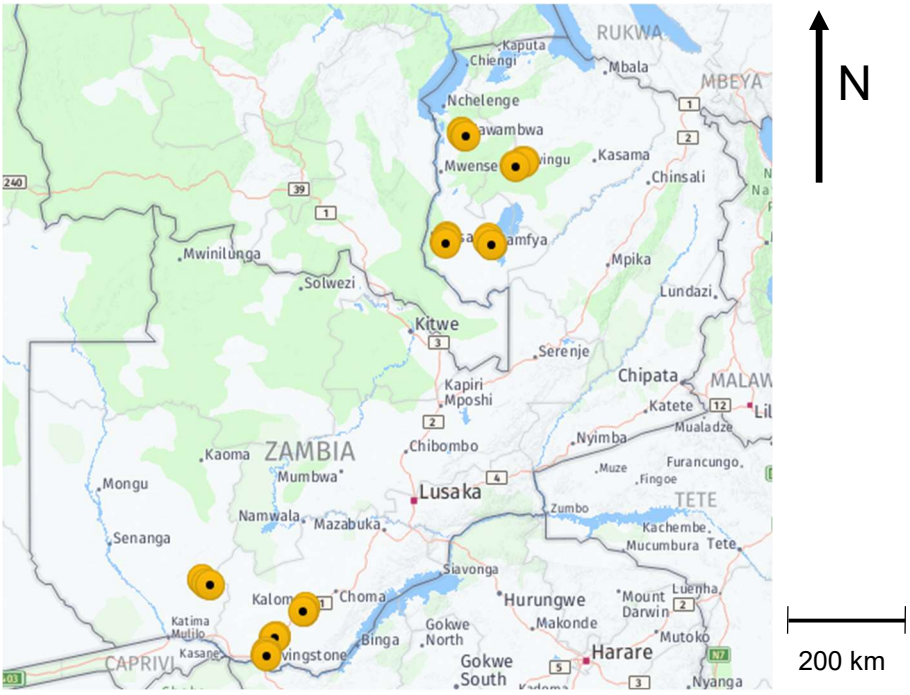


Image acquired with Heremaps™

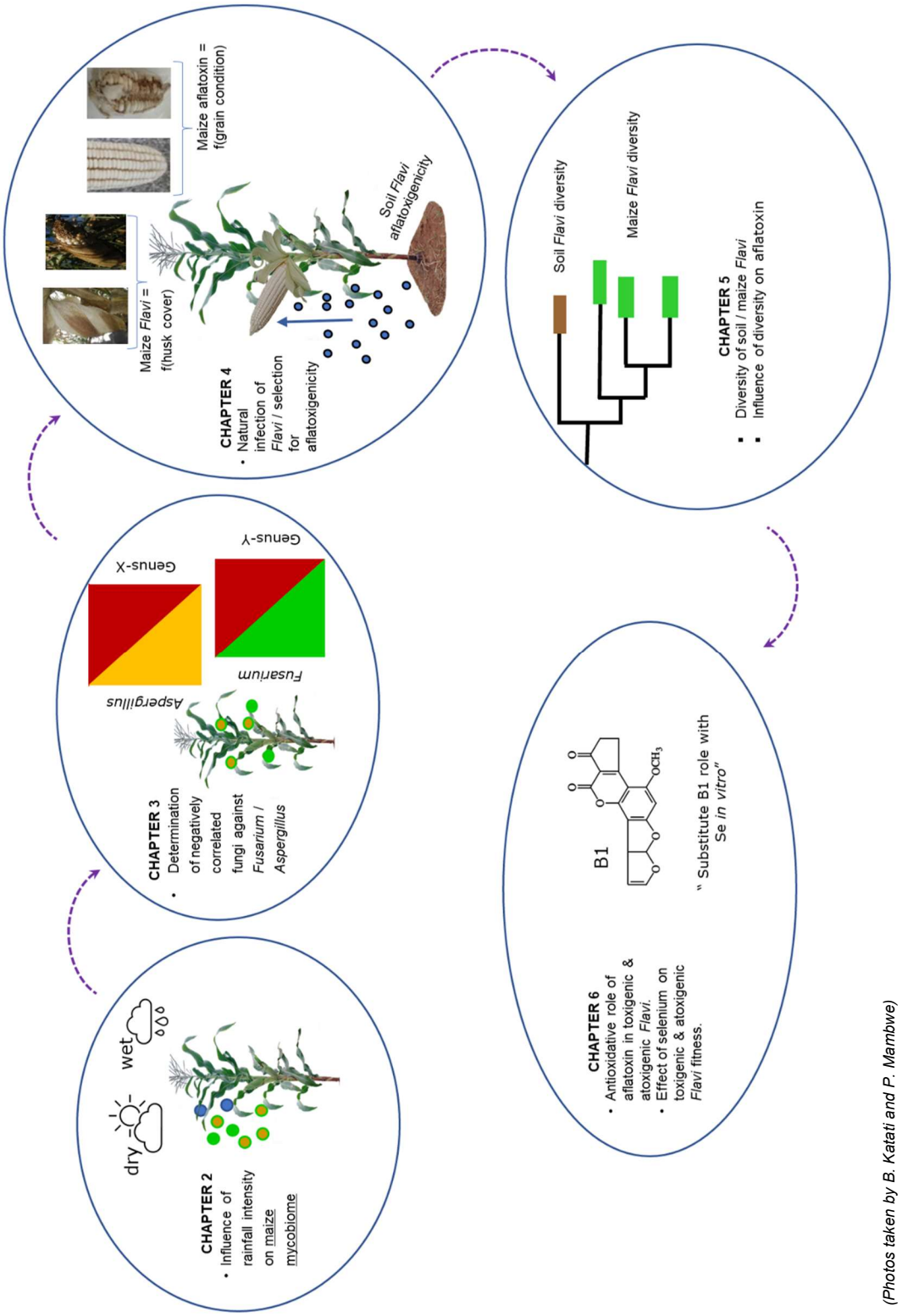
Figure 2. The map of Zambia with the eight selected districts earmarked for field maize sampling (yellow discs). The southerly sampling points are in the drier agroecological zone (AEZ) 1 receiving on average < 800 mm rainfall per annum (p.a.), while the northerly sampling points are in the wetter AEZ3 with average rainfall > 1000 mm p.a.

Table 1. Research objectives, questions and predictions

Objective	Question	Hypothesis and prediction
a) To describe the spectrum of fungi contaminating maize under different rainfall patterns.	- What is structure of preharvest maize mycobiome in Zambia.	- The structure of the preharvest mycobiome is largely composed of *Fusarium* and *Stenocarpella*.
	- What is mycobiome composition in relation to low and high rainfall patterns?	- Rainfall pattern influences the preharvest maize mycobiome composition.
b) Elucidate evidence of influence of niche partitioning on fungal genera abundances and subsequently FB and AF in maize.	- Which fungi in maize show negative correlation, by abundance, with *Fusarium* and *Aspergillus* irrespective of prevailing weather conditions?	- Fungal genera, whose niche is maize, exist with negative correlation between *Fusarium* or *Aspergillus* irrespective of prevailing weather conditions.
	- Which fungi show negative correlation with FB and AF?	- Fungal genera with a negative correlation with *Fusarium* (or *Aspergillus*) lead to lower levels of FB1 (or AF), when *Fusarium* (or *Aspergillus*) is in low abundance.
c) To investigate the risk for natural infection of preharvest maize with *Flavi* and AF.	- Does loose husk cover condition always lead to *Flavi* infection and subsequently aflatoxin contamination of preharvest maize?	- Loose husk cover does not lead to higher infection of preharvest maize with *Flavi* even when *Flavi* are present in soil.
	- What is selection frequency for mycotoxigenicity of soil *Flavi*.	- Soil isolates from the drier Agroecological zone (AEZ) 1 in Zambia, have a higher selection frequency for mycotoxigenicity than those from the wetter AEZ3.
d) To investigate the *Flavi* community structure between maize and soil, and how maize *Flavi* diversity may influence AF in maize.	- How is community structure of *Flavi* in soil and maize related?	- The *Flavi* community structure between maize and soil is different. Maize has a lower diversity than soil.
	- How does maize *Flavi* diversity affect its preharvest Zambian maize aflatoxin?	- The diversity of aflatoxigenic *Flavi* in Zambia influences the AF levels in preharvest maize.
e) Determine the role in which AF is utilised in toxigenic and atoxigenic *Flavi*, and the effect of antioxidant on fitness of atoxigenic and atoxigenic *Flavi*.	- Is AF utilised in the antioxidative mechanism by both toxigenic and atoxigenic *Flavi*?	- *Flavi* degrade AF in an antioxidative mechanism.
	- Does increasing antioxidant in *Flavi* environment lead to better fitness of toxigenic than atoxigenic *Flavi*?	- Toxigenic *Flavi* are fitter than atoxigenic counterparts under antioxidant environment.

1.4 Thesis outline

In **Chapter 1**, I introduce the context of the study, giving a background on the maize fungal infection. This is in the context of niche partitioning in fungi with maize as their ecological niche. Particular attention is paid to *Aspergillus* and its mycotoxin aflatoxin (AF). In addition, *Fusarium* is also considered with its mycotoxin fumonisin (FB). The research objectives as well as related research questions, and their hypotheses/predictions have been laid down. In **Chapter 2**, I explore the maize mycobiome in relation to different rainfall patterns of low and high precipitation in Zambia, as the study model (Figure 1 in Chapter 2). This lays the foundation for **Chapter 3** in which I describe the fungal correlations in terms of their relative abundance. The fungi have maize as their ecological niche, and I explore to identify negatively correlated genera with *Fusarium* and *Aspergillus*. The relations of how unrelated genera may influence FB and/or AF levels in maize is then explored and described. This gives prospects for application of such genus in competitive exclusion of genus like *Fusarium* for the mitigation of FBs. In **Chapter 4**, I investigate the natural infection of *Flavi* in maize by understanding the presence or absence of *Aspergilli* on preharvest maize in relation to presence in soil. Furthermore, I investigate influence of husk cover condition on *Aspergillus* infection of maize ears as well as the selection for toxigenicity in soil *Flavi*. This lays down the foundation for evaluating the risk of *Flavi* infection in maize due to soil *Flavi*. In **Chapter 5**, I describe the diversity of *Flavi* from a region prone to maize AFs in Zambia through a cross sectional study. I investigate the community structure of soil and maize *Flavi*. Furthermore, I investigate influence of toxigenic *Flavi* species on AF levels in preharvest maize. In **Chapter 6**, I explore the mechanism of AF degradation by atoxigenic isolates and its modulation by toxigenic isolates. Furthermore, I investigate differences in fitness between atoxigenic and toxigenic *Flavi* over different treatments with selenium as antioxidant. Lastly, in **Chapter 7,** I wrap up the study as a whole and offer the prospective future research and applications of the research findings on how they may contribute to solving the problem of AF as well as FB contamination in a key staple crop.



(Photos taken by B. Katati and P. Mambwe)

Figure 3. An overview of the thesis introduction, laying down the key subjects of investigation. Abbreviations: AF – aflatoxin; B1 – Aflatoxin-B1; Se - Selenium.

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If fungi were a human world, the earth would have had a very diverse human race

Chapter II

Preharvest maize fungal microbiome: case of Zambia's different rainfall patterns

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2.0 Abstract

The preharvest maize mycobiome may be crucial in defining the health of the crop in terms of potential disease burden and mycotoxins. We investigated the maize mycobiome structure at harvest including the influence of weather patterns, in terms of rainfall intensity, on its composition. Forty maize fields from selected districts in the wetter northern (N) and drier southern (S) agroecological zones of Zambia were sampled over two seasons (1 and 2). The defined weather variables over two sampling seasons were low rainfall with dry spell (S1), low rainfall (S2) and high rainfall (N1 and N2). High-throughput DNA amplicon sequencing of ITS1 was used to determine the mycobiome structure as well as the composition in relation to rainfall patterns. We detected 61 genera with *Fusarium* and previously unreported *Sarocladium* in Zambia having the highest frequency of detection on the maize samples. There was a significant difference in fungal genera composition between S1 and S2, but no difference between N1 and N2. The weather pattern with dry spell, S1, had a strong proliferation of *Meyerozyma* and moderately xerophilic *Penicillium*, *Kodamaea* and *Aspergillus*. The four genera drove the difference in composition between S1 and S2 and the significantly higher fungal diversity in S1 when compared with N2. Of the mycotoxin-important fungi, dry conditions (S1) seemed the key driver of proliferation of *Aspergillus*, while *Fusarium* proliferation occurred irrespective of weather patterns.

Key words: Fungi, ITS1 , Maize, Mycobiome.

2.1 Importance

Fungi contaminate various crops world-wide. Maize, an important human staple and livestock cereal, is susceptible to contamination with fungi in the field. Fungi are drivers of plant disease and can compromise yield. Some species of fungi are known to produce chemical compounds (mycotoxins) which are carcinogenic to humans and impair livestock productivity. It is important to understand the spectrum of fungi on maize and how weather conditions can impact their abundance. This is because the abundance of fungi in the field can have a bearing on the health of the crop as well as potential for mycotoxins contamination. By understanding the spectrum of the preharvest fungi it becomes possible to know the key fungi adapted to the maize and subsequently the potential for crop disease as well as mycotoxins contamination. The influence of weather conditions on the spectrum of preharvest fungi on maize has not been fully explored.

2.2 Introduction

Maize (*Zea mays*) is an important and widely propagated cereal (Shahbandeh, 2021) providing essential calories for both human food and livestock feed world-wide. In Zambia, for example, maize is the principal staple and cash crop (Tembo & Sitko, 2013) and approximately 65% of the arable fields are covered by maize. Annual per capita consumption of maize in Zambia is estimated at 105 kg ((De Groote et al., 2019); https://www.yieldgap.org/zambia).

Unfortunately, maize is often contaminated with an array of fungal genera in the field. *Aspergillus*, *Fusarium, Penicillium* and *Stenocarpella* have a world-wide spread and have been commonly reported as fungi contaminating maize (Ekwomadu *et al.*, 2018; Fandohan *et al.*, 2003; Mukanga *et al.*, 2010; Simpasa *et al.*, 2018). The importance of fungal genera to crop is that some species may be agents of plant disease. For example, genera such as *Ustilago* are known for causing smut in maize (Regine Kahmann *et al.*, 2018). Furthermore, *Fusarium* and *Stenocarpella*, which have been reported in Zambia (Mukanga *et al.*, 2010) and several studies elsewhere are associated with maize ear rot (Duan *et al.*, 2016; García-Reyes *et al.*, 2022; Luna *et al.*, 2016; Williams *et al.*, 2019). Members of the genus *Fusarium* are well-known pathogens both in the tropics and temperate zones (van Diepeningen *et al.*, 2015) and commonly occur on maize in the field. *Aspergillus* and *Penicillium* are known to be

post-harvest opportunistic pathogens especially proliferating during storage. The other importance of fungi to crop is that some species of certain genera are also known to produce metabolites termed mycotoxins (Daniel Barug *et al.*, 2006; Munkvold *et al.*, 2019) which are harmful to human health and impair livestock productivity. For example, *Fusarium*, *Aspergillus*, and *Penicillium* are the most infamous producers of the important mycotoxins, fumonisin (FB; *Fusarium*), aflatoxin (AF; *Aspergillus*) and ochratoxin (OTA; *Penicillium*) (Frisvad *et al.*, 2019; Gil-Serna *et al.*, 2014; Perera *et al.*, 2021) as well as other compounds such as cyclopiazonic acid.

Temperature and water availability are important factors in fungal contamination of crops such as maize, wheat and rice (Perrone *et al.*, 2020). In tropical climates such as Zambia, the climatic conditions can promote fungal growth and subsequently fungal contamination of crop. In the face of climate change, Southern African countries are expected to encounter increased frequencies of dry spells, droughts as well as a higher frequency of periods of heavy precipitation, resulting in unevenness of the rainfall patterns (Nicholson *et al.*, 2018; Phiri *et al.*, 2013). Dry spells during plant growth may translate into higher chances of contamination of grain in the field with xerophilic and moderately xerophilic fungi including *Aspergillus* and *Penicillium*.

Although studies have indicated *Penicillium*, *Fusarium* and *Aspergillus* to be the common contaminants of maize, it remains possible that other genera could be of equal importance in the contamination of preharvest maize. The importance would be in terms of their potential plant-disease burden due to their high frequency of contamination of the maize in the field. Furthermore, the maize could be host to less reported or indeed rare genera which may be of phytosanitary importance in terms of plant disease or mycotoxins. Knowledge of the preharvest maize mycobiome structure is vital as the structure has a bearing on prevalence and relative abundance of pathogenic and mycotoxigenic fungi. For example, the prevalence of *Ustilago* in the mycobiome may have an impact on plant diseases like smut (Regine Kahmann *et al.*, 2018) or maize ear rot (Duan *et al.*, 2016; García-Reyes *et al.*, 2022; Luna *et al.*, 2016). *Ustilago* is seldom reported in sub-Saharan Africa (SSA) despite such a plant disease threat. Despite the known importance of weather patterns in shaping the fungal contamination of crops, it remains unknown how such patterns may influence the maize mycobiome composition in general. In view of the knowledge gaps on seldom or unreported genera, the objectives of this study were to: (1) Establish the structure

of the preharvest maize mycobiome in Zambia. We anticipate a higher diversity of preharvest maize fungi than previously reported, highly contested by *Fusarium* and *Stenocarpella*, as the commonly reported non-xerophilic field fungi. (2) Determine the influence of prevailing weather conditions on the preharvest maize mycobiome composition. We hypothesise that the composition of the mycobiome is influenced by weather conditions.

2.3 Materials and Methods

2.3.1 Study area and assignment of weather variables

Two regions of Zambia with known contrasting climatic patterns, dry and hot versus wet and comparatively cooler, were selected for the study. The first region was to the south (S) of Zambia (within Lat: 16° 36' 54" S to 17° 46' 40" S; Long: 24° 49' 57" E to 26° 31' 35" E) from which four districts were selected for the study (Figure 1) and is part of Zambia's agroecological zone (AEZ) 1. The second region was in the north (N) of Zambia (within Lat: 9° 44' 55" S to 11° 30' 21" S; Long: 28 °46' 35" E to 30° 5' 40" E) from which four districts were selected for the study and is part of AEZ3. The two AEZs are climatically contrasting regions such that on average AEZ1, characterised as a low rainfall area, receives less rainfall (600 - 800 mm) per annum (p.a.) and is hotter (30 – 36 ºC) than AEZ3. AEZ3, characterised as a high rainfall zone, receives higher rainfall (1000 – 1500 mm) p.a. and has comparatively lower average temperature (30 – 33 ºC) ((Bunyolo *et al.*, 1995; Phiri *et al.*, 2013); https://www.yieldgap.org/zambia; live rainfall and temperature data available at Zambian online weather portal http://41.72.104.142:8080/secure/common/main.vm). For example, between 2010 and 2012 from this historic data, Mansa district (under AEZ3 in our study) received 1367 (± 189) mm rainfall with mean maximum annual temperature of 32.5 (± 0.4)°C. In the same period, Livingstone district (under AEZ1 in our study) received 695 (± 137) mm rainfall with mean maximum annual temperature of 36.3 (± 0.5)°C. The collection of preharvest field maize was carried out over two maize cropping seasons in 2018/2019 (season-1) and 2020/2021 (season-2). For this study, AEZ with interaction of maize cropping season was defined as weather variable 'S1' (AEZ1 in Season1), 'N1' (AEZ3 in Season1), 'S2' (AEZ1 in Season2) and 'N2' (AEZ3 in Season2). The variables were defined by rainfall pattern as low rainfall with dry spell (S1), low rainfall (S2) and high rainfall (N1 and N2). During the study S1 had a prolonged dry spell during the maize growth season-1 from 15th February to early

April 2019, followed by about one to three precipitations for about a week. S2 had the stable rains according to normal conditions of AEZ1. Similarly, N1 and N2 had stable rains according to average weather conditions of AEZ3.

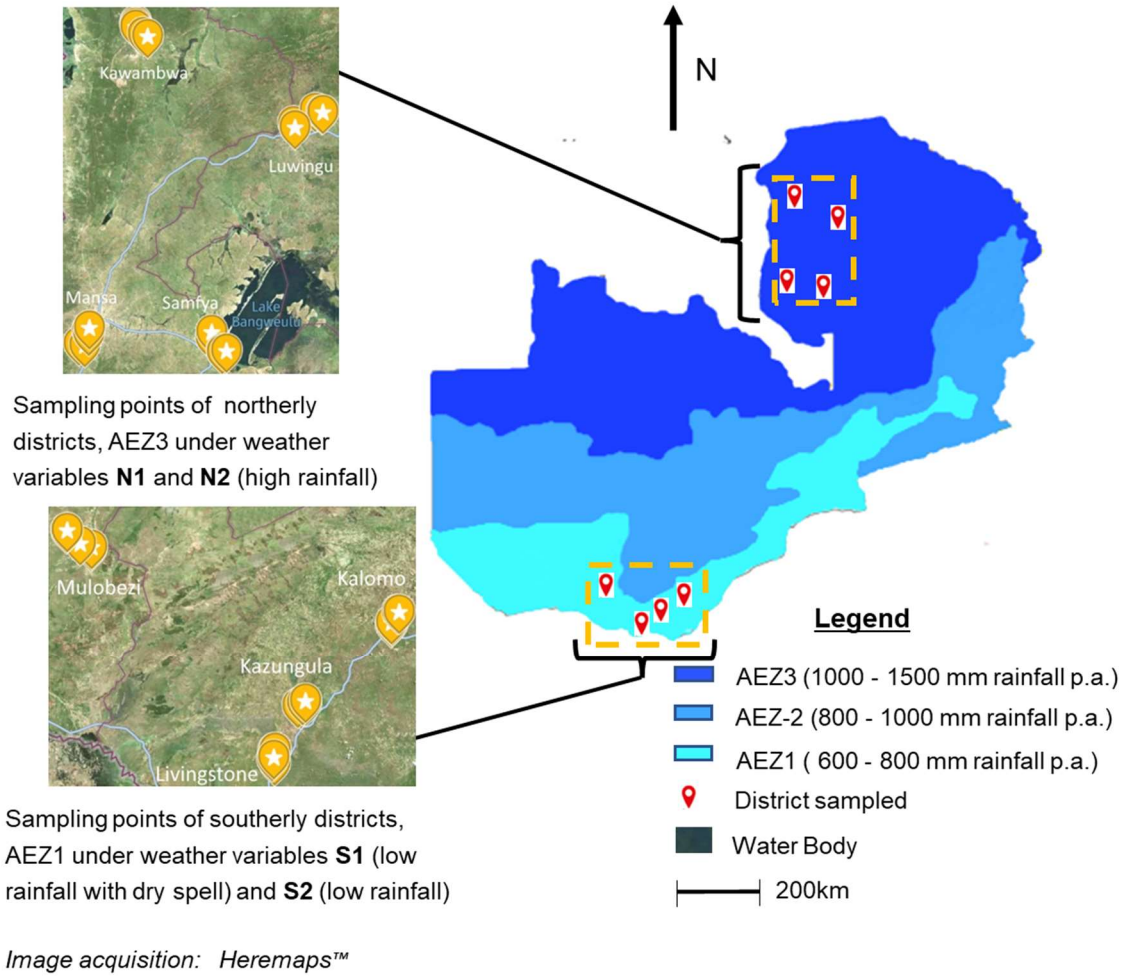


Figure 1. Zambia's agroecological zones and sampling points from selected districts.

2.3.2 Sampling

Sampling per AEZ was carried out immediately farmers began harvesting, mid-April for AEZ1 (low rainfall zone) and early June for AEZ3 (high rainfall zone). The timing of the sample collection was done with prior consultation with field agricultural extension officers. Per sampling period, four districts were selected from each AEZ. Selection of districts was on the basis that they were of agricultural importance by production tonnage of maize, human population, as well as their accessibility to commerce in terms of sales of harvest to commercial crop marketing agencies/ enterprises. Per district, five maize fields were purposively selected for sampling. Therefore, 20 fields

were sampled in each AEZ per sampling period. Consequently a total of 80 maize fields were sampled for the entire study.

In both study regions, maize field sizes varied between 1.0 – 2.5 acres. A minimum of 1 km between selected sampling fields was observed to avoid possible influence of neighbouring fields' mycobiomes on each other. The collection of maize cobs per selected field was executed according to the method described by (Jaime-Garcia & Cotty, 2010). A total of 30 maize cobs per field were collected along two diagonal transects. For season-1, cobs with poor husk cover were substituted with nearest well covered cobs to avoid chances of superficial fungal contamination from the soil or air. For season-2 sampling, an equal number of good husk covered and poor husk covered cobs (n = 15 per set) per field were collected. For all collections, the maize cobs were retained in their husks and placed in permeable potato bags and weighed. On-site moisture determination (Supplemental Table 1) was carried out using a portable moisture meter (model MT-Pro+, AgraTroinx, OH, USA). This was done on two extra cobs collected randomly from two separate points in the field over the two diagonals.

2.3.3 Sample preparation

Samples were dried without removing husks at (42 ± 2°C, 48hrs) to a moisture content of about 8 - 13% in a Forced Draft Oven (model D-6450, Heraeus, Hanau, Germany). This was done to prevent any potential growth of fungi or mycotoxin build up. The dried samples were then shelled, without touching the kernels, with a sterilised clean rod in a sterile PVC bag. For both seasons, the shelled grains from one transect with good-husk cover cobs were thoroughly homogenised and split into two approximately equal parts and stored at -35°C in freezer (model 3565 S, Scientemp, Michigan 49221, USA) pending further analyses. One portion was reserved for mycotoxins analysis that would be done in other investigations while the other part was for mycobiome assays.

2.3.4 DNA extraction

All the water used in the assays was Milli-Q type-1 water from a deioniser (Integral-3 Milli-Q water Deioniser, Merck, Darmstadt, Germany), and sterilised by autoclaving at 120°C for 15 min. Prior to DNA extraction, a pellet from the maize surface wash containing target fungal DNA was generated. Like in a similar study, we used centrifugation to pelletise the wash (Lane *et al.*, 2018).

In our protocol, for each sample 100 g kernels were weighed into a sterile 250 ml Erlenmeyer flask. Next, 35 ml of sterile 0.05% Triton-X solution was added to the flask. The flasks were shaken for 3 min at amplitude 12 on a shaker (Burrell Wrist-action shaker, Model 75, Burrell Scientific, LLC, Pittsburgh, PA, USA). The contents were then swirled by hand for about 10 s to homogenise and the solution passed through a sterilised 1.2 mm nylon mesh into a sterile 50 ml polypropylene tube to separate kernels from the wash. The tube was centrifuged at 12,751 *g* for 10 min in a temperature-controlled centrifuge (Suppra 22K, Hanil Scientific Inc., Gimpo 10136, South Korea) set at 10℃. All the 1st supernatant was removed, leaving about 3 ml with the pellet that had formed at the bottom of the tube. The pellet was resuspended by briefly vortexing and two equal portions of the suspension transferred to two 2 ml microcentrifuge tubes. The microcentrifuge tubes were centrifuged at 14,000 rpm for 5 min in a temperature-controlled centrifuge (Model 5403, Eppendorf, Hamburg, Germany) set at 4℃. The 2nd supernatant was removed without agitating the pellet. To wash off the residue surfactant (Triton-X) 1000 µl of sterile water was added to the microcentrifuge tubes. The tubes were then vortexed to resuspend the pellet and recentrifuged at 4°C as in the previous step. The 3rd supernatant was removed, the washing step repeated, and final 4th supernatant separated from pellet. The possible loss in spore quantity due to the multiple pellet washing was estimated by dilution plating on PDA (Supplemental Table 2).

DNA, which included target fungal DNA, was extracted from the pellet using the PowerSoil™ DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA). A 400 µl of CD1 lysis buffer was added to each of the two tubes containing sample pellet. The contents were vortexed and the pellet suspensions from both tubes transferred to one bead-beating tube. The prescribed PowerSoil DNA isolation kit protocol (https://qiagen.com/nl, kit catalogue No. 47014) was used to isolate the DNA. Tube beating was carried out using a homogeniser (Model MM200 Retch, Haan, Germany) set at 25 beats/second for 12 min continuously. The bead-beating tube was then centrifuged at room temperature in a microcentrifuge unit (model 5403, Eppendorf, Hamburg, Germany). The isolated DNA concentration was read on a spectrometer (Nanodrop model 2000, ThermoScientific, Wilmington, DE, USA).

To test the robustness of the amplicon sequencing, a mock community of fungal DNA was generated and treated as samples alongside the season-2 samples. This was for

quality control purposes to determine accuracy of the DNA recovery from PCR amplification, sequencing, raw data pipeline clean up and finally the generation of amplicon sequence variants (ASVs) and fungal genera abundance. The mock community consisted of a pure *Fusarium verticillioides* isolate DNA as the genus with a high ASV relative abundance on maize and pure *Aspergillus flavus* isolate DNA as the genus with a lower ASV relative abundance on maize. The DNA for spp. *verticillioides* and spp. *flavus*, upon dilution of each to 10 ng/µl, were mixed in percentage ratios: 95:05, 85:15, 50:50, respectively, in duplicate.

Furthermore, we cultured *Aspergillus* from maize kernels by dilution plating to compare fungal abundance by culture based analysis with DNA amplicon sequencing. In this approach, *Aspergillus* section *Flavi* was quantified from kernels washes on modified rose Bengal agar (MRB) by dilution plating as previously described by (Cotty, 1994). Briefly, 40 g of kernels were transferred to a 250 ml sterile bottle. Next, 40 ml 0.05% sterile Triton-X was added. Contents were shaken on a sideways shaker (GFL model 3018, Society for Laboratory Technology, Burgwedel, Germany) at 200 rpm for 10 min This made a 1x initial extract. For the plating, a 1 ml of the initial extract was diluted to a 0.5x solution then serially diluted up to 1 x 10^{-3}, with agitation during each pipetting. A 150 µl suspension was plated on 90 mm petri dishes of MRB and spread with the help of sterile 3 mm glass beads (20 to 30 beads per plate). MRB plates were incubated at 31°C (3 days, dark). Colonies were enumerated as CFU/g kernels based on their characteristic morphology for *Flavi* (Figure 2, (Cotty, 1994)). The culturing, in order to compare *Aspergillus* abundance by dilution plating with DNA amplicon sequencing, was also done on all the 80 sample collections representing the different weather variables (S1, low rainfall with dry spell; S2 low rainfall; N1, high rainfall; N2 high rainfall).

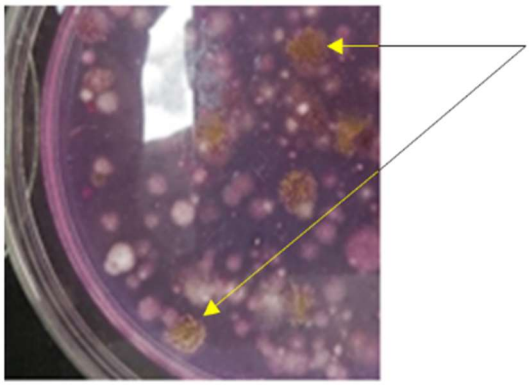


Figure 2.
Colonies characteristic of *Aspergillus* section *Flavi* on MRB medium pending passage to PDA for isolate cleaning.

2.3.5 High-throughput DNA Sequencing and bioinformatic analysis

The purified template DNA concentration per sample was normalised to 10 ng/µl from initial concentrations determined on the Nanodrop spectrometer. The DNA was sequenced on the platform Illumina, Miseq V3, by paired-end amplicon sequencing (2 x 300bp) at LGC Genomics (Biosearch Technologies, Berlin, Germany). The targeted region for the bi-directional sequencing was the ITS1 (Nuclear Ribosomal Internal Transcribed Spacer 1) region of the fungal genomic DNA. Amplification of the ITS1 region, partly overlapping into 5.8S region, was achieved using the following primer set: ITS1F_Kyo2 (forward) TAG AGG AAG TAA AAG TCG TAA and ITS86R (reverse) TTC AAA GAT TCG ATG ATT CAC. We selected ITS1 in preference to ITS2 for its higher specificity for fungal DNA against non-fungal DNA (Scibetta *et al.*, 2018). This is considering that environmental DNA samples would be highly laden with non-target plant DNA.

The adaptor- and primer-clipped raw data after the sequencing was in form of demultiplexed samples with an average sequencing depth of 131,351 (± 26,239) total reads per sample for season-1, and 229,027 (± 31,270) total reads per sample for season-2. All reads of final length <100 bp had been discarded. The raw data was then processed in the bioinformatics pipeline DADA2 (Divisive Amplicon Denoising Algorithm version 2) (Callahan *et al.*, 2016). This was for reads quality adjustment and assembly, generation of amplicon sequence variants (ASVs) from the raw reads, and the assigning of taxa to the ASVs. Briefly, the forward and reverse reads upon filtering were set to a precautionary minimum length of 50 bp to avoid any possible low quality reads as an alternative to read-end truncation. Read-end truncation was avoided due to high within species ITS sequence length variations. Maximum expected error rate in the algorithm was set at ‘2’ and all ‘N’ nucleotides forbidden. From an input of 6,512 unique sequences which had been generated from 40,182 total sequences read, the pipeline generated 92 true sequence variants. The denoised forward and reverse reads were then merged and a sequence table matrix constructed, composed of sample IDs (rows) and ASVs (columns). Chimeric sequences were then removed to exclude artifact fungal sequences. The chimeras comprised 10% of merged sequence reads, giving an acceptable final 90% recovery of the raw input sequence variants. Taxonomy was then assigned to the generated non-chimeric ASVs by naive Bayesian classifier using the UNITE ITS fungal database, version 8.0 of 2019

(UNITE_Communtiy, 2019). The generated sequence table matrix was then processed in Phyloseq (McMurdie & Holmes, 2013) for the data analysis.

2.3.6 Data analysis

All statistical computations were conducted in software R (R_Core_Team, 2017) version 4.1.0 using the below packages and their functions:

Phyloseq (McMurdie & Holmes, 2013) was used to generate the ASV relative abundances of fungal genera. Vegan ("Vegetation analysis") (Oksanen *et al.*, 2010) was used for computation of taxa diversity and composition. The package ggplot2 (Wickham, 2016) was deployed for visualisations.

Fungal genera abundance/composition - Seasonal fungal composition over the weather variables was evaluated using pairwise adonis2, with Bonferroni correction of *P* value. Permanova was used for analysis of the ecological data (species composition) due to highly heterogenous distribution of such data (Anderson & Walsh, 2013). Fungal diversity was determined over the weather variables' (S1, low rainfall with dry spell; S2 low rainfall; N1, high rainfall; N2 high rainfall) using the Shannon diversity measure. This was on basis of the precision of the inferred species across the four variables (S1, S2, N1 and N2). Differences in computed fungal alpha diversity across the weather variables were evaluated by ANOVA (applied for the normal distributed alpha diversity data). The Wilcoxon rank sum test was used to determine changes in fungal genera abundances between weather variables for non-normal distribution or ANOVA for normal distribution data. Similarly, for comparisons of specific genera between AEZ, Kruskal-Wallis rank sum test was used for non-normal distributions (Shapiro Wilk test, $P < 0.05$) and *t*-test for normal distributions. To compare *Aspergillus* relative abundance/quantity between HDSeq (%) and dilution plating (CFU/g), the ranked test (Spearman rank correlation) was used. This is given that the two methods did not produce the same abundance measurement, but were rather expected to produce a good level of positive correlation. For example, expectation is that absence of *Aspergillus* by HDSeq should show an absence of the *Aspergillus* by plating.

Mycobiome composition in relation to weather variables - The weather factor considered in the test was rainfall. The dependent variable, genera relative abundance,

was computed against the four weather variables S1 (low rainfall with dry spell), S2 (low rainfall), N1 (high rainfall) and N2 (high rainfall).

For data availability, DNA Sequences of the fungal amplicon sequence variants (ASVs) were deposited to GenBank. Code for mycobiome census as well as GenBank accession numbers for ASVs are found at https://github.com/bkatati/mmycobiome. Additional data is available upon request from corresponding authors.

2.4 Results

To map the fungal microbiome by DNA amplicon sequencing, we used the commonly employed method of targeting the ITS region. Considering the high accuracy of amplification, sequencing and ASVs generation in the mock community, (Supplemental Table 3), we assume that all generated taxa in the samples amplified similarly. This is given the fact that the mock and test samples were both ITS1 DNA. We furthermore assume that the generation of sample ASVs and genera abundances was as accurate as that for the mock community such that the output sequencing data reflects relative abundance of the genera in the total population. Furthermore, by using the PowerSoil kit, we assumed that the DNA of all fungal species was efficiently extracted, to represent the fungal community on kernels. This is coupled with the non-significant loss of viable fungal spores (< 0.6% loss, at 95% C.L) due to washing during the pellet preparation (Supplemental Table 2). In addition to this, the robustness of the sequencing was tested on basis of *Aspergillus* by pairing HDSeq data with dilution plating data. It should be noted that despite the two being different methods of fungal enumeration, they are expected to produce a good level of positive correlation of abundances. The result showed a significantly strong positive correlation in *Aspergillus* between HDSeq abundance (%) and dilution plating quantities (CFU/g) (Spearman rank correlation rho = 0.78, $P < 0.001$). Abundances between plating and sequencing are provided in Supplemental Figure 1).

2.4.1 Preharvest maize mycobiome structure

Based on pooled data from agroecological zone (AEZ) 1 and AEZ3, and mycobiome census, a total of 61 fungal genera were detected. *Sarocladium*, previously unreported on Zambian maize, and seldom reported to occur on maize from other sub-regional studies, was detected in high frequency (100%) throughout the fields. Furthermore,

with respect to mycotoxin-important fungi, *Fusarium* was the dominant genus similarly with a 100% field frequency of detection like *Sarocladium*. *Fusarium* and *Sarocladium* had the highest ASV representation of 45% and 37%, respectively (Figure 3). Although *Fusarium* and *Sarocladium* had the highest ASV representations across locations (districts) which were also balance (*Fusarium* 44.8 ± 13.8%; *Sarocladium* 36.5 ± 13.1%), an exception was observed with Samfya district. The district had lower average relative abundance of ASVs of *Fusarium* (17%) and higher average for *Sarocladium* (56%). The exception is further demonstrated by positions of the field's principle coordinates for *Sarocladium* under district Samfya which are highly biased towards the arbitrary positive direction from the zero-axis (Figure 4B). For the rest of the districts, coordinates for *Fusarium* and *Sarocladium* evenly distributed across the zero-axis towards the arbitrary positive and negative values. *Aspergillus*, a mycotoxin-important genus like *Fusarium*, had a 10 - 100% field detection frequency which was weather variable dependent, highly detected under the low rainfall with dry spell variable S1 (Table 1). *Penicillium*, a reported common maize contaminant, had a high frequency of field contamination with average 76% across the weather variables.

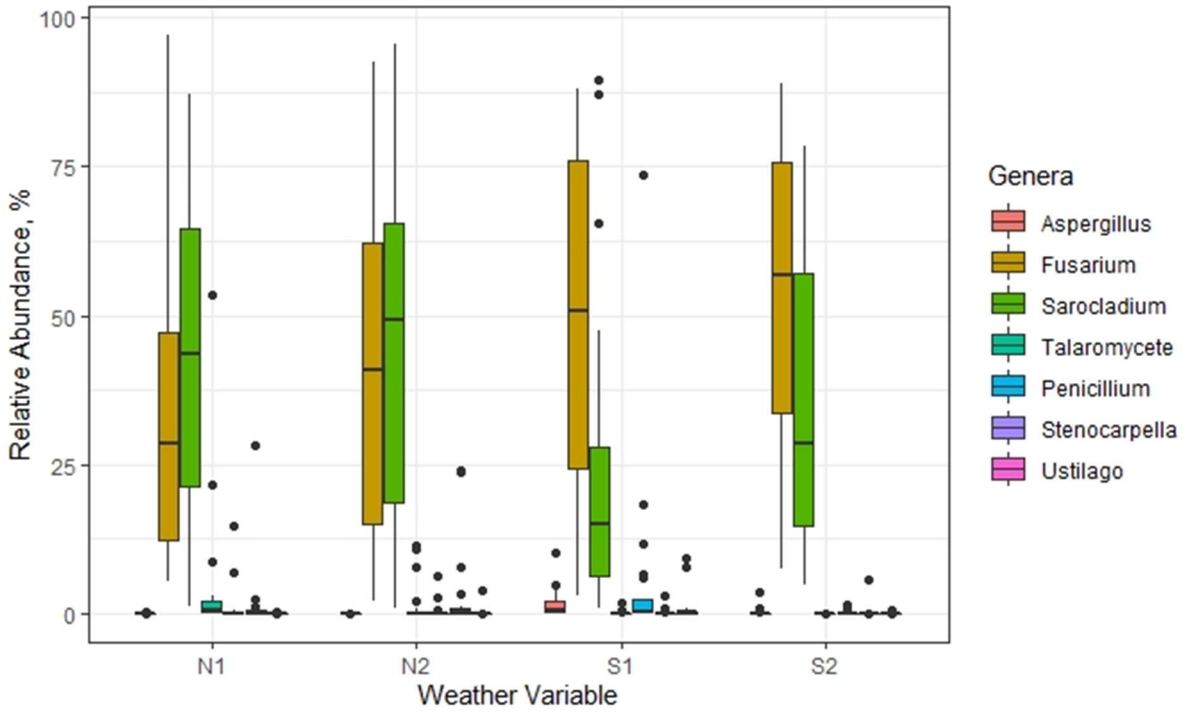


Figure 3. Boxplot of relative abundance representation of ASVs of the mycotoxin-important genera (*Aspergillus*, *Fusarium* and *Penicillium*); the high ASV abundance *Sarocladium*; and three genera harbouring common plant pathogens, *Stenocarpella*, *Talaromyces* and *Ustilago*. N1 and N2 are high rainfall weather variables under AEZ3, season-1 and season-2, respectively. S1 and S2 are low rainfall variables under AEZ1, season-1 and season-2, respectively. S1 is in addition accompanied with a dry spell.

In combination with its former teleomorphic state, now separate genus *Talaromyces*, field frequency was 88%. We analysed *Penicillium* and *Talaromyces* as separate genera considering their distinction in phylogenetic clades, with *Talaromyces* (member of Leotiomycetes (Egidi *et al.*, 2019)) having seven sections and *Penicillium* (member of Eurotiomycetes) having 26 sections (Houbraken *et al.*, 2020).

In Table 1, the common disease agent *Stenocarpella* as well as *Ustilago* were also frequently detected in fields with overall frequencies of 59% and 53%, respectively.

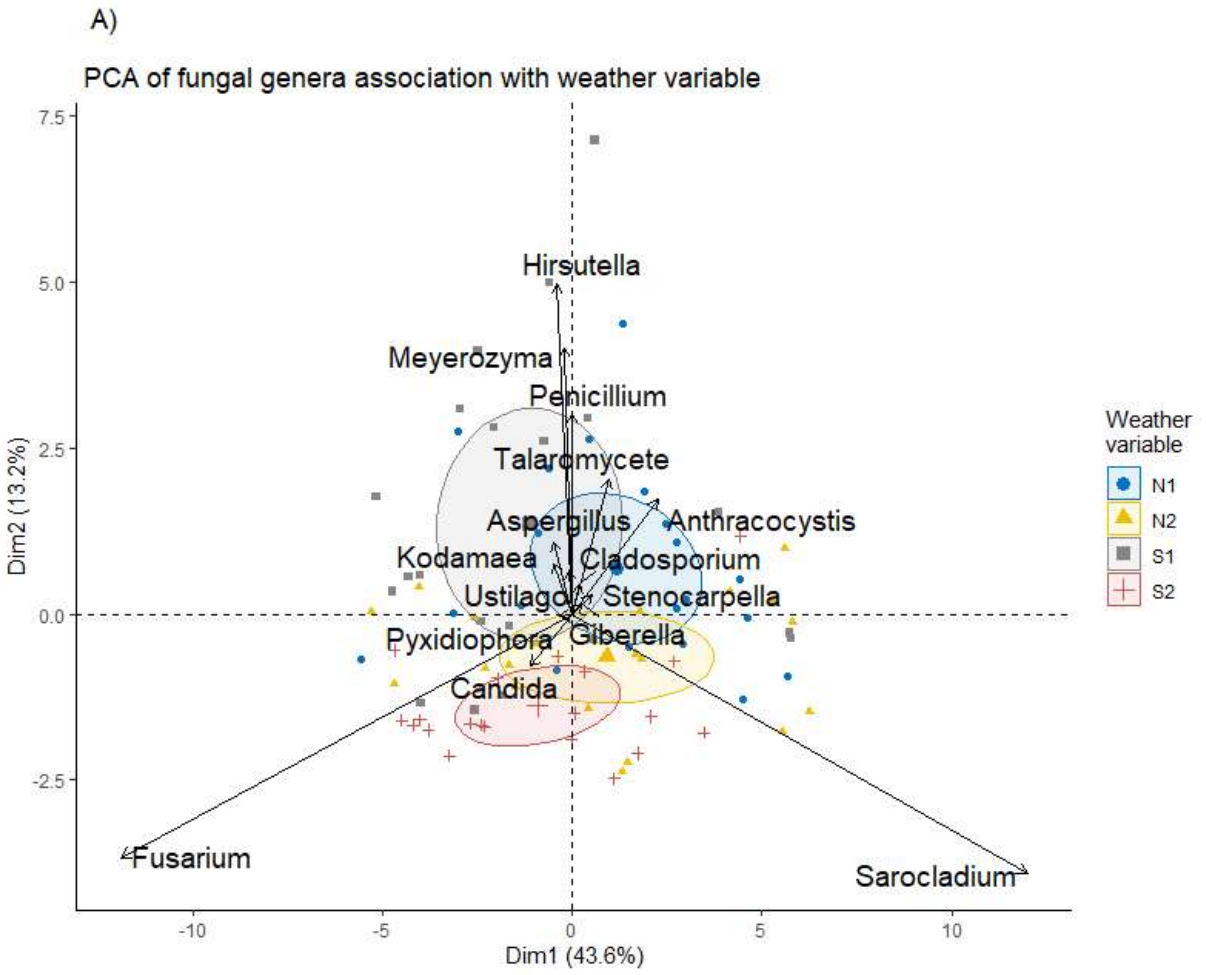
Table 1. Frequency and abundance of genera that were frequently detected including the mycotoxin and disease-burden important taxa ‘

Genus	Weather variable	Frequency %	Relative Abundance %	Mean relative abundance (%)	Relative Abundance range %	
					Min	Max
Fusarium	S1	100	48.4	45	2.9	87.9
	N1	100	33.8		5.4	97.0
	S2	100	55.0		7.5	89.0
	N2	100	42.1		2.2	92.7
Sarocladium	S1	100	24.8	37	0.9	89.7
	N1	100	42.6		1.3	87.3
	S2	100	34.1		4.8	78.4
	N2	100	44.8		0.8	95.7
Talaromyces♯	S1	65	0,2	1.7	0,0	1,7
	N1	100	4,8		0,0	53,5
	S2	20	0,0		0,0	0,1
	N2	70	1,7		0,0	11,3
Penicillium♯	S1	95	6,0	2.0	0,0	73,7
	N1	80	1,2		0,0	14,7
	S2	65	0,1		0,0	1,3
	N2	65	0,5		0,0	6,3
Stenocarpella	S1	60	0.2	1.4	0.0	3.0
	N1	75	1.7		0.0	28.3
	S2	20	0.3		0.0	5.8
	N2	80	3.2		0.0	23.9
Ustilago	S1	90	1.0	0.3	0.0	9.3
	N1	45	0.0		0.0	0.3
	S2	65	0.0		0.0	0.7
	N2	10	0.2		0.0	3.9
Aspergillus	S1	100	1.7	0.5	0.0	10.3
	N1	40	0.0		0.0	0.1
	S2	40	0.3		0.0	3.4
	N2	10	0.0		0.0	0.0

♯ *Talaromyces* and *Penicillium* were analysed separately considering the distinct phylogenetic clades, despite some morphological resemblance. N1 and N2 are high rainfall weather variables under AEZ3, season-1 and season-2 respectively. S1 is low rainfall with dry spell weather variable under AEZ1, season-1. S2 is low rainfall weather variable under AEZ1, season-2.

2.4.2 Mycobiome composition in relation to weather patterns

The weather variables S1, N1, S2 and N2 are shown in Figures 4, 5, 6 and 7, and Table 2. A difference in fungal composition was detected between S1 (low rainfall with dry spell) and S2 (low rainfall) (Figure 5, $P = 0.006$). However there was no difference in fungal composition between the high rainfall variables N1 and N2 ($P = 0.492$). A comparison of weather variables, N1, N2, S1 and S2, within an AEZ showed that there was no difference in alpha diversity between N1 and N2 as well as between S1 and S2. However, a significant difference in alpha diversity was observed between N1 and S2 (Figure 6, pairwise Tukey's HSD, $P = 0.03$).



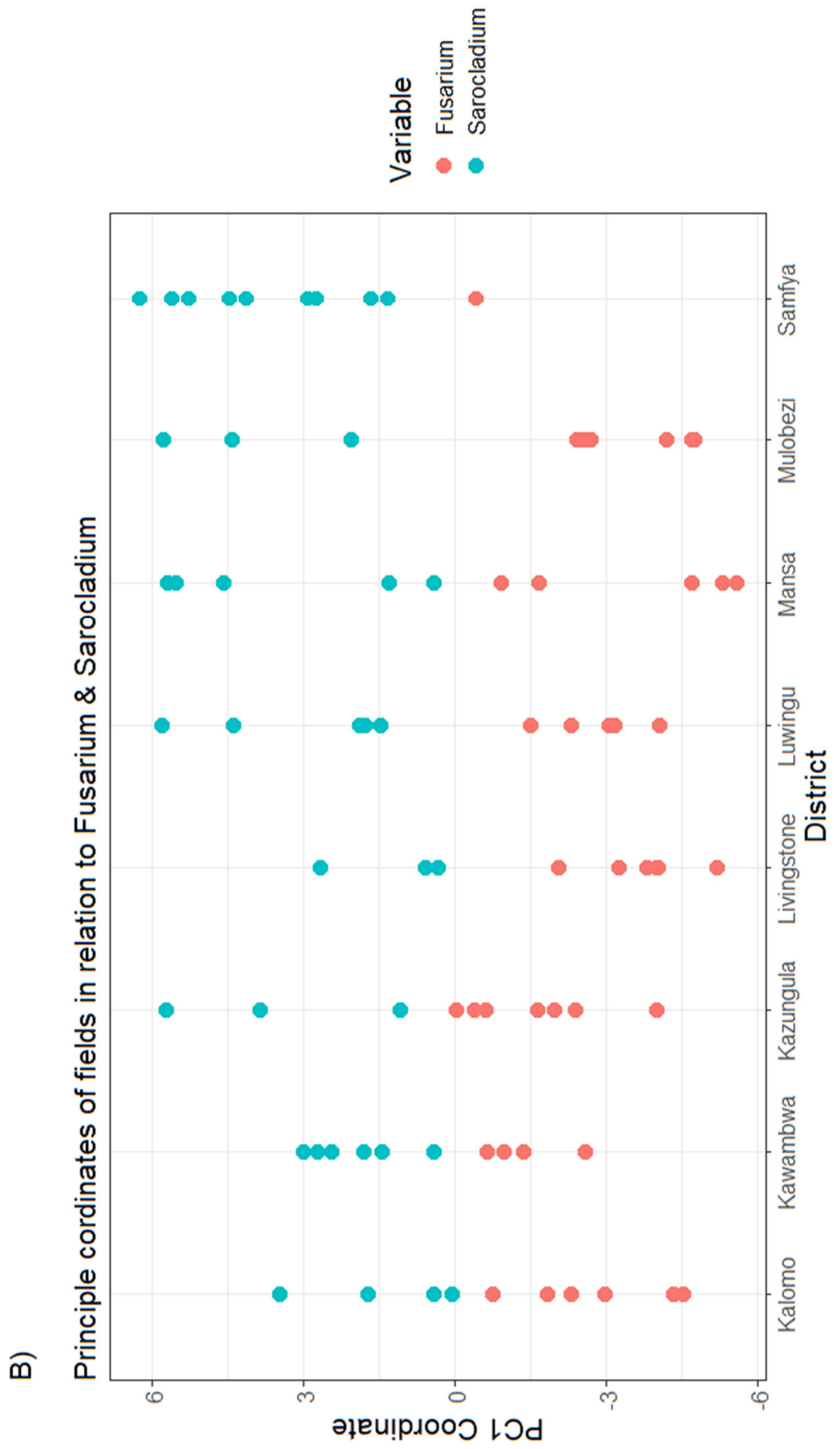


Figure 4. A) PCA biplot of fungal genera inclinations in response to weather variables. N1 and N2 are high rainfall weather variables under AEZ3, season-1 and season-2 respectively. S1 and S2 are low rainfall weather variables under AEZ1, season-1 and season-2, respectively. S1 is in addition characterised with a dry spell. **B)** Spatial niche partitioning based on district field's PCA coordinates for the two highest ASV abundance genera (*Fusarium* and *Sarocladium*) that had no weather variable orientation on the biplot in "A." Samfya district PCA coordinates for fields are biased in positive direction, as exceptionally dominated by *Sarocladium* (9/10 fields) than *Fusarium*.

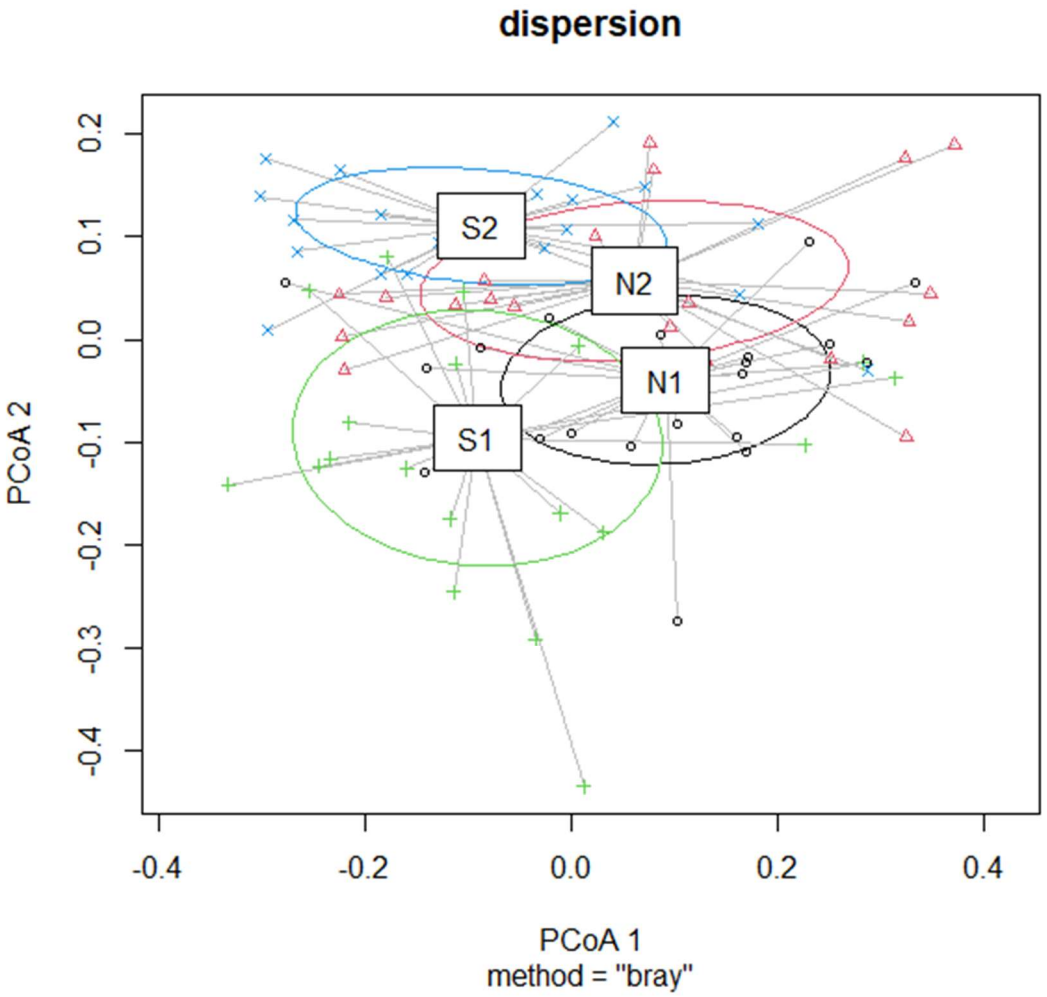


Figure 5. Principle Coordinate Analysis of fungal genera composition across weather variables. N1 and N2 are high rainfall weather variables under AEZ3, season-1 and season-2, respectively. S1 is low rainfall with dry spell weather variable under AEZ1, season-1. S2 is low rainfall weather variable under AEZ1, season-2. Difference in composition between S1 and S2 by pairwise adonis2 Permanova test was significant, $P = 0.006$. N1 v N2 not significant.

Between AEZs, combined data showed that the drier AEZ1 had a comparatively higher *Fusarium* relative abundance (52%) than AEZ3 (38%) (student *t*-test, $P = 0.028$; Figure 7). However, there was no difference in relative abundances of *Fusarium* over the weather variables, S1 (48.4%), S2 (55.0%), N1 (33.8%) and N2 (42.1%) (pairwise *t*-test, $P > 0.05$). Similarly, *Aspergillus*, an important mycotoxins genus like *Fusarium*, was substantially more relatively abundant in AEZ1 than in AEZ3 (Kruskal Wallis rank sum test, $\chi^2 = 22.71$, $P < 0.001$, df = 1). A comparison of weather variables showed that, *Aspergillus* abundance was higher under the S1 compared to all three variables N1, S2 and N2 (pairwise Wilcoxon rank sum test, $P < 0.001$).

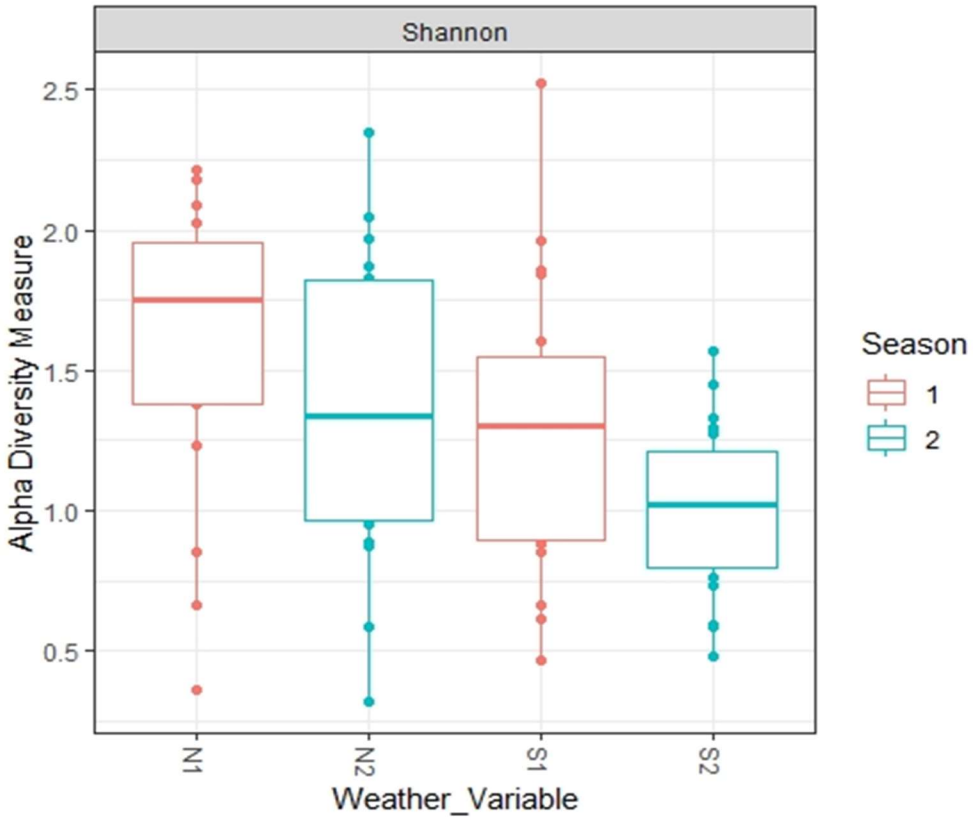


Figure 6. Alpha diversity measured by precision of detected species in mycobiomes of the weather variables. N1 and N2 are high rainfall weather variables under AEZ3, season-1 and season-2 respectively. S1 and S2 are low rainfall weather variables under AEZ1, season-1 and season-2, respectively. S1 is in addition characterised by a dry spell.

The differences between AEZ1 and AEZ3 are further elaborated in Figure 4A: Dimension (Dim) -1 explains more the difference in mycobiome variation in AEZ1 and AEZ3. Dim-2 explains more the difference between season-1 and season-2, indicating *Hirsutella* to be more associated with Season-1 than Season-2. Overall, Dim-1 explains variations better (43.6%) than Dim-1 (13.2%). It is evident from Figure 4A that *Penicillium*, *Kodamaea*, *Aspergillus* and *Meyerozma* were more associated with S1, whereas *Fusarium* and *Sarocladium* are not associated with a particular weather variable.

Within the AEZ1, some genera had significantly higher relative abundance ($P < 0.01$) in the first season characterised by a dry spell (weather variable S1) compared to following sampling season (weather variable S2) (Table 2). In particular, *Aspergillus* relative abundance was higher under the S1 than S2. Furthermore, the moderate xerophiles were more associated with the S1 (Table 2). *Meyerozyma* was more associated with season-1 than 2 and proliferated the most under S1, while rest of

genera were either associated with AEZ3 or AEZ1. No such significant differences were observed between the two high rainfall variables, N1 and N2.

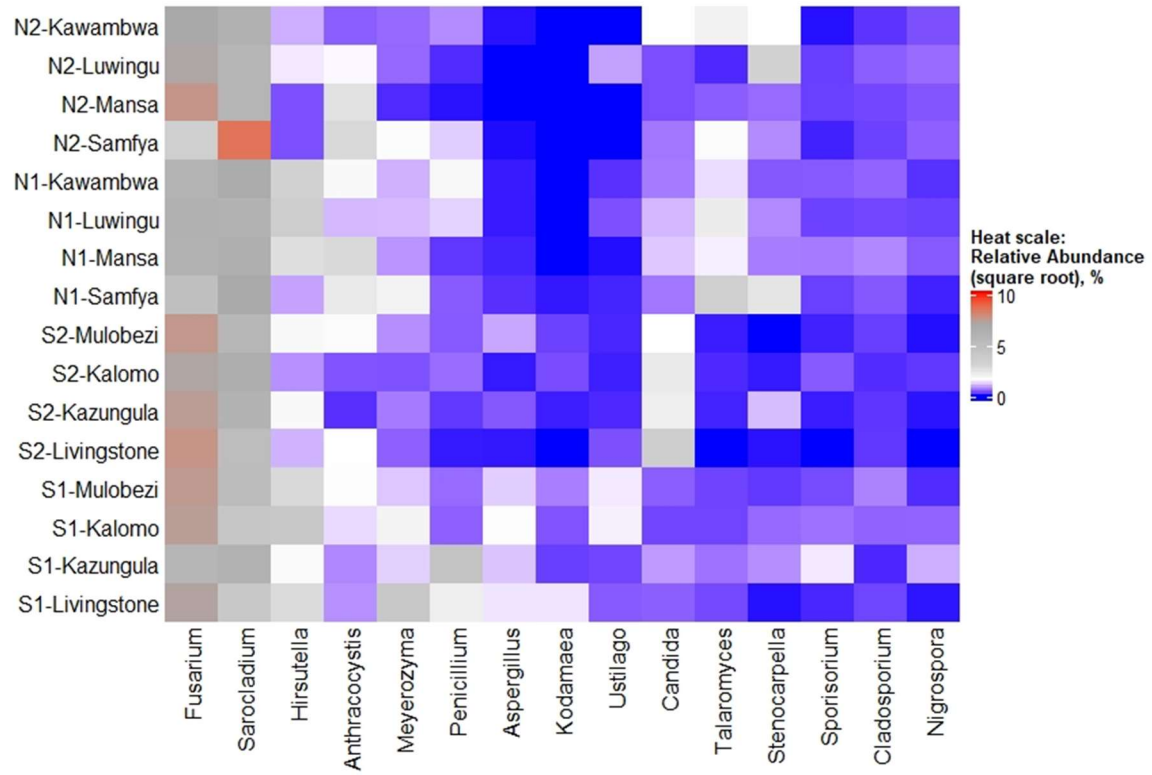


Figure 7. Unclustered heatmap of top 15 fungal genera (x-axis) relative abundances across districts over two seasons (y-axis). N1 and N2 are high rainfall weather variables under AEZ3, season-1 and season-2 respectively. Suffixes are districts. S1 and S2 are low rainfall weather variables under AEZ1, season-1 and season-2, respectively. S1 is in addition characterised by a dry spell.

Table 2. Mean relative abundance (%) describing the distinct fungal genera over weather variables.

Genus	S1	S2	N1	N2
Aspergillus[a,b]	1.7	0.3	0.0	0.0
Kodamaea[a]	0.6	0.1	0.0	0.0
Meyerozyma	5.4	0.3	1.6	0.8
Penicillium[a]	6.0	0.1	1.2	0.5
Talaromyces	0.2	0.0	4.8	1.7
Stenocarpella	0.2	0.3	1.7	3.2
Ustilago	1.0	0.0	0.0	0.2
Hirsutella	8.7	1.9	7.2	0.8
Fusarium equiseti[c]	0.2	0.1	2.0	2.7

Table 2. Changes in relative abundance of fungi over the different weather variables. ([a]) are xerophilic (or moderately xerophilic), while the rest have some agroecological zone inclination.
([b]) *Aspergillus* was detected in the range S1 = 0 - 10.3%; S2 = 0 - 3.4%; N1 = 0 - 0.1%; N2 = 0%.
([c]) detected as a distinct anamorph of the former *Gibberella intricans*.

2.5 Discussion

Deploying high-throughput DNA sequencing (HDSeq) of ITS1 region, we describe and show a diverse fungal microbiome on preharvest Zambian maize in comparison to previous findings. In addition, we demonstrate the dominance of the previously unreported *Sarocladium* on Zambia preharvest maize, which is also seldom reported in other regions. We furthermore demonstrate for the first time how rainfall pattern may potentially impact a diverse mycobiome by composition.

2.5.1 Preharvest maize mycobiome structure

Although the previous study done in Zambia by dilution plating revealed 13 genera on the maize at harvest (Mukanga *et al.*, 2010), we reveal a 5-fold higher number of genera (*n* = 61) (excluding genera that only appeared in one field with a relative abundance of < 0.1%, Supplemental Data 1). This demonstrates a higher diversity of preharvest maize mycobiome than previously reported. We attribute the higher number of preharvest fungal genera detected to the use of a higher resolution method (HDSeq) compared to dilution plating. Standard techniques for quantifying fungal populations such as dilution plating, although quite effective, may tend to favour quiescent structures, particularly conidia, compared to other actively growing parts of the population (Klich *et al.*, 1992). Furthermore, although effective in fungal enumeration, dilution plating may favour the most abundant species over the less abundant and slower growers, which may result the underrepresentation of the latter (Lane *et al.*, 2018; Lane & Woloshuk, 2017; Taylor *et al.*, 2014). Hence, using HDSeq would likely give a more realistic picture of fungal diversity than plating methods (Baldrian *et al.*, 2021; Buee *et al.*, 2009; Taylor *et al.*, 2014; Xia *et al.*, 2016). In a previous study by (Lane *et al.*, 2018; Lane & Woloshuk, 2017) similarly demonstrated a higher number of detected genera by HDSeq compared to plating. Although we do observe a common pattern of the common maize contaminants *Aspergillus*, *Fusarium*, *Penicillium*, and *Stenocarpella* from our study and the previous on US and Kenyan maize using HDSeq (Lane *et al.*, 2018) our study does report unique genera in high frequency. These include the maize pathogen *Ustilago* (Regine Kahmann *et al.*, 2018) (53% frequency) *Kodamaea* (31% frequency), *Meyerozyma* (88% frequency) and *Anthracocystis* (94% frequency).

Out of 61 genera detected on Zambian preharvest maize, *Fusarium* and *Sarocladium* were the most frequently detected genera across all fields (100%), Table 1). Furthermore, we demonstrate that although *Sarocladium* has previously not been reported on Zambian maize, it was a highly frequent contaminant of maize in the fields like *Fusarium*. Our findings contrast expectations of a preharvest mycobiome dominated by *Fusarium* and *Stenocarpella*. A past dilution plating study of Zambian maize collected from AEZ1 and 2 demonstrated *Fusarium* to be the fungus of highest abundance on the preharvest maize out of the 13 genera that were detected (Mukanga *et al.*, 2010). However, *Sarocladium* was not detected. *Sarocladium* is also rarely reported in regional studies of similar climatic pattern as Zambia, but may be a fungus of importance in contaminating preharvest maize. For example, a recent regional study by plating similarly revealed *Fusarium* as the main contaminant of maize in Zimbabwe (Akello *et al.*, 2021), but *Sarocladium* was, however, not reported. In a different climatic region, (Lane *et al.*, 2018) reported similar high relative abundances of OTUs for *Sarocladium* in Kenyan and USA maize using HDSeq. Although *Sarocladium* may be important to the maize mycobiome according to our study, it should also be noted that overall, the genus is largely a rice pathogen (Sakthivel *et al.*, 2002). Compared to *Fusarium*, *Sarocladium* has no such reported level of pathogenicity or mycotoxigenicity on maize. As regards *Fusarium*, our findings for AEZ3 as well as the previously studied AEZ1 by (Mukanga *et al.*, 2010), confirm that *Fusarium* is readily detected on preharvest Zambian maize. The dominance of *Fusarium* by frequency of detection is also demonstrated by (Lane *et al.*, 2018) on Kenyan and USA maize. This further demonstrates that *Fusarium* is a widely important field maize contaminant considering that in combination, our study and the previous by (Lane *et al.*, 2018) were done under three different geographical regions. Although the actual abundance of *Fusarium* may not have been ascertained in this study, we reveal a similar pattern of the comparatively high abundance of *Fusarium* by ASVs (45%) as that of previous study of *Fusarium* OTUs using HDSeq (Lane *et al.*, 2018).

Although our study shows that *Fusarium* was a genus present at high frequency in the field throughout weather variables, better climatic associations would be clearer if the genus were split into various species based on ecological adaptations. For example, accounting for about 3% of *Fusarium* in this study, *Fusarium equiseti,* (genetically distinct in this study as the former *Gibberella intricans* (Jurado *et al.*, 2005)), was more

associated with districts in AEZ3 (2.35%) than AEZ1 (0.17%). However, it's unclear from this study if the assigned taxon *Fusarium* were split further into species, that certain *Fusarium* species would be seen to incline towards a particular AEZ or weather variable studied. Furthermore, a similar association of genus with specific weather variable is observed with *Candida*, which was more associated with the southern hotter and drier region (AEZ1) under normal rainfall pattern (S2) (Figure 4A). We also see a pattern in which over the two maize growing seasons the state *Talaromyces* (64% frequency) was found to be more commonly associated with the areas of AEZ3 than in AEZ1 similar with *Stenocarpella* (Figure 7; Figure 4A). It should be noted that *Talaromyces* has a distinct phylogenetic clade (with seven sections) from its asexual anamorph *Penicillium* (with 26 sections) (Houbraken *et al.*, 2020), hence assessed separately from *Penicillium* in our study. *Talaromyces* is a genus recently reported as causative in destructive maize ear rot (Liu *et al.*, 2021). An earlier country study has demonstrated *Fusarium* and *Stenocarpella* to be causative fungi of maize ear rot in Zambia (Mukanga *et al.*, 2010). The higher relative abundance of *Talaromyces* observed in the wetter northerly districts (AEZ3) compared to the drier southerly districts (AEZ1) may augment the maize ear rot caused by *Fusarium* and *Stenocarpella*. This warrants the need to further explore species within *Talaromyces* and establish further links between maize ear rot and this genus in AEZ3. It should also be noted that some species belonging to this genus are known for the production of the mycotoxin rubratoxin (Yilmaz *et al.*, 2012) like some *Penicillium* species (Otero *et al.*, 2020).

Of the mycotoxin-important genera other than *Fusarium, Aspergillus* was part of the most frequently detected genera in fields investigated (Table 1). The study by (Mukanga *et al.*, 2010) which partly investigated districts in AEZ1 showed *Aspergillus* abundance to be the second highest after *Fusarium*. In our study, however, we detected an additional four genera between *Fusarium* and *Aspergillus* in order of reducing frequency of appearance in AEZ1 namely *Sarocladium* (100%) > *Hirsutella* (95%) > *Penicillium* (80%) > *Ustilago* (78%), with *Penicillium* being important for the mycotoxin Ochratoxin. *Aspergillus* frequency in AEZ1 was 70% indicating its importance in this AEZ. It's also worth noting that although *Aspergillus* was in comparatively lower relative abundance in the field for the low rainfall variable S2 compared to the low rainfall with dry spell variable S1 (Tables 1 and 2), propagules of

the xerophile carried over from field to storage can proliferate during storage (Ding *et al.*, 2015) especially when conditions become favourable.

2.5.2 Mycobiome composition in relation to weather patterns

We demonstrate that although generally stable, the preharvest mycobiome composition may possibly be altered by such conditions as dry spell. The stability of the mycobiome composition in our study is demonstrated in Figure 5. It's further exemplified by the non-significant difference in the relative abundance of the high ASV abundance *Fusarium* over the weather variables, S1 (48.4%), S2 (55.0%), N1 (33.8%) and N2 (42.1%) (pairwise *t*-test , $P > 0.05$; Table 1). The stability in mycobiome composition is despite specific inclinations of certain genera like *Talaromyces* and *Stenocarpella*, towards AEZ3 and *Candida* towards S2 (Figure 7). As regards the change in composition, the low rainfall variable with dry spell (S1) had a demonstrable different composition from that of the normal low rainfall variable (S2). The difference in composition between S1 and S2 is attributed to the proliferation of the xerophiles (or moderately xerophilic) *Aspergillus* and *Penicillium* as well as the less reported *Kodamaea* in S1, similarly with *Meyerozyma*. When rainfall normalised in the same locality the following sampling season (Season-2, 2020/2021) the genera relative abundances declined (Table 2). The three genera, as moderate xerophiles, as well as *Meyerozyma*, would be more adapted to the dry spell conditions (S1). Considering that certain genera like *Kodamaea* appeared under the dry weather pattern, it may be imperative for future studies to consider the dynamics of the relative abundance of such species during storage. In addition, consideration should be taken to explore if at all such genera are specific to certain geographic regions or are truly weather pattern dependent (S1) as seen in our study.

As regards the mycotoxins important genera *Aspergillus* and *Fusarium*, the strong influence of the dry spell, was the key factor that may have led to the *Aspergillus* proliferation (Figure 4A) as was shown in earlier studies (Giorni *et al.*, 2016; Payne & Widstrom, 1992). The *Aspergillus* contamination observed under S2 and its strong proliferation under S1 demonstrates the potential risk of field contamination of maize with *Aspergillus*, a fungus generally associated with the maize storage phase. It would be worth exploring the potential correlation of the genus *Aspergillus* as a whole with field aflatoxin, generally considered a storage mycotoxin. On the contrary, this study revealed that *Fusarium* abundance on the preharvest maize was not influenced by the

dry spell conditions, implying that maize ear colonisation by *Fusarium* may likely have occurred early before onset of the dry spell (about three months after sowing). An exception was, however, observed in Samfya district where the comparatively lowest relative abundance of *Fusarium* and inversely the highest relative abundance of *Sarocladium* was observed. This may be attributed to the fact that Samfya's agroecological terrain is, in part, comprised of water bodies like lakes and plains (Figure 1) conducive for the better adapted rice pathogen *Sarocladium* (Musonerimana *et al.*, 2020; Pramunadipta *et al.*, 2021). This also suggests that, although in our study and that by (Lane *et al.*, 2018) showed *Fusarium* to have the most abundant ASVs/OTUs on maize, *Fusarium* may probably not always be the most abundant taxon on maize in comparison to *Sarocladium*. This argument applies for regions such as the rice growing areas with abundant water bodies, such that *Sarocladium* would instead likely dominate. For Example, the study by (Gromadzka *et al.*, 2019) recorded incidences of *Sarocladium* infection of maize without concurrent infection by *Fusarium*. Considering that *Sarocladium* and *Fusarium* were found to be the main contaminants of preharvest maize, it is worth exploring whether or not the *Sarocladium* relative abundance across the weather variables could have a bearing on levels of fumonisin (FB) in preharvest maize

2.6 Conclusion

The preharvest maize mycobiome in Zambian maize is diverse compared to previous country studies. Furthermore, the previously unreported *Sarocladium* may be a key contaminant of preharvest maize besides commonly reported *Fusarium*. Although the fungal composition and diversity were similar over the different rainfall patterns, severe weather conditions, such as dry spell, may likely alter the mycobiome composition due to the proliferation of xerophilic (or moderately xerophilic) genera adapted to such conditions. The high prevalence of *Sarocladium* and *Fusarium* warrants the need for further study the species of *Sarocladium* as it could be a fungus of interest from a phytosanitary aspect with regard to *Fusarium* and FBs.

2.7 Acknowledgement

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2.8 Conflict of interest

Authors declare no conflict of interest.

2.9 Supplemental material

Supplemental data 1.

Very low frequency and abundance genera - Genera appearing only in one field out of the 80 samplings with relative abundance < 0.1%, (*n* = 24) were as follows:

Articulospora, Ascochyta, Chaetomium, Chalara, Clonostachys, Colacogloea, Coniothyrium, Cryptococcus, Cyanodermella, Cyphellophora, EuPenicillium, Exophiala, Kwoniella, Melampsora, Neoascochyta, Olpitrichum, Periconia, Phaeosphaeria, Pseudopithomyces, Pyricularia, Saturnispora, Simplicillium, Sphaeronaemella, Tremella. Due to their very low relative abundance and frequency, it could not be completely ascertained if they belonged to the mycobiome.

Supplemental Table 1. Maize moisture percentage in maize samples at harvest.

Field	%(S1)	Field	% (N1)	Field	% (S2)	Field	%(N2)
1-01	34.0	1-21	15.7	3-41	28.0	3-61	14.6
1-02	25.1	1-22	15.4	3-42	17.5	3-62	14.1
1-03	28.0	1-23	15.0	3-43	20.7	3-63	18.1
1-04	20.2	1-24	17.4	3-44	17.8	3-64	18.8
1-05	25.2	1-25	13.5	3-45	22.5	3-65	19.9
1-06	29.7	1-26	13.2	3-46	22.7	3-66	19.2
1-07	15.8	1-27	19.0	3-47	15.6	3-67	17.8
1-08	24.8	1-28	17.1	3-48	13.1	3-68	14.9
1-09	23.2	1-29	17.5	3-49	22.7	3-69	18.8
1-10	21.5	1-30	14.3	3-50	31.9	3-70	14.3
1-11	34.0	1-31	13.6	3-51	12.9	3-71	16.2
1-12	34.5	1-32	13.0	3-52	24.8	3-72	15.1
1-13	19.3	1-33	15.8	3-53	22.8	3-73	18.4
1-14	22.9	1-34	13.8	3-54	18.1	3-74	20.5
1-15	17.9	1-35	15.7	3-55	18.2	3-75	17.6
1-16	25.3	1-36	13.6	3-56	13.5	3-76	14.4
1-17	17.0	1-37	13.5	3-57	14.2	3-77	22.5
1-18	21.0	1-38	13.1	3-58	33.1	3-78	15.2
1-19	14.6	1-39	11.5	3-59	21.4	3-79	17.3
1-20	17.1	1-40	15.1	3-60	14.6	3-80	14.5
Average	*23.6*	Average	*14.8*	Average	*20.3*	Average	*17.1*
Std_Dev	*6.1*	Std_Dev	*1.9*	Std_Dev	*5.9*	Std_Dev	*2.4*
Min	*14.6*	Min	*11.5*	Min	*12.9*	Min	*14.1*
Max	*34.5*	Max	*19.0*	Max	*33.1*	Max	*22.5*

Supplemental data 2. Evaluation of loss of viable fungal mass during kernel wash pelleting.

The protocol described in materials and methods for generation of pellet of mycobiome DNA extraction was followed. It was evaluated for extent of loss of viable fungal spores during the multiple washing steps to wash off the surfactant, Triton-X. Maize kernels were weighed into a 250 ml flask and spores extracted by shaking in 0.05% Triton-X at concentration 1 g kernels /ml as described in the protocol under materials and methods section. The first supernatant of the maize grain wash from the first centrifuge step (materials and methods section) was pipetted off into a 1.5 ml microcentrifuge tube for plating, and assigned 1st supernatant. The remaining ≈ 2 ml lower fraction was transferred to a microcentrifuge tube, as described in materials and methods, and centrifuged. About 500 µl of the second supernatant was transferred to a microcentrifuge tube for plating and assigned 2nd supernatant. The remaining lower fraction of approximately 500 µl was assigned 2nd lower fraction. It was pipetted off from the pellet into another 1.5 ml microcentrifuge tube prior to addition of further washing water (MQ sterile water) to the pellet. There was no 1st lower fraction as all lower fraction was transferred to microcentrifuge tubes. A total of four supernatants and three lower fractions were therefore prepared and plated by the dilution plate technique on 90 mm petri dishes of PDA medium and incubated at 25°C (7 days, dark).

Supplemental Table 2. Estimation of viable fungal spore loss while pelleting maize grain surface wash.

Fraction	Sample1			Sample2		
	C, sp/ml	V, ml	Q, sp	C, sp/ml	V, ml	Q, sp
1st supernatant	0.0	31.0	0.00	0.0	35.0	0.00
2nd supernatant	0.0	0.5	0.00	0.0	0.4	0.00
2nd lower fraction	0.0	0.5	0.00	3.3	0.4	1.35
3rd supernatant	0.0	0.5	0.00	0.0	0.4	0.00
3rd lower fraction	0.0	0.5	0.00	0.0	0.4	0.00
4th supernatant	0.0	0.5	0.00	3.3	0.4	1.35
4th lower fraction	3.3	0.5	1.65	10.0	0.4	4.05
Total (lost spores):			1,65			6,75
Pellet (spores):	2000	0.48	960	2833	0.72	2040
% pellet spore loss:			0.17			0.33

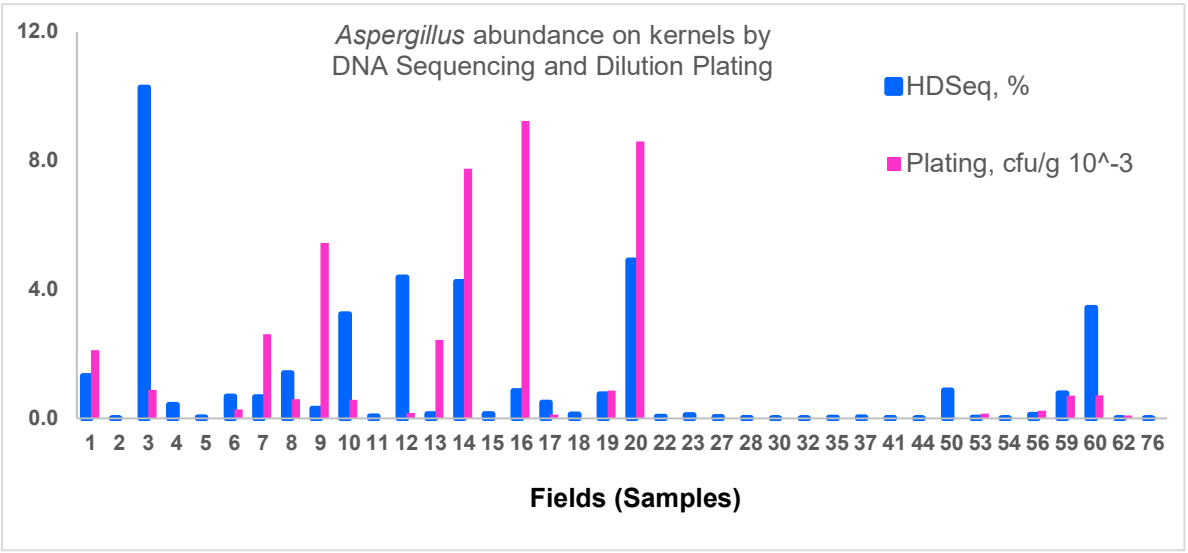
Supplemental Table 2. Spore loss from supernatant fractions during centrifuge. C = spore concentration per fraction (spore/ml); V = approximate volume of fraction in tube (ml). Q = estimated quantity of spores per fraction, C x V. Total spore loss from pellet through fractions = 0.25 ± 0.11%.

The quantity of spores (Q, spores) was determined as product of volume of suspension (V, ml) x concentration of spores (C, spore/ml) in the suspension (Supplemental Table 2). The concentration of the spores had been determined by spore count from the PDA plates.

Supplemental Table 3. DNA recovery in mock fungal community

Pre-sequencing spike ratio, %		**post ASV clean-up abundance, %**		**Standard deviation**	**Recovery, %**	
Aspergillus	*Fusarium*	*Aspergillus*	*Fusarium*		*Aspergillus*	*Fusarium*
5	95	5.1	94.9	± 0.2	101.1	99.9
15	85	16.5	83.5	± 0.0	110.2	98.2
50	50	48.0	52.0	± 1.7	96.0	103.9

Supplemental Table 3. Amplicons Sequence Variant recovery of spiked DNA of *Fusarium verticillioides* and *Aspergillus flavus* as mock community.



Supplemental Figure 1. Comparison of *Aspergillus* abundance on maize kernels by high-throughput DNA amplicon Sequencing (HDSeq) and plating. Fields (samples) shown had *Aspergillus* detected by HDSeq only or HDSeq and plating. All fields positive for *Aspergillus* by plating were positive by sequencing. Number of fields positive for *Aspergillus*: Sequencing = 38/80; Plating = 19/80. Fields without *Aspergillus* by both methods are not shown. Fields 1 – 20 = Season1, AEZ1 (S1, low rainfall with dry spell); Fields 21 – 40 = Season1, AEZ3 (N1, high rainfall); Fields 41 – 60 = Season2, AEZ1 (S2, low rainfall); Fields 61 – 80 = Season2, AEZ3 (N2, high rainfall).

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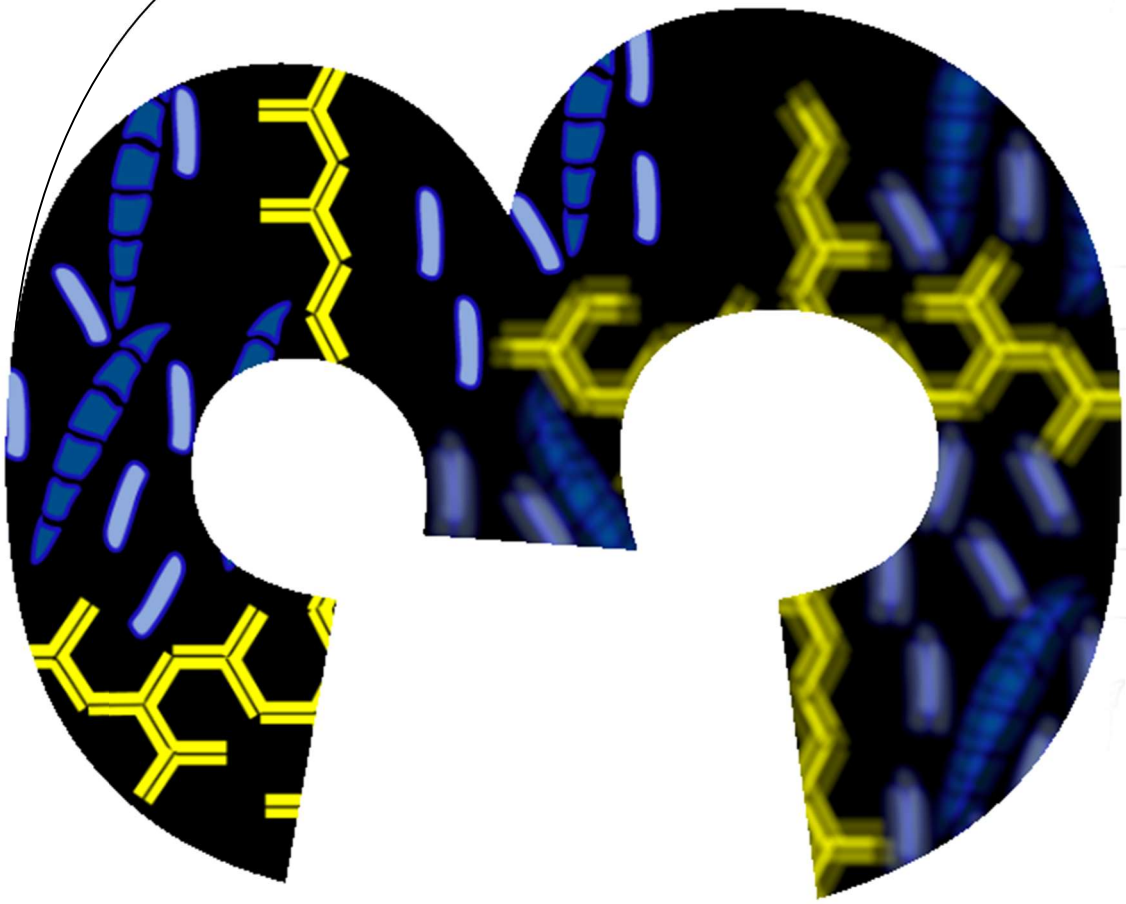
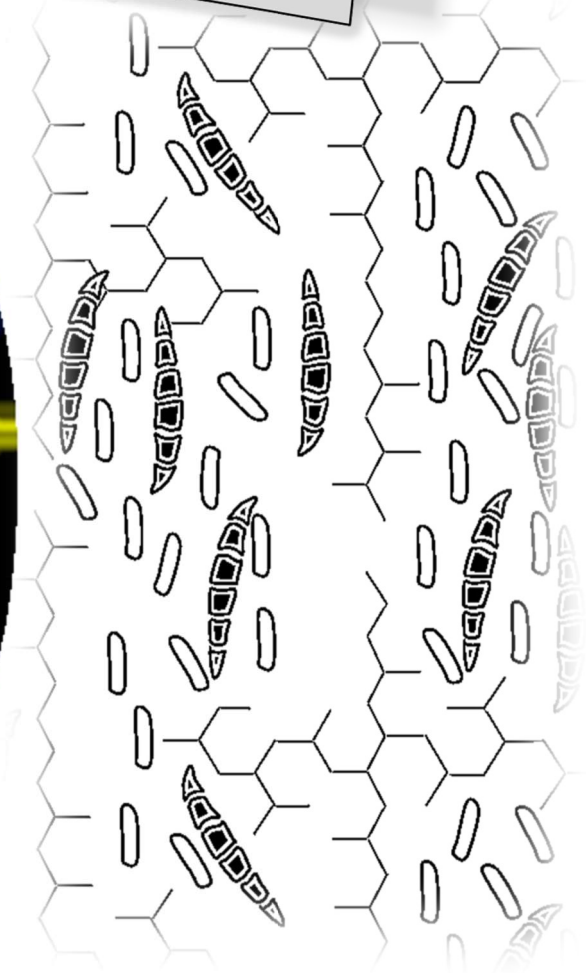
If fungi were a human world, Sarocladium and Fusarium would be sworn foes





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Chapter III

Niche partitioning reveals negative correlation of *Sarocladium* with *Fusarium* and fumonisin-B1 levels in preharvest maize

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3.0 Abstract

Through partitioning of ecological niches several fungi are able to coexist, for example, on the same host crop. In (partial) absence of niche partitioning, competitive exclusion among fungi can occur. Ecological niches are formed by abiotic and biotic factors. Perturbations in abiotic factors, can alter how niches are partitioned, for example favouring of certain genera over others. Specifically, we investigated the fungal correlations, in terms of relative abundance of the fungi, in preharvest maize under different rainfall patterns, in order to identify possible co-existence and competition in fungi. When species compete within one niche (competitive exclusion), we may expect to detect higher levels of mycotoxins – since mycotoxins are considered antagonistic agents aimed at defending or invading an ecological niche. In this investigation, positive or absence of correlations between presence of fungal types signified good niche partitioning, whereas negative correlations signified poor niche partitioning and potential for competitive exclusion. The correlations of presence of fungal species were established by deploying DNA amplicon sequencing to estimate the relative abundances of the fungal genera. This was done for both internal (within maize kernels) and external (on the surface of maize kernels) mycobiomes. The investigation was done on preharvest maize fields (n = 80) under different rainfall patterns, high rainfall, low rainfall and low rainfall with dry spell. To estimate the importance of mycotoxins in competitive exclusion, we measured the influence of the fungal correlations on levels of fumonisin-B1 (FB1) and aflatoxin (AF) in the maize. FB1 and AF levels were determined by HPLC. Results showed that *Sarocladium* and *Fusarium* had the strongest negative correlation irrespective of the weather variable (rainfall pattern) suggesting poor niche partitioning between the two genera. Furthermore, higher levels of *Sarocladium* resonated with lower levels of FB1 and vice versa. Although *Hirsutella* had a negative correlation with *Aspergillus*, we could not ascertain its effect on AF levels.

3.1 Introduction

Fungi are ubiquitous and diverse. In nature fungi evolve into diverse communities residing in such ecological systems as soil or host plant such as maize. Diversity in such communities of fungal species may depend on niche partitioning. Through niche partitioning, living organisms can coexist for example in one space without competing for the same resource (Chesson, 2000). In fungi, several different genera can infect the same host crop and coexist through niche partitioning. The niche partitioning in fungi can be driven by various abiotic factors including temperature and rainfall as well as carbon source among others (Arroyo *et al.*, 2008; Giorni *et al.*, 2009; Medina *et al.*, 2017; Mellon *et al.*, 2000; Mellon *et al.*, 2005; Sejakhosi Mohale *et al.*, 2013; Perrone *et al.*, 2020; Samsudin *et al.*, 2016). Where the niche partitioning is 'good' fungi will likely coexist and on the contrary, where the partitioning is 'poor' the fungi will likely undergo competition (Chesson, 2000). Competition may take the form of reduction in abundance of the less competitive genus or species relative to another. In quantitative terms, the result can be a negative correlation between the two organisms in their abundance due to change in abundance of one relative to the other. Among the deterministic ecological mechanisms related to 'good' or 'poor' niche partitioning among microorganisms include neutralism (no effect on abundance of the existing populations); mutualism (co-existing population abundances increasing); predation/parasitism (with part of existing population increasing and other reducing); competition (generally the existing populations reducing), etc. (Abdullah *et al.*, 2017; Panikov, 2010; West *et al.*, 2006) (Figure 1).

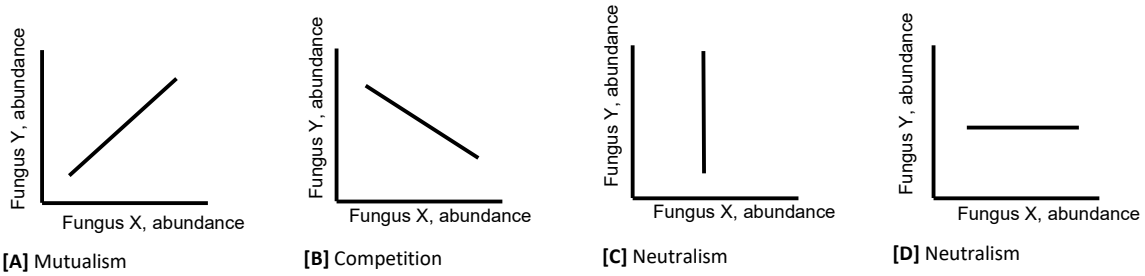


Figure 1 demonstrates idealistic correlations between two fungal genera with (A) mutualism, (B) competition, and (C) or (D) lack of correlation.

Resource partitioning may make it possible for different fungi to occupy a single host in their demand for different carbon sources (Arroyo *et al.*, 2008; Giorni *et al.*, 2009; Medina *et al.*, 2017; Mellon *et al.*, 2000; Mellon *et al.*, 2005; Sejakhosi Mohale *et al.*, 2013; Samsudin *et al.*, 2016), while finite nutrient availability may trigger competition

between microorganisms. Resource partitioning therefore can allow crops to be contaminated with an array of fungal genera. Furthermore, resource partitioning may lead to differentiation in genera that will infect a specific crop. For example, of the mycotoxigenic fungi, groundnuts in the field are known to be more prone to metabolite aflatoxin (AF) contamination due to its susceptibility to *Aspergillus* infection (Hulikunte Mallikarjunaiah *et al.*, 2017; Kachapulula *et al.*, 2017b; Ndisio *et al.*, 2017) compared to contamination with the metabolite fumonisin (FB) due to *Fusarium* infection. On the contrary, maize in the field is more prone to the metabolite FB contamination due to *Fusarium* infection, compared to *Aspergillus* infection (Akello *et al.*, 2021; Lane *et al.*, 2018). However, both *Fusarium* and *Aspergillus* will contaminate preharvest maize besides genera like *Stenocarpella* and *Penicillium*.

Lack of resource partitioning will result in niche exclusion due to the competition for specific niche space (Dorner JW *et al.*, 1999). In competitive exclusion one fungal genus or indeed one species outcompetes the fungus or species effectively reducing its population. For example, the out-competition of *Ustilago* by *Fusarium* (Alvarado-Serrano & Knowles, 2014) may suggest that under the specific conditions, *Fusarium* and *Ustilago* would be occupying the same niche. Some important antagonisms in cereals include the antagonism between *Fusarium* and *Trichoderma*. *Trichoderma* is a ubiquitous soil dweller (Bacon *et al.*, 2001) and is non-pathogenic to maize (Gromadzka *et al.*, 2019). It is a prospective fungus in the competitive exclusion of wheat's pathogenic *Fusarium* (Filizola *et al.*, 2019; Juan Palazzini *et al.*, 2018).

Besides the finite nutrient availability of host crop, rainfall and temperature are important abiotic factors that can determine levels of niche partitioning. For example, alteration in rainfall intensity may result in shifts in fungal composition (Perrone *et al*., 2020). This may signify out-competition or exclusion of certain fungal genera while others thrive. Under dry conditions, as an example, the fungal contamination dynamics may shift in favour of moderately xerophilic members such as *Aspergillus* (Payne & Widstrom, 1992) and *Penicillium*.

As part of competition for niche space, fungi can produce biochemicals. For example, it has been demonstrated *in vitro* that outcompeting of *Ustilago* by *Fusarium* may be through generation of cell wall-degrading secondary metabolites by *Fusarium*, competitively excluding *Ustilago* (Alvarado-Serrano & Knowles, 2014). Similar antagonistic mechanisms have been demonstrated for example in the production of

antagonistic agents such as bacteriocins in bacteria as a mechanism of antagonism (Schoustra *et al.*, 2012).

Furthermore, studies have demonstrated the production of the biochemical aflatoxin in *Aspergillus* as a defence response to invasion of its ecological space (Drott *et al.*, 2017; Trienens *et al.*, 2010). More generally, mycotoxins could be important compounds when species compete within an ecological niche. In mixed fungal communities, it is plausible that the way the presence of genera in an ecological niche correlate either negatively or positively by relative abundance, could be linked to subsequent levels of FB and AF in the fungal environment. Some species belonging to the genus *Fusarium* produce FB whereas some species belonging to the genus *Aspergillus* produce AF. Of the FB variants (FB1, FB2, FB3, FB4), FB1 is known to form the major compound (70-80%) on average of the total unmasked fumonisin in maize grain (Gil-Serna *et al.*, 2014) and has also been found to be the most toxic one (Wentzel *et al.*, 1992; Yu *et al.*, 2020). Where poor niche partitioning, and thus high levels of competitive exclusion is expected, it would be anticipated that certain genera may negatively correlate. In addition, it would also be expected that competing fungi would secrete anti-competitor toxins under such a negative correlation. Specifically, negative correlation between *Fusarium* and another genus, or *Aspergillus* and another genus would be expected to lead to higher levels AF or FB in maize, respectively, in this case. The importance of the identification of such genera with negative correlations with *Fusarium* or *Aspergillus* would suggest that these species are in direct competition for the specific niche. This would predict prospects for competitive exclusion by genera with negative correlations in their presence with either *Fusarium* or *Aspergillus*. This would provide prospects for combat of FBs and AFs, where the *Fusarium* and *Aspergillus* are able to be effectively excluded, respectively.

Aim of this study was to elucidate existence of evidence that niche partitioning has an effect on competitive exclusion in fungi. Our approach investigated negative and positive correlations in fungal genera present on maize kernels to identify such correlations from which reliable predictions can be made. The correlations were tested under different rainfall patterns, as a level of niche partitioning, and identify such fungi whose correlations with either *Aspergillus* or *Fusarium* would not be influenced by such an important abiotic factor, rainfall. In addition, similarity would have to be shown either in composition between internal and external mycobiomes or similarity in relative

abundance between important genera such as *Fusarium* and *Aspergillus* in internal and external mycobiomes. This is considering that besides the external mycobiome, the internal mycobiome may also play a role in the contamination of the grain with mycotoxins. Once consistent correlations would be observed, directed experimental studies could be designed to test if antagonism between strains is indeed a mechanism by which competitive exclusion takes place.

The study had two specific objectives: 1) To determine correlations in fungal abundance between co-existing fungal genera on maize in order to identify potential mutualistic and antagonistic relations between the fungi, under different weather patterns. We suggest that absence of correlations among fungi demonstrates good niche partitioning on maize grain; whereas negative correlation may indicate poor niche partitioning, signifying potential for competition between such negatively correlated genera. We specifically predict that other fungal genera with negative correlation with *Fusarium* and *Aspergillus* exist irrespective of prevailing weather condition. Importance for this is identification of genera for potential use in the competitive exclusion of *Fusarium* and *Aspergillus*, respectively. 2) to evaluate influence of fungi that are negatively correlated with *Fusarium* and *Aspergillus* on the levels of the mycotoxins FB1 and AF, respectively. Our specific hypothesis was that negatively correlated fungal genera with *Fusarium* or *Aspergillus*, lead to a decrease in FB and AF in maize, when *Fusarium* or *Aspergillus* are subsequently in low abundance, respectively. This is on basis that where *Fusarium* or *Aspergillus* is outcompeted, the outcompeted fungus will have a reduced competitive edge for their ability to produce FB and AF. In the opposite scenario (high abundance of both *Fusarium* or *Aspergillus* with their negatively correlated genera), we hypothesise that higher levels of FB and AF are likely to occur in the substrate (maize). This is on basis that the two competing fungi (*Fusarium* or *Aspergillus*) will secrete anti-competitor toxins (including FB or AF) due to the competition with the other fungi.

3.2 **Materials and Methods**

3.2.1 Determination of fungal correlations

3.2.1.1 Sample collection and preparation

We investigated correlations within external and internal mycobiomes. Internal mycobiome was for purposes of comparing consistence in fungal correlations between

external and internal mycobiomes as well as to check possible changes in their relative abundances. Data for external mycobiome had previously been generated by DNA amplicon sequencing in Chapter 2 (B. Katati *et al.* (in press)). Internal mycobiome data was generated in this study:

Maize grains were retrieved from freezer at -35°C (Scientemp, model 3565 S, Michigan 49221, USA) as part of previously studied material prepared as described in Chapter 2 for mycobiome assays. In all, the samples represented maize fields grown under four weather variables described in Chapter 2. The variables were on basis of rainfall pattern during the maize growth season. The variables were high rainfall (N1 and N2), low rainfall (S1 and S2), were S1 was in addition characterised by a dry spell. The retrieved grain samples were equilibrated to room temperature prior to analysis. For each sample, the entire portion of sample (150 to 200 g) was sterilised in 10% (v/v) sodium hypochlorite solution (household grade) by dipping for 3 min to destroy all surface unwanted DNA (Prince & Andrus, 1992) and microbials. The kernels were then rinsed in sterile water three times and dried inside sterilised cotton bags. The bags were then placed in a Forced Oven Dryer (Heraeus, model D-6450, Hanau, Germany) to rapidly drive out surface moisture (42 ± 2°C, 24 hrs). The dried kernels were then milled using an Ultra Centrifugal Mill (model ZM200, Retsch, Haan, Germany) fitted with a 1.0 mm ring sieve, all material passing through mill. The pot and rings were cleaned between each mill with sterile H_2O. The milled material was placed into a sterile polyethylene bag and stored at -35°C in a freezer pending the DNA extraction.

Additional agronomic factors with potential to influence *Fusarium* proliferation are presented in Supplemental questionnaire. Questionnaire was administered with the help of agricultural field Extension Officers who routinely work with the farmers.

3.2.1.2 DNA extraction and bioinformatic analysis

The extraction of internal mycobiome DNA from the maize mill was done according to the Qiagen PowerSoil quick-start spin protocol (detailed at www.qiagen.com, product catalogue number HB-2495 of 2019) as follows: milled maize grain samples were retrieved from freezer (-35°C) and equilibrated to room temperature. Each sample was thoroughly homogenised by hand and portions of the maize mill scooped carefully from different positions of the sample, collecting about 200 mg into a bead beating tube for DNA extraction. Next, 800 µl of CD1 lysis buffer was added to the tube and the

prescribed PowerSoil quick-start spin protocol followed. The tube beatings were done using a sideways homogeniser (Model MM400 Retch, Haan, Germany) set at 25 beats/second for 12 min continuously. Centrifugations were done at room temperature in a microcentrifuge unit (model 5424, Eppendorf, Hamburg, Germany). The isolated DNA concentration and quality was read on spectrometer (Nanodrop model 2000, Thermo Scientific, Wilmington, DE, USA).

3.2.1.2.1 DNA amplicon sequencing and bioinformatic analysis

The DNA, which included the target internal mycobiome metagenomic DNA, was normalised to 10 ng/µl per sample. The DNA was sequenced on Illumina platform, Miseq V3 by paired-end amplicon sequencing (2 x 300bp) at LGC Genomics (Biosearch Technologies, Berlin, Germany). This is as described in Chapter 2 (B. Katati *et al.,* (in press)) including the bioinformatic analysis.

3.2.2 Influence of fungal abundance correlations on aflatoxin (AF) and fumonisin-b1 (FB1)

3.2.2.1 Sample preparation

From the retrieved samples, we determined levels of FB1 and AF in the corresponding samples of mycobiome assays. The previously stored sample portion (Chapter 2) was retrieved from freezer at -35°C (model 3565 S, Scientemp, Michigan 49221, USA) for the mycotoxins analysis. Prior to milling, sample was equilibrated to room temperature. The entire grain sub-sample was then further homogenised in a 10 kg tumble blender (model MB015, Pharmatech, Warwickshire, UK) for 10 min at 20 rpm. About 1 kg homogenised grain sample was milled using an Ultra Centrifugal Mill (model ZM200, Retsch, Haan, Germany) fitted with a 1.0 mm ring sieve. Prior to mycotoxin analysis, the milled material was further homogenised using a 1 kg tumble blender (model MB005, Pharmatech, Warwickshire, UK). Analysis of the mycotoxins FB1 and AF was done by extraction, derivatisation of extracted material followed by chromatographic analysis as described below.

3.2.2.2 Mycotoxin extraction

The aflatoxin widebore immuno-affinity columns (IAC) were purchased from Romer Labs (Romer Labs Inc., Newark, DE 19713, USA). Other consumables including the fumonisin (FB) IAC (FumoniTest™ narrow bore IAC) were purchased from Vicam™.

Methanol and the Vicam™ AOZ IAC were purchased through the International Atomic Energy Agency. All mycotoxin extractions were matrix matched for generation of a calibration curve compensating for recoveries. The AF and FB1 technical standards had been purchased from Sigma Aldrich (currently Merck Millipore). Centrifugations were carried out at room temperature in a centrifuge (Suppra 22K, Hanil Scientific Inc., Gimpo 10136, South Korea).

3.2.2.2.1 Aflatoxin extraction

For season-1, samples were extracted according to standard AflaTest™ Vicam™ method for determination of aflatoxins by High Pressure Liquid Chromatography (HPLC) (www.vicam.com). We used 50 g sample with 1 min blending at high speed in a 1 L blender (Waring® Commercial, Stamford, CT, USA) as described in the protocol.

For season-2 the standard AOZ Vicam method for simultaneous extraction of Aflatoxins, Ochratoxins and Zearalenone was used. Derivatisation of AFs was post-column automated. Briefly, 5 g homogenised sample portion was weighed into a 50 ml polypropylene tube and 10 ml of 3:2 (v/v) acetonitrile:water added. The tubes were clamped onto a shaker (Burrell Wrist-action shaker, Model 75, Burrell Scientific, LLC, Pittsburgh, PA, USA) and shaken at amplitude 12 for 30 min. The contents were centrifuged at 6000 *g* for 2 min, then 8 ml of the supernatant was diluted with 32 ml 0.01% tween-phosphate buffered saline, pH 7.0, (0.01%-TPBS). Next, 10 ml diluted extract (unfiltered) was immediately passed through an AOZ immunoaffinity column. The column was washed with 10 ml 0.01%-TPBS, followed by washing with 10 ml Milli-Q water, allowing airflow through the IAC bed prior to elution. The AF was eluted from the column into a 5 ml glass tube using 1.5 ml LC-MS grade MeOH followed by a further 1.5 ml 0.1% acetic acid.

3.2.2.2.2 Fumonisin-B1 extraction

For both maize cropping seasons, extraction was done according to the standard Vicam™ method for determination of FBs in maize by HPLC (https://vicam.com/products/fumonitest). A slight modification of the extraction method was made: 5 g homogenised sample portion was weighed into a 50 ml polypropylene tube followed by the addition of 0.5 g sodium chloride (analytical reagent). Next, 10 ml 4:1 (v/v) methanol:water was added to each sample and the tubes were shaken for 30 min at amplitude 12 on a shaker (Burrell Wrist-action shaker, Model 75, Burrell

Scientific, LLC, Pittsburgh, PA, USA). The contents were centrifuged at 6000 *g* for 2 min, then 8 ml of the supernatant diluted with 32 ml phosphate buffered saline, pH 7.0, (PBS) and mixed well. The prescribed protocol was then followed, filtering diluted contents through a 1.5 µm 11 cm microfiber filter paper (Vicam™ part number 31955) prior to passing 10 ml of the filtrate through a FumoniTest™ immunoaffinity column.

3.2.2.3 Mycotoxin derivatisation

The extracted Mycotoxins were derivatised to enable AF-B1, AF-G1 and FB1 be detected on fluorescence detector.

3.2.2.3.1 Aflatoxin-B1, -G1 derivatisation

For season-1, prior to the HPLC analysis AF was derivatised pre-column. The eluted material in a 5 ml glass tube was dried down under a stream of nitrogen at 50°C in a sample concentrator workstation (part number 133718/09, Biotage™, Uppsala, Sweden). The residue was reconstituted by adding 200 µl acetonitrile:water 86:14 v/v and vortexing for 7 s continuously. Next, 300 µl 70:20:10 v/v mixture of water:trifluoroacetic acid:glacial acetic acid was added to the 200 µl reconstituted material and vortexed for 3 s. The mixture was allowed to react for 25 min at 65°C in the sample concentrator's waterbath (without streaming nitrogen), with the top of the 5 ml glass tube covered with parafilm. The contents were then cooled, while covered in light protective aluminium foil. Next, 500 µl deionized water was added to contents and vortexed for 3 seconds. Contents were filtered (0.22 µm, Ø 13 mm, nylon) into a 2 ml screwcap glass HPLC amber vial and immediately injected into HPLC as described further below.

For Season-2, the filtered (0.22 µm, nylon) AF eluate was derivatised post-column after HPLC injection from a 2 ml screwcap glass amber vial. Derivatisation was done using an automated online derivatisation system (part number 10617, LCTech, Obertaufkirchen 84419, Bayern, Germany) coupled to the HPLC unit.

3.2.2.3.2 Fumonisin-B1 derivatisation

The eluate in a 5 ml glass tube was evaporated to dryness at 50°C under a stream of nitrogen in a sample concentration workstation (part number 133718/09, Biotage™, Uppsala, Sweden). The residue was then reconstituted with 200 µl 1:1 methanol:water. Next, 60 µl portion of reconstituted eluted material was transferred into a 2 ml screwcap

glass HPLC amber vial containing 540 µl o-phthaldialdehyde reagent (Vicam™ parts number G5004 and G5003, mixed). The mixture was vortexed for 30 s and allowed to stand for a further 1 min for the derivatisation reaction, and immediately 80 µl was injected into the HPLC as described below.

3.2.2.4 Mycotoxin chromatographic quantification

All analyte (AF/FB1) separations were reversed-phase and achieved on a 4.6 i.d x 100 mm x 3.5 µm particle size Eclipse C18 column, (Zorbax, Agilent Technologies, Santa Clara, CA, USA). An HPLC unit (Agilent Infinity II 1260 Series, Agilent Technologies, CA, USA) was used for the mycotoxins analysis. Detailed HPLC conditions are provided in Supplemental Table 1.

3.2.2.4.1 Aflatoxin analysis

For season-1 samples, 50 µl of filtered, derivatised material was injected into the HPLC unit equipped with a fluorescence detector (FLD) and autosampler. Analyte elution was in isocratic mode at flow rate 0.9 ml/min with acetonitrile:water 1:4 v/v.

For season-2 samples, 50 µl of filtered eluate was injected into HPLC unit as above except that the mobile phase consisted of methanol:water 45:55 v/v (Supplemental Table 1). Post-column derivatisation of AF-B1 and -G1 was achieved using an online UVE™ photochemical reactor (part number 10617, LCTech, Obertaufkirchen 84419, Bayern, Germany).

3.2.2.4.2 Fumonisin-B1 analysis

For both cropping seasons, the same analytical method was used. Elution was done in isocratic mode at flow rate 0.8 ml/min. Mobile phase was Methanol:0.1M sodium dihydrogen phosphate (23:77, v/v), buffered to pH 3.5 with o-phosphoric acid.

3.2.3 Data analysis

AF (season-1 and -2) and FB1 concentration data was acquired and generated using the HPLC Agilent ChemStation™ software. Prior to the data analyses analytes were quantified by matrix-assisted calibration curve based on AF/FB1 fluorescence peak area (as response factor) and corresponding spiked-AF/FB1 concentration (as known variable).

All statistical computations were conducted in software R (R_Core_Team, 2017) version 4.1.0 using the following packages and their functions as described in Chapter 2: Phyloseq (McMurdie & Holmes, 2013) for internal mycobiome abundance; Vegan ("Vegetation analysis") (Oksanen *et al.*, 2010) for internal and external mycobiome composition; Reshape2 (Wickham, 2007) for generation of Pearson's Correlation matrix in order to study the fungal relative abundance correlations; ggplot2 (Wickham, 2016) for data visualisation. *P* values were adjusted by Bonferroni method.

3.2.3.1 Determination of fungal correlations

For assessment of correlations of the fungi, the top 40 genera out of the full external mycobiome (*n* = 61 genera, Chapter 2) were used, given that the top 19 were the most abundant members likely to drive significant correlations. Criterion for potential mutualism or antagonism was that two genera must, respectively, have a positive or negative relative abundance correlation under all the four tested weather patterns, N1, S1, N2 and S2. Criterion for identification of a potential biocontrol agent for AF and FB was that the genus should have a negative relative abundance correlation with *Fusarium* or *Aspergillus* under all four weather variables including the control internal mycobiomes of N1 and S1. Further the organism must successfully occupy the external mycobiome, judged on basis that the genus appears in at least a third of the fields as previously described (Chapter 2), and appearing in all four weather variables. The correlation were generated by Pearson's Correlation matrix using the function Reshape (Wickham, 2007).

3.2.3.2 Influence of fungal correlations on AF and FB1

To associate influence of fungal genera identified as negatively correlated with *Fusarium* and *Aspergillus* relative abundances and on mycotoxin levels, FB1 and AF levels, fungal abundance data was obtained from previous Chapter 2. Fungal genus abundance was ordinated into abundance levels while the mycotoxin (AF or FB1) was response factor, and data analysed by Spearman rank correlation ρ. Due to heterogeneity of the data, relative abundance for *Sarocladium* was ordinated as categorical value as "very low" (0 – 9%, *n* = 18), "low" (10 – 29%, *n* = 23), "medium" (30 – 49%, *n* = 11), "high" (50 – 69% , *n* = 17) and "very high" (≥ 70%, *n* = 11). The corresponding FB1 data was used as response. Principle Coordinate Analysis (PCoA) was used to assess composition of genera between internal and external mycobiome.

Due to a highly heterogenous distribution of such ecological data, *P* values were determined by Permanova test (Anderson & Walsh, 2013).

Wilcoxon rank-sum test was used to determine changes in fungal genera abundances between internal and external mycobiomes. Furthermore, it was used to determine differences in mycotoxins levels between weather variables for non-normal distribution, while ANOVA was used for normal distribution data. Similarly, for comparisons between AEZ, Kruskal-Wallis rank sum was used for non-normal distributions (Shapiro Wilk test, $P < 0.05$) and *t*-test for normal distribution. The FB1 data was log-transformed to satisfy conditions for normality of distribution (Shapiro Wilk Test, $P > 0.05$). Considering that not all *Fusarium* species in the genus produce FBs, FB1 data was spread into six quantiles (x-axis) according to levels of the FB1. Corresponding *Fusarium* abundances were hence auto-generated similarly into six quantiles as well (y-axis). The correlation between *Fusarium* and FB1, and *Sarocladium* and FB1 was then analysed by Spearman rank correlation rho. Similarly, corresponding genera with a negative correlation to *Fusarium* and FB1 were assessed by Spearman rank correlation rho in similar manner. The same principle was applied for AF and *Aspergillus*.

3.3 Results

3.3.1 Fungal relative abundance correlations

For both the external and internal mycobiome, *Fusarium* and *Sarocladium* had the strongest negative correlations in relative abundance throughout weather variables (Figure 2A and 2B). The next most consistent negative correlation throughout tested weather variables was between *Aspergillus* and *Hirsutella*.

Besides the correlations, we detected a difference in composition between internal and external mycobiomes for both S1 and N1 (Figure 3). This was attributed to some genera whose abundances changed from external to internal mycobiome as follows: four genera including *Hirsutella* significantly reduced in abundance with another four reducing to undetectable levels (Table 2); *Stenocarpella* drastically increased in abundance; *Lasiodiplodia* marginally increased. *Aspergillus*, *Fusarium* and *Sarocladium* did not change in abundance.

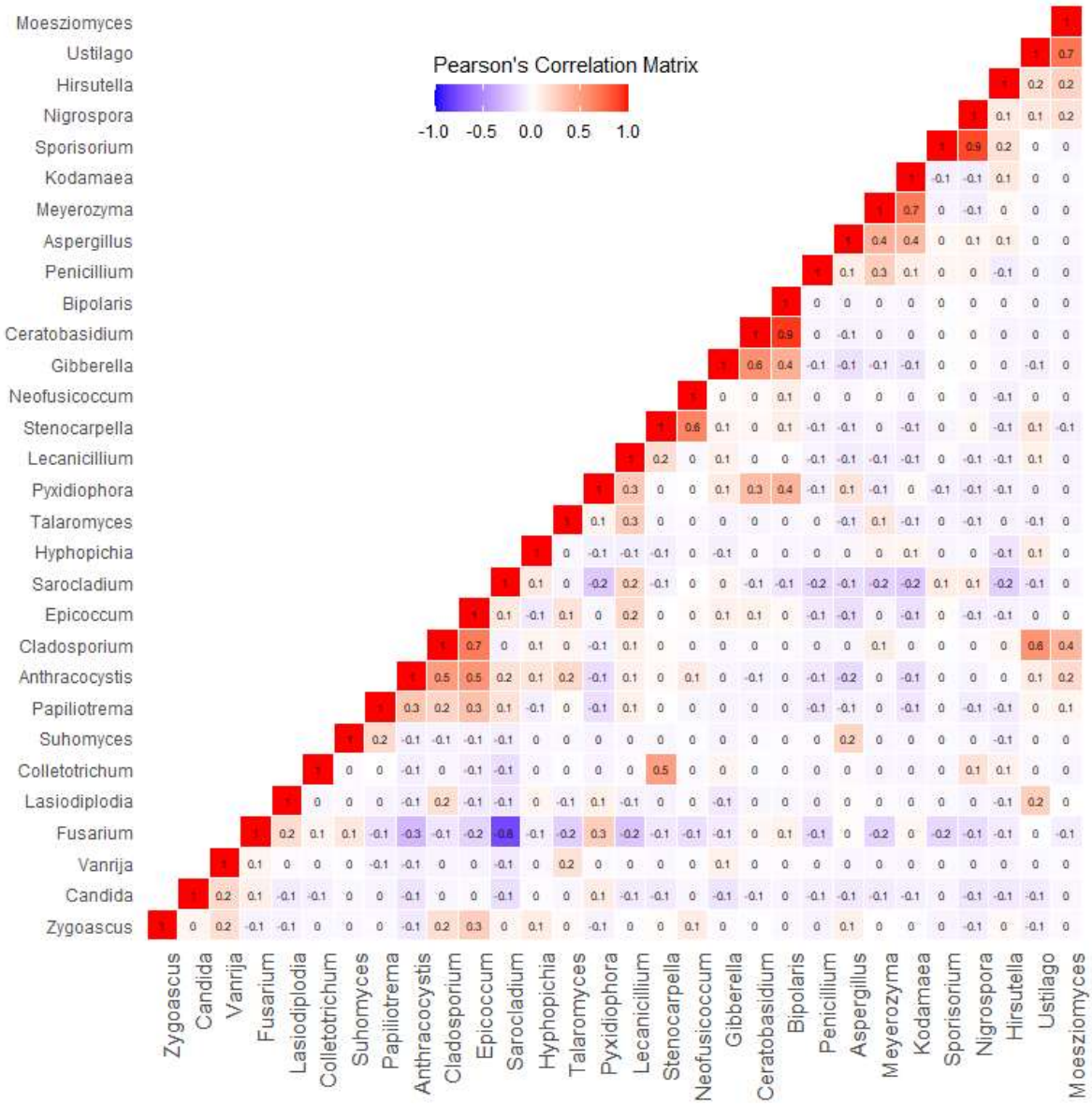


Figure 2A. Pearson's correlation matrix, top 30 most abundant genera. The correlations are the overall combining abundances of all external mycobiome weather variables (N1, S1, N2 and S2) abundances. N1 and N2 are high rainfall weather variables under AEZ3, season-1 and season-2 respectively. S1 and S2 are low rainfall variables under AEZ1, season-1 and season-2, respectively, were S1 is in addition accompanied with a dry spell. Negative values (blue) are negative correlations and positive values (red) are positive correlations in fungal relative abundances. Individual correlations of fungi by weather variables for external mycobiome are presented in Table 1.

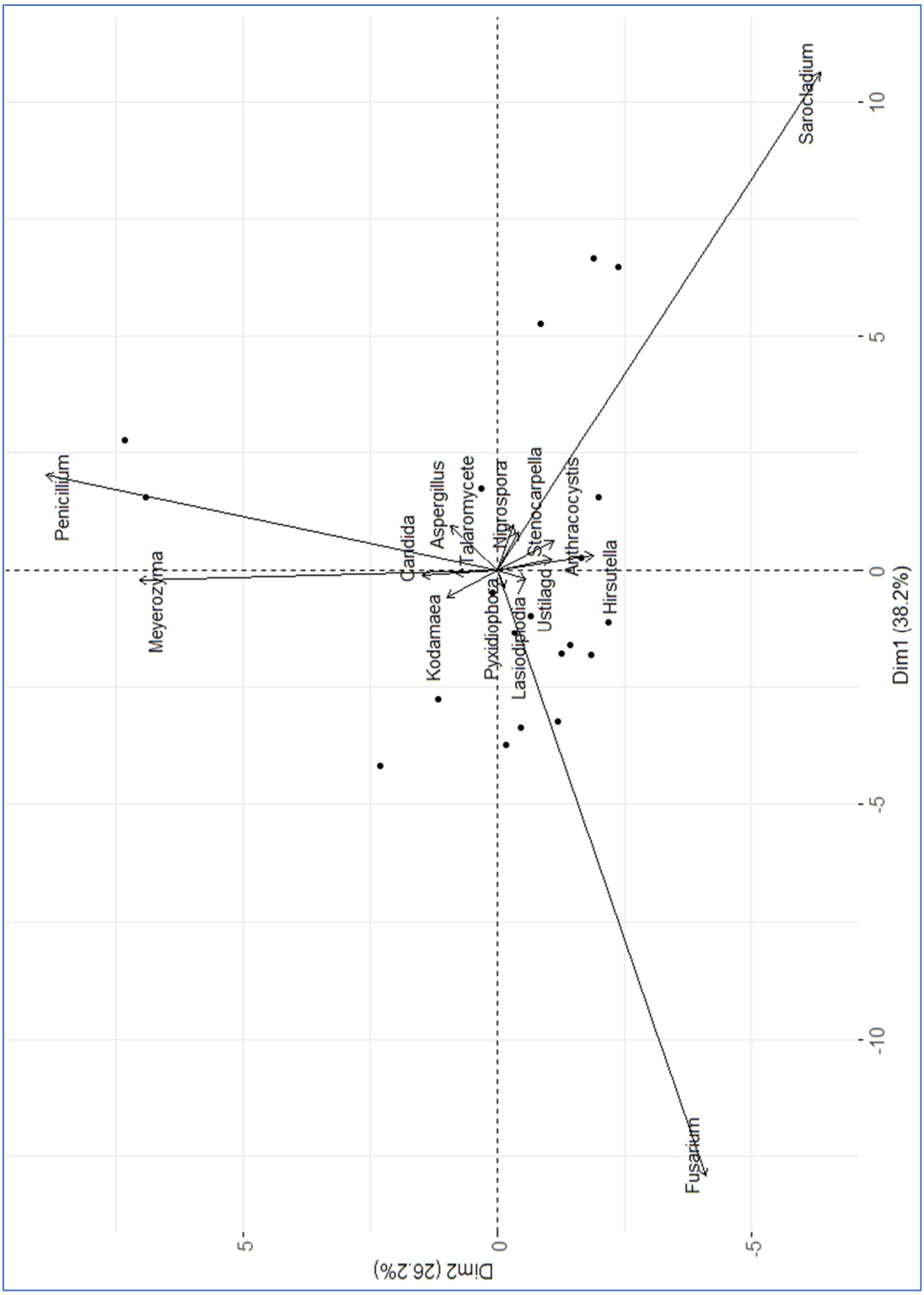


Figure 2B. PCA of external mycobiome fungal relations under weather variable S1 (low rainfall with dry spell) as demonstration example. A negative correlation between *Sarocladium* and *Fusarium* is explained by dimension-1 (Dim-1); Negative correlation between *Hirsutella* and *Aspergillus* is explained by Dim-2. Correlation coefficients for all weather variables are presented in Table 1. A positive correlation is shown between *Penicillium* and *Meyerozyma*. There's no clear correlation between *Meyerozyma* and *Fusarium*, while *Pencillium* has a negative correlation with *Fusarium*. There's no clear correlation between *Fusarium* and *Ustilago*.

Table 1 Genera with positive or negative correlations throughout weather variables (external mycobiome).

Genus 1	Genus 2	Rainfall pattern	Correlation coefficient
Aspergillus	*Hirsutella*	High (N2)	-0.12**
		High (N1)	-0.30**
		Moderate (S)	-0.30**
		Dry spell (S1)	-0.01**
Candida	*Stenocarpella*	High (N2)	-0.21**
		High (N1)	-0.08**
		Moderate (S)	-0.06**
		Dry spell (S1)	-0.18**
Cladosporium	*Fusarium*	High (N2)	*ND*
		High (N1)	-0.09**
		Moderate (S)	-0.34**
		Dry spell (S1)	-0.21**
Cladosporium	*Meyerozyma*	High (N2)	*ND*
		High (N1)	0.26
		Moderate (S)	0.02
		Dry spell (S1)	0.00
Fusarium	*Talaromycete*	High (N2)	0.00
		High (N1)	-0.33**
		Moderate (S)	-0.15**
		Dry spell (S1)	-0.09**
Fusarium	*Sarcoladium*	High (N2)	-0.93**
		High (N1)	-0.74**
		Moderate (S)	-0.92**
		Dry spell (S1)	-0.62**
Fusarium	*Ustilago*	High (N2)	-0.05**
		High (N1)	-0.06**
		Moderate (S)	-0.21**
		Dry spell (S1)	-0.03**
Hirsutella	*Talaromycete*	High (N2)	-0.03**
		High (N1)	-0.09**
		Moderate (S)	-0.14**
		Dry spell (S1)	-0.20**

Table 1. Positive or negative correlations of genera on external mycobiome. (**) indicates negative correlation. *ND* means not ascertained.

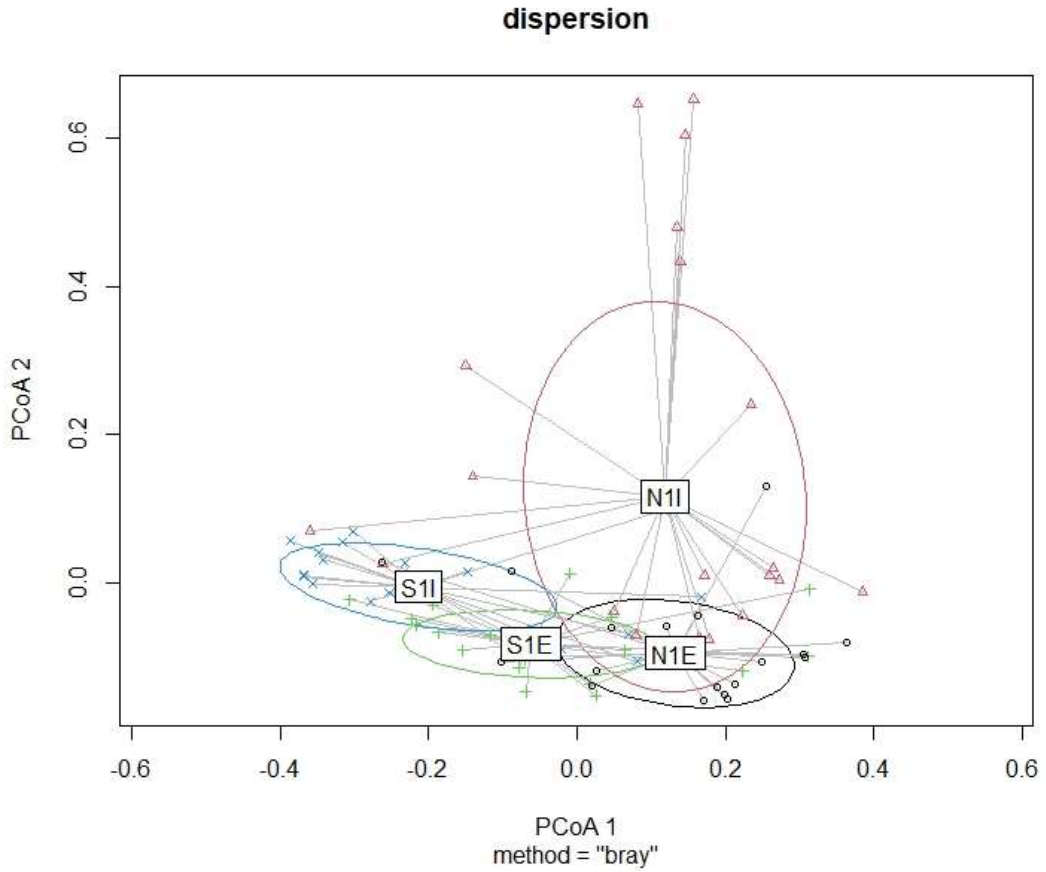


Figure 3. Principle Coordinate Analysis for dispersion of genera abundance between internal and external mycobiomes. 'S' means samples collected from the southern agroecological zone 1, while 'N' means samples collected from the northern agroecological zone 3. 'I' means internal mycobiome, 'E' external mycobiome. Genera composition are compared by Latitude: S-internal v S-external, $P = 0.006$; N-internal v N-external, $P = 0.006$.

Table 2. Genera that changed in relative abundance from external to internal mycobiome

Genus /	**Wilcox pairwise test, internal v external**		**Relative abundance (%) in ext. and int. mycobiome**			
Weather variable	SE-SI	NE-NI	SE	SI	NE	NI
Anthracocystis	0.0055*	0.0010*	1.29	0.13	4.20	0.75
Candida	0.0000*	0.0004*	0.31	0.01	0.80	0.14
Hirsutella	0.0003*	0.0000*	8.67	0.61	7.20	0.14
Kodamaea	0.0010*	1.0000	0.61	0.03		
Lasiodiplodia	1.0000	0.0360*			0.00	0.21
Meyerozyma	0.0003*	0.0001*	5.55	0.13	1.60	0.05
Papiliotrema	0.0510	0.0001*			0.07	0.00
Stenocarpella	1.0000	0.0050*			1.71	25.48
Talaromyces	0.0840	0.0392*			4.82	3.89
Ustilago	0.0838	0.0318*			0.05	0.00

Table 2. 'S' means samples collected from the southern agroecological zone 1, while 'N' means samples collected from the northern agroecological zone 3. 'I' means internal mycobiome, 'E' external mycobiome. * Significant change in relative abundance.

3.3.2 Influence of fungal genus abundance on mycotoxin levels

Higher levels of *Sarocladium* correlated with lower levels of FB1 (Figure 4; Spearman rank correlation rho, *P* = 0.02, rho ~ -1). Although *Hirsutella* and *Aspergillus* were negatively correlated (Table 1), the influence of *Hirsutella* on levels of aflatoxin could not be ascertained as only 11 out of 80 samples were positive for field AF. *Ustilago* relative abundance did not have a significant correlation with *Fusarium* relative abundance (Spearman rank correlation rho, *P* = 0.4972, rho ~ -0.371.) or any correlation with levels of FB1 (Spearman rank correlation rho, *P* = 0.2417, rho ~ -0.6.). The lack of correlation is also demonstrated by the negligibly negative correlation between *Ustilago* and *Fusarium*, (Table 1; Figure 2B).

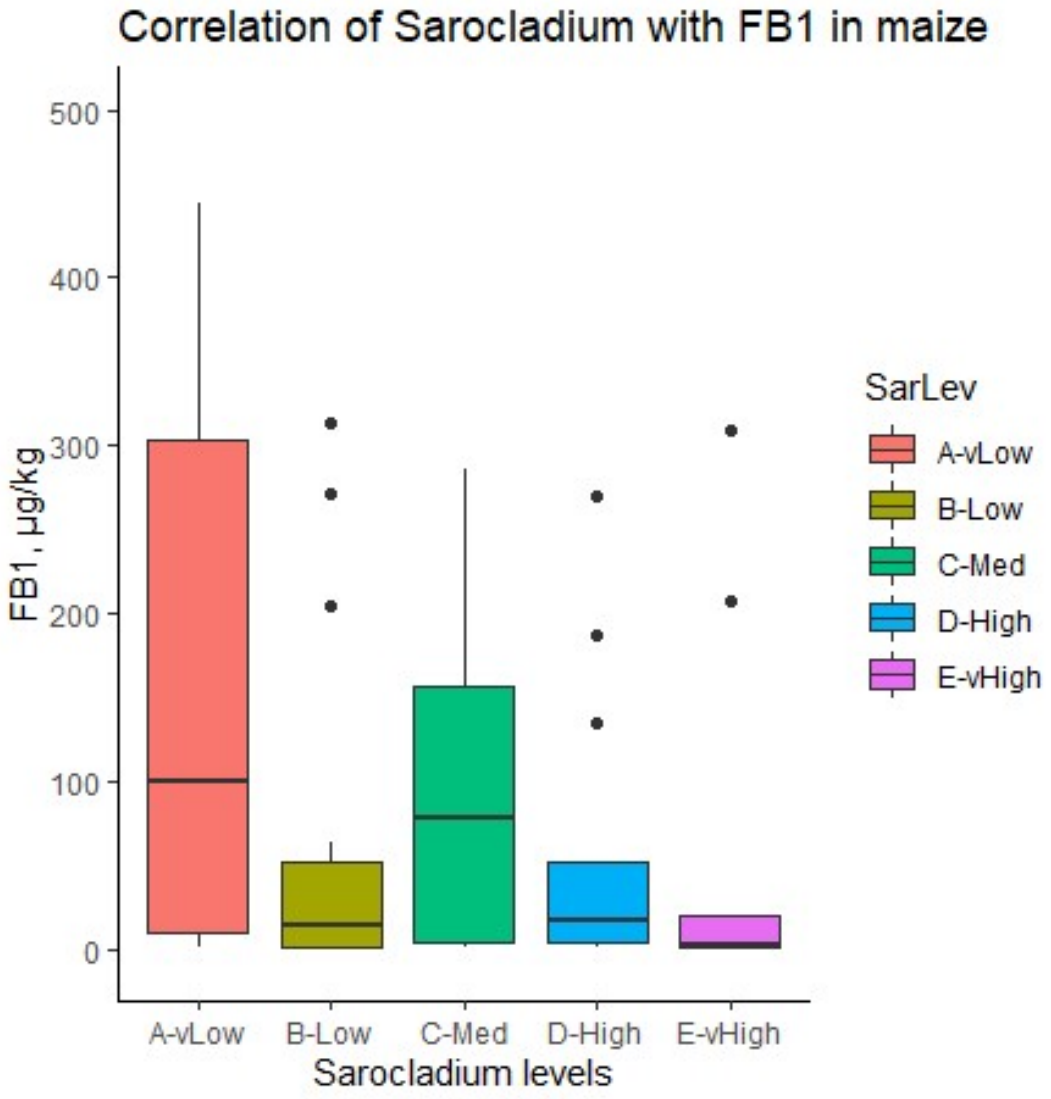


Figure 4. Ordinal data showing FB1 concentration in maize (y-axis limit 500 µg/kg) against *Sarocladium* levels categorised as 'A-vLow' = 0 – 9%, 'B-Low' = 10 – 29%, 'C-Med' = 30 – 49%, 'D-High' = 50 – 69%, E-vHigh ≥ 70%. Higher levels of *Sarocladium* were associated with lower levels of FB1 and vice versa (Spearman rank correlation rho, *P* = 0.02, rho ~ -1.0).

Table 3. Aflatoxin and fumonisin-B1 levels in preharvest maize, µg/kg.

Field №	Season	AEZ	Weather Variable	District	AF-B1 range,	Total, AF, range	FB1 * range
1 – 5	One	I	S1	Kalomo (a= 1; b = 1)	0.9 – 33.9	13.5	18.7 - 357.4
6 - 10	One	I	S1	Kazungula (a = 1; c = 1)	0.9 - 118.2	1.7 - 208.2	32.1 - 938.3
11-15	One	I	S1	Livingstone (b = 1; c = 1 ; a = 1; d =1)	1.1 – 306.8	1.9 – 348.4	15.1 – 1787.1
16-20	One	I	S1	Mulobezi (b = 1 ; a = 1; d = 1; e = 1)	0.9 – 35.8	1.7 – 58.7	21.4 - 1715.9
21-25	One	III	N1	Samfya	ND	ND	20.9 - 493.9
26-30	One	III	N1	Mansa	ND	ND	12.6 - 488.1
31-35	One	III	N1	Luwingu (e = 1)	ND	ND	28.7 - 1454.9
36-40	One	III	N1	Kawambwa (d = 1)	ND	ND	11.4 - 1487.0
41-45	Two	I	S2	Kalomo (d = 1; e = 1)	ND	ND	1.0 - 4328.5
46-50	Two	I	S2	Kazungula (d= 2; e = 1)	ND	ND	1.0 - 4764.8
51-55	Two	I	S2	Livingstone (d= 1)	ND	ND	1.0 - 3681.5
56-60	Two	I	S2	Mulobezi	ND	ND	1.0 - 962.2
61-65	Two	III	N2	Samfya (d = 1)	ND	ND	1.0 - 1694.8
66-70	Two	III	N2	Mansa	ND	ND	1.0 – 51.1
71-75	Two	III	N2	Luwingu	ND	ND	1.0-1.0
76-80	Two	III	N2	Kawambwa	ND	ND	1.0 - 109.5

Table 3. District fields aflatoxin (AF) and fumonsin-B1 (FB1) levels in preharvest maize. The letters 'a,' 'b' and 'c' indicate levels of AF in fields as follows [(a)] = 10 – 50 µg/kg; [(b)] = 50 – 100 µg/kg; [(c)] > 100 µg/kg. ND = below limit of detection: AF-B1 = 0.29 µg/kg, Total AF = 1.09 µg/kg as sum of detection limits for AF-B1, -B2, -G1 and -G2. AF was detected only under fields in S1 except fields number 4, 5, 6, 17, as well as, 12 and 18. Weather variables are N1 and N2 = high rainfall, under AEZ3, season-1 and season-2 respectively; S1 = low rainfall with dry spell under AEZ1 season-1; S2 is low rainfall weather variable under AEZ1, season-2. The letters 'd' and 'e' indicate levels of FB1 in the field as follows: [(d)] = FB1 > 1000 µg/kg (EU regulatory limit); [(e)] = FB1 > 4000 µg/kg (US Food and Drug Administration regulatory limit). ND FB1 = 1 µg/kg**.** * FB1 adjusted value by multiplication factor x1.67 taking into consideration analytical recovery value for FB1 of 60% based on value of reference material extracted alongside samples.

3.4 Discussion

3.4.1 Fungal correlations defined by niche partitioning

We demonstrate in this study that niche partitioning has an effect on relative abundance correlations of fungal genera. As *Sarocladium* had a strong negative

relative abundance correlation with *Fusarium*, this suggests poor niche partitioning between the two genera, characteristic of competing fungi for niche space as a likely consequence of similarity in niche utilisation. The demonstrated negative relative abundance correlation between *Fusarium* and *Sarocladium* resonates with the fact that *Sarocladium* has been demonstrated to be antagonistic with *Fusarium*, inhibiting its growth *in vitro* studies (Comby *et al.*, 2017; Kemp *et al.*, 2020). The second consistent negative correlation between genera was detected between *Hirsutella* and *Aspergillus* irrespective of weather variable. Contrary to *Fusarium* and *Sarocladium*, we did observed that there was no significant negative correlation between *Fusarium* and *Ustilago*. This may suggest that the partitioning between *Fusarium* and *Ustilago*, under the given weather variables in this study, or the overall biotic environment, was fairly good for these fungal genera which may be expected to have a negative correlation. However, it should be noted that *Fusarium* in competition with *Ustilago* has been demonstrated *in vitro* to lead to the production of secondary metabolites by *Fusarium* (Alvarado-Serrano & Knowles, 2014). Irrespective of the prevailing weather conditions, the abundance of *Sarocladium* was not significantly affected as previously shown in Chapter 2 (B. Katati *et al.* (in press)). The above argument takes into account that rainfall can be a strong driver in determining stability in niche partitioning, such that dry conditions can destabilise the balance to favour the occurence of xerophilic fungi. Our findings demonstrate that rainfall pattern was not a factor to alter the niche partitioning for *Sarocladium* on kernels. The same pattern for *Sarocladium* applies for *Fusarium*, whose niche partitioning with *Sarocladium* was not determined by rainfall pattern. We do however observe in the biotic environment that abundance of *Sarocladium* was strongly negatively correlated with *Fusarium* (Figure 2A). This may suggest potentially antagonistic relationship between *Sarocladium* and *Fusarium* that can be attributed to poor niche partitioning between the two genera. In this case, the two fungi could be utilising similar niches. The consequence of poor niche partitioning is competitive exclusion, which may be observed as reduction in abundance of one genus relative to the other, leading to a negative correlation between such genera (Figure 2A).

It should also be noted that although maize was the ecological niche for both *Sarocladium* and *Fusarium* under the different weather variables, an exception is seen with Samfya district which was dominated by *Sarocladium* throughout fields as previously described (Chapter 2). The terrain of Samfya is characterised by high

number of water bodies (plains and lakes) characteristic of rice growing areas. In line with this, *Sarocladium* is known as a rice pathogen, suggesting that the terrain of Samfya supported its stronger existence in the environment, potentially displacing *Fusarium* on maize. This does demonstrate that the deterministic factor in niche partition for *Sarocladium* and *Fusarium* was not rainfall, but may rather be another abiotic factor related to high terrain water availability in environment, favouring one genus in the environment over the other. This would have to be investigated further.

While studies have demonstrated that *Trichoderma* is a ubiquitous soil dweller (Bacon *et al.*, 2001; Egidi *et al.*, 2019), it was not detected on the maize. As soil is regarded the natural reservoir for fungi, we may attribute the absence of *Trichoderma* on maize in our study to its poor colonisation of the maize in presence of other genera under this study's weather conditions. Similarly, in a previous Zambian study, *Trichoderma* was only detected in one district out of 11 studied (Mukanga *et al.*, 2010). This demonstrates that niche partitioning driven by our weather variables may potentially not support the *Trichoderma* natural habitation of maize, or that the genus would be excluded by other genera. Importance of *Trichoderma* to the maize mycobiome is its non-pathogenicity to maize (Gromadzka *et al.*, 2019) which would make it the desirable resident on maize compared to maize-pathogenic *Fusarium*. For example, *Trichoderma* is a prospective fungus in the competitive exclusion of *Fusarium* in wheat and maize (Filizola *et al.*, 2019; Galletti *et al.*, 2020; Lu *et al.*, 2020; J. Palazzini *et al.*, 2018; Yassin *et al.*, 2021).

3.4.2 Influence of genera correlations on mycotoxin (FB1/AF) levels

With respect to antagonism that may be mediated by presence of mycotoxins, a negative correlation was detected between *Fusarium* and *Sarocladium* with a consequence on levels of the mycotoxin fumonisin-B1 (FB1) produced by *Fusarium*. In terms of production of secondary metabolites in fungi in defence of their ecological niche due to competition with other fungi, the production of cell-degrading metabolites by *Fusarium* in competition against *Ustilago* has been demonstrated (Alvarado-Serrano & Knowles, 2014). More importantly perhaps may be the fate of levels of FB1, a known carcinogen (Yu *et al.*, 2021) largely produced by *Fusarium*, in the maize as a direct result of the negative correlation with *Sarocladium*. We do demonstrate for the first time the (indirect) negative influence of *Sarocladium* on levels of FB1 in preharvest maize. The lower levels of FB1 due to higher levels of *Sarocladium* may be linked to

the reduced competitive growth ability of *Fusarium* against *Sarocladium*. This is demonstrated by the lower relative abundance of *Fusarium* as the relative abundance of *Sarocladium* increased, based on the negative correlation between the two genera (Figure 2A, Table 1). This is the case of poor niche partitioning between two genera, leading to reduction of population of one species compared to the other. We did not detect any correlation between *Ustilago* abundance and FB1 levels in maize (Spearman rank correlation rho ~ -0.6, P = 0.2417). This is despite the fact that confrontation between *Fusarium* and *Ustilago* may lead to upregulation of excretion of (other) secondary metabolites and cell wall-degrading proteins by *Fusarium* (Alvarado-Serrano & Knowles, 2014). Although *Sarocladium* is a reported rice pathogen (Sakthivel *et al.*, 2002), it was capable of naturally and extensively infecting maize through the tested weather variables, satisfying preliminary conditions as a genus with prospects for the competitive exclusion of *Fusarium* for the biocontrol of FB in maize. It should, however, also be noted that some strains of *Sarocladium zeae* and *S. strictum* are able to produce secondary metabolites such as beauvericin and enniatin B1 (Blaszczyk *et al.*, 2021). Nonetheless, *Sarocaldium zeae* is an endophyte of maize, and the two metabolites (beauvericin and enniatin B1) are of much less concern in the food safety landscape when compared to AF and FBs, which have track record of human carcinogenicity (IARC, 2012) with subsequent strong trade regulations. Some species of *Sarocladium* can, however, be human opportunistic pathogens (Perez-Cantero & Guarro, 2020; Tabliago *et al.*, 2022).

As regards *Aspergillus*, some species belonging to the genus are known to produce the secondary metabolite aflatoxin (Frisvad *et al.*, 2019). This study has demonstrated a negative relation in both external and internal mycobiome between *Aspergillus* and *Hirsutella* across the investigated weather variables (Table 1). Although this was the case, our investigation was not conclusive in terms of identifying a negative influence of *Hirsutella* abundance on levels of aflatoxin in the maize. This was due to the low number of AF-positive samples above quantifiable limit in the field (11/80, Table 3). However, it should be noted that a successful displacement of *Aspergillus* by *Hirsutella* would imply reduction in the aflatoxin levels at preharvest.

3.4.3 Prospects in biocontrol of AF and FB.

The observed negative correlations in fungal relative abundances are a useful attribute in the prospect for competitive exclusion of important mycotoxigenic genera such as

Aspergillus and *Fusarium*. The biocontrol of mycotoxins such as AF, FB and deoxynivalenol is premised on competitive exclusion of one fungus by another (Agbetiameh *et al.*, 2020; Bandyopadhyay *et al.*, 2016; Błaszczyk *et al.*, 2017; Busko *et al.*, 2008; Cotty & Bayman, 1993; Filizola *et al.*, 2019; Tian *et al.*, 2016). Therefore the observed negative correlation between *Fusarium* and *Sarocladium* as well as *Aspergillus* and *Hirsutella* helps define prospects in utilisation of these genera for the biocontrol of FB and AF, respectively. It should be noted that one of the attributes for successful biocontrol of mycotoxigenic fungi is that the biocontrol agent should be able to effectively and widely contaminate the crop and thrive in the environment in which the target pathogen thrives. This attribute has been displayed by *Sarocladium* in this investigation, with *Fusarium* as a target pathogen (Figure 2A, Table 1). This then suggests identification of strains of *Sarocladium* that are non-pathogenic to humans and maize for the prospective competitive exclusion of *Fusarium*. As regards *Hirsutella*, however, some early warning limitations observed for its strong candidature in the competitive exclusion of *Aspergillus* was the strong reduction in its abundance from external to internal mycobiome (Table 2); coupled with the lack of an overall strong negative correlation with *Aspergillus* (< -0.25) (Table 1).

3.5 Conclusion

The present work is a study on correlations between fungal genera relative abundances, under the assumption that these correlations may be driven by niche partitioning. We found evidence for these correlations using fungal populations on sampled maize, grown under different weather patterns. The approach was by investigating the fungal microbiome on the surface of kernels (external mycobiome) as well as inside the kernels (internal mycobiome). It's demonstrated that the niche partitioning can affect the ability for competitive exclusion of fungi on maize. A critical next step in our work is to design directed experiments to assess if these correlations are indeed caused by niche partitioning and that mycotoxins play a critical mechanistic role. Experiments using pairs of strains grown under various levels of niche partitioning (e.g. different types of nutrients) could be used to test direct and specific predictions on what strains my outcompete others under what levels of niche partitioning and whether or not mycotoxins such as AFs and FBs would be important antagonistic agents.

3.6 Supplemental data

Supplemental Table 1. HPLC conditions for mycotoxins detection

Analyte	**Mycotoxin**	Aflatoxin	Aflatoxin	Fumonisin
Method	**™**	Vicam AOZ	RomarLabs WB	Vicam FumoniTest WB
Column	**Reversed-phase, C18**	4.6 I.D x 100 mm x 3.5 µm	4.6 I.D x 100 mm x 3.5 µm	4.6 I.D x 100 mm x 3.5 µm
Mobile phase	**v/v**	Methanol:water 45:55	Acetonitrile:water 20:80	Methanol:0.1M sodium dihydrogen phosphate (23:77)
	Flow rate, ml/min	0.5	0.8	0.9
Column Oven	**°C**	30		30
Detector	**Excitation, nm**	365		335
	Emission, nm	455		440
Matrix Calibrants	**µg/kg**	1, 5, 10, 50, 100 and 500		7.5, 15, 30, 60, 150 and 250
Limit of Detection	**µg/kg**	B1 = 0.29; B2 = 0.22; G1 = 0.30; G2 = 0.28		1.0

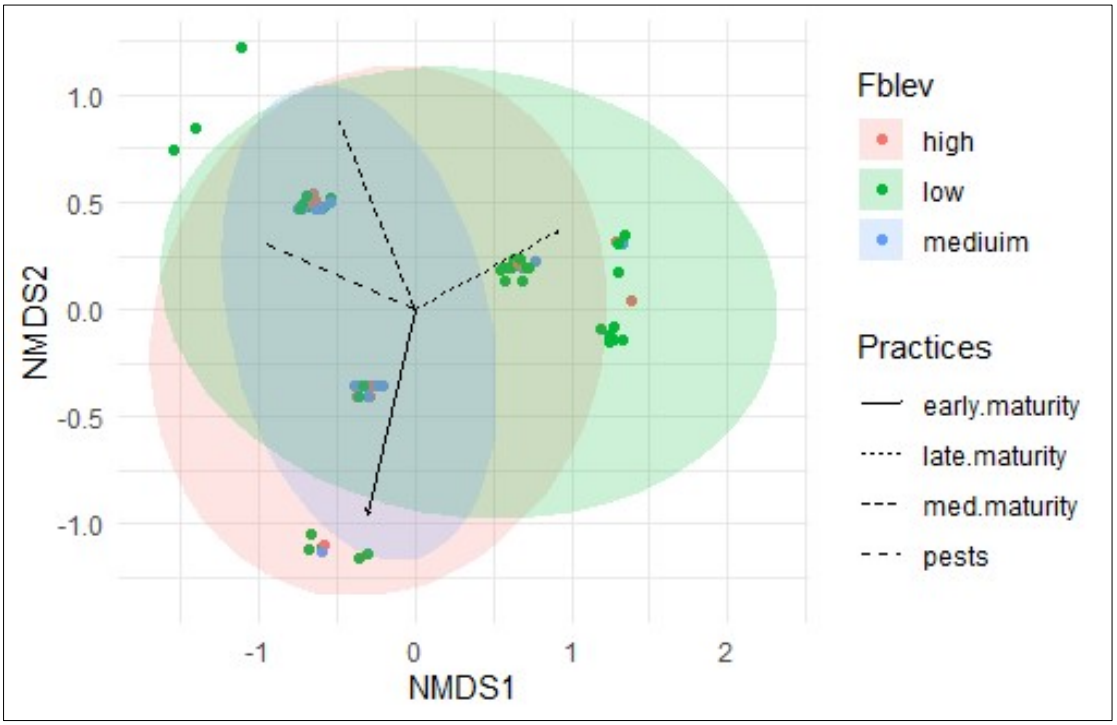
Supplemental questionnaire

(Questionnaire with questions pertaining to agronomic factors that could influence the fungal microbiome).

1. What breed of maize was planted?
   1.1. Was this breed drought resistant?
2. Were pesticides used?
   2.1. If so, what pesticides were applied?
   2.2. Please indicate for each pesticide the application regime (e.g. one-off or continuous application, when was the pesticide applied, how much)
3. Did field experience any pests?
   3.1. If any, please name them (e.g. stalk borer, army worms)
   3.2. Were pests eradicated? If so, please indicate how.
4. Was any fertilizer applied to field?
   4.1. If yes, was the fertilizer a one-off or continuous application, and what was the regime?
5. What was the previous crop planted?
6. Was residual plant material prior to sowing burned?
7. What type of seed was planted? (early, late maturing, or recycled)

8. When was sowing done?
9. NA
10. NA
11. NA
12. NA

Supplemental Figure 1



Supplemental Figure 1. NMDS relating levels of FB1 in maize grain against seed type by maturity, pest or cropping type. No correlation was observed as shown by ellipse overlaps.

To check any possible influence of seed maturity type (early or late variety), cropping type, as well as presence and absence of pests on fumonisin levels (Roucou *et al.*, 2021), Non-Metric Multi-Dimensional Scaling (NMDS) was used. For the NMDS, levels of FB1 were ordinated as low (0 – 49 µg/kg, n = 42), medium (50 – 499 µg/kg, n = 24) and high (≥ 500 µg/kg, n = 14) across the 80 sampling points. The independent variables (seed maturity type and pests) were tested against the FB1 levels in the NMDS.

3.7 Acknowledgement

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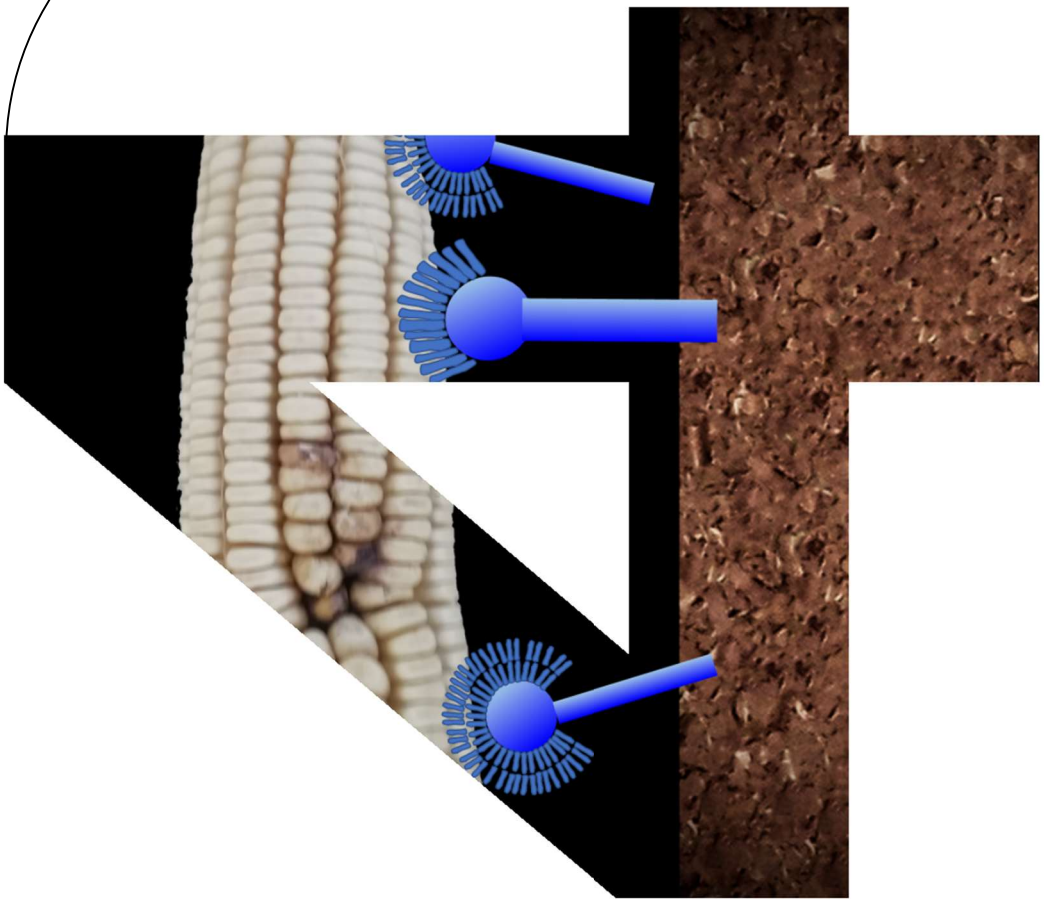
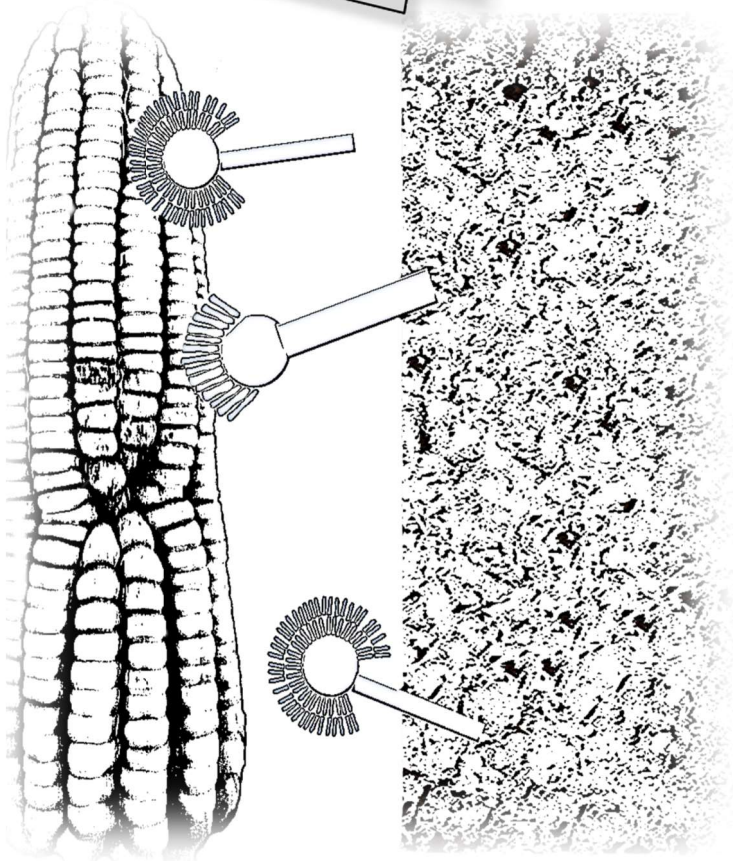
If fungi were a human world, soil would be their home and maize their holiday resort





Chapter IV

Natural infection of preharvest maize with *Aspergillus* section *Flavi* and aflatoxin contamination

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4.0 Abstract

Soil is a reservoir for *Aspergillus* section *Flavi* (*Flavi*, for short), where they dwell as opportunistic pathogens, becoming opportunistic saprophytes on maize as their niche. We investigated the natural infection of preharvest maize by *Flavi* and the contamination with aflatoxins (AFs). We furthermore investigated the selection frequency of soil *Flavi* for toxigenicity with respect to rainfall conditions during the maize growth season. Factors investigated that may influence maize *Flavi* infection or aflatoxin contamination were ear husk condition, rainfall intensity (dry spell), maize genotype, field burning and pest infestation. With respect to toxigenicity we investigated the ability of *Flavi* to produce AF-B and -G variants as well as cyclopiazonic acid (CPA). *Flavi* include common members like *A. flavus* and *A. parasiticus*. Maize and soil samples were collected over two seasons from two climatically contrasting regions of Zambia as the study model. Dilution plate technique on modified rose Bengal agar was used to quantify *Flavi* abundances in maize and soil, while Thin Layer Chromatography (TLC) was used to qualitatively determine AF and CPA production of isolates on Yeast Extract Sucrose medium. Absence of AF by TLC was verified by HPLC. Results showed that loose husk cover did not lead to *Flavi* infection of ears. Furthermore, although *Flavi* were detected in all soils, we did not detect it in all preharvest maize samples. Most *Flavi* incidences on preharvest maize were detected during a dry spell, the key factor likely to lead to the observed *Flavi* ingress. Pest incidence was the second most important factor increasing *Flavi* infection. Aflatoxins were only detected in fields (n = 11/20) under a dry spell. Furthermore, most soil isolates were aflatoxigenic (83 to 97%) which may suggest the potential risk for maize contamination with aflatoxin, should maize be the niche for such soil isolates, for example *A. parasiticus*.

4.1 Introduction

The maize grain is a perfect substrate for an array of fungal genera including *Aspergillus*. Maize contaminated with aflatoxigenic species of *Aspergillus* section *Flavi* (*Flavi*, for short) becomes susceptible to contamination with aflatoxins (AFs) – a group of known human carcinogens also implicated in impairing livestock productivity (Cao *et al.*, 2022; Ivanovics *et al.*, 2021; Robens & Richard, 1992). The infection mechanism of *Flavi* in maize is a complex process which involves colonisation of silks (Payne, 1987) and is also enabled by damaged kernels in combination with insects (Barry *et al.*, 1985; Marsh & Payne, 1984; Widstrom, 1979; Widstrom *et al.*, 1975; Windham *et al.*, 1999). Several crop infection drivers are known and include host plant resistance against infection (Badu-Apraku & Fakorede, 2017; Brown *et al.*, 2016; Gupta *et al.*, 2022); cropping systems, (Bhatnagar-Mathur *et al.*, 2015; WHO, 2018a); insect damage; plant/grain condition, which is directly linked to factors like drought stress (Xu *et al.*, 2022); planting date, weed control, et cetera (Venkateswarlu *et al.*, 2011). Although these factors can partly be controlled by breeders and growers to prevent infection (Lillehoj, 1983; Venkateswarlu *et al.*, 2011) environmental pressures still lead to the infection of crop with *Flavi* and AFs.

One major factor influencing the infection of maize by *Flavi* is the climate, as has been demonstrated by previous studies (Battilani *et al.*, 2016; Medina *et al.*, 2014). Increase in temperature, drought stress, and late rain, have been identified as major factors aggravating *Flavi* and aflatoxin contamination in crops (Cast, 2003; Liu *et al.*, 2016; Njeru *et al.*, 2019; Vardon, 2003). In addition, a study by Horn showed that soil population densities of *Aspergilli* in the United States appeared to be correlated with the frequency of drought in aflatoxin-susceptible crops (Horn & Dorner, 1998). Furthermore, *Flavi* infection is chronic in the USA's southeast, a region of perpetual drought and hot conditions (Payne & Widstrom, 1992). Such climatic patterns can directly affect the plant condition, such as grain condition and its defence against microbial attack (Payne, 1998), including *Flavi* attack. With respect to fungal infection due to insect attack on the crop, husk cover of the maize ear has been linked to insect access to kernels, with tight husks preventing insect access (Lillehoj, 1983), subsequently reducing aflatoxin contamination (Barry *et al.*, 1986; Demissie *et al.*, 2008).

The source of *Flavi* contaminating maize, soil, is known to be a reservoir for *Aspergilli* (Boyd & Cotty, 2001; Cotty, 1997; B. Horn, 2005; Horn, 2003; Jaime-Garcia & Cotty, 2004; Jaime-Garcia & Cotty, 2010; Kachapulula *et al.*, 2017b), and is a relevant abiotic factor to the infection of maize in two ways: 1) as a source of aflatoxin-producing (toxigenic) *Flavi,* and 2) as a source of non-aflatoxin-producing (atoxigenic) *Flavi*. For example, a previous Zambian study demonstrated that soils from areas cultivated with maize and groundnuts, harboured *Flavi* species, amongst them toxigenic strains of *A. parasiticus* and *A. flavus* (Kachapulula *et al.*, 2017b), and atoxigenic species. This illustrates the relevance of soil as a factor in the etiology of contamination of maize with *Aspergilli* and aflatoxins. Furthermore, the selection pressure in *Flavi* to produce or not produce aflatoxin (aflatoxigenicity) is linked to *Flavi* confrontation with fungivores, such that toxigenic *Flavi* may better survive in such an environment (Drott *et al.*, 2017). This may suggest an advantage for *Flavi* to select for toxigenicity over atoxigenicity.

Taking advantage of the presence of different species of *Flavi* in soil, biocontrol of aflatoxin premised on competitive exclusion, artificially applies to the soil atoxigenic isolate(s) (Abbas, 2005; Atehnkeng *et al.*, 2022; Bandyopadhyay *et al.*, 2016; Cotty & Bhatnagar, 1994), giving such isolate(s) an early growth advantage. This enables the atoxigenic *Flavi* isolate(s) to contaminate the crop before toxigenic *Flavi* , subsequently excluding maize ear colonisation by toxigenic strains and preventing aflatoxin contamination.

In this study we investigated: (1) the natural infection of maize with *Flavi* and the subsequent aflatoxin contamination of the maize; and (2) the mycotoxigenicity of the soil isolates based on aflatoxin (AF) and cyclopiazonic acid (CPA) profiles. Although husk condition has been associated with fungal ingress into maize, we hypothesise that: a) within natural infection of maize with *Flavi*, loose-husk cover does not lead to higher infection of preharvest maize with *Flavi*. We base our hypothesis on the grounds that *Flavi* are xerophilic (moderately xerophilic) and are likely to proliferate during storage unlike at preharvest, unless conditions are pre-disposing for their proliferation at preharvest, such as dry weather conditions; b) loose husk cover, like poor grain health, correlates with higher aflatoxin contamination under dry weather conditions; We furthermore hypothesise that c) soil *Flavi* from the drier and hotter southern agroecological zone (AEZ1) districts of Zambia have a higher selection for

aflatoxigenicity compared to soil *Flavi* from the wetter and comparatively cooler northern (AEZ3) districts. We base the last hypothesis on higher environmental stress for the fungal isolates in the drier conditions normally associated with increased aflatoxin production (Angel Medina *et al.*, 2015) leading to evolutionary selection for higher aflatoxigenicity.

4.2 Materials and Methods

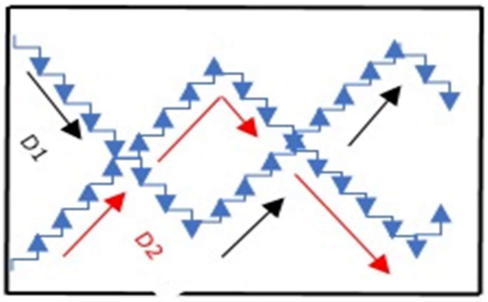
4.2.1 Sampling and sample preparation

We collected soil and maize samples, as described in Chapter 2 (B. Katati *et al.* (in press)). The two sets were from five fields per district (n = 80 per set) over two seasons from selected districts of two climatically contrasting agroecological zones (AEZs) of Zambia, The regions were AEZ1 and AEZ3 as described in Chapter 2 on sampling of maize. Briefly, prevailing weather conditions (variables) were: AEZ1 low rainfall with a dry spell (assigned as S1), low rainfall (assigned as S2); and AEZ3 high rainfall (assigned as N1 and N2). The digits '1' and '2' correspond to sampling season-1 (2018/2019) and sampling season-2 (2020/2021). Variable S1 is a maize growth stressful weather condition due to a two-month dry spell that occurred during flowering of the maize in the field. For each field from which maize was collected, two composite soil samples were also collected (Figure 1). Fifteen soil subsamples, about 30 to 70 g each, were collected per transect at a depth of 2 to 3 cm. The soil subsamples from each transect were combined into one composite soil sample of approximately 1.5 kg. The soil subsamples were collected from points at which a maize cob was also collected. Maize samples were processed as described in Chapter 2. For soil samples, each composite sample was dried, while in cotton bags, at 42 ± 2°C for 48hrs in a Forced Oven Dryer (model D-6450, Heraeus, Hanau, Germany). This was done to prevent any growth of fungi. The dried samples were then thoroughly homogenised by coning and quartering, then scooping from different positions about 40 to 45 g subsample into a sterile 50 ml polypropylene tube.

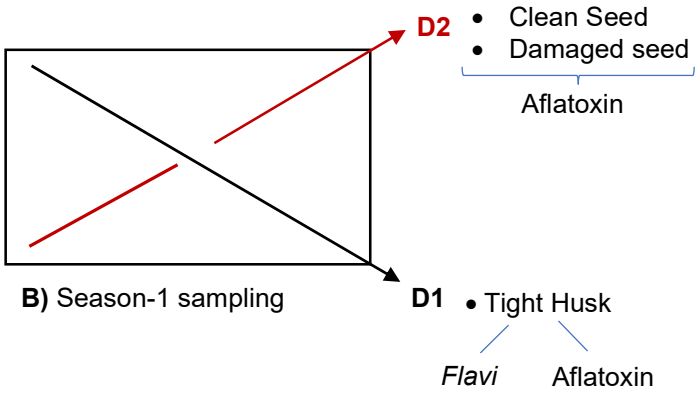
4.2.1.1 Classification of maize by husk cover and grain condition

Cobs from each maize field were sorted into tightly covered cobs and loosely covered cobs. Tight husk cover was defined as a cob having no visible kernels out of the husk. Loose husk cover was defined as a cob having visible kernels, usually at the tip. The

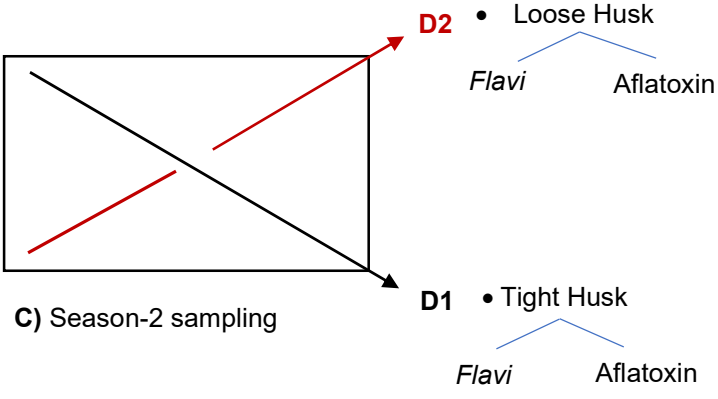
grain condition was gauged by visual sorting, observing kernels for presence or absence of insect damage and/or fungal growth and/or discoloration (Aoun *et al.*, 2020). The entire cob was graded as 'poor' (loose cover) whenever a single grain (or more) on the cob was found to have visible fungus, grain discoloration and/or evidence of insect tampering (for example insect boring; a dead insect). A cob was graded as 'good' (tight cover) if and only if all kernels were without visible fungal growth, discolouration or insect tampering.



A) Overall sampling scheme



B) Season-1 sampling



C) Season-2 sampling

Figure 1A. Sampling scheme for maize and soil. Black boundary shows an example of a maize field perimeter (not drawn to scale; fields varied in size between 1.0 and 2.5 acres) within which soil and maize samples were collected. 'D1' and 'D2' with black and red arrows represent transects from which cobs and soil portions were collected to generate a sample for the field. Blue arrow heads indicate stop point from which a cob and soil were collected (not drawn to exact number of steps). A total of 15 cobs were collected per transect. We assume transect D1 an D2 are same; **B)** Sampling scheme for season-1 across transects D1 and D2 for determination of *Flavi* in maize and the soil, as well as aflatoxin in kernels. **C)** Sampling scheme for season-2 across transects D1 and D2 for determination of *Flavi* in maize and the soil, as well as aflatoxin in kernels. For both Figures 'B' and 'C', The perimeter shown is same as that in 'A' within which cobs and soil subsamples were collected.

4.2.2 Determination of the natural infection of *Aspergillus* section *Flavi* (*Flavi*) in maize and AF contamination

4.2.2.1 Influence of weather variables on *Flavi* infection

To determine the natural infection of *Flavi* with weather as a predisposing factor for infection, we enumerated both the soil and maize *Flavi* in corresponding fields across the weather variables S1, N1, S2 and N2.

Additional factors investigated which could further influence the maize *Flavi* infection were mono-cropping, pest incidences (lack of their control) and breed type of the maize seed, all assigned as 'agronomic factors' (see Supplemental questionnaire in Chapter 3). The data on the agronomic factors to which fields were subjected was acquired with assistance from the agriculture field Extension Officers who routinely work with farmers in the districts.

Cobs were sought from transect D1 of season 1 and 2 as shown in Figures 1B and 1C above. The cobs from D1 had tight husk cover. Tight husk cover was assigned on the basis of a cob having no visible kernels out of the husk. The soil sample per field was collected as a subsample of the composite of transects D1 and D2.

4.2.2.1.1 Isolation and enumeration of *Flavi* from soil and maize

Isolation of *Flavi* for both soil and maize kernels was achieved using dilution plate technique on modified rose Bengal agar (MRB) as described by (Cotty, 1994).

For maize kernels, 40 g of kernels were transferred to a 250 ml sterile bottle. Then 40 ml 0.05% sterile Triton-X was added, and the contents were shaken on a sideways shaker (GFL model 3018, Society for Laboratory Technology, Burgwedel, Germany) at 200 rpm for 10 min, making a 1x initial extract. For the plating, 1 ml of the initial extract was diluted to a 0.5x solution, and then serially diluted up to 1 x 10^{-3}, with agitation during pipetting.

For soil samples, 15 g of a homogenised subsample was transferred to a sterile 250 ml bottle and 2 ml of 0.05% sterile Triton-X solution was added per gram soil (0.5x dilution) to make an initial extract. The contents were shaken on a sideways shaker (GFL model 3018, Society for Laboratory Technology, Burgwedel, Germany) at 200 rpm for 15 min. Next, 1 ml of soil suspension was transferred, while agitating by hand,

to a sterile 10 ml glass test tube containing 4 ml sterile deionised water (0.1x dilution). A further 1 ml of initial extract was transferred to a 1.5 ml microcentrifuge tube for later downstream enumeration of *Flavi*. The 0.1x dilution was serially diluted up to 1 x 10^{-3}.

For inoculation of both soil and maize serial dilution (0.5x to $1x10^{-3}$) suspensions on MRB, 150 µl of was plated on MRB and spread with the help of sterile 3 mm glass beads (20 to 30 beads per plate). All MRB incubations were carried out at 31°C (3 days, dark). Colonies characteristic of *Flavi* were enumerated as CFU/g of soil or maize based on their characteristic morphology for *Flavi* (Cotty, 1994).

For isolate cleaning, 30% (or at least three isolates if fewer than 10) of maize isolates on MRB with characteristics of *Flavi* were randomly selected and sub-cultured on potato dextrose agar (PDA). For soil, > 50% isolates were transferred to PDA. Isolates were sub-cultured at least twice on the PDA for purification by single spore passaging. Spores from purified isolates were transferred using a sterile cotton swab dipped in a sterile 0.8% saline solution for downstream determination of toxigenicity by Thin Layer Chromatography (TLC). The *Flavi* per sample were enumerated as CFU/g soil or maize.

4.2.2.2 Influence of husk cover condition on maize *Flavi* infection

Cobs were sought from transects D1 and D2 of season 2 as shown in Figure 1C. The cobs from the D1 of season 2 were tight husk cover and had been processed in previous step. Cobs from D2 were loose husk cover. The cobs were processed as below.

4.2.2.2.1 Isolation and enumeration of *Flavi* from soil and maize

The maize *Flavi* for D2 season 2, were isolated as described in previous section. Similarly the isolates were enumerated as CFU/g.

4.2.2.3 Influence of husk cover condition on preharvest maize aflatoxin

Cobs were sought from transects D1 and D2 of season 2 as shown in Figure 1C. Aflatoxin from tight husk cover grain was determined previously in Chapter 3. For loose husk cover, the grain was analysed for aflatoxin by HPLC, similarly as described in Chapter 3 for tight husk cover.

4.2.2.4 Influence of grain condition on preharvest maize aflatoxin

Cobs were sought from transect D2 of season 1 as shown in Figure 1C. The kernels were processed as described in Chapter 3. Aflatoxin was determined by HPLC as described in Chapter 3.

4.2.3 Determination of selection for toxigenicity of soil *Flavi* isolates

Toxigenicity of the *Flavi* isolates from soil was based on the assessment of the isolates ability to produce aflatoxins (AF) and/or cyclopiazonic acid (CPA).

The purified isolates from maize and soil were induced on Yeast Extract Sucrose (YES) medium for production of metabolites (Abdollahi & Buchanan, 1981), including AFs as well as CPA. The YES medium was prepared in bulk (200 g/l sucrose; 20 g/l yeast extract autolysate), amended to 50 mg/l chloramphenicol. The pH was adjusted to 6.0 with concentrated HCl. Aliquots of 2 ml YES medium were transferred to 15 ml test tubes and autoclaved (120°C for 15min). The test tubes were then inoculated with 100 µl of a $1x10^{-4}$ spore/ml pure isolate spore suspension. Test tubes were then incubated in a rotary incubator (Innova 4330, New Brunswick Scientific, CT, USA) set at 150 rpm, for 7 days (31°C, dark).

For extraction of aflatoxins, the tubes were first vortexed for one second and 2 ml dichloromethane was added. The mixture was then vortexed for 15 s, allowed to stand for about 15 to 30 min, then vortexed again for 30 s and homogeneously transferred into two 2 ml microcentrifuge tubes. The microcentrifuge tubes were centrifuged at 25,000 *g* in a microcentrifuge unit (model 5424, Eppendorf, Hamburg, Germany) for 5 min. The supernatant (aqueous phase) was pipetted off and discarded. Next, 500 µl of the dichloromethane phase (lower fraction) was transferred into a 4 ml screw neck vial (45 x 14.7 mm, VWR, cat № 548-0051, https://www.vwr.com).

The AF and CPA levels were qualitatively determined by Thin Layer Chromatography (TLC) as follows: The contents of the 4 ml glass vial were evaporated to dryness in a fumehood, with vials protected from light using aluminium foil. Vial contents were reconstituted in 250 µl 1:1 methanol:water (v/v), vortexed for 10 s continuously. Next, 400 µl extract was spotted on a 20 x 20 cm TLC plate (Supelco, Mo., USA) alongside aflatoxin-B1, -B2, -G1, -G2 and CPA standards. The plate was developed in 150 ml toluene:ethyl acetate:90% formic acid (5:4:1, v/v) solvent system. The TLC plate was

dried for 40 to 60 min under a fume hood and visualised on a UV transilluminator (Model TFX-35M, Vilber Lourmat, France) at 312 nm (Figure 4). Toxigenicity was determined as percentage quantity of isolates producing AF and/or CPA.

4.2.4 Data analysis

All statistical computations were conducted in software R version 4.1.0 (R_Core_Team, 2013).

The package ComplexHeatmap (Z. Gu *et al.*, 2016) was used for generation of an interactive heatmap (Figure 2) of *Flavi* levels (dependent variable) in maize and soil; and aflatoxin (dependent variable) in maize. In the heatmap, S1, N1, S2 and N2 were the (independent) weather variables. Measurements for dependent variables were scaled in square root (sqrt) for visibility by column as: [A] – *Flavi* in soil, sqrt CFU/g x 10^2; [B] - *Flavi* on tight husk cover cob, sqrt CFU/g x 10^2; [C] - *Flavi* on loose husk cover cob, sqrt CFU/g; [D] - Aflatoxin in tight husk cover cob, sqrt CFU/g x 10^2; [E] - Aflatoxin in loose husk cover cob, sqrt µg/kg; [F] - Aflatoxin in good grain, sqrt µg/kg x 10^2; [G] - Aflatoxin in poor grain, sqrt µg/kg x 10^2. Gray shade meant 'not sampled.' Total aflatoxin contamination benchmark value was 10 µg/kg, which is the current regulatory limit for total aflatoxin in maize in Zambia, and other sub-Saharan African countries (Akello *et al.*, 2021).

To determine influence of the additional factors (agronomic factors) to the weather variables that would be associated with *Flavi* infection in maize, Non-metric Multi-Dimensional Scaling (NMDS) was used. NMDS stress factor acceptance criterion was set at the standard ≤ 0.20 (Dexter *et al.*, 2018).

4.3 Results

4.3.1 Natural infection of *Flavi* in maize and Aflatoxin (AF) contamination

4.3.1.1 Influence of weather variables on *Flavi* infection

Natural infection of maize by *Flavi* is shown in Figure 2*. Flavi* was detected in all soils (column A) but not all maize fields (columns B and C). There was no significant difference in soil *Flavi* quantities (CFU/g) among the weather variables S1, N1, S2 and N2 (column [A], pairwise Wilcoxon test, $P > 0.05$). However, there was a significantly higher number of *Flavi* on maize grown under S1 (AEZ1 low rainfall with dry spell

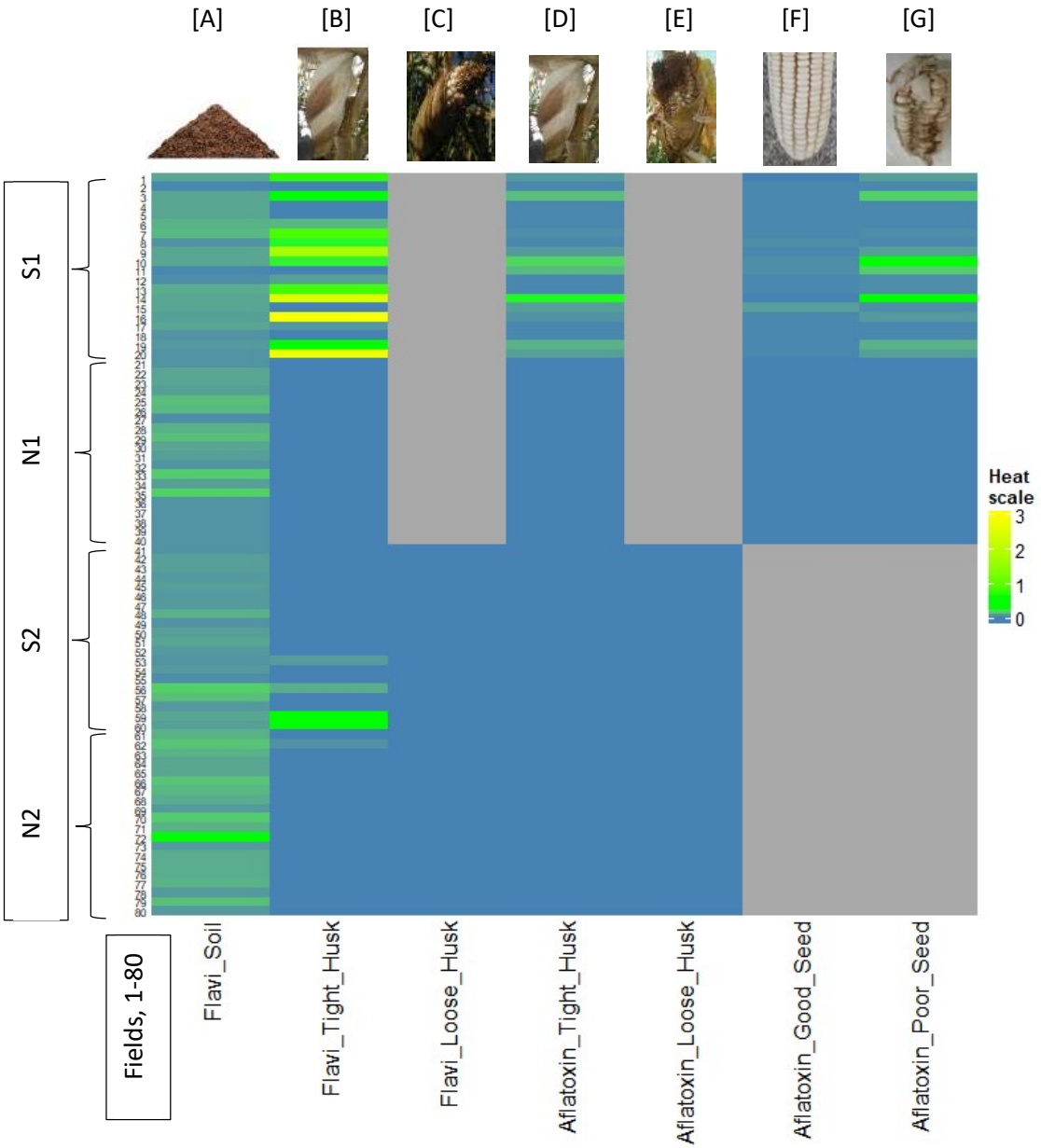


Figure 2. Quantitative unclustered heatmap of *Flavi* abundance over the gradient from soil (column A) to maize under different weather variables and maize husk cover condition (columns B & C); then aflatoxin contamination of the maize cobs under different weather variables and husk cover and grain conditions (columns D to G).

conditions) compared to the normal AEZ1 low rainfall conditions (S2) or the normal AEZ3 high rainfall conditions (N1 and N2). (Figure 2, column B and Table 1).

Table 1. Wilcox pairwise *t*-test for *Aspergillus* section *Flavi* in maize over weather variables:

	N1	**N2**	**S1**
N2	1.000	-	-
S1	< 0.001	< 0.001	-
S2	0.241	0.866	0.004

Of the investigated factors leading to *Flavi* infection, the dry spell in AEZ1 (P = 0.002) was the key factor associated with *Flavi* proliferation in the preharvest maize (Figure 3; NMDS1 and NMDS2). Of the additional factors that could compound the *Flavi* infection noted as agronomic factors, pest incidence (lack of control of pests that appeared) was the second important factor of the maize *Flavi* ingress (P = 0.010). Similarly, not destroying previous crop residues (no burning of crop residues) (P = 0.007) contributed to *Flavi* infection under S1 (Figure 3 NMDS1). On the other hand, monocropping did not influence *Flavi* proliferation (P = 0.960). A summary of agronomic factors is presented in Supplemental Table 1.

4.3.1.2 Influence of husk cover condition on maize *Flavi* infection.

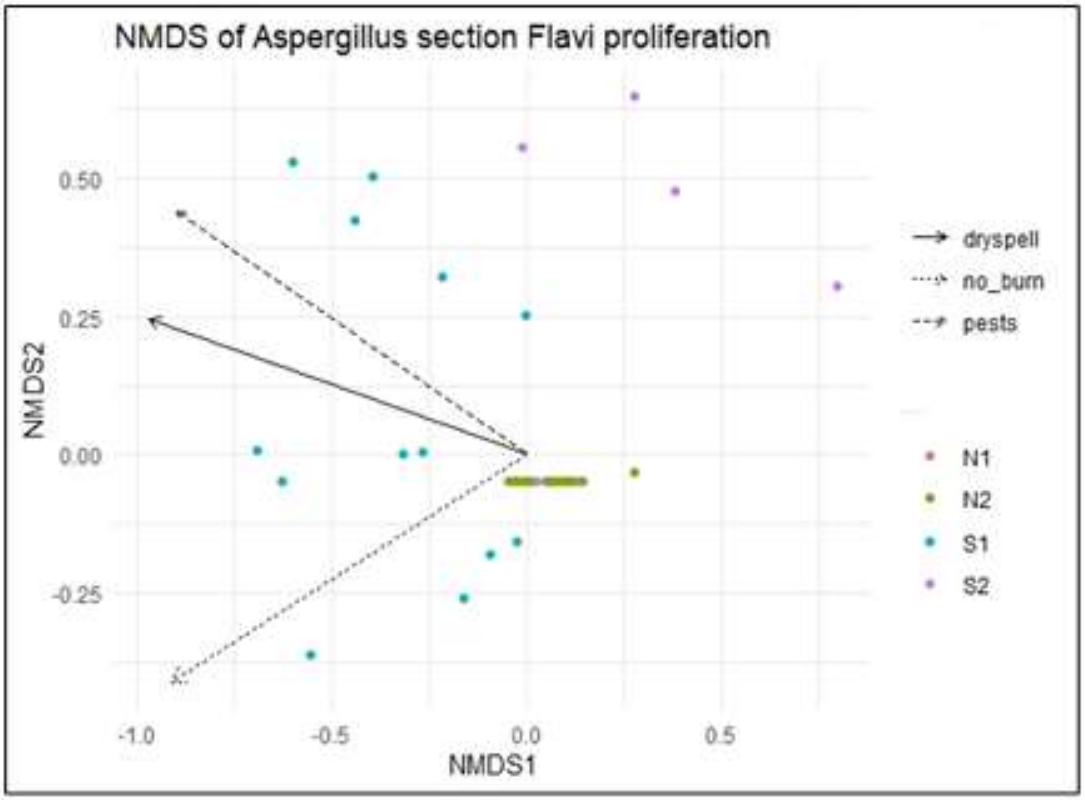
Flavi was not detected on all loose husk cover cobs (Figure 2, column C). However, *Flavi* was detected on maize with tight husk cover from corresponding 4 out of 20 fields of season-2 variables N2 and S2 (Figure 2, column B).

4.3.1.3 Influence of husk cover condition on aflatoxin (AF) contamination.

Loose husk cover and tight husk cover cobs collected in season 2 from both agroecological zones did not contain AF (Figure 2, columns [D] and [E]). Similarly, AF was not detected in the corresponding tight husk cover cobs.

4.3.1.4 Influence of grain condition on aflatoxin (AF) contamination.

Poor condition grain (visible fungal contamination and/or insect boring), under a dry spell weather condition (S1), showed a high accumulation of aflatoxin (Wilcoxon signed rank test P = 0.006, df = 19) with a geometric mean of 11.0 µg/kg, (range ND - 1288.3; ND = limit of detection, AF-B2 = 0.28, -G1 = 0.30, -B2 = 0.22, -B1 = 0.29). Maize from 9 out of 20 fields that experienced the dry spell had aflatoxin levels that exceeded benchmark value of 10 µg/kg total aflatoxin. This is in comparison with good condition grain (good cobs) from the dry spell season which had a lower geometric mean of 2.2 µg/kg, and range ND - 27.7 µg/kg, of which only 1 out of 20 fields exceeded 10 µg/kg aflatoxin (Figure 2, columns [F] and [G]). In the high rainfall AEZ3 (N1), both poor and good condition grain from all 20 fields had no aflatoxin contamination.



Agronomic factors linked to preharvest maize *Flavi* proliferation.

Agronomic factor	R	P
Dry spell *	0.215	0.002
No field burning *	0.268	0.007
Pests incident *	0.177	0.010
No pesticide use	0.115	0.068
Monocropping	0.001	0.960

* *factor significant*

Figure 3. Agronomic factors and dry spell with influence on proliferation of *Flavi* in maize over the different weather variables, N1, N2, S1 and S2.

4.3.2 Selection for toxigenicity of soil *Flavi* isolates

The selection frequency (percentage proportion) for aflatoxigenicity in the soil isolates was high (> 80%, Table 2A). Producers of aflatoxin-B and -G, a metabolic profile characteristic of *A. parasiticus* (Frisvad *et al.*, 2019), a key fungus contaminating grain in Zambia, were most frequently detected irrespective of the AEZ (83 ± 11% AEZ1; 85 ± 12% AEZ3) or weather variable. Non-producers of aflatoxin were in very low frequency accounting for an average presence of only 7%. The results also show a very low frequency of CPA producers (< 3%). The highest selection for aflatoxigenicity in strains (97.4% aflatoxin positive) corresponded with the dry spell conditions (S1) during the maize growing season of the normally low rainfall AEZ. In terms of quantity

(CFU/g) of toxigenic isolates, N2 had the highest proliferation (60.5 CFU/g) of the aflatoxigenic *Flavi* in the soil compared to the other three seasons (S1, S2 and N1) which ranged from 8.4 to 18.2 CFU/g.

Table 2A. Proportion of mycotoxigenic soil *Flavi* Isolates based on aflatoxin (AF) and cyclopiazonic acid (CPA) production

District	Weather Variable	Average *Flavi* density (CFU/g soil)	Std. dev	AF-B, %	AF-B+G, %	AFB+G+C, %	CPA, %	None, %
Kalomo	S1	33.6	9.0	0.0	75.3	24.7	0.0	0.0
	S2	20.8	1.2	0.0	64.5	22.6	0.0	12.9
Kazungula	S1	43.9	10.2	8.5	91.5	0.0	0.0	0.0
	S2	24.7	13.7	15.0	85.0	0.0	0.0	0.0
Livingstone	S1	28.2	13.2	0.0	100.0	0.0	0.0	0.0
	S2	15.8	8.2	0.0	87.8	0.0	0.0	12.2
Mulobezi	S1	16.0	7.3	0.0	83.0	0.0	9.2	7.8
	S2	63.3	47.1	0.0	77.7	0.0	0.0	22.3
Samfya	N1	45.8	27.1	9.5	62.7	0.0	9.5	18.3
	N2	60.3	33.4	1.5	89.2	1.5	1.5	6.2
Mansa	N1	57.2	18.1	0.0	91.1	8.9	0.0	0.0
	N2	79.2	8.2	8.2	69.2	2.3	2.3	18.1
Luwingu	N1	77.2	22.8	0.0	81.0	7.9	0.0	11.0
	N2**	185.6	189.3	2.7	95.9	1.4	0.0	0.0
Kawambwa	N1	8.5	1.0	6.0	94.0	0.0	0.0	0.0
	N2	51.3	6.5	0.0	93.0	0.0	0.0	7.0

** *Distribution not normal due to high variation between camps.*

Table 2B. Summary of soil *Flavi* mycotoxigenicity and abundance

Weather Variable	Toxigenic. *Flavi*; Geometric Mean CFU/g, (% aflatoxigenic)	AFB, %	AFB+G, %	AFB+G+CPA, %	CPA, %	NONE, %
S1[a]	12.4 (97.4)	3.4	86.9	7.1	1.4	1.2
N1	18.2 (88.8)	2.5	80.3	6.1	2.3	8.9
S2[b]	8.4 (84.4)	2.7	78.0	3.7	0.0	15.6
N2[a,b]	60.5 (93.6)	3.2	88.9	1.4	0.8	5.6

In **Table 2B** same letters between weather variables indicate significance of difference in quantity (CFU/g) of toxigenic isolates (pairwise Wilcox test, $P < 0.05$) between the weather variables.

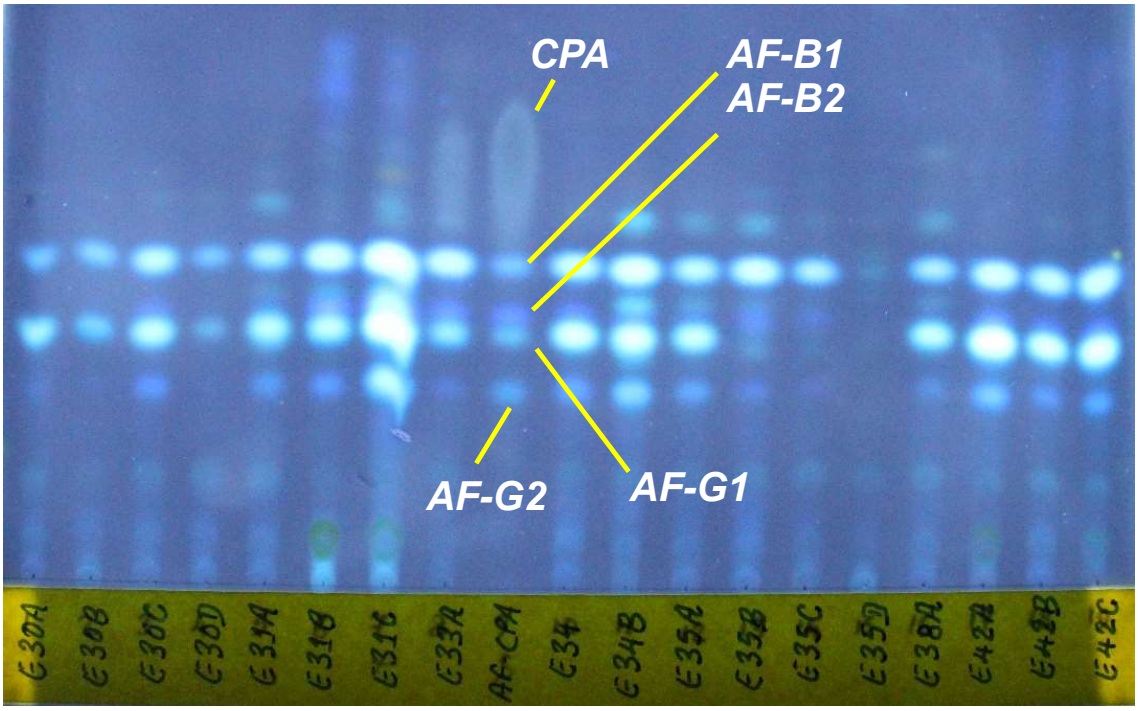


Figure 4. A developed TLC plated visualised under UV (312 nm) for AFs and CPA. Lanes show soil isolate codes. Bands show metabolites including aflatoxins B and G, as well as CPA.

4.4 Discussion

4.4.1 Natural Infection of *Flavi* in maize and AF contamination

4.4.1.1 Influence of weather variables on *Flavi* infection

We demonstrate the comparatively higher vulnerability of maize fields under the AEZ1 areas (S1 and S2) to natural *Flavi* infection compared to AEZ3 (N1 and N2). The highest vulnerability to *Flavi* infection on kernels was recorded under conditions that experienced a dry spell (weather variable S1). This illustrates the threat that erratic weather patterns or uneven rainfall pattern in areas where rainfall is normally low can increase crop susceptibility to *Flavi* infection. This observation is in conformity with previous study findings (Payne & Widstrom, 1992). It is worth further pointing out that although there was an increase in the abundance of *Flavi* on maize in the dry spell year (S1) compared to the other weather variables (Table 1, pairwise Wilcox *t*-test, $P < 0.05$), there was no correspondingly higher significance of *Flavi* abundance in soil of S1, suggesting that a higher density of *Flavi* on maize may not be a direct result of higher density of *Flavi* in soil. This may be so particularly if maize is not the niche for the soil *Flavi*. The weather pattern may possibly play the more critical role in the soil-to-maize gradient for the infection of *Flavi* as seen with S1. From the findings, it is clear

that despite *Flavi* being present in the soil in all the maize fields, it may not always be present (in detectable levels) on the maize grain within the same evaluated field.

With respect to other factors with potential to influence maize *Flavi* proliferation, our findings show that a dry spell coupled with pest incidences may increase the proliferation of *Flavi* on maize. The majority of the farmers reported the presence of army worms and stalk borers during the maize growing seasons. Both pests belong to the group Lepidoptera which is highly implicated in *Flavi* infestation and aflatoxin contamination (Barry *et al.*, 1985; Marsh & Payne, 1984; Widstrom, 1979; Widstrom *et al.*, 1975; Windham *et al.*, 1999). Considering the three factors in this study that significantly contributed to maize *Flavi* ingress, dry spell, lack of field burning and pests, the incidence of pests was the most important factor in *Flavi* infection under normal low rainfall conditions (S2). The contrary is seen with AEZ3 (N1 and N2) which had no observed *Flavi* proliferation on maize despite also having reports of pest incidents and absence of field burning. This demonstrates that the areas under AEZ1 are potentially more prone to *Flavi* infection even in the absence of dry spell conditions (S2). Although it was shown that field burning had some negative effect on *Flavi* proliferation for S1, it showed no orientation for S2 maize *Flavi* (Figure 3). This suggests a weak link of field burning with maize *Flavi* proliferation, hence the need for effective means to control pests by farmers.

4.4.1.2 Influence of husk cover condition on maize *Flavi* infection.

We demonstrate that loose husk covering may not necessarily influence infection of ears with *Flavi*. However, loosely husk-covered cobs had visually more incidences of fungal contamination than the tightly covered cobs. Although previous studies have indicated that loose husk covering in maize led to an increase in insect infestation and subsequently *Flavi* (fungal) infection (Barry *et al.*, 1986; Dowd, 2003; Ni *et al.*, 2011), our results show that loose husk covering did not lead to an increase in *Flavi*. In addition to this, the observed non-infection of corn with *Flavi* under AEZ3 supports our hypothesis that natural infection of *Flavi* in preharvest maize may not necessarily occur as a result of husk condition. We attributed our findings of non-infection of maize with *Flavi* under AEZ3 to the absence of drought-related plant stress (dry spell) in AEZ3 during growth of the maize. Considering that insects were also prevalent in AEZ3 and that *Flavi* was present in all soils, it shows that AEZ3, by virtue of its weather conditions,

may have supported a biological barrier to effectively exclude *Flavi* translocation from soil to maize in detectable quantities. This consolidates the argument that husk cover alone may not be the key factor for *Flavi* infection of ears. Rather, conditions predisposing for *Flavi* infection of ears such as a dry spell should be present for the *Flavi* to manifest in detectable levels in preharvest maize. Other investigators have put forward a similar hypothesis (Magagnoli *et al.*, 2021; Ni *et al.*, 2011).

4.4.1.3 Influence of husk cover and grain condition on preharvest maize aflatoxin

With regard to aflatoxin contamination due to poor maize grain condition, the findings in this study show that the contamination may likely be the case in combination with dry weather conditions, such as a dry spell (S1). This consolidates the arguments that weather conditions play a crucial role in contamination of field grain with aflatoxin and that this contamination is not just caused by visible damage through insect attack or fungal growth on kernels. Similar inferences were made by other investigators (Ni *et al.*, 2011; Widstrom *et al.*, 1990). This also illustrates the threat that a climate change scenario may pose of increased preharvest maize contamination with AF (Battilani *et al.*, 2016). The typical example of this case is the frequent contamination of preharvest maize with aflatoxin in the southeast of the USA, a region known for frequent droughts (Seager *et al.*, 2009) during the maize growing season.

Considering that: a) loose husk cover ears had kernels with a physically poorer condition (more visible fungi and at times insect damage, in most cases compared to tight husk cover ears); and b) that ears with poor condition kernels accumulated higher aflatoxin levels compared to good condition kernels under the dry weather condition (S1); we postulate that loose husk cover ears may more likely accumulate higher levels of aflatoxin under conditions such as S1 that are predisposing for *Flavi* ingress into maize ears and higher AF contamination thereof.

4.4.2 Selection for mycotoxigenicity of soil *Flavi* isolates

We report a high frequency (proportion percentage) of aflatoxigenic isolates in the soil over the two seasons sampled across the weather variables (83 to 97%). This frequency is high compared to other studies that showed proportions of soil toxigenic *Flavi* isolates in the range of 30 to 70% (Donner *et al.*, 2009; Kachapulula *et al.*, 2017b; Razzaghi-Abyaneh *et al.*, 2006; Sebők *et al.*, 2016). We attribute this higher selection

frequency for aflatoxigenicity to the probable predominance in the soil by the species *A. parasiticus*, which in nearly all known cases is aflatoxigenic. This is based on the TLC profiles which showed that most of the isolates had a metabolic pattern characteristic of *A. parasiticus*, characterised by production of aflatoxins B and G but without CPA (Figure 4). This would have to be genetically proofread to ascertain the actual dominance of *A. parasiticus* in the soil. In the previous study in Zambia by (Kachapulula *et al.*, 2017b), the *A. parasiticus* frequency in cultivated soils was found to be about 58%, while the remaining portion can contain both toxigenic and atoxigenic strains. Similarly, (Donner *et al.*, 2009) showed an 85% frequency of *A. flavus* isolates, a species that can have both toxigenic and atoxigenic isolates, hence the lower frequency for aflatoxigenic *Flavi* compared to our study. It should also be noted that *Flavi* species frequency can potentially change between different years (Ortega-Beltran & Cotty, 2018). We do also demonstrate that the dry weather conditions of S1 (dry spell) did not necessarily favour higher proportions of aflatoxigenic *Flavi* in soil. This is contrary to expectations and to a report by Shearer and co-workers, which showed that the proportion of aflatoxigenic strains in aflatoxin outbreak years was higher than in non-epidemic years (Shearer *et al.*, 1992). We attribute our scenario to the fact that the soil in this study was dominated by *A. parasiticus*, which is unlikely to proliferate under hot drought conditions as comparatively cooler temperatures favour this species (Ching'anda *et al.*, 2021; B. W. Horn, 2005). Furthermore, the dry spell in our study was a one-off event for (S1) over four years and for a period of two months. This could not have been sufficient for the proliferation of *A. flavus* in a soil community dominated by *A. parasiticus*, despite the fact that *A. flavus* is favoured by higher temperatures characteristic of our dry conditions of S1.

The observed high selection frequency for aflatoxin-B and/or -G over the weather variables (83 to 97%) may demonstrate the potential risk for maize aflatoxin contamination when conditions become favourable, should maize be the niche for such soil *Flavi* species. It is, however, imperative that for cases where *Flavi* was present in the soil and maize, the genetic characteristics of the *Flavi* are described at strain level for both the soil and maize isolates. This is in order to help better understand the species responsible for the observed aflatoxin contamination under weather variable S1 and the role that soil would play as their reservoir.

4.5 Conclusion

The detection of *Flavi* from all soils of maize fields irrespective of weather variables (AEZ and maize cropping season) shows the importance of soil as a reservoir of *Flavi* in maize fields. Although all soils had *Flavi* present, it was not detected from all fields on maize. In addition, although loose husk cover may lead to higher fungal load on kernels, including *Flavi* infection, our findings show that it may not necessarily always lead to higher *Flavi* infection. Instead, conditions may have to be pre-disposing for the natural infection of maize by *Flavi* to occur in maize of either tight or loose husk cover. Therefore, based on our findings, it may not be recommended to sort preharvest maize ears on basis of husk cover as an attempt to minimise risk of AF exposure, provided that storage observes Good Agricultural Practices to minimise chances of AF accumulation. This is considering that the sorting exercise is laborious. Rather, grain condition, as seen in this study and others, would give a better prediction of possible AF contamination of ears, particularly when conditions are pre-disposing for aflatoxin contamination of the crop, such as a dry spell.

4.6 Acknowledgement

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4.7 Supplemental materials

Supplemental Table 1. Summary of agronomic factors as factors associated with *Aspergillus* section *Flavi* infection of maize cobs.

Weather variable	S1(%)	N1(%)	S2(%)	N2(%)	average	Std. dev.
Fields with *Flavi*	70.0	0.0	25.0	5.0		
Fields with pest uncontrolled	90.0	60.0	80.0	60.0	72.5	15.0
Field burning	35.0	60.0	50.0	35.0	45.0	12.2
Monocropped fields	55.0	35.0	40.0	10.0	65.0	18.7

Supplemental Table 2. *Flavi* abundance in kernels and soil and aflatoxin in maize grain. Gray shade means not sampled. Values indicted '0.0' are lower bound values meaning below detection limit for aflatoxins or not detected for CFUs.

Field code	Weather variable	*Flavi*, CFU/g			Total aflatoxin, µg/kg.			
		Soil.	Tight husk cover, Maize	Loose Husk Cover, Maize	Tight husk cover, Maize	Loose husk cover, Maize	Good Seed, Maize	Poor seed, Maize
S1_KA01	S1	40.0	4466.7		13.5		0.0	21.3
S1_KA02	S1	1.7	0.0		2.0		2.0	2.0*
S1_KA03	S1	40.0	764.4		91.1		2.0	170.4
S1_KA04	S1	40.0	0.0		1.8		1.7	2.1*
S1_KA05	S1	40.0	0.0		1.7		1.7	1.7*
S1_KZ06	S1	66.7	73.3		1.7		1.7	1.7*
S1_KZ07	S1	76.7	6777.8		2.7		2.2	3.9
S1_KZ08	S1	10.0	351.1		2.2		2.5	1.7*
S1_KZ09	S1	33.3	29555.6		14.1		2.2	21.6
S1_KZ10	S1	40.0	326.7		208.2		2.6	567.5
S1_LV11	S1	1.7	0.0		89.7		5.3	145.0
S1_LV12	S1	3.3	26.7		1.9		1.7	2.6*
S1_LV13	S1	53.3	5900.0		2.2		2.0	2.3*
S1_LV14	S1	33.3	60000.0		348.4		0.0	1288.3
S1_LV15	S1	41.7	0.0		23.0		27.7	2.3
S1_ML16	S1	23.3	85166.7		9.2		1.9	18.7
S1_ML17	S1	33.3	13.3		1.7		1.7	1.7*
S1_ML18	S1	6.7	0.0		2.0		1.9	2.1*
S1_ML19	S1	13.3	742.2		58.7		1.7	62.6
S1_ML20	S1	8.3	73833.3		21.0		2.1	22.0
N1_SF21	N1	6.7	0.0		0.0		0.0	0.0
N1_SF22	N1	33.3	0.0		0.0		0.0	0.0
N1_SF23	N1	40.0	0.0		0.0		0.0	0.0
N1_SF24	N1	23.3	0.0		0.0		0.0	0.0
N1_SF25	N1	106.7	0.0		0.0		0.0	0.0
N1_MS26	N1	73.3	0.0		0.0		0.0	0.0
N1_MS27	N1	3.3	0.0		0.0		0.0	0.0
N1_MS28	N1	56.7	0.0		0.0		0.0	0.0
N1_MS29	N1	100.0	0.0		0.0		0.0	0.0
N1_MS30	N1	40.0	0.0		0.0		0.0	0.0
N1_LG31	N1	30.0	0.0		0.0		0.0	0.0
N1_LG32	N1	6.7	0.0		0.0		0.0	0.0
N1_LG33	N1	146.7	0.0		0.0		0.0	0.0
N1_LG34	N1	23.3	0.0		0.0		0.0	0.0
N1_LG35	N1	163.3	0.0		0.0		0.0	0.0
N1_KW36	N1	10.0	0.0		0.0		0.0	0.0
N1_KW37	N1	6.7	0.0		0.0		0.0	0.0

N1_KW38	N1	6.7	0.0		0.0		0.0	0.0
N1_KW39	N1	10.0	0.0		0.0		0.0	0.0
N1_KW40	N1	8.3	0.0		0.0		0.0	0.0
S2_KA41	S2	6.7	0.0	0.0	0.0	0.0		
S2_KA42	S2	30.0	0.0	0.0	0.0	0.0		
S2_KA43	S2	23.3	0.0	0.0	0.0	0.0		
S2_KA44	S2	13.3	0.0	0.0	0.0	0.0		
S2_KA45	S2	30.0	0.0	0.0	0.0	0.0		
S2_KZ46	S2	16.7	0.0	0.0	0.0	0.0		
S2_KZ47	S2	16.7	0.0	0.0	0.0	0.0		
S2_KZ48	S2	70.0	0.0	0.0	0.0	0.0		
S2_KZ49	S2	6.7	0.0	0.0	0.0	0.0		
S2_KZ50	S2	23.3	0.0	0.0	0.0	0.0		
S2_LV51	S2	40.0	0.0	0.0	0.0	0.0		
S2_LV52	S2	16.7	0.0	0.0	0.0	0.0		
S2_LV53	S2	8.3	20.0	0.0	0.0	0.0		
S2_LV54	S2	16.7	0.0	0.0	0.0	0.0		
S2_LV55	S2	3.3	0.0	0.0	0.0	0.0		
S2_ML56	S2	173.3	53.3	0.0	0.0	0.0		
S2_ML57	S2	100.0	0.0	0.0	0.0	0.0		
S2_ML58	S2	16.7	0.0	0.0	0.0	0.0		
S2_ML59	S2	33.3	486.7	0.0	0.0	0.0		
S2_ML60	S2	26.7	497.8	0.0	0.0	0.0		
N2_SF61	N2	70.0	0.0	0.0	0.0	0.0		
N2_SF62	N2	121.7	6.7	0.0	0.0	0.0		
N2_SF63	N2	60.0	0.0	0.0	0.0	0.0		
N2_SF64	N2	33.3	0.0	0.0	0.0	0.0		
N2_SF65	N2	40.0	0.0	0.0	0.0	0.0		
N2_MS66	N2	125.0	0.0	0.0	0.0	0.0		
N2_MS67	N2	75.0	0.0	0.0	0.0	0.0		
N2_MS68	N2	55.0	0.0	0.0	0.0	0.0		
N2_MS69	N2	13.3	0.0	0.0	0.0	0.0		
N2_MS70	N2	133.3	0.0	0.0	0.0	0.0		
N2_LG71	N2	61.7	0.0	0.0	0.0	0.0		
N2_LG72	N2	876.7	0.0	0.0	0.0	0.0		
N2_LG73	N2	20.0	0.0	0.0	0.0	0.0		
N2_LG74	N2	53.3	0.0	0.0	0.0	0.0		
N2_LG75	N2	50.0	0.0	0.0	0.0	0.0		
N2_KW76	N2	51.7	0.0	0.0	0.0	0.0		
N2_KW77	N2	71.7	0.0	0.0	0.0	0.0		
N2_KW78	N2	16.7	0.0	0.0	0.0	0.0		
N2_KW79	N2	95.0	0.0	0.0	0.0	0.0		
N2_KW80	N2	16.7	0.0	0.0	0.0	0.0		

Supplemental Table 2. The lower bound values were used for purposes of heatmap scaling (Figure 2) e.g. 0.0 for aflatoxin-B1 in place of upper bound value (limit of detection, LOD) of 0.29 µg/kg. (*) is less than limit of quantitation (3 x LOD) for total aflatoxin.

Supplemental Table 3. Season-associated agronomic characteristics of maize from the study

Agroecological zone	Weather variable	Average total kernels wt (g) / 30 cobs[ǂ]
AEZ-I	S1	1602
	S2	2491
AEZ-III	N1	4197
	N2	4322

[ǂ] 30 cobs were collected per field at harvest.

Supplemental Table 3. The plant stressful conditions for maize that ensued from the during the growth of the maize under weather variable S1 is evident from the low grain weight shown in the Table, supported by previous literature (Barutçular *et al.*, 2016; Hussain *et al.*, 2019). Average grain weight under S1 is about 64% that of the weight of grain from the same fields in the following year S2. We define S1 as 'dry weather condition with dry spell.' Conversely, N1, N2 and S2 were conducive for maize growth and are defined in text as 'high rainfall' (N1, N2) and 'low rainfall' (S2).

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If fungi were a human world, the calmodulin gene would differentiate the human race by locations

Chapter V

Diversity in soil and maize *Aspergillus* and influence on aflatoxin levels in Zambian maize

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5.0 Abstract

Aspergillus section *Flavi* (*Flavi*, for short) is quite a diverse group of fungal species whose common members include *A. flavus* and *A. parasiticus*. These are well-known for the production of aflatoxin B and G and other toxic metabolites, like cyclopiazonic acid (CPA). They are saprophytic soil dwellers but may also occur on crops. A consequence of this is possible contamination of harvested products with mycotoxins, like carcinogenic aflatoxins (AF). Deploying a triphasic characterisation and dilution plate technique, we investigated the *Flavi* community structure of maize samples and that of their surrounding soil samples, and studied the link of the *Flavi* diversity with preharvest maize aflatoxin levels. The studied *Flavi* populations originated from four selected districts of Zambia in a low rainfall season characterised by a dry spell, which was the apparent driver of proliferation of *Flavi* on preharvest maize. The triphasic characterisation involved use of morphological (colony colour and sclerotia formation), metabolic (AF and CPA production) and genetic (calmodulin gene polymorphism) data. *Flavi* abundance was determined by a dilution plate technique on modified rose Bengal agar. Our results showed that *Flavi* communities on maize and in surrounding soil differed, with maize having a higher *Flavi* species diversity than soil. The four main species identified on maize, by frequency of infection, were *A. minisclerotigenes* (45%), followed by *A. parasiticus* (35%), *A. flavus* (30%) and *A. oryzae* (25%). *A. parasiticus* dominated the soil community by frequency of field detection (95%), followed by *A. flavus* (15%) and *A. oryzae* (5%). Rare members in either soil or maize were *A. krugeri, A. sergii, A. sojae*, and *A. transmontanensis*. The ratio between *A. parasiticus* and *A. minisclerotigenes* seemed to influence the levels of aflatoxins in maize, with a ratio close to 1:1 having higher levels than a pure community of either *A. parasiticus* or *A. minisclerotigenes*.

5.1 Introduction

Aspergillus section *Flavi* (*Flavi*, for short) is a group of quite diverse fungal species belonging to the genus *Aspergillus*. Common members include species *Aspergillus flavus* and *Aspergillus parasiticus*, which are known for the production of aflatoxins B and G, as well as other metabolites (Amaranta *et al.*, 2021; Frisvad *et al.*, 2019) such as cyclopiazonic acid and kojic acid. They are saprophytic soil dwellers that may become epiphytic on crops like maize, groundnuts or cotton (Diener *et al.*, 1987). The result is contamination of such crops with mycotoxins such as the carcinogenic aflatoxins (AF) (IARC, 2012).

Soil is the main reservoir for *Flavi* (Boyd & Cotty, 2001; Jaime-Garcia & Cotty, 2004; Jaime-Garcia & Cotty, 2010; Kachapulula *et al.*, 2017b). *Flavi* communities on crops and in soil have been explored before (Donner *et al.*, 2009; Elsie *et al.*, 2016; Thathana *et al.*, 2017). Considering that soil is a niche for mycotoxigenic *Flavi* that may infect crops growing on those soils, *Aspergillus* biocontrol agents (non-aflatoxin producing isolates of *Aspergillus flavus*) are generally applied to the soil, to outcompete mycotoxigenic strains after transfer to the crop. This therefore can be translated as the ability to define the *Aspergilli* that could be present on the maize cob on basis of the isolates present (including those artificially applied) in the soil. Previous studies have extrapolated the risk of *Aspergillus* and AF contamination on crops, based on the *Aspergillus* population of the (untreated) soil in which those crops would be planted (Donner *et al.*, 2009; Kachapulula *et al.*, 2017b; Njoroge *et al.*, 2016). In Zambia, for example, it has been demonstrated that the cultivated areas for maize and groundnuts generally harbour *Flavi* species, including toxigenic strains of *A. parasiticus* and *A. flavus*, which pose a risk to maize AF contamination (Kachapulula *et al.*, 2017b). Similarly, a study by (Donner *et al.*, 2009) indicated that the frequency of AF contamination of crops in Nigeria is likely related to the soil propagules of *Flavi*. However, the predictability of the risk of maize infection with *Aspergillus* on basis of soil *Flavi* population may pose a scientific conundrum as it is unclear to what extent the *Flavi* population structure in soil, without artificial perturbation of the soil through biocontrol, will mirror the population structure of *Flavi* on maize. Soil and maize are two different ecological niches for fungi. For example, propagules of *Flavi* that could potentially be difficult to detect in the soil by dilution plate techniques due to low

numbers, may in fact proliferate on the maize. This could result in differences in *Flavi* community structures between soil and maize.

With respect to maize in Zambia, the community structure of *Flavi* has been investigated at the time of maize storage (Kachapulula *et al.*, 2017b; Kankolongo *et al.*, 2009; Mukanga *et al.*, 2010). *Aspergillus flavus* and *A. parasiticus* were found to be the two most important species contaminating maize and groundnuts. The *Flavi* species reported in these three studies were *A. flavus*, *A. parasiticus*, *A. niger* and *A. tamarii*. *Aspergillus flavus* and *A. parasiticus* were the most important members (Kachapulula *et al.*, 2017b; Kankolongo *et al.*, 2009; Mukanga *et al.*, 2010) and are both species of concern with regards to the production of AFs. Although aflatoxigenic species contaminating maize in Zambia have been documented, other currently unreported species in the *Flavi* section also produce AFs, including *A. nomius*, *A. pseudotamarii*, *A. bombycis*, *A. toxicarius*, *A. parvisclerotigenus* and *A. minisclerotigenes (Frisvad et al., 2019)*. Whether or not these could be species of concern depends on their ability to significantly infect maize and thereby contribute to the severity of AF contamination of the crop, especially in the field. For example, infection of grains with species like *A. minisclerotigenes* and *A. aflatoxiformans* would be a subject of concern due to the species' ability to produce AF B and G variants as well as cyclopiazonic acid (CPA) (Frisvad *et al.*, 2019). CPA is known to have potential for human toxicity (Chang *et al.*, 2009; Goeger *et al.*, 1988; Riley *et al.*, 1992) and has documented negative effects on livestock (Burdock & Flamm, 2000; Byrem *et al.*, 1999). In order to better assess the *Flavi* population in soil and maize, it is imperative to employ a polyphasic approach of characterisation of isolates, using genetic, morphological and metabolic identification means (triphasic). Resolving species with aid of their genetic architecture, supported by preliminary morphological screening and metabolic characterisation, would provide a higher resolution in defining the species. For example *A. oryzae* has been found to be a closer relative of *A. minisclerotigenes* (Kjaerbolling *et al.*, 2020), while previously thought to be a closer relative of *A. flavus* (Chang *et al.*, 2006; Cleveland *et al.*, 2009). Some studies have reported misidentification of some *Flavi* species whose naming was later corrected by genetic proofreading (Balajee *et al.*, 2006; Tam *et al.*, 2014).

With respect to AF contamination of maize, it is known that abiotic factors like temperature and water activity influence the contamination level. However, maize

batches from the same germplasm in a given area, under the same climatic conditions, can still have different AF levels. Attributing this simply to heterogeneity in fungal infection of crop by AF-producing strains could be an oversimplification of the infection dynamics. Recent explanations for the discrepancy in AF levels within grain has been attributed partly to interaction between toxigenic and atoxigenic *Flavi* isolates. In this case, the latter could potentially degrade AF produced by the former, leading to lower AF contamination in some maize batches as compared to others (Maxwell *et al.*, 2021). However, it is not known whether or not the *Flavi* species abundances on maize relative to each other could also contribute to differences in contamination levels with AF in the maize.

Objectives of this study were: 1) To determine differences between the *Flavi* community in soil and on maize. We hypothesise that the community structures are different from each other, with maize having a lower diversity than soil. We base our hypothesis on the fact that maize as niche and nutrient source is more homogenous than soil which is laden with heterogeneous organic matter, therefore the latter may accommodate a broader spectrum of fungal species. 2) To investigate the influence of *Flavi* diversity on AF levels in preharvest maize. We hypothesise that *Flavi* species on Zambian preharvest maize are more diverse than previously reported, and expect to find additional aflatoxigenic species. Furthermore, we hypothesise that the ratio between aflatoxigenic species influences the resulting AF levels in maize. The latter hypothesis is based on the assumption that due to the competition for nutrients and space on the kernel, the mycotoxigenic strains may produce the metabolite AF in both inter- and intraspecies competition.

5.2 Materials and methods

5.2.1 Determination of *Flavi* community structure

To determine the community structures, both maize and soil *Flavi* were characterised by a triphasic approach. As detailed below, this involved macro-morphological determination, metabolic profiling based on aflatoxin (AF) and cyclopiazonic acid (CPA), and genetic characterisation based on partial sequencing of the calmodulin gene and, for some verifications, the beta-tubulin gene. The *Flavi* abundances and frequencies were determined by dilution plate technique.

The *Flavi* were isolated from the maize and soil samples as described in Chapters 2 and 4. For this study we considered all locations where *Flavi* was present in both soil and maize in the same field (*n* = 25). Most of the samples that had *Flavi* in both soil and maize of the same field were under weather variable S1 (low rainfall with dry spell), as described in Chapter 2 (B. Katati *et al.* (in press)), making it the study area of choice for this investigation. Hence, the study area covered locations in the four districts under agroecological zone 1 namely, Kalomo, Kazungula, Livingstone and Mulobezi. For each field, an isolate was considered non-clonal if there was sequence divergence detected based on the calmodulin gene. The previously isolated and purified maize and soil isolates were retrieved from storage at -20°C in glycerol-agar.

5.2.1.1 Characterisation of the *Flavi* isolates

Macro-morphological characterisation – Pure wildtype isolates from maize were plated on AFPA medium (Pitt *et al.*, 1983) on 35 mm petri dishes for preliminary screening of *Flavi*. Both soil and maize pure isolates were plated on MEA medium in 90 mm petri dishes and incubated at 25°C (7 days, dark). All isolates were scored for colony colour on PDA medium as well as production or non-production of brown sclerotia on the medium. The maize isolates producing an orange colour on the reverse side of the AFPA plates (Figure 1C) were ready for genetic and metabolic characterisation without further screening, as this confirmed them to be subsets of *Aspergillus* section *Flavi*.

Metabolic characterisation – The metabolic profiles were established based on production of AF and CPA by the isolates. The metabolic profiles for soil isolates are described in Chapter 4. For maize isolates, the metabolites were generated by inducing isolates on Yeast Extract Sucrose (YES) medium (Abdollahi & Buchanan, 1981) and AF was extracted by dichloromethane partitioning with methanol:water (1:1 v/v) reconstitution, as described in Chapter 4. For all isolates, the term “toxigenic” referred to isolates that produced any of the three metabolites, AF-B, AF-G or CPA. CPA-toxigenic means an isolate producing CPA as only metabolite of the three. Aflatoxigenic refers to isolates producing aflatoxin B and/or G, with or without CPA. Non-producer refers to isolates not producing any of the three metabolites AF-B, AF-G or CPA. For both maize and soil, AF and CPA profiles were generated by Thin Layer Chromatography (Figure 2) as described in Chapter 4. Isolates were qualitatively assigned metabolic profiles as positive or negative by production of aflatoxin B, -G and

CPA. All TLC negative isolates were verified by HPLC (Agilent Infinity II 1260 Series, Agilent Technologies, CA, USA) using metabolites generated from the YES medium. All isolates that produced AF bands by TLC were diluted 10-fold and filtered (0.22 µm, nylon) prior to HPLC injection, and 10 µl injected with autosampler. Isolates that did not produce bands were diluted to total volume 500 µl sufficient for HPLC vial, and 20 µl injected into HPLC. Extracts of isolates MLV12A and MLV13A were diluted 100-fold due to them exceeding the instrument's superior detection limit, and 10 µl used as injection volume. Reversed phase analyte separation was achieved with a Cortecs C18 column 3.5 i.d x 100 mm x 2.7 µm particle size (Waters, Milford MA, USA) fitted with a guard column. The mobile phase consisted of an 0.1%-acetic acid:methanol:acetonitrile: 70:20:10 (v/v) at a flow rate of 0.45 ml/min. The column oven was set at 35°C. Eluted AFs were detected on the fluorescence detector (λ_{excit} = 365 nm; λ_{emit} = 440 nm).

Genetic characterisation – For both maize and soil samples, the stored spore suspension of each isolate was retrieved from -20°C, equilibrated to room temperature and diluted to 1 x 10^{-4} spores/ml with sterile Milli-Q water. The diluted spore suspension of each isolate was spotted on a sterilised cellophane disc immersed on the surface of MEA medium in a 60 mm petri dish. Samples were incubated for 2 to 3 days (25°C, dark) allowing just enough fresh mycelia to grow and to be harvested. Fresh dry mycelia on top of the disc were carefully harvested with a sterile spatula and transferred to a 2 ml sterile microcentrifuge tube (Eppendorf, Hamburg, Germany) containing about five 3 mm sterile glass beads. DNA from the mycelia was extracted using the CTAB breaking buffer (2% hexadecyl trimethyl ammonium bromide, 1.4M NaCl, 20mM EDTA, 100 mM Tris HCl, pH=8.0) as follows: the tubes of mycelia were placed for 3 to 5 min in liquid nitrogen. The tubes were immediately clamped on a vortex beater (FastPrep-24™ 5G, M.P. Biomedicals, LLC, Santa Ana, USA) and beating was done at 30 beats/second for 10 s. The tubes were placed back in liquid nitrogen for 2 to 3 min and the beating was repeated for 10 s. Next, 500 µl CTAB breaking buffer was immediately added to each tube after the second beating, and the contents were briefly vortexed. Next, 2 µl proteinase-K solution (15-20 mg/ml, Qiagen, Hilden, Germany) was added per tube and briefly vortexed. Tubes were incubated at 65°C for 1 hour with mild shaking (300 rpm) on a thermomixer (Comfort, Eppendorf, Hamburg, Germany). Next, 500 µl chloroform:isoamyl alcohol (24:1, v/v) solution was

added per tube in a fume hood and mixed gently by inversion 10 times. Tubes were centrifuged in a fume hood for 15 min at 25,000 *g* in a microcentrifuge unit (model 5417R, Eppendorf, Hamburg, Germany) at room temperature. The separated water phase was carefully pipetted off (300 µl) into a new microcentrifuge tube and the remaining liquid and chloroform phase discarded. Next, 300 µl of ice-cold isopropanol was added per tube in open air. Contents were briefly mixed by inversion, five times. The tubes were placed in a freezer at -20°C for 20 min (or overnight when a pause in extraction was unavoidable). Upon withdrawal from the freezer, tubes were immediately centrifuged at 25,000 *g* for 15 min in a temperature-controlled microcentrifuge unit (model 5417R, Eppendorf, Hamburg, Germany) at 4°C. All liquid was decanted by inverting the tube. Next, 300 µl of absolute ethanol was added per tube and the contents were centrifuged at 25,000 *g* at room temperature for 5 min. Liquid was decanted as in the previous step and 300 µl 70% ethanol was added per tube. Tubes were centrifuged again at 25,000 *g* at room temperature for 5 min and the liquid decanted as in the previous step. Tubes were dried overnight, protected from light with aluminium foil, with caps open to ensure all ethanol evaporates prior to the DNA reconstitution. The extracted DNA was dissolved in 50 µl sterile DNA-/RNA-ase free Milli-Q water and stored at 4°C prior to DNA concentration determination (or -20°C for several days storage). DNA concentrations were measured with a Nanodrop spectrometer (model 2000 ThermoScientific, Wilmington, DE, USA) and stored at -20°C pending Sanger sequencing.

For the Sanger sequencing, the purified template DNA concentration of each sample was normalised to 10 ng/µl with sterile Milli-Q water. The DNA was then amplified by PCR for the partial calmodulin gene using primer pairs Cmd5/Cmd6 (Forward: 5′-CCG AGT ACA AGG AGG ARG CCT TC-3′; Reverse: 5′-CCG ATR GAG GTC ATR AGG TGG-3′) (Hong *et al.*, 2006) and alternative primer set CF1/CF4 (Forward: 5’-GCC GAC TCT TTG ACY GAR GAR-3’; Reverse: 5’-TTT YTG CAT CAT RAG YTG GAC-3’) (Peterson *et al.*, 2005) used whenever Cmd5/Cmd6 did not amplify. The two primer sets have different binding sites but amplify a homologous target site (PCR product sizes: Cmd5/Cmd6 ~580 bp; CL1/CL4 ~ 750 bp). For partial sequencing of the beta-tubulin gene for verification of identity of isolates were necessary, the following primer set was used: T10/ Bt2b (Forward: 5′-ACG ATA GGT TCA CCT CCA GAC-3’; Reverse: 5′-ACC CTC AGT GTA GTG ACC CTT GGC-3′). The 25 µl PCR reaction mixture per

tube was composed of 15.9 µl H_2O (sterile Milli-Q) and the following reagents from Promega™ (Madison, WI, USA): 5 µl buffer (5X Go green), 1 µl dNTPs, 0.1 µl Taq polymerase (GoTaq) and the above primers from Biolegio™: 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM). As template DNA, 1 µl of the fungal DNA solutions (10 ng/µl) was used. The amplification was done in a thermal cycler (T100™, Biorad Laboratories Inc., CA, USA) under the following conditions: 1 step of hot start at 95°C for 3:00 min; 31 steps of DNA denaturing at 95°C for 0:45 min; 31 steps of primer annealing at 57°C (primer set Cmd5/Cmd6) or 47°C (primer set CL1/CL4) or 55°C (primer set T10/Bt2b) for 0:30 min; 31 steps of strand elongation at 72°C; 1 final step of elongation at 72°C for 4:00 min. Gel electrophoresis was performed on 1% agarose gel amended with ethidium bromide to aid DNA visualisation. The electrophoresis was performed at 70 V in an electrophoresis chamber. Visualisation of DNA bands was done on a Biorad gel imaging system (Biorad Laboratories Inc., CA, USA) at 312 nm.

All PCR products were purified by Qiagen PCR product purification kit as prescribed in the protocol. Unlike PCR products amplified with the Cmd5/Cmd6 primer set, multiple bands instead of the desired one band were produced by the PCR products generated with the CF1/CF4 primers set. Therefore, for purification of the PCR product of CF1/CF4, the target band was excised from the agarose gel before the product clean-up. Hence, for the clean-up: 1) For non-excised PCR products (from primer pair Cmd5/Cmd6), 10 µl of the product was transferred to a 0.5 ml microcentrifuge tube containing 90 µl sterile Milli-Q water. Next, 200 µl of undiluted NT1 buffer was added to the tube and the contents were briefly vortexed to mix. The mixture was transferred to a NucleoSpin® Gel and PCR Clean-up column attached to a 2 ml collection tube. Contents were centrifuged at 11,000 *g* for 30 s (model 5424, Eppendorf, Hamburg, Germany). Flow-through was discarded from the collection tube, the spin column was re-attached and 700 µl NT3 buffer was added to the spin column to wash the silica membrane. Contents were centrifuged again at 11,000 *g* for 30 s. The washing step with NT3 was repeated, and then the spin column was placed in a new 2 ml collection tube. The tube was centrifuged at 11,000 *g* for 1 min to remove the buffer completely. Tubes were placed at 70°C in an incubator (Comfort, Eppendorf, Hamburg, Germany) for 5 min with caps open to allow all ethanol to evaporate, then left to stand for a further 15 min at room temperature. The spin column was transferred to a DNA/RNA-ase-free 2 ml microcentrifuge collection tube. DNA was eluted by adding 30 µl of NE elution

buffer directly to the silica membrane and the contents were incubated at room temperature for 2 min. The contents were centrifuged at 11,000 *g* for 1 min to collect the amplified DNA, after which the spin column was discarded. 2) For excised PCR-products, a clean scalpel was used to excise about 40 to 80 mg of the target band from the agarose gel. Excision was done with the aid of UV visualisation of the ethidium bromide-stained band, limiting the total exposure time of the DNA to UV to 5 *s*. Next, 2 µl NT1 buffer was added per 1 mg agarose in a sterile 1.5 ml microcentrifuge tube. Tubes were incubated for 10 min at 50°C, vortexing the contents every 3 min. The dissolved mixture was transferred to a NucleoSpin® Gel and PCR Clean-up column attached to a 2 ml collection tube as described above for non-excised DNA. Contents were centrifuged at 11,000 *g* for 30 s and the same protocol was followed as described above for the purification of non-excised DNA.

All PCR-product DNA was stored at -20°C pending Sanger sequencing. The Sanger sequencing was done at Eurofins Genomics (85560 Ebersberg, Germany, https://eurofinsgenomics.eu/) with the same primers used for the PCR amplification.

For the bioinformatics analysis, the raw FASTA files from the sequencing data were inspected and cleaned in MEGA7 version 7.0.26 (Kumar *et al.*, 2016). Front and back ends of the sequence outputs of low quality were denoised, based on inconsistent peak width and height as well as peak overlap, trimming off such peaks that overlap with each other and resulting in inconsistent distances between nucleotide letters. The evolutionary analyses were also conducted in MEGA7, the evolutionary history inferred using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993). All positions with less than 95% site coverage were eliminated, which means fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. Assignment of taxa to the sample FASTA files was achieved using the GenBank database (https://www.ncbi.nlm.nih.gov) with the help of reference isolates (Supplemental Table 1) to verify the assignment of species identities to the isolates. The alignment was saved for downstream processing.

5.2.1.2 Frequency and abundance of *Flavi* in soil and maize and community structure

Flavi abundance was determined as colony quantities (CFU/g soil or maize). This was determined by dilution plate technique (Chapter 3). The frequency of each species was

determined as proportion, in percentage, of number of times a species appeared across the sampled fields to total number of species.

For determination of the community structure, a phylogenetic tree was constructed in MEGA 7 from the saved sequence alignments described above. For generation of bootstrap support, 500 replicates were used. The constructed tree was further processed as an "nwk" file in software R (R_Core_Team, 2013) version 4.1.0 using package ggtree (Yu, 2020). The metabolic profiles were linked to the phylogenetic tree in ggtree using the packages ComplexHeatmap (Zuguang Gu *et al.*, 2016) and gplots (Warnes *et al.*, 2016). The community diversity of *Flavi* in soil and maize was determined using the Shannon index, deduced from seven species detected, namely *A. flavus, A. oryzae, A. minisclerotigenes,* and *A. parasiticus,* as common members and *A. sojae, A. transmontanensis,* and *A. krugeri* as the less common members.

5.2.2 Influence of *Flavi* diversity on preharvest aflatoxin contamination.

5.2.2.1 Abundance of aflatoxigenic species

From the characterised *Flavi*, we determined the abundance (%) of aflatoxigenic isolates in maize across the sampled locations described in Chapter 2 Figure 1, under the low rainfall with dry spell variable (S1). This was determined as the percentage of aflatoxigenic species against overall abundance of *Flavi* on maize. It was presumed that aflatoxin (AF) in maize will only originate from the aflatoxigenic species present on the maize. Determining the proportion of aflatoxigenic isolates may, however, be only used to assess the risk of the AF contamination as not all AF producers detected may possibly be responsible for the detected AF contamination on the maize. Therefore, the link between diversity and observed levels of maize AF was investigated as described immediately below.

5.2.2.2 Influence of aflatoxigenic species ratio on maize aflatoxin levels

The species ratio of the two most abundant aflatoxigenic species on maize was determined. Aflatoxigenicity was based on TLC profiles as described in Chapter 4. Abundance was based on CFU counts, with species identification aided by Sanger sequencing. We selected samples from fields that produced at least six confirmed *Flavi* CFUs per MRB petri dish. The number of isolates per petri dish were counted without dereplication of clonal isolates. The isolates were then categorised as aflatoxigenic or

non-aflatoxigenic based on the TLC aflatoxin B/G profiles. To fit a model to explain the influence of the species ratio on aflatoxin levels, we generated the independent variable 'X' as sum of the proportion ratio of the two most aflatoxigenic species on the plate. 'X' was determined by adapting the Shannon diversity index to our scenario as below and defined as a modified Shannon index (H_m):

$$X = -\sum_{i=1}^{2} [(n_i/N)*Ln(n_i/N)] \quad \text{----------} \quad [1]$$

where, *n* = number of *A. parasiticus* and *A. minisclerotigenes* as the most aflatoxigenic isolates of *Flavi* in fields overall; N = number of the total *Aspergillus* section *Flavi* in a specific field. The *Flavi* were determined by triphasic characterisation.

The dependent variable Y is the total aflatoxin level in maize (S1) on a logarithmic scale. Aflatoxin levels were determined by HPLC (Chapter 3).

5.2.3 Data analysis

Using a combination of morphological, metabolic and genetic characteristics (triphasic characterisation), the isolates were identified as species. This was followed by the quantification of species abundance based on their CFU counts to determine abundance of each species type across the sampled districts as percentage on basis of CFUs of spp/g kernels or soil. Furthermore, soil and maize community structures were similarly determined using CFU counts to determine the *Flavi* abundances and to build a phylogenetic tree to describe the distributions in soil and maize. Maize *Flavi* toxigenicity was determined as the proportion of species in a district producing a particular combination of the metabolites AF and CPA in relation to the total number of *Flavi* species in the district. CFU counts to determine *Flavi* abundance were also used to determine the influence of the *Aspergillus* species ratio on field aflatoxin (AF) contamination between *A. minisclerotigenes* and *A. parasiticus* (equation 1). For the influence of species abundance ratios (X) on maize AF levels (Y), a linear model of Y = f(X) was fitted using Microsoft® Excel® version 365 and significance of the model (*P*–value) and the value of its regression line (R^2) determined. For statistical comparisons, CFU/g data were log-transformed, and zeros converted to a calculated Limit of Detection as 0.152 CFU/g, derived on the basis of lowest detected value and blank values adapted to our scenario from (Magnusson, 2014).

The triphasic characterisation for the maize and soil *Flavi* isolates comparison was done for low rainfall with dry spell weather variable, S1 only (Chapter 2). This is due to the fact that under S1 we found 32 non-clonal isolates compared to S2 with only 5 non-clonal isolates per field, N2 with 1 non-clonal isolate and N1 with none detected. These are included in the supplemental data files and only discussed briefly in the following sections.

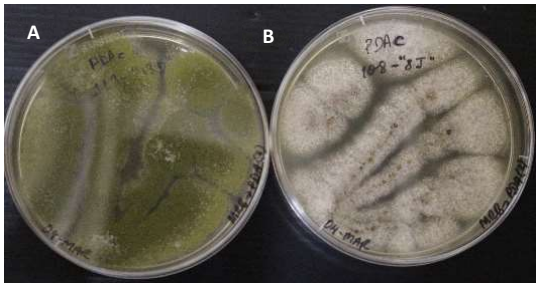
5.3 Results

5.3.1 Determination of *Flavi* community structure between maize and soil

Based on the isolates' genetic lineages, as inferred from their partial calmodulin gene sequences, we report 55 *Flavi* isolates without clones per field (maize, *n* = 32 ; soil, *n* = 23) isolated from S1 (low rainfall with dry spell weather variable). Results, as described below, were on basis of colony morphology for the morphological characteristics; AF-B, AF-G and CPA profiles for metabolic characterisation; then DNA sequence clustering of the partial calmodulin gene for genetic characterisation. The identified isolates were mainly *A. parasiticus*, *A. minisclerotigenes*, *A. flavus* and *A. oryzae*. Other isolates with very low abundances as well as frequency of detection across sampled fields were *A. krugeri, A. sergii, A. sojae, A. transmontanensis,* and *A. tubingensis*.

5.3.1.1 Characterisation of *Flavi* in soil and on maize

Morphological characterisation – Morphologies of the isolates are described in Table 1. Overall, *A. flavus* isolates produced a green shade on their mycelium due to formed green conidiospores, except for isolate MKZ07D, which produced a cream-white shade (Figure 1). *A. flavus* isolates also produced brown sclerotia, except for MKA01K. *A. Oryzae* produced a green shade like *A. flavus*, but in contrast, it predominantly did not produce brown sclerotia, except for one isolate, MML19A. *A. minisclerotigenes* produced a cream-white shade, except for MML19G, with its cream-white shade and green in the middle. All *A. minisclerotigenes* isolates produced brown sclerotia. All *A. parasiticus* produced a green shade. Production of brown sclerotia was not a distinctive trait for *A. parasiticus*, as some isolates produced the brown sclerotia while others did not.



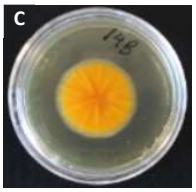


Figure 1. Macro-morphology of *Aspergillus* section *Flavi* on a 90 mm Petri dish at the end of a 7-day incubation (25°C, dark) on PDA from MRB, during isolation passaging. **[A]** *A. parasiticus* isolate MLV13D, with characteristic green colour. **[B]** *A. minisclerotigenes* isolate MKZ08J, with characteristic cream-white colour and visible sclerotia. **[C]** AFPA medium (36-hour incubation, 31°C, dark) on 35 mm petri dish. Orange reverse characteristic of macro-morphology of all isolated species in maize belonging to *Aspergillus* section *Flavi*. Picture shows isolate MKZ14B.

Table 1. Maize and soil *Aspergillus* section *Flavi* isolates' morphology on MEA

Source	Species & morphology	G	G/W	W/G	W	Sclerotia	Sclerotia absent
Maize	*A. flavus*	5	-	-	1	4	2
	A. oryzae	6	-	-	-	1	5
	A. minisclerotigenes	-	-	1	8	9	0
	A. parasiticus	8	-	-	-	3	5
Soil	*A. flavus*	1	-	-	-	1	-
	A. oryzae	1	-	-	-	-	1
	A. parasiticus	12	11	-	-	12	11
	A. sergii	2	-	-	-	-	2
	A. transmontanensis	1	-	-	-	-	1

W = Cream-white; G = Green; G/W = predominantly green with white outer ring; W/G = predominantly white with some middle green shade. All colony diameters on PDA (7 days, 25°C) were ≥ 25 cm.

Genetic and metabolic characterisation – In maize, 32 *Flavi* isolates without clones (having distinct sequence divergence on calmoudlin gene) per field were detected from 14/20 fields of S1 (low rainfall with dry spell). Isolates were identified as *A. minisclerotigenes* x 10, *A. parasiticus* x 9, *A. oryzae* x 7, and *A. flavus* x3. The other three isolates detected were one each for *A. tubingensis*, *A. transmontanensis* and *A. Krugeri*. In this study, we found *A. minisclerotigenes* to be a closer relative of *A. flavus*, followed by *A. oryzae* (Figure 4). Metabolically, *A. parasiticus* characteristically produced aflatoxin B and G, and did not produce CPA (Figure 4). On the other hand, *A. minisclerotigenes* was a producer of aflatoxin B, G and CPA, and was exclusive to maize only. Most CPA producers were aflatoxigenic (> 90%, Table 2), except for isolates in the district Mulobezi. All metabolites that were found negative by TLC were also found negative by HPLC verification, except for isolate MML19K (*A. flavus*, AF-

B1), MML19E (*A. flavus*, AF-B1) and MLV14K (*A. parasiticus*, AF-B1 & -G1) all of which were found to produce relatively low amounts of AF (< 5 ng/ml YES broth).

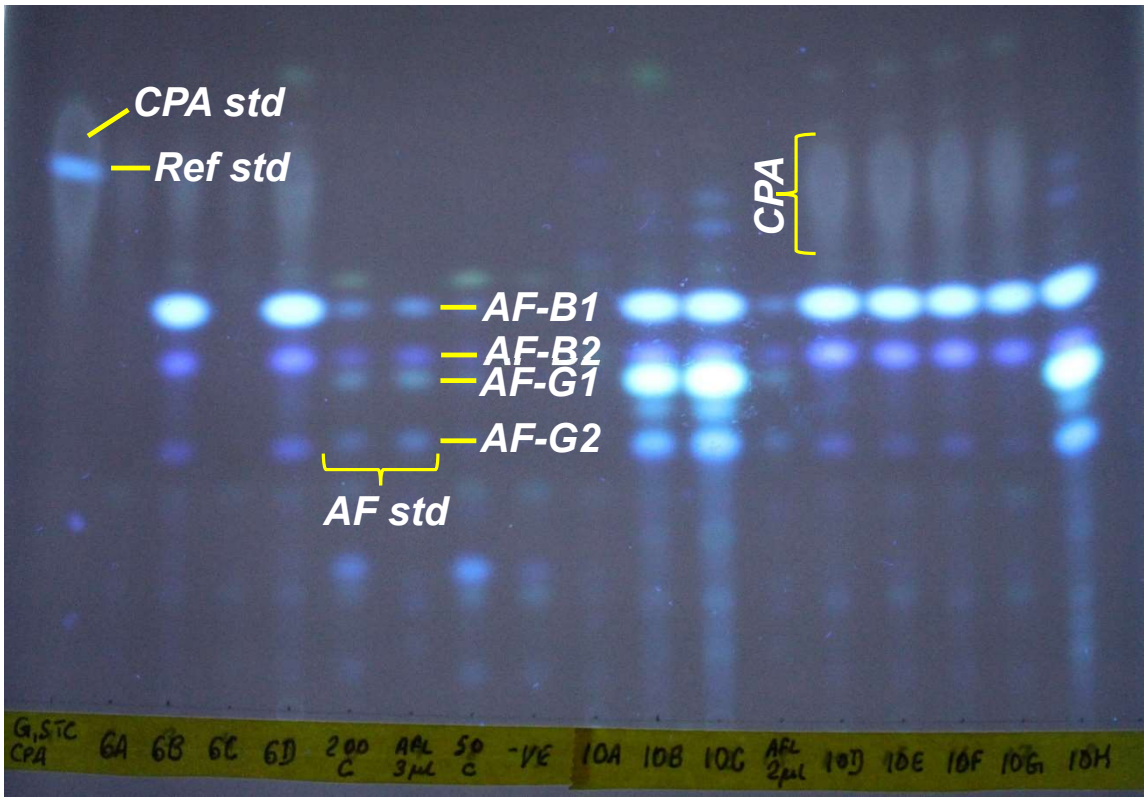
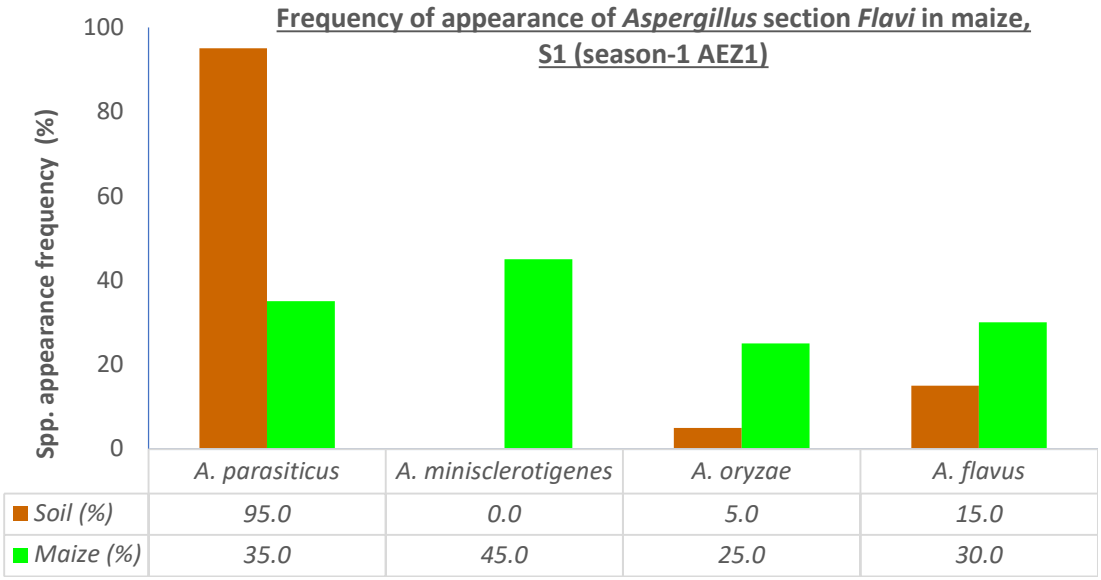


Figure 2. Developed Thin Layer Chromatography plate visualised under UV (312 nm) to qualitatively determine aflatoxins B1, G1, B2, G2 as well as cyclopiazonic acid (CPA) from metabolite extracts of maize *Flavi*. *A. parasiticus* bands of AF-B and -G toxins without CPA are shown in lane '10A' and '10B.' *A. minisclerotigenes* bands are shown in lanes '10F' and '10G,' producing AF-B and -G (faint) and CPA bands. Toxigenicity levels of soil isolates were similarly analysed. Lane '200C' is a spiked aflatoxin standard in medium that was not inoculated with spores. Lane 'Afl3ul' contains pure aflatoxins B1, G1, B2, G2 standard, mixed. Lane 'G, STC_CPA' is a pure CPA standard spotted together with a griseofulvin standard. (Griseofulvin is normally used for the determination of the rf value of the chromatography).

In soil, 23 *Flavi* isolates without clones (having distinct sequence divergence on calmoudlin gene) per field were detected from all fields of S1 (Figure 4). From these non-clonal isolates, 19 were *A. parasiticus*, and one each as *A. oryzae*, *A. sergii*, *A. transmontanensis* and a highly divergent *A. sojae*. Metabolically, all the soil isolates (including clones) produced both aflatoxin B and G, without CPA, with the exception of isolates EKZ07A (*A. parasiticus*, AF-B only) and EML16B (*A. Oryzae*, CPA only) (Figure 4). Some CPA producers were detected in soil (Supplemental Table 2). The proportion of CPA producers on maize was significantly higher than in soil ($P = 0.020$). A high percentage of toxigenic isolates was detected in soil (83 to 100%).

5.3.1.2 Soil and maize *Flavi* relative abundance and community structure

The overall spread of the *Flavi* species between the two ecological niches, soil and maize, was as follows: *A. parasiticus* was the most dominant soil species detected, surpassing all other species encountered in soil combined (Figure 3). In contrast, *A. minsiclerotigenes* was exclusive to maize, with none detected in soil (Figure 4). Overall, there was a higher species variation in maize compared to soil, as shown in Figure 3. This is supported by a higher Shannon diversity index for maize (1.34) compared to soil (0.53), despite soil having a higher abundance of *A. parasiticus* than maize. Some species were detected in very low abundance with only 1 isolate in a field. These included *A. krugeri, A. sojae, A. transmontanensis, A. tubingensis* as well as *A. sergii* (not shown in Figure 3; see Supplemental Tables 5A and 5B).



	A. parasiticus	*A. minisclerotigenes*	*A. oryzae*	*A. flavus*
Soil (%)	95.0	0.0	5.0	15.0
Maize (%)	35.0	45.0	25.0	30.0

Figure 3. Most frequently detected *Aspergillus* section *Flavi* species on maize and in soil.

While *A. parasiticus* was predominant in soil, CPA producers, with or without aflatoxin, including *A. minisclerotigenes*, were predominant on maize (Figures 3 and 4), with only two isolates (EKA04B, EML16A) detected in soil in this region (Figure 4). *A. parasiticus*, however, was abundantly detected in both the soil and maize grain. *A. oryzae* and *A. flavus* were all detected on maize, except for one isolate of *A. oryzae* (EKA16A) in soil and one *A. flavus* (EKA04B) in soil as well. The non-producer isolates, like the CPA producers, were reported more on maize than in soil. The four predominant species on maize in order of decreasing frequency of field infection were: *A. minisclerotigenes* (4/4 districts; 9/20 fields), *A. parasiticus* (4/4 districts; 7/20 fields), *A. oryzae* (3/4

districts; 6/20 fields) and *A. flavus* (3/4 districts; 4/20 fields). In this sampling season and area, *A. minisclerotigenes* and *A. parasiticus* were the most common species found on maize (Figures 3 and 4), with *A. parasiticus* also being the most common species found in soil.

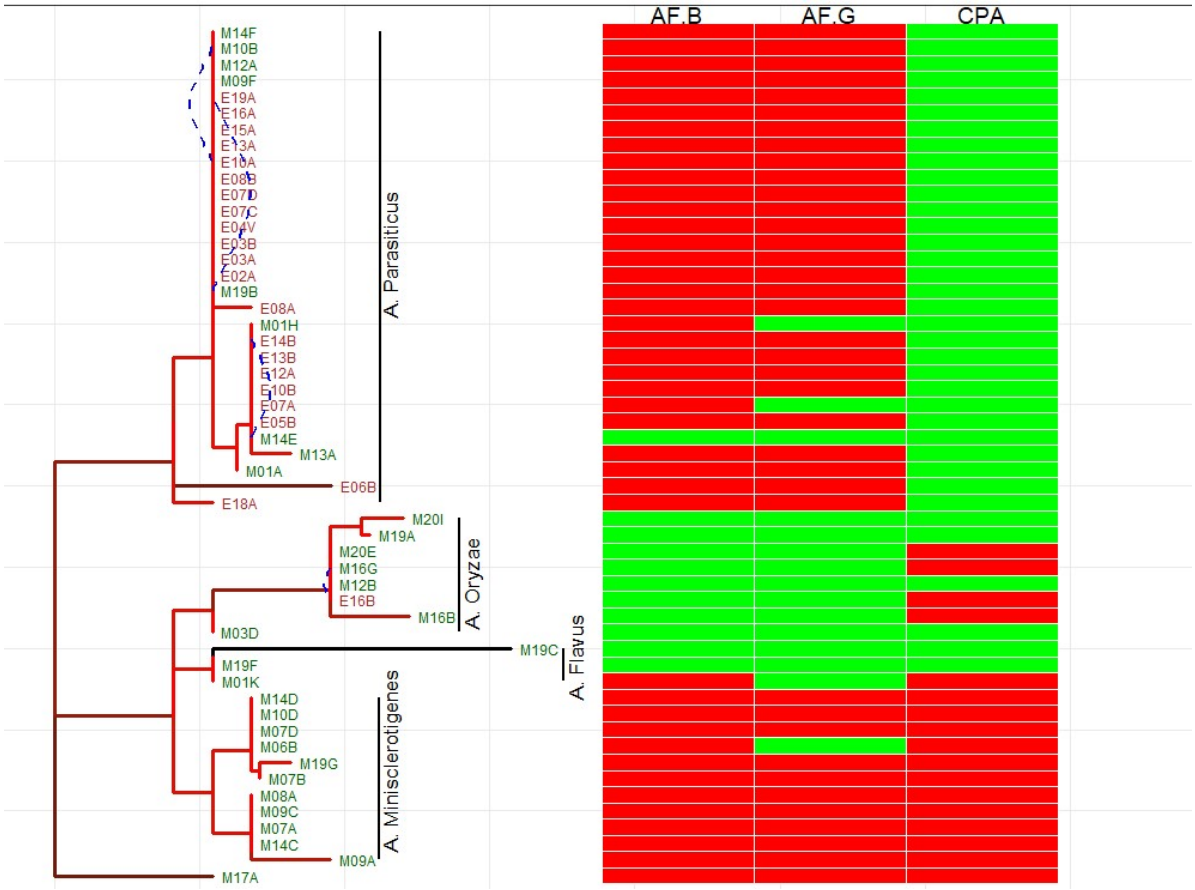


Figure 4. Phylogeny of *Aspergillus* section *Flavi* isolates detected in soil (brown with starting letter 'E') and maize (green with starting letter 'M') of selected districts of Zambia under low rainfall pattern (S1). Blue dotted lines show corresponding soil and maize isolates from the same field (same numerical value) having no sequence divergence based on partial sequencing of the calmodulin and b-tubulin genes. The metabolic characteristics of each species based on aflatoxin-B1 and/or B2 (AF-B), aflatoxin-G1 and/or G2 (AF-G) and cyclopiazonic acid (CPA) are qualitatively indicated as **red** (**produced** by the species) or **green** (**not produced** by the species). Unclustered species on the tree are M03D - *A. flavus*, MML17A – *A. krugeri*. Dark red for tree branch indicate a longer branch on x-axis than light red.

Overall for maize and soil, the metabolic profile for the production of AF-B and -G without CPA was exclusive to *A. parasiticus* (Figure 4). The production of all three metabolites, AF-B, -G and CPA, was exclusive to *A. minisclerotigenes*, with the exception of one isolate of *A. krugeri* (MML17A). The production of CPA only, was exclusive to *A. oryzae* and *A. flavus*, with the exception of isolate MKA06A (*A. tubingensis*). Non-producers (producing none of the three metabolites) were mainly *A. oryzae* and *A. flavus*, with an exception of one maize isolate of *A. parasiticus*

(MLV14E). There was a significantly higher population of CPA-toxigenic isolates in maize compared to soil, $P = 0.020$).

5.3.2 Influence of *Flavi* diversity on preharvest aflatoxin contamination

5.3.2.1 Frequency of aflatoxigenic species

We detected the species *A. minisclerotigenes*, a species previously unreported on Zambian maize, with aflatoxin (AF) producing potential in high frequency on Zambian maize. The overall frequency of species mycotoxigenicity is presented in Table 2. Most isolates on the maize across the sampled districts were producers of AF, except for one location (Mulobezi, Table 2) with a longitude to the west of the other three locations (Chapter 2, Figure 1), which had a low frequency of AF producers.

Table 2. Frequency of maize isolates' mycotoxigenicity determined by aflatoxin B (AF-B) and G (AF-G), and cyclopiazonic acid (CPA).

District*			**Toxigenic species**						**Non-toxigenic**
	Aspergillus density, (CFU/g, x 10^{-3})		**Aflatoxigenic**						
							CPA-toxigenic		
	Min	Max	Total aflatoxigenic species, %	AF-B, %	AF-B+G, %	AF-B+G +CPA, %	CPA, %	Total CPA producing species, %	%
Kalomo	0.0	4.5	92.7	0.0	61.0	31.7	0.0	31.7	7.3
Kazungula	0.1	29.6	99.8	0.0	34.5	65.3	0.1	65.4	0.1
Livingstone	0.0	60.0	100.0	0.0	9.0	91.0	0.0	91.0	0.0
Mulobezi	0.0	73.8	0.3	0.0	0.3	0.0	90.3	90.3	9.5

Table 2. *Fields per district $n = 5$; Toxigenicity of soil isolates is presented in Supplemental Table 2. Supplemental Data for maize *Flavi* species abundances and mycotoxigenicity of sampling season-2 AEZ1 (S2) is presented in Supplemental Table 3 and Supplemental Figure 1.

5.3.2.2 Influence of aflatoxigenic species ratio on maize aflatoxin levels

Looking at the influence of species diversity (ratio between *A. parasiticus* and *A. minisclerotigenes*) on the levels on aflatoxins (AFs) produced in maize, a linear regression model fits the data significantly (Figure 5, $P = 0.001$). The lowest AF content in *Flavi*-contaminated maize was recorded at a 'modified' Shannon Diversity index (H_m) of '0' (only one species between *A. parasiticus* and *A. minisclerotigenes* present, fields 08 and 13). On the other hand, the highest content of AF was recorded when H_m had

the highest value (0.72, Figure 5), also signifying a closer value to a 1:1 ratio between *A. parasiticus* and *A. minisclerotigenes* relative to the total *Flavi* on a maize sample.

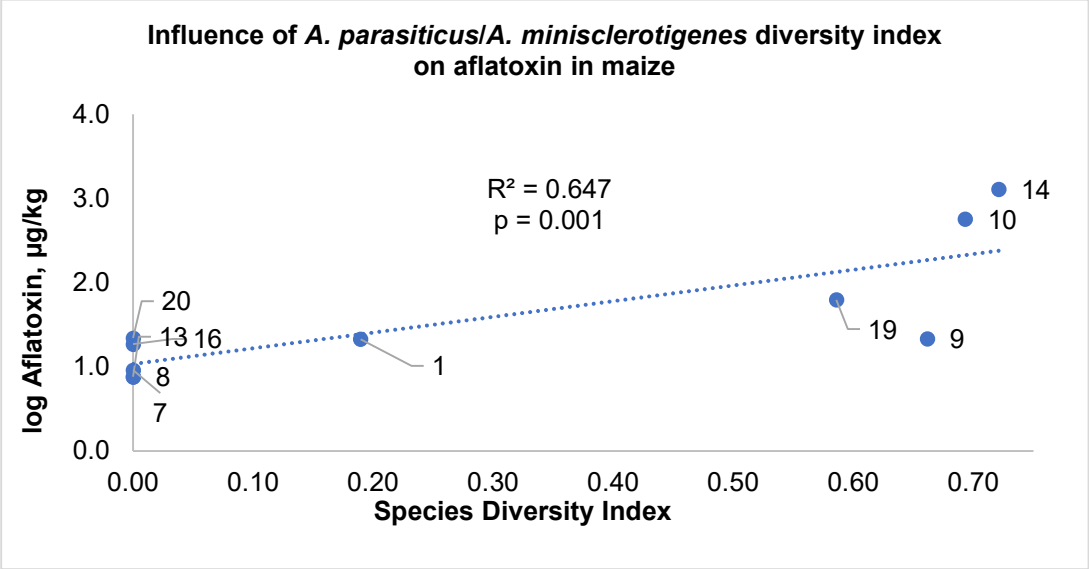


Figure 5. Influence of species diversity on levels of aflatoxin in maize. A modified Shannon diversity index (Hm) equation-[1] (material and methods) was used to calculate species diversity. (In an ideal situation, excluding other species, the highest possible Hm value would be 0.69, when the *A. parasiticus* to *A. minisclerotigenes* ratio is 1:1).

5.4 Discussion

5.4.1 *Aspergillus* section *Flavi (Flavi)* community structure in maize and soil

There were differences in *Flavi* population structures between soil samples and their corresponding maize samples. On maize, the previously unreported *Aspergillus minisclerotigenes* in Zambia influenced the aflatoxin (AF) levels in the preharvest maize. Although risk of AF contamination has at times been estimated based on the *Flavi* community in the soil, we demonstrate that this should be done with precaution as the two community structures can differ. This is evidenced by the fact that we detected only four maize-soil isolate pairs with no sequence divergence within a field, out of the 55 per-field-unique strains detected in 20 fields (Figure 4).

5.4.2 Characterisation of *Flavi* isolates (soil and maize)

Employing a triphasic characterisation, we detected additional important *Flavi* species on Zambian preharvest maize and soil across the four sampled districts than previously reported. These included *A. oryzae* and *A. minisclerotigenes*. We detected *A. minisclerotigenes* and *A. oryzae* in relatively high frequencies on maize (Figure 3).

Morphologicaly, *A. minisclerotigenes* resembled *A. flavus* strains in this study, but the isolates consistently produced brown sclerotia on PDA and MEA, with cream-white vegetative mycelium, sparse conidial heads. Hence, *A. minisclerotigenes* can morphologically be misidentified as *A. flavus* (Dooso Oloo *et al.*, 2019; Karimizadeh Esfahani *et al.*, 2019). Similarly, *A. oryzae* can easily be morphologically misidentified as *A. flavus*, or indeed reported as an atoxigenic variant of *A. flavus*, as it is a close relative of *A. flavus* (Chang *et al.*, 2006). Although *A. flavus* isolates often produced brown sclerotia in our study, it should be noted that this does not imply *A. flavus* is always a producer of sclerotia. As population distributions of *Flavi* are bound to change with season (Ortega-Beltran & Cotty, 2018), isolates of *A. flavus* that do not produce brown sclerotia could, for example, be found to be predominant on preharvest maize or be less dominant in another season. However, from the production of distinct brown sclerotia on PDA by *A. minisclerotigenes* detected in this study, we may infer that the morphology is predominant of this species as described in a previous study of *Aspergilli* (Frisvad *et al.*, 2019).

We demonstrate that *A. flavus* is a close relative of *A. oryzae*, based on sequence divergence (Figure 4) in part of the calmodulin gene. While this resonates with earlier findings by (Chang *et al.*, 2006; Cleveland *et al.*, 2009), we do demonstrate in addition that *A. minisclerotigenes* was further away from *A. oryzae* and *A. flavus* (with 85% bootstrap support) than was *A. flavus* from *A. oryza*e. This is contrary to findings by (Kjaerbolling *et al.*, 2020) who demonstrated closeness of *A. minisclerotigenes* with *A. orzyae* than *A. flavus*. We attribute these differences to the choice of loci used in the genetic differentiation. However, consensus is that in section *Flavi*, *A. parasiticus* would cluster away from *A. flavus*, *A. orzyae* and *A. minisclerotigenes*.

Metabolically, *A. minisclerotigenes* has been implicated in the notorious levels of AFs detected in Kenyan maize east of the country (Dooso Oloo *et al.*, 2019). Identified on Zambian maize in this sampling season, this may sound an alarm bell on the potential danger for high maize AF contamination in Zambia. However, we cannot directly implicate *A. minisclerotigenes*, in the observed field contamination of maize with AF under weather variable S1 (low rainfall with dry spell) as it may not have been the single species producing the AFs. This is in spite of the fact that one of the four most virulent isolates appraised by AF inducing medium was *A. minisclerotigenes* and the rest were *A. parasiticus* (Supplemental Table 4). It is also worth noting that *A.*

minisclerotigenes is also a categorical producer of CPA (Figure 4) (Frisvad *et al.*, 2019; Pildain *et al.*, 2008). Beyond plant pathology, an *A. minisclerotigenes* isolate was recently implicated as human Keratitis agent in Iran (Karimizadeh Esfahani *et al.*, 2019).

The sampled southerly (S) Zambian agroecological zone AEZ1 as shown in Figure 1 of Chapter 2 (B. Katati *et al.* (in press)) was under a dry spell during our first sampling season. It may be suggested that such severely dry and hot conditions may have benefited *A. minisclerotigenes*, just like hotter temperatures seem conducive for its closer relative *A. flavus*, which performs better than *A. parasiticus* under those conditions (Ching'anda *et al.*, 2021). This is consolidated by the fact that *A. minisclerotigenes* was not detected under the normal low rainfall conditions (weather variable S2) from the exact same fields in the 2020/2021 sampling season (Supplemental Figure 2), a season that generally had a lower frequency of detected *Flavi.* This also suggests that the observed preharvest contamination pattern under weather variable S1 could have been seasonal, with the potential to recur. It's also plausible that *A. minisclerotigenes* may be present on stored maize, but is unreported due to absence of molecular surveillance. The detection of other previously unreported species, namely *A. transmontanensis* and *A. sergii*, detected in the southerly (S) agroecological zone 1, in year 2 (S2) in high abundance (Supplemental Figure 1) suggests that the diversity of *Flavi* in Zambian maize is beyond the commonly reported *A. flavus* and *A. parasiticus*. This warrants the need for further seasonal studies by triphasic characterisation of the *Flavi* community structure, particularly on preharvest maize, in order to measure the extent of the diversity of the *Flavi* and subsequently the AF contamination risk. The observed high frequency of *A. minisclerotigenes* coupled with its production of both B and G aflatoxins and CPA shows that this is a fungus deserving attention in the maize *Flavi* infection scenario.

Although earlier country studies showed that *A. flavus* was the major contaminant of maize (Kachapulula *et al.*, 2017b; Kankolongo *et al.*, 2009; Mukanga *et al.*, 2010), the likely carry-over of the other detected species in this study from harvest to storage would imply that more species beyond *A. flavus* can contaminate the crop during storage, in contrast to what has been reported in earlier studies. We attribute the higher number of species on maize detected in this study compared to earlier country studies to close genetic relationships and morphological similarities of *A. flavus* to *A.*

minisclerotigenes and *A. oryzae*. This shows the importance of genetic species identification as well as metabolic screening of the different important mycotoxins. Our overall findings are in agreement with previous findings indicating that *A. parasiticus* is (one of) a major contaminant of maize (Kachapulula *et al.*, 2017b). Although our study was, in contrast to previous studies, done on preharvest grain, it is likely that the *Flavi* species would be carried over to storage. From other studies of *Aspergilli* on maize, we do note that the previously reported *Flavi* species *A. parasiticus*, *A. flavus*, *A. tamarii* and *A. niger* are canonically non-producers of CPA, except for some strains of *A. flavus*. In contrast, the detected maize isolates in our study season, except for *A. parasiticus*, were all capable producers of CPA (*A. minisclerotigenes*, most strains of *A. oryzae*, and some strains of *A. flavus*).

Although in S2 fewer *Flavi* non-clonal isolates were isolated, a CPA-producing species (*A. transmontanensis*) was detected, and this species had a 48% relative abundance in district Mulobezi. From our findings, the significant frequency of detection of CPA producers of *Flavi* on preharvest maize (10/17 fields, in S1) may suggest a potential risk of maize contamination with CPA other than AFs, particularly when conditions become favourable. Furthermore, two non-aflatoxigenic isolates of *A. flavus* were found to produce CPA. Such isolates producing CPA are not uncommon. It therefore remains imperative when selecting candidates as non-aflatoxigenic biocontrol strains for AF, that such candidates are monitored for non-production of CPA

5.4.2.1 Comparison of soil and maize community structures

Soil is the reservoir for *Aspergilli*. In line with this, for example, past studies have suggested AF contamination events to be likely driven by the toxigenic isolates from soil (Donner *et al.*, 2009; Kachapulula *et al.*, 2017b; Njoroge *et al.*, 2016). However, it should not be inferred, for example, that observed high frequency of detection of *A. parasiticus* in soil would mirror the frequency of this species on maize. We demonstrate in our studied districts that the *Aspergillus* community structure on maize greatly differed from that in soil, both quantitatively and qualitatively, with maize having a higher frequency of *Flavi* species compared to soil (Figure 3). Furthermore, while all species found in soil were also found on maize, some species in maize were not detected in soil. In 4/20 fields, a soil isolate with no sequence divergence between itself and the corresponding maize isolate was found (Figure 4). Therefore, attempts to

predict the source of AF contamination of maize on basis of soil *Flavi* structure in the case of non-biocontrol agent-treated soil should be done with caution. Some isolates not detected in soil may proliferate on maize if that happens to be their conducive niche. This could explain, for example, the dominance of the soil-absent *A. minisclerotigenes* on maize, compared to dominance of *A. parasiticus* in all soils (Figure 4) of the same fields under weather variable S1. *A. parasiticus*, for example, is a poor coloniser of maize compared to *A. flavus* (Dorner JW *et al.*, 1999; Horn, 2003; Zummo & Scott, 1990) and generally produces lower spore densities in air compared to *A. flavus*. As a result, high proportions of *A. parasiticus* in soil may likely not mirror the proportions on maize.

Although *A. parasiticus* is demonstrated as the most commonly found soil fungus in this study, it should also be noted that the *Flavi* soil community structure can be latitude-dependent, with higher temperatures likely to favour *A. flavus* over *A. parasiticus* (Ching'anda *et al.*, 2021). For example, studies in Kenya showed that the density of *A. flavus* in soil was higher than that of *A. parasiticus* (Benard *et al.*, 2013; Elsie *et al.*, 2016). This is the opposite with the Zambian scenario ((Kachapulula *et al.*, 2017b); Figure 3), which has a different climate and is at higher latitude than Kenya.

Our findings also show that CPA producers may have a propensity towards maize colonisation as their niche compared to soil dwelling, considering that CPA producers were more frequently detected on the maize compared to soil (Figure 4). These findings are supported by a previous study on maize, which demonstrated that maize isolates produced more CPA than soil isolates and that CPA producers had a higher propensity to colonise maize kernels (Chalivendra *et al.*, 2017). In line with this, *A. minisclerotigenes*, detected in high numbers in this study, may be a species of concern, being better armed with the accessories (CPA, AF) to both penetrate the maize ear easier and contaminate the crop in field or post-harvest with genotoxic aflatoxins-B and -G as well as CPA.

5.4.2.2 Influence of *Flavi* diversity on aflatoxin contamination

The high frequency of aflatoxigenic *Flavi* found on maize (Table 2) demonstrates the risk for aflatoxin (AF) contamination of maize when conditions become favourable. Furthermore, *A. minisclerotigenes*, a species previously unreported on Zambian maize, was detected in high frequency, and all its isolates were producers of genotoxic

aflatoxins B and G and neurotoxic CPA (Figure 4). This warrants it to be a species of interest and concern in the Zambian food safety landscape. Although the species contributed to the AF contamination risk of the maize based on its field frequency (Figure 3) and aflatoxigenicity, this was not sufficient to conclude causal relation between *A. minisclerotigenes* and the AF contamination of the field maize. This is considering that some fields that had *A. minisclerotigenes* did not have detectable AF levels, despite being under the same dry weather pattern as some AF-heavily contaminated fields. The dry spell had been found to be the major driver of the AF contamination (Chapter 3). As an alternative explanation to the differences in AF contamination due to species present, we report on a simple model correlating the influence of the abundance ratio between *A. parasiticus* and *A. minisclerotigenes* on field maize AF contamination. There was no significant AF contamination observed when the ratio between *A. parasiticus* and *A. minisclerotigenes* was close to 0 (actual ≤ 0.2), even under high CFU count of a species. On the other hand, high AF levels were recorded in fields with higher ratios described by the modified species diversity index in equation 1. This may suggest that as the two most aflatoxigenic species became more equal in relative abundance to each other, a trigger of higher overall AF production occurred. This could also imply the potential for AF to be a signature of competition between similar species for colonising the kernel. This warrants further investigations. Findings show that within the diversity of *Flavi* in preharvest Zambian maize, *A. minisclerotigenes* can be an important contributor to the AF contamination of the maize.

5.5 Conclusion

The difference in the *Flavi* community structure between paired soil and maize samples demonstrates that the different *Flavi* species may preferentially be adapted differently to the ecological niches, soil and maize. On this basis, the risk of infection of maize with *Flavi* should not be anchored on the community structure of the soil, but rather on the maize community structure. Deploying a triphasic characterisation, we demonstrate that the maize *Flavi* landscape on Zambian preharvest maize is quite diverse, while high frequencies of the previously unreported *A. oryzae* and the AF and CPA-toxigenic *A. minisclerotigenes* were observed. This study further suggests that CPA producers may have a higher propensity for maize dwelling than soil. We attribute the higher *Flavi* species diversity found on the maize compared to soil to the higher

number of species that produced CPA compared to the non-CPA producer *A. parasiticus*, which dominated the soil in this study.

We postulate that AF levels in the preharvest maize may have been driven by the ratio of the two aflatoxigenic species *A. minisclerotigenes* and *A. parasiticus*, as demonstrated by a simple linear model.

5.6 Supplemental material

Supplemental Table 1. Reference *Aspergillus* section *Flavi* isolates from GenBank (FASTA).

Species	GenBank accession number *
A. flavus	CBS501.65 MG517996.1 MG517989. MG518125.1
A. minisclerotigenes	MG518009.1 MG518021.1 MG518022.1 MG518083.1
A. aflatoxiformans	MG518076.1 MG518075.1 MG518079.1 MG518090.1
A. oryzae	EF661506.1 EF661507.1
A. parasiticus	MG518030.1 MG518019.1 MG518097.1 MG518126.1
A. sergii	MG518059.1
A. sojae	EF202041.1 EF661517.1 MG518028.1
A. transmontanensis	HM803020.1 HM803021.1 HM803023.1 HM803028.1

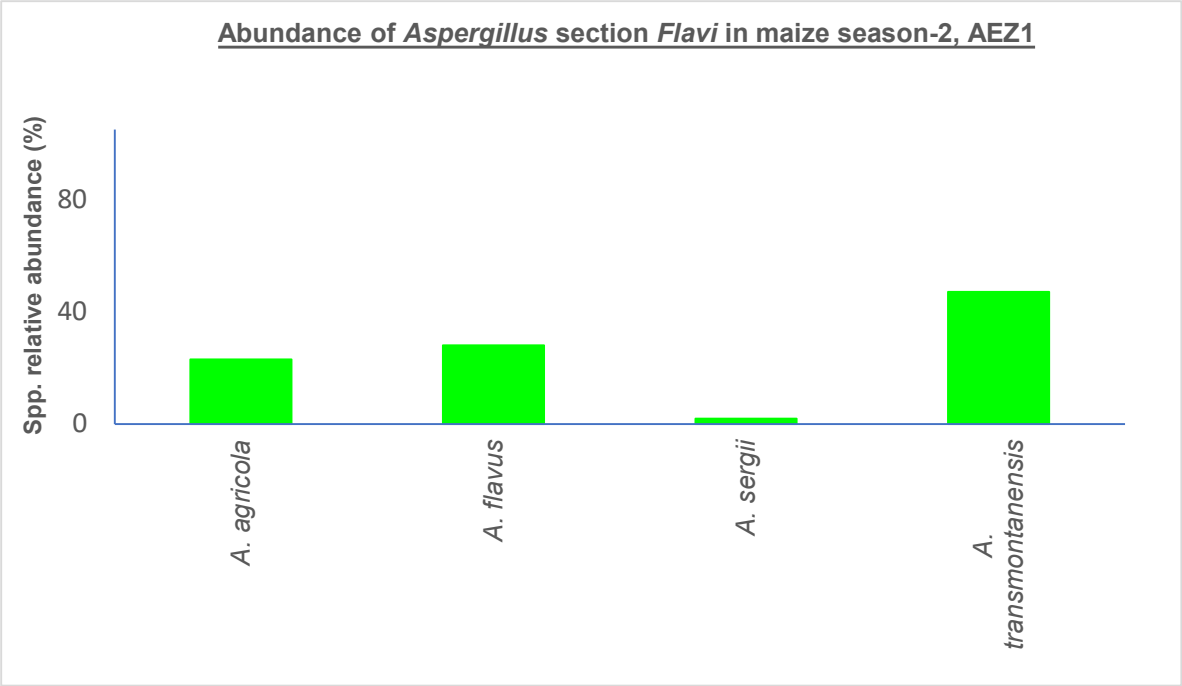
**Reference for isolates*: (Frisvad *et al.*, 2019).
The following *A. flavus* isolates were added to the analysis as live laboratory reference cultures: “01MS7,” “125GF7,” “125FG8” and “126MS6” obtained from the International Institute of Tropical Agriculture (Zambia).

Supplemental Table 2. Season-1 soil isolates' mycotoxigenicity determined by aflatoxin B (AF-B) and G (AF-G), and cyclopiazonic acid (CPA).

District	*Aspergillus* density		Toxigenic species						Non-toxigenic
			Aflatoxigenic						
						CPA-toxigenic			
	(CFU/g)	Std. Dev	Total aflatoxi-genic species, %	AF-B, %	AF-B+G, %	AF-B+G +CPA, %	CPA, %	Total % CPA producing species	%
Kalomo	32.3	17.1	100.0	0.0	75.3	24.7	0.0	24.7	0.0
Kazungula	45.3	26.7	100.0	8.5	91.5	0.0	0.0	0.0	0.0
Livingstone	26.3	23.6	100.0	0.0	100.0	0.0	0.0	0.0	0.0
Mulobezi	17.0	11.2	83.0	0.0	83.0	0.0	9.2	9.2	7.8

Supplemental Table 3. Season-2 maize isolates' mycotoxigenicity determined by aflatoxin B (AF-B) and G (AF-G), and cyclopiazonic acid (CPA).

District	*Aspergillus* density, (CFU/g, x 10^{-3})		Toxigenic species						Non-toxigenic
			Aflatoxigenic						
						CPA-toxigenic			
	Min	Max	Total aflatoxi-genic species, %	AF-B, %	AF-B+G, %	AF-B+G +CPA, %	CPA, %	Total CPA producing species, %	%
Kalomo	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Kazungula	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Livingstone	0.0	0.02	100.0	100.0	0.0	0.0	0.0	0.0	0.0
Mulobezi	0.0	0.50	50.5	2.6	0.0	48.0	0.0	48.0	49.5



Supplemental figure 1. Abundance of *Flavi* detected on maize from four selected districts of AEZ1 in season-2 (S2) under which *A. agricola*, *A. flavus, A. sergii and A. transmontanensis* were detected.

Supplemental Table 4. Aflatoxigenicity (ng/ml) in selected isolates of *A. parasiticus* and *A. minisclerotigenes*.

Code	Species	G2	G1	B2	B1	Total
MML19G	*A. minisclerotigenes*	0.8	110.6	4.4	224.7	340.5
MKZ09C	*A. minisclerotigenes*	10.0	137.5	4.0	850.5	1002.0
MKZ06B	*A. minisclerotigenes*	13.3	0.3	97.2	2684.2	2795.0
MKZ08H	*A. minisclerotigenes*	22.7	0.3	138.9	4545.5	4707.3
MLV14D**	*A. minisclerotigenes*	344.1	13262.0	149.0	4507.7	18262.8
MLV14K	*A. parasiticus*	0.3	7.3	0.3	13.6	21.4
MKA01H	*A. parasiticus*	0.3	0.3	1.9	20.0	22.3
MVL14F	*A. parasiticus*	1.9	346.0	2.1	138.4	488.5
MML19B	*A parasiticus*	4.0	288.0	9.7	383.5	685.2
MKA01H	*A. parasiticus*	18.1	822.4	27.8	1053.2	1921.5
MKZ09F	*A. parasiticus*	35.7	1265.6	15.5	836.7	2153.5
MKZ09H**	*A. parasiticus*	241.6	8731.2	100.2	2972.8	12045.9
MLV13A**	*A. parasiticus*	191.2	5898.6	247.4	6167.0	12504.2
MLV12A**	*A. pParasiticus*	275.3	11334.7	241.3	8025.3	19876.6

Aflatoxin levels were determined by HPLC with fluorescence detector. Limit of detection (G2 = 0.28, G1 = 0.30, B2 = 0.22, B1 = 0.29, ng/ml). (Superior detection limit AF-B1 ~ 16 µg/ml). ** Four most virulent strains on basis of total aflatoxin generated per ml Yeast Extract Sucrose broth medium (7-day incubation, 31°C, dark).

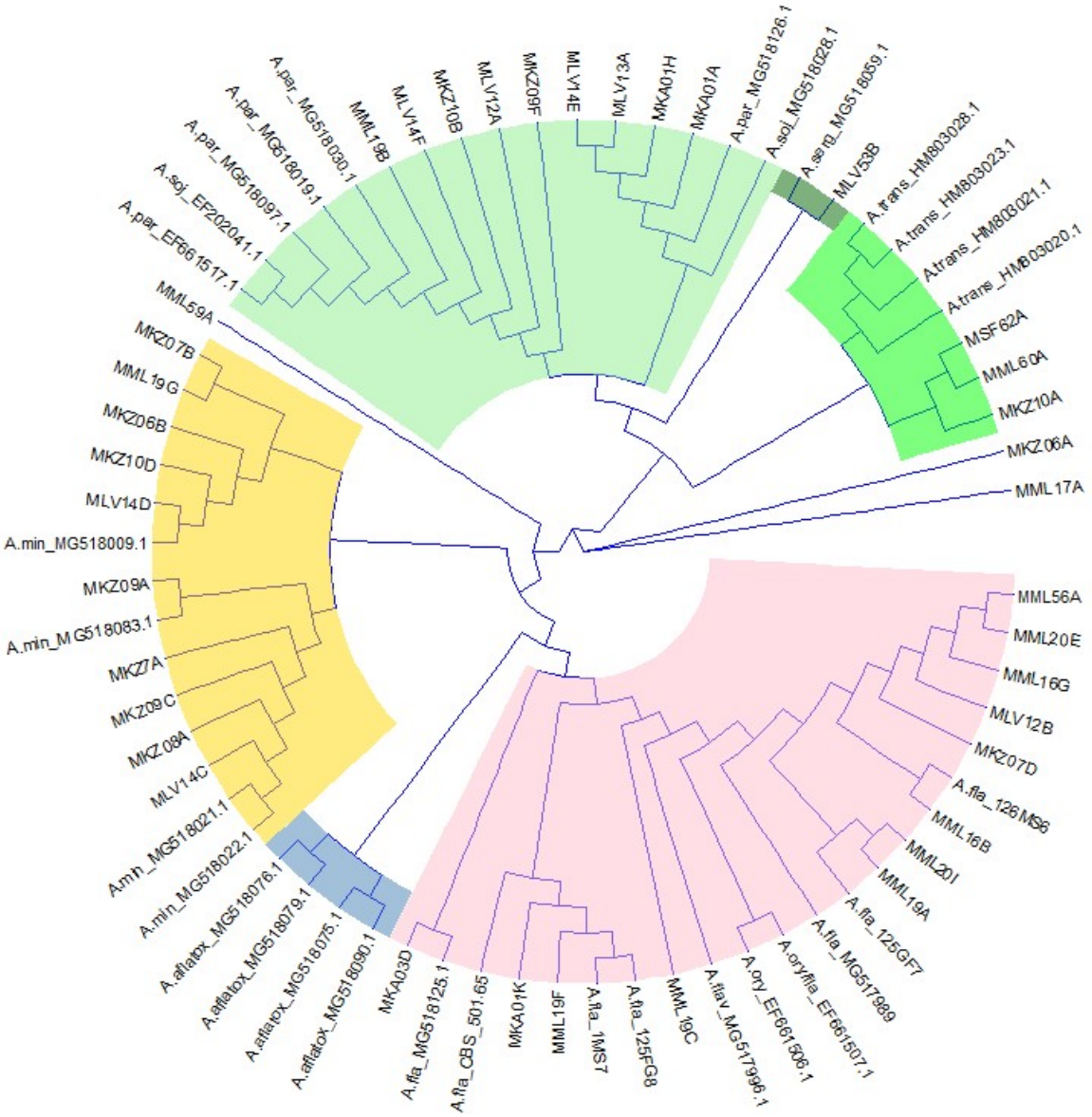
Supplemental Table 5A. Frequency of detection of *Aspergillus* section *Flavi* in soil across fields of S1 (weather variable low rainfall with dry spell).

Field	CFU/g	*A. flavus*	*A. oryzae*	*A. minisclerotigenes*	*A. parasiticus*	Other*	*
1	40.0	100.0	0.0	0.0	0.0	0.0	
2	1.7	0.0	0.0	0.0	100.0	0.0	
3	40.0	0.0	0.0	0.0	100.0	0.0	
4	40.0	33.3	0.0	0.0	66.7	0.0	
5	40.0	0.0	0.0	0.0	50.0	50.0	*A. Sojae*
6	66.7	0.0	0.0	0.0	100.0	0.0	
7	76.7	0.0	0.0	0.0	100.0	0.0	
8	10.0	0.0	0.0	0.0	100.0	0.0	
9	33.3	0.0	0.0	0.0	100.0	0.0	
10	40.0	0.0	0.0	0.0	100.0	0.0	
11	1.7	0.0	0.0	0.0	100.0	0.0	
12	3.3	0.0	0.0	0.0	100.0	0.0	
13	53.3	0.0	0.0	0.0	100.0	0.0	
14	33.3	0.0	0.0	0.0	100.0	0.0	
15	41.7	0.0	0.0	0.0	100.0	0.0	
16	23.3	0.0	33.3	0.0	66.7	0.0	
17	33.3	0.0	0.0	0.0	100.0	0.0	
18	6.7	0.0	0.0	0.0	100.0	0.0	
19	13.3	50.0	0.0	0.0	50.0	0.0	
20	8.3	0.0	0.0	0.0	100.0	0.0	

Supplemental Table 5B. Frequency of detection of *Aspergillus* section *Flavi* in maize across fields of S1 (weather variable low rainfall with dry spell).

Field	CFU/g	*A. parasiticus*	*A. minisclerotigenes*	*A. oryzae*	*A. flavus*	Other*	*Asp. spp
1	4.47	78.6	7.1	0.0	14.3	0.0	
2	0.00	0.0	0.0	0.0	0.0	0.0	
3	0.76	0.0	50.0	0.0	50.0	0.0	
4	0.00	0.0	0.0	0.0	0.0	0.0	
5	0.00	0.0	0.0	0.0	0.0	0.0	
6	0.1	0.0	50.0	25.0	0.0	25.0	*A. Tubingensis*
7	6.8	0.0	100.0	0.0	0.0	0.0	
8	0.4	0.0	100.0	0.0	0.0	0.0	
9	29.6	37.5	62.5	0.0	0.0	0.0	
10	0.3	37.5	50.0	0.0	0.0	12.5	*A. Transmontanensis*
11	0.0	0.0	0.0	0.0	0.0	0.0	
12	0.0	50.0	0.0	50.0	0.0	0.0	

13	5.9	100.0	0.0	0.0	0.0	0.0	
14	60.0	64.3	28.6	0.0	7.1	0.0	
15	0.0	0.0	0.0	0.0	0.0	0.0	
16	85.2	0.0	0.0	80.0	20.0	0.0	
17	0.01	0.0	0.0	0.0	0.0	100.0	*A. Krugeri*
18	0.0	0.0	0.0	0.0	0.0	0.0	
19	0.7	36.4	9.1	9.1	45.5	0.0	
20	73.8	0.0	0.0	30.0	70.0	0.0	



Supplemental Figure 2. Phylogenetic tree shows the clustering of the maize isolates from season-1 and season-2. **Long codes (> 6 characters) with prefix 'A' are GenBank reference isolates** to estimate correctness in assignment of species identity to our isolates generated in the study. Shorter codes with 2-digit figure < 20 are season-1 isolates, and > 20 are season-2 isolates.

Abbreviations: *A.aflatox = Aspergillus aflatoxiformans; A.fla = Aspergillus flavus; A.min = Aspergillus minisclerotigenes; A.soj = Aspergillus sojae; A.trans = Aspergillus transmontanensis*

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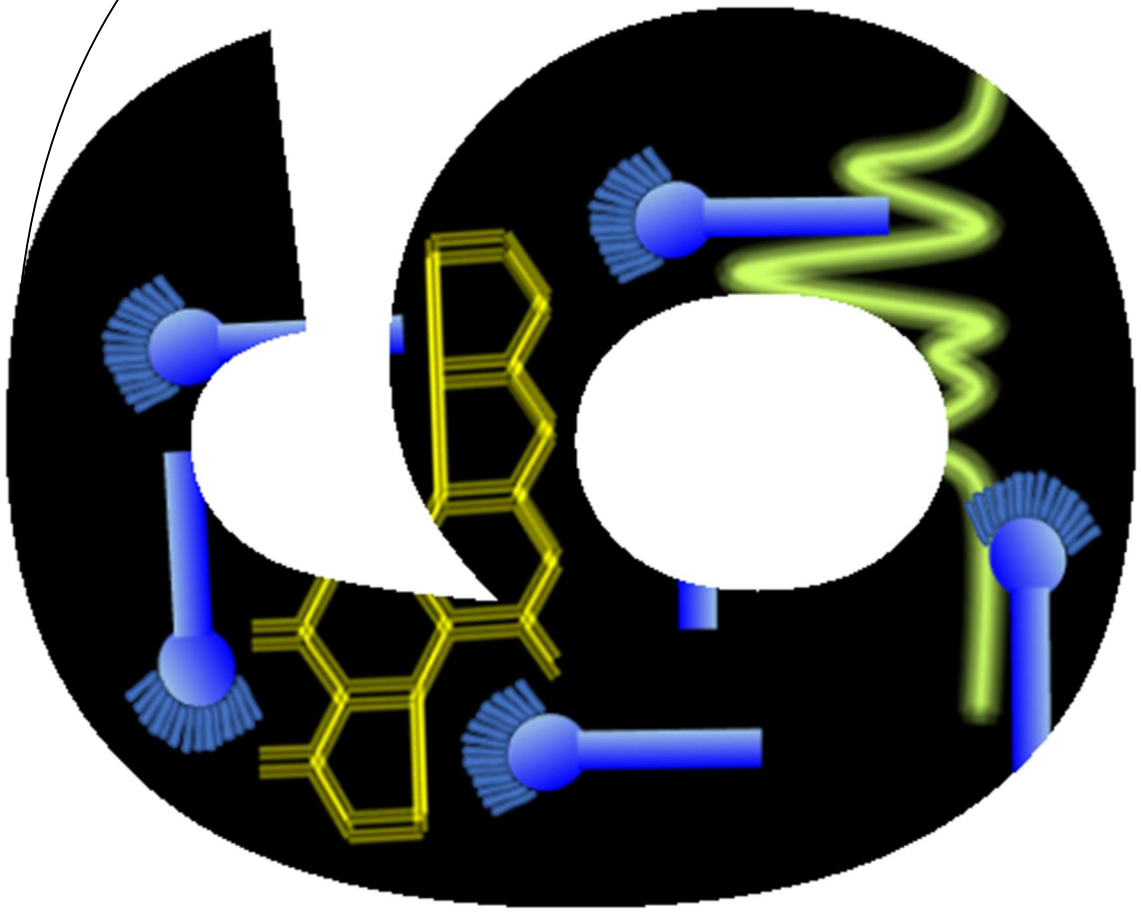
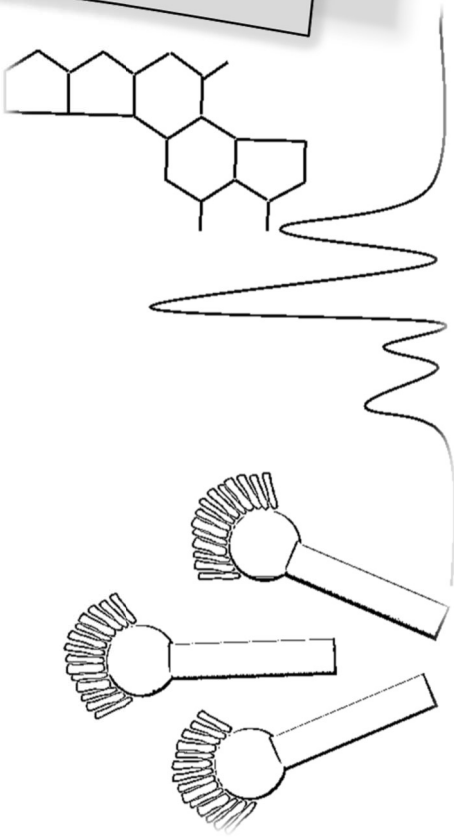
If toxigenic Aspergilli were a human world, antioxidant would be their stress-relieving supplement





Chapter VI

Aflatoxigenic *Aspergillus* modulates aflatoxin-B1 levels through an antioxidative mechanism

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6.0 Abstract

Aflatoxins (AF) are considered to play important functions in species of *Aspergillus* section *Flavi* (*Flavi*, for short) including: an antioxidative role, a machinery against fungivorous insects, and antibiosis. Atoxigenic *Flavi* are known to degrade AF-B1 (B1). To better understand the purpose of AF degradation, we investigated the degradation of B1 and AF-G1 (G1) in an antioxidative role in *Flavi*. Atoxigenic and toxigenic *Flavi* were treated with crude extracts of B1 and G1 with or without the antioxidant selenium (Se), expected to affect levels of AF. After incubations, AF levels were measured by HPLC. To estimate which population would likely be favored between toxigenic and atoxigenic *Flavi* under Se, we investigated the fitness, by spore count, of the *Flavi* as a result of exposure to 0, 0.40 and 0.86 µg/g Se in 3%-sucrose cornmeal agar (3gCMA). Results showed that levels B1 in medium without Se were significantly reduced in all isolates, while G1 did not significantly change. When medium was treated with Se, toxigenic *Flavi* significantly digested less B1, while levels of G1 significantly increased. Se did not affect digestion of B1 in atoxigenic *Flavi*, and also did not alter levels of G1. Further, atoxigenic strains were significantly fitter than toxigenic strains at Se 0.86 µg/g 3gCMA. Findings show that while atoxigenic *Flavi* degraded B1, toxigenic *Flavi* modulated its levels through an antioxidative mechanism to levels less than they produced. Furthermore, B1 was preferred in the antioxidative role compared to G1, in the toxigenic isolates. The higher fitness of atoxigenic over toxigenic counterparts at plant non-lethal dose of 0.86 µg/g would be a useful attribute for integration in the broader biocontrol prospects of toxigenic *Flavi*.

6.1 Introduction

Aflatoxins (AFs) are a group of secondary metabolites produced by *Aspergillus* section *Flavi* (*Flavi*, for short) members such as *A. parasiticus* and *A. flavus*. The group of fungi infect important cereals like maize and groundnuts (Akello *et al.*, 2021; Hulikunte Mallikarjunaiah *et al.*, 2017; Kachapulula *et al.*, 2017b; Munkvold *et al.*, 2019; Ndisio *et al.*, 2017; WHO, 2018c) resulting in crop AF contamination. Of the known AFs produced by *Flavi*, AF-B1 (B1) is the most carcinogenic (IARC, 2012; Li *et al.*, 2022; Rushing & Selim, 2019). The production of AF by *Flavi* has been demonstrated to be linked to useful biological functions including the competitive inhibition of other microbes such as fungivores (Drott *et al.*, 2019; Drott *et al.*, 2017), antibiosis against bacteria (Arai *et al.*, 1967) and oxidative stress alleviation (Doyle & Marth, 1979; J. C. Fountain *et al.*, 2016; Jake C. Fountain *et al.*, 2016; Jayashree & Subramanyam, 2000; Narasaiah *et al.*, 2006) (Figure 1).

The link of AF to oxidative stress alleviation suggests that AF may be responsible for the scavenging of reactive oxygen species (ROS) (Fountain *et al.*, 2014) which results in the subsequent degradation of the AF molecule in reaction with the ROS. In this regard it has been suggested that an increase in the antioxidative capacity of a plant could alleviate the increase in AF production by aflatoxigenic species such as A. *flavus* (Jake C. Fountain *et al.*, 2016). Hence, It would be estimated that introducing an antioxidant in the growth environment of the fungus (the crop) would reduce ROS thereby reducing the need for the fungus to produce AF. A number of antioxidants elements are known, which include copper, manganese, selenium, zinc. Selenium (Se) is important to human health (Rayman, 2000) as well as playing an anti-carcinogenic role (Kursvietiene *et al.*, 2020). The element is also known to play a defence role against AF in animals (Alsuhaibani, 2018; Mughal *et al.*, 2017; Wang *et al.*, 2019). In right amounts Se can enhance plant growth (Kaur *et al.*, 2022).

Se can be introduced into a plant by biofortification through conventional breeding, genetic modification, mineral fertilisation of soil or through foliar application. In crop protection, the biofortification of plants with such an element would play the role of indirect control of AF accumulation in important cereals like maize and wheat while improving the crop's micronutrient status. However, it should also be noted that Se can be toxic in elevated levels to the plant itself (Kaur *et al.*, 2022; Naseem *et al.*, 2021) other than inhibiting fungal sporulation at high levels.

Atoxigenic *Flavi* strains have been demonstrated to degrade B1 (Maxwell *et al*., 2021). With respect to this, colonisation of a crop by atoxigenic *Flavi* has been demonstrated to be able to reduce AF contamination in crops like maize, cotton and groundnuts (Bock & Cotty, 1999; Medina *et al.*, 2017; Raksha Rao *et al.*, 2020). Although degradation of AF by atoxigenic *A. flavus* has been demonstrated (Maxwell *et al.*, 2021), the reason behind the phenomenon is still unclear. For example, considering that atoxigenic *Flavi* do not produce AF, could the degradation of AF have a link to antioxidative alleviation in the non-aflatoxin producing *Flavi*, and would the mechanism be the same with aflatoxin producers?

Some studies have demonstrated that the mechanism of inhibition of AF production involves the downregulation of regulatory genes *aflS*, reducing abundance of the ratio of *aflS* to *aflR* (Xing *et al.*, 2017). This would imply that there is actual reduction in the amount of AF produced by the *Flavi* due to reduced messenger RNA from structural genes such as *aflD* and not necessarily the reduction of AF through its degradation. The *aflD* is a structural gene responsible for the early conversion of AF precursor molecules in the aflatoxin biosynthetic pathway (Yu *et al.*, 2004; Yu *et al.*, 2011) and is regulated by the regulatory genes *aflR* (Chang, 2004; Georgianna & Payne, 2009; Price *et al.*, 2005; Woloshuk *et al.*, 1994) and *aflS*. To further understand the potential role of AF production and utilisation in *Flavi*, two related questions need to be answered: 1) is AF degraded by atoxgenic *Flavi* a part of oxidative stress alleviation? 2) is AF also degraded by aflatoxin-producing *Flavi* as part of the mechanism of oxidative stress alleviation.

Introducing a natural antioxidant such as Se to the environment of toxigenic and atoxigenic members of section *Flavi*, and observing the associated levels of aflatoxin produced in its environment might provide some insights into the potential role aflatoxin plays as an antioxidant in both toxigenic and atoxigenic *Flavi*. It would also help to understand in which direction the population dynamics between toxigenic and atoxigenic *Flavi* would likely shift in an Se-fortified environment. It is known that fungi respond to abiotic stimuli, and that the exact nature of the response will depend on the fungal strain and abiotic condition. With respect to an antioxidative environment, if aflatoxin were an antioxidant for both toxigenic and atoxigenic *Flavi*, then one would expect that toxigenic *Flavi* would be fitter (e.g. produce more spores) in an environment with antioxidant such as Se, compared to atoxigenic *Flavi*. It would, however, be

desirable that atoxigenic strains are fitter in a Se-fortified environment compared to toxigenic counterparts (Figure 1) such that population dynamics are not in favour of the toxigenic over atoxigenic strains.

The aim of this investigation was to explore the potential involvement of AF as an antioxidant in the growth environment for toxigenic and atoxigenic *Flavi*. Specific objectives of this study were to 1) determine whether AF is broken down by both atoxigenic and toxigenic *Flavi* in the role of an antioxidant. 2) to establish differences in fitness levels between atoxigenic and toxigenic isolates as a result of antioxidant (Se) fortification. We hypothesise that both toxigenic and atoxigenic *Flavi* utilise AF in an antioxidative role. We also hypothesise that toxigenic strains are fitter when treated with antioxidant Se compared to atoxigenic counterparts. We base our hypothesis on grounds that toxigenic isolates expend more energy producing AF presumably in an antioxidative role, compared to the atoxigenic strains. This would imply that introduction of a substitute antioxidant (Se), relieves more energy for the aflatoxin-producing fungus to utilise for other purposes including sporulation (fitness).

Understanding the role of AF in both toxigenic and atoxigenic *Flavi* from an antioxidative role will better guide efforts aimed at improving the micronutrient status of maize without the risk of promoting toxigenic isolates over atoxigenic counterparts if integrated into the broader biocontrol programme. In addition such knowledge will help to better understand how antioxidant would help in preventing spikes of AF in maize.

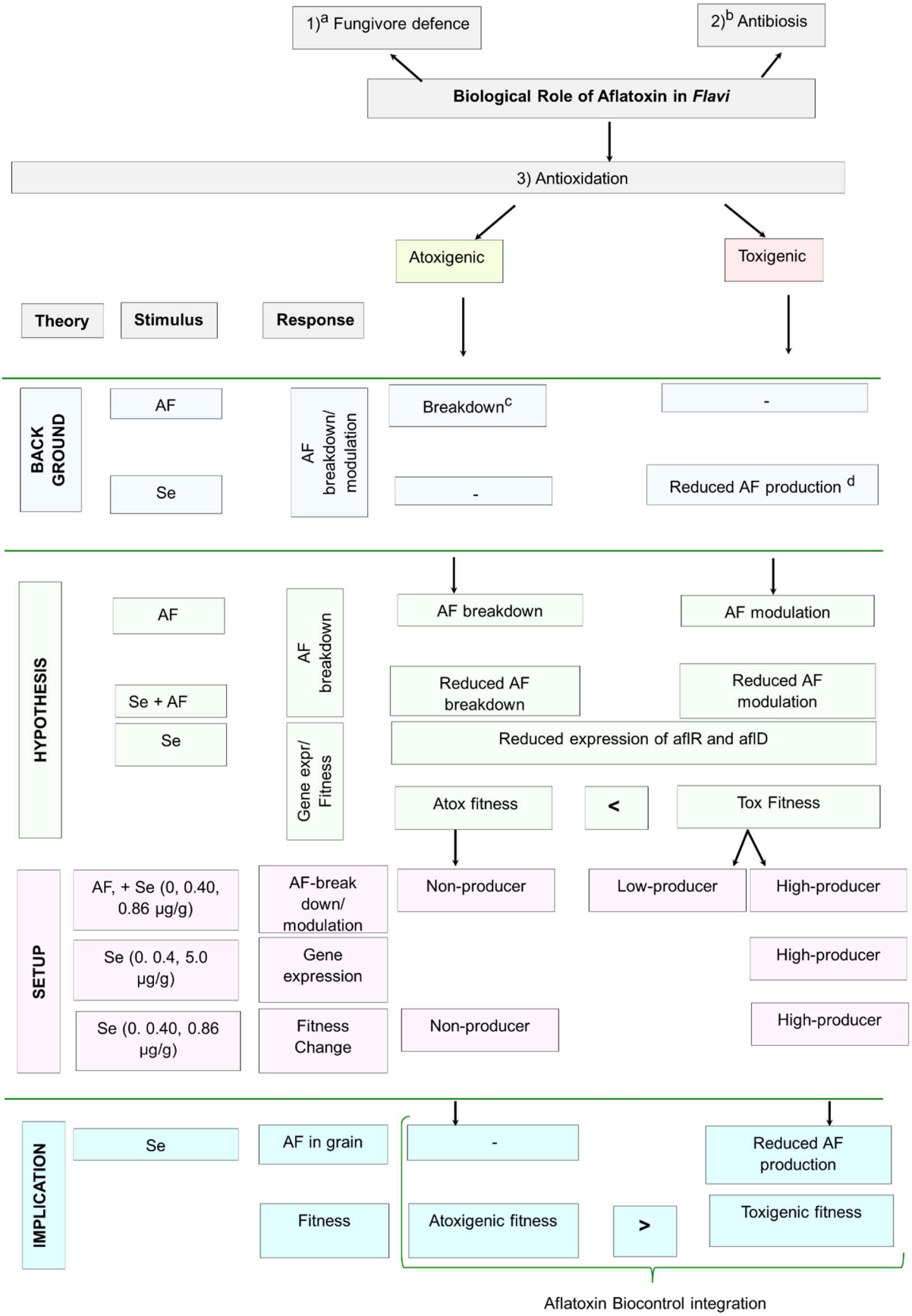

Figure 1. Schematic representation of the investigation of the role of aflatoxin (AF) degradation in an antioxidative mechanism. The scheme includes: the Background of what is known/presumed;

breakdown of AF by atoxigenic *Flavi*; reduction in AF production under increased antioxidant Se (selenium) in environment; **Hypothesis**, the experimental **Setup** and **implication** on control of AF. [a](Drott *et al.*, 2019; Drott *et al.*, 2017); [b](Arai *et al.*, 1967); [c](Maxwell *et al.*, 2021); [d](Doyle & Marth, 1979; J. C. Fountain *et al.*, 2016; Jake C. Fountain *et al.*, 2016; Jayashree & Subramanyam, 2000; Narasaiah *et al.*, 2006)

6.2 Materials and methods

All isolates used had previously been isolated from maize and soil (Chapter 3 and 4) stored in glycerol at -70°C. Isolates were retrieved from storage and regenerated on Malt Extract Agar medium (MEA) for 7 days (25°C, dark). Spores from regenerated cultures were suspended in 0.8% sterile saline solution, as stock culture solution. The stocks were diluted with sterile Milli-Q water to 1 x 10^4 spores/ml prior to inoculations. Chromatographic analyses for aflatoxin (AF) were done by HPLC (Agilent Infinity II 1260 Series, Agilent Technologies, CA, USA). Technical standards for AF-G2, G1, B2 and B1 were obtained from Merck (Merck Life Science NV, Amsterdam, The Netherlands).

6.2.1 Determination of antioxidative degradation of aflatoxin levels by toxigenic and atoxigenic *Flavi*

To determine the antioxidative role that aflatoxin (AF) might be involved in for its degradation, we specifically used an antioxidant (selenium) in *lieu* of previously used oxidative compound such as H_2O_2 (Jake C. Fountain *et al.*, 2016; Narasaiah *et al.*, 2006). This would enable the direct determination of a substitute compound for AF, if the AF is used in an antioxidative role.

6.2.1.1 Assignment of atoxigenic and toxigenic isolates

From the bulk of maize and soil *Flavi* isolates assessed by TLC for mycotoxigenicity (Chapter 4 and 5), we selected 12 wildtype laboratory cultures based on the qualitatively assessed amount of aflatoxin they could produce. The first four isolates were initially assigned as non-producers (atoxigenic) based on absence of TLC bands for AFs. Another four isolates were initially assigned high producers based on very high intensity of AF bands on TLC plates. Then, four isolates were assigned low producers based on less intense bands of Aflatoxins by TLC compared to the high-producers. All the 12 isolates were then verified by HPLC for levels of AF produced. To determine this, a 100 µl spore suspension (1 x 10^4 spores/ml) per isolate was inoculated into test tube containing 2 ml autoclave sterilised YES broth (Abdollahi & Buchanan, 1981) (pH 6.0). Tubes were incubated at 31°C (7 days, dark). Aerobic liquid

fermentation was then carried out with shaking at 150 rpm in a temperature-controlled incubator (Innova 4330, New Brunswick Scientific, CT, USA). AF was extracted by dichloromethane partitioning as described in Chapter 4. AF quantification was carried out by HPLC as described in Chapter 5, diluting the crude extracts 10-fold and injecting 10 µl into HPLC. The isolates were hence assigned as high- (> 300 ng/ml YES medium), low- (LOQ – 299 ng/ml YES) and non-producers (Table 1). Limit of quantitation (LOQ) in crude extracts was assigned as 2.5 ng/ml. All values below LOQ were converted to 0 as unquantifiable noise.

Table 1: *Flavi* isolates used In determination of B1 and G1 Degradation.

Isolate	toxigenicity	Mean aflatoxin produced, ng/ml	
		B1	G1
1MS7[a]	None	0	0
125GF8[a]	None	0	0
ESF24B	None	0	0
MLV12B	None	0	0
ESF62A[b]	Low	0	0
ELV13C	Low	3.2	8.0
MKZ06B	Low	33.8	0
EKZ10A	Low	52.1	146.3
MKA01K	High	342.4	83.9
EKW36B	High	164.6	323.3
EKW40A	High	1340.2	527.6
ELG33C	High	6749.8	10.4

[a] Obtained as reference isolates from other upstream experiments.

[b] Was assigned as low producer considering that AF was produced in presence of antioxidant. Strain coding first letter is ‘E’ for soil and ‘M’ for maize. The aflatoxins B1 and G1 concentrations were determined by High Pressure Liquid Chromatography.

6.2.1.2 Aflatoxin (AF) degradation assay

Crude AF was first prepared from one aflatoxin-producing isolate “EKZ10A.” For this, a 100 µl of isolate EKZ10A spore suspension (1 x 10^4 spores/ml) was inoculated into test tube containing 2 ml autoclave sterilised YES broth (Abdollahi & Buchanan, 1981) (pH 6.0). Fifty tubes were incubated at 31°C (7 days, dark) for the liquid fermentation with shaking at 150 rpm in a refrigerated incubator (Innova 4330, New Brunswick Scientific, CT, USA). AF was extracted as described in Chapter 4, collecting the centrifuged dichloromethane (CH_2Cl_2) extracts from each tube into one pooled sterile 50 ml tube to ensure homogeneity of AF concentration. Next, 1000 µl of AF extract in CH_2Cl_2 was transferred per 10 ml glass test tube (x 104 tubes) for x 96 test samples, x 4 positive controls, and x 4 negative controls. The CH_2Cl_2 was vapourised under

fumehood to dryness and reconstituted with 300 µl absolute ethanol to re-sterilise crude AF. Tubes were then placed in laminar flow hood to vaporise the ethanol. Sterilised YES agar medium was dispensed warm at 50°C per tube, then vortexed for 20 s to redissolve the crude extract of AF for inoculation with isolates. The YES had been prepared as described in Chapter 4 but with addition of agar at 0.2% concentration to generate a paste. One set of YES agar had been fortified with anhydrous analytical reagent sodium selenite (99.75% purity, cas 10102-18-8, Alfa Aesar, Haverhill, MA, USA) at concentration 0.40 µg/g. The concentration was selected on basis of recommended daily intake for selenium (Se) at 55 µg/day (Monsen, 2000). Considering the estimated food consumption rate of about 310 g/capita/d of maize (without maize products) in Zambia (FAO, 2017), the recommended Se intake rate of 55 µg/day from maize only, would then be achieved. In this experiment, the selenite salt was the antioxidant. The YES Tubes were set up for the 12 isolates with each isolate receiving all four of the following treatment permutations: i) isolate only ("Spp") ii) isolate with AF ("Spp + Afla"), iii) isolate with antioxidant ("Spp + AntiOx") iv) isolate with AF and antioxidant ("Spp + Afla + AntiOx"). Each isolate was duplicated (total 96 test samples). Positive controls were AF extract without isolate ("Afla") as well as AF extract with antioxidant ("Afla+AntiOx), in duplicate. Negative controls were YES as well as YES with antioxidant, in duplicate. Each tube with the YES medium was inoculated with 50 µl of 1 x 10^4 spores/ml isolate suspension for each of the 12 test isolates. The positive controls to determine base AF levels in tubes were inoculated with 50 µl sterile Milli-Q H_2O in place of isolate inoculation. All tubes were incubated at 31°C (7 days, dark). The liquid fermentation was carried out in an incubator (Innova 4330, New Brunswick Scientific, CT, USA) with mild shaking at 100 rpm. After the incubation, AF was extracted by dichloromethane partitioning as described in Chapter 4. No biomass growth ensued in the positive controls for AF, confirming integrity in sterility of the crude AF extract medium during preparation.

AF quantification was carried out by HPLC as described in Chapter 5, diluting the crude extracts 10-fold and injecting 10 µl into HPLC using autosampler.

6.2.1.3 Gene expression (*aflR* and *aflD*) response to antioxidant (Se)

To determine the possible correlation response of gene expression to antioxidant treatment, three randomly selected high aflatoxin-producing isolates (EKZ10B, ELG33C and MLV14F) were tested for their gene expression correlation response.

(Isolate EKZ10B fell out of the experiment). Target genome loci were *aflR* and *aflD* (Table 2) as part of the aflatoxin biosynthetic pathway genes. The ß-tubulin was used as the endogenous control in the analyses to normalise the qPCR cycle threshold (Ct) values.

6.2.1.3.1 Generation of RNA

For generation of the genomic RNA with response to antioxidant, the spore suspensions from isolates ELG33C and MLV14F were plated on cellophane discs immersed on YES agar fortified with Se. YES was used as it induces AF production. The antioxidant was introduced as mineral Na_2SeO_3 at concentrations in µg/g YES of 0 (RNA calibrator), 0.40 (equivalent recommended Se daily intake) and 5.0 (elevated concentration). Treatments were incubated in triplicate at 31°C (12 days, dark).

6.2.1.3.2 Isolation and quantification of RNA

Mycelia was aseptically harvested from top of cellophane disc into an RNA- /DNA-ase free (sterile) 1.5 ml screw cap tube containing four sterile 3 mm glass beads. Each tube upon harvest of mycelia was immediately placed into liquid N_2. The frozen tubes were clumped on a sideways beater (model MM400, Retsch, Haan, Germany) and beating done at 30 beats/second for 30 s for the cell disruption. Immediately, 800µl TRI lysis solution (RNA lysis buffer) was added to the tube and inverted three times to suspend all material in the tube. For the rest of the isolation steps, the Zymo Research Kit was used, available at (https://zymoresearch.com, catalogue № R2073), following the RNA extraction protocol for “Tough-to-Lyse Samples.” The vortex was done at max speed of 10 (Genius model VG 3 S000, IKA, Staufen, Germany) for 15 s. Centrifuge was done at 16,000 *g* (centrifuge model 5424, Eppendorf, Hamburg, Germany) at room temperature. Extracted RNA was eluted from column with 100 µl sterile DNase/RNase-free Milli-Q water. The RNA was stored at -80°C for long term storage or -20°C for short term storage for downstream analysis.

Purified total RNA concentration was measured using the Qubit fluorometer (Invitrogen, MA, USA) using the RNA BR Assay Kit (ThermoFisher Scientific) (see Supplemental procedure 1). The RNA quality was analysed directly on the nanodrop (model 2000, ThermoScientific, Wilmington, DE, USA) using 1.0 µl extracted RNA per sample (A260:A280 ratios: ELG33C = 1.91 ± 0.07; MLV14F = 1.67 ± 0.00). Furthermore, the RNA integrity was checked with 1% gel electrophoresis, mixing 8 µl

RNA with 2 µl loading dye. Two clear bands were obtained indicating the ribosomal material had not degraded.

6.2.1.3.3 cDNA synthesis (Reverse-transcriptase PCR)

The complementary DNA (cDNA) was synthesised using the SensiFast cDNA synthesis kit (SensiFast®) in a 20 µl reaction mixture as follows, working on ice throughout: Added 15 µL (~ 500 ng ELG33C; ~ 200 ng MLV14F;) of mRNA template to a 0.5 µL PCR tube. Next, 4 µL 5X TransAmp buffer was added per 0.5 PCR tube followed by addition of 1 µL enzyme Reverse Transcriptase. Contents were gently mixed by up-down pipetting. The cDNA was synthesised from the mRNA in a Thermal cycler (T100™, Biorad Laboratories Inc., CA, USA) under the following conditions: Primer annealing, 25°C x 10 min; Reverse transcription, 42°C x 15 min; Optional step (in case of highly structured RNA), 48°C x 15 min; Inactivation, 85°C x 5 min. The synthesised cDNA concentration was then extrapolated from the RNA concentration prior to the cDNA synthesis. The cDNA concentration was then diluted to 3.5 ng/µl.

6.2.1.3.4 Quantitative PCR (Real-time PCR)

Real-time PCR *(*qPCR*)* - was carried out in a Real-Time Thermal Cycler (model CFX96™, Biorad Laboratories Inc., CA, USA) with the iQ SYBR Green Supermix kit. For test samples, the qPCR was carried out in triplicate in total reaction volume of 10 µl per sample in a MicroAmp optical 96-well reaction plates, sealed with optical adhesive covers (Applied Biosystems). Each reaction was performed using 5 µl of 2X iQ SYBR Green supermix, 2 µl of 2µM reverse primer (400 nM), 2 µl of 2 µM forward primer (400 nM), and 1 µl of cDNA template of 3.5 ng/µl. For primer efficiency check, DNA from isolate EKA03C, already verified positive for *aflR* and *aflD*, was used, serial diluted to 3.50 ng/µl, 1.75 ng/µl and 0.875 ng/µl. Negative control with sterile Milli-Q water in place of DNA was used. The thermal cycling conditions were executed with an initial denaturing step of 95°C x 2 min followed by 40 cycles of 95°C x 15 s (denature), 55°C x 20 s (anneal), 72°C x 20 s (elongate). Melting curve analysis of the PCR product was performed by ramp heating from 50°C to 95°C in steps of 0.5°C per increment, continuously measuring the fluorescence. Each ramp per increment was held at 20 s, and Ct values acquired from instrument. The housekeeping gene β-Tubulin was used as the endogenous expression control against which normalisation of Ct values for *aflR* and *aflD* was done.

Table 2. Primers used in quantitative PCR assays for determination of gene expression in *aflD* and *aflR*.

Gene	Primer name	Primer pair Nucleotide sequence	Position *	GenBank accession №	Reference
aflR	AflR taq1	(F) - TCG TCC TTA TCG TTC TCA AGG	1646	AF441435.2	(A Medina *et al.*, 2015)
	AflR taq2	(R) - ACT GTT GCT ACA GCT GCC ACT	1735		
aflD	Nor taq1	(F) - GTC CAA GCA ACA GGC CAA GT	516	XM_002379908.1	(Abdel-Hadi *et al.*, 2010)
	Nor taq2	(R) - TCG TGC ATG TTG GTG ATG GT	562		
ß-tubulin	Ben taq 1	(F) - CTT GTT GAC CAG GTT GTC GAT	65	AF036803.1	(A Medina *et al.*, 2015)
	Ben taq 2	(R) - GTC GCA GCC CTC AGC CT	99		

* Positions are according to the published sequences of the above genes of *A. flavus*.

6.2.1.4 Corresponding aflatoxin production with gene expression

Pieces of YES agar under the cellophane on which RNA generation had been done were collected into a pre-weighed test tube. The agar with crashed to paste with clean rod and test tube re-weighed. Clean 5 glass beads (3 mm) were added per tube and 600 µl of 0.05% Triton-X added per tube. Contents were vortexed for 10 s continuously at max speed 10 on a vortex unit (Genius model VG 3 S000, IKA, Staufen, Germany). Next, 3 ml dichloromethane was added per gram agar and the AFs extracted by dichloromethane partitioning as described in Chapter 4.

6.2.2 Fitness response of atoxigenic and toxigenic isolates under antioxidant (Se)

6.2.2.1 Isolates Collection and preparation of inoculation medium

From the retrieved and regenerated isolates of maize and soil (Chapter 4), we selected five toxigenic and five atoxigenic isolates on basis of their high sporulation ability without Se treatment. Spore count was used as the proxy for isolate fitness. CMA amended to 3% w/v sucrose (3gCMA) was used for isolates fitness assays. The 3gCMA was prepared by adding 17 g cornmeal agar per litre demi water according to manufacture specifications. Next, 3% w/v sucrose was added to the medium, configuring the medium to more or less mimic the kernel including its sucrose-rich endosperm. Three levels of Se concentration in the 3gCMA was prepared by fortifying the medium with Se as: 0 µg/g, 0.40 µg/g and 0.86 µg/g. Maximum Se concentration for maize, such that the crop's homeostasis with elements like Zn and Cu are not affected is about 5 mM (0.86 µg/g). Both 0.4 µg/g and 0.86 µg/g selenite salt values

were obtained as corrected values for Se using a multiplication factor of 2.19 for the molecular weight difference between pure selenium (78.96 g/mole) and its salt sodium selenite (172.94 g/mole). The medium was then amended to 50 mg/l chloramphenicol then boiled to dissolved the contents. Medium was dispensed warm and viscous (50°C) to 50 ml glass bottles at 3 ml / bottle. Bottles were autoclaved at 120°C for 15 min.

6.2.2.2 Fungal fitness assay under antioxidant (Se) treatment

Isolate spore suspensions, adjusted to 1 x 10^4 spore/ml, were inoculated in triplicate at 20 µl per sterile 3gCMA bottle with or without Se. The spore suspension was spread on the agar using 3 mm glass beads while shaking bottles sideways by hand. Beads were discarded from bottles after 30 min stand, then bottles incubated at 30°C (7 days, dark).

After incubation, the spores were harvested from the medium with 0.05% Triton-X (surfactant). For the harvest, 3 ml surfactant was added per bottle and contents placed on sideways shaker (GFL model 3018, Society for Laboratory Technology, Burgwedel, Germany) and agitated at 200 rpm per minute for 2 min. Next, 1000 µl spore suspension was transferred by pipettor while swirling to a 1.5 ml microcentrifuge tube. Spore concentration was analysed using an automated cell counter (Casy™ TT, Omni Life Sciences, Bremen, Germany). Briefly 10 µl of the collected spore suspension was added to 10 ml of Casy Ton dilution solution and contents gently inverted 10 times, avoiding build-up of bubbles. Immediately, the count was done on the unit. If spore count was below 200 counts detected, 100 µl spore suspension was used in the dilution in place of 10 µl.

6.2.3 Data analysis

To determine the degradation of AF with or without antioxidant treatment, the pairwise Wilcoxon Rank Sum Exact Test was used on the treatments. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method as described by (Livak & Schmittgen, 2001). For gene expression we considered either the upregulation or downregulation of *aflR* and *aflD* by relative number of times the expression increased or reduced in relation to calibrator (0 Se treatment). The simultaneous upregulation or downregulation of *aflD* and *aflR* was tested by Spearman rank correlation rho.

Fungal fitness was determined as spore concentration and analysed across treatments by Wilcoxon rank sum exact test, significant if $P < 0.05$. Mean spore counts between atoxigenic and toxigenic isolates was determined by geometric mean. Data computations were executed in software R (R_Core_Team, 2013) version 4.1.0. Visualisations were computed with aid of the R package ggplot2 (Wickham, 2016).

6.3 Results

6.3.1 Determination of antioxidative degradation of aflatoxin

Without antioxidant, the results on the reduction/change in AF (B1 and G1) are shown across the variables “Afla,” “Spp” and “Spp+Afla” (Figure 2), summarised in Table 3A. With antioxidant, the reduction/change in B1 or G1 is shown across the variables “Spp+Afla+AntiOx” and “Spp+AntiOx” (Figure 2), summarised in Table 3B. “Afla,” is the extrinsically introduced B1 or G1, while “Spp” is the toxigenic or atoxigenic *Flavi*. “AntiOx” is the antioxidant selenium (Se).

6.3.1.1 Degradation of aflatoxin in non-aflatoxin producers without antioxidant

There was a significant reduction in added B1 (“Afla”) when exposed to the atoxigenic strains (“Spp+Afla”) (Figure 2A; Table 3A) with all isolates digesting the toxin. The greatest reduction was observed with isolate 1MS7 (60%). There was however, no significant reduction in G1 added. All isolates except 1MS7 did not reduce the added G1. Isolate 1MS7 reduced G1 by 14%.

6.3.1.2 Change in aflatoxin in low-aflatoxin producers without antioxidant

There was a significant reduction in B1 due to exposure of added AF (“Afla”) to low AF producer strains (“Spp+Afla”) (Figure 2 [B]; Table 3A). All isolates reduced the B1. There was no significant overall reduction or change in G1 levels compared to added amount (“Afla”) due to exposure to low AF producer strains (“Spp+Afla”). The levels remained significantly higher than what the isolates produced overall (“Spp” v “ Spp+Afla”, $P = 0.029$). Two isolates reduced G1 with ELV13C having the highest G1 reduction (73%). The two other isolates did not reduce G1. Further, the maximally produced levels of G1 (“Spp” = 146 ng/ml) was exceeded when extrinsic AF (G1 “Afla” = 649 ng/ml) was introduced (G1 “Spp + Afla” = 976 ng/ml).

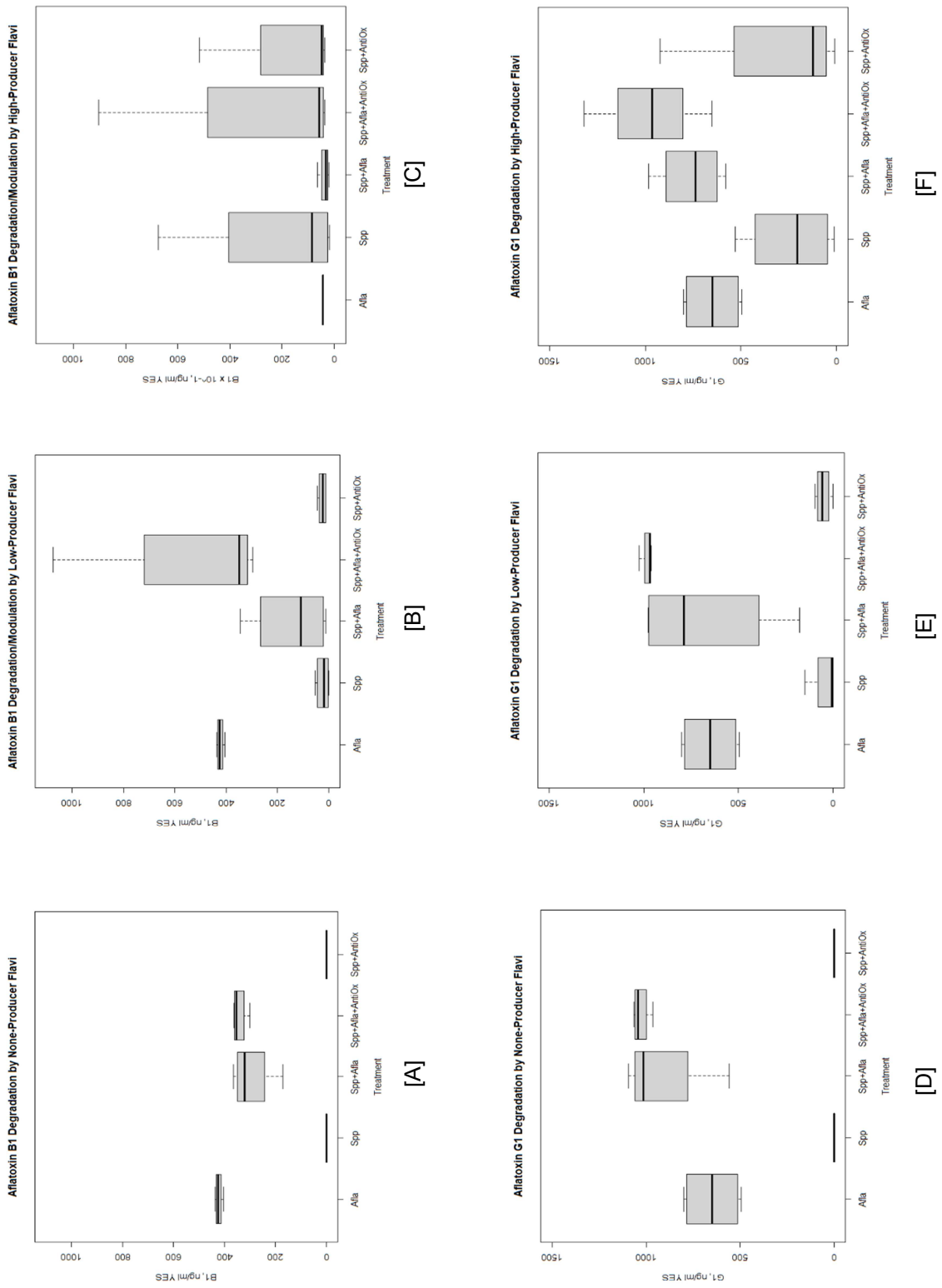

Figure 2. Boxplot showing change in quantity of AFs B1 ([A], [B], [C]) and G1 ([D], [E], [F]) exposed to toxigenic and atoxigenic *Flavi* with or without the antioxidant (Se). [A] and [D] is change in B1 and G1 in none-producers; [B] and [E] is change in B1 and G1 in low-producers; [C] and [F] is change in B1 and G1 in high-producers. Note: The B1 amount on y-axis for high producers **[C] has been scaled to 1x10^{-1} to fit y-axis.**

6.3.1.3 Change in aflatoxin in high-aflatoxin producers without antioxidant

There was no significant change in added B1 ("Afla") due to exposure to high AF producer strains ("Spp+Afla") (Table 3A). However, the B1 maximum levels produced ("Spp + Afla": 633 ng/ml) was modulated to levels closer to added amount ("Afla": 423 ± 14 ng/ml) than maximum levels the species ("Spp") would produce without extrinsic B1 ("Spp": 6750 ng/ml) (Figure 2 [C]). (Modulate implies maintaining the level of the aflatoxin variant to within what the Flavi produces).

With G1, however, significantly higher levels were produced ("Spp+Afla": 758.6 ± 175.7 ng/ml) when extrinsic G1 ("Afla" = 648.7 ± 157.3 ng/ml) was introduced than the species would have produced ("Spp" v "Spp + Afla" P = 0.029; Figure 2 [F]). This is like an augmentation of added amount ("Afla") with amount normally produced ("Spp").

Overall the aflatoxin-producing isolates modulated B1 in their environment by reducing the extrinsically added amount or limiting overall amount to levels added. This was however not the case with G1 which by overall increased (Table 3A).

Table 3A. Aflatoxin digestion in *Flavi* according to levels of B1 and G1, ng/ml (without antioxidant treatment).

Toxigenicity	**AF digestion: [A] "Afla" v "Spp+Afla"**		**[B] Effect on AF max levels if AF not reduced in [A]: ["Spp" v "Spp + Afla"]**	
	B1	**G1**	**B1**	**G1**
None	**Reduced**, P = 0.016	**No reduction**, P = 0.064	NA	NA
Low	**Reduced**, P = 0.029	**No reduction**, P = 0.686	NA	NA
High	**No reduction**, P > 0.05	**Increased** P = 0.029	Max produced **reduced** (6750 to 192)	Max produced **exceeded** (527.6 to 982.8)

Table 3B. Aflatoxin digestion in *Flavi* according to levels of B1 and G1, ng/ml due to antioxidant (Se) treatment.

Toxigenicity	**AF utilisation: [C] "Spp+Afla" v "Spp+Afla+AntiOx"**		**AF suppression: [D] Effect on Se on max levels ["Spp" v "Spp + AntiOx"]**	
	B1	**G1**	**B1**	**G1**
None	Se no effect, **B1 reduction** same P > 0.05	Se no effect, **No G1 reduction** same P > 0.05	NA	NA
Low	**B1 Utilisation reduced** by Se, P = 0.029; (143 to 517)	**G1 non-utilisation** unaffected by Se, P = 0.686; (682 to 980)	No effect, P = 0.886; B1 Max produced **not exceeded** (52.1 to 42.7)	No effect, P = 0.657; G1 Max produced **not exceeded** (146 to 93)

High	**B1 Utilisation reduced** by Se, *P* = 0.029; (360 to 2629)	**G1 non-utilisation** unaffected by Se, *P* = 0.486; (759 to 974)	No effect, *P* > 0.05; B1 Max produced **not exceeded** (6750 to 5168)	No effect, *P* > 0.05; G1 Max produced is **exceeded** (528 to 922)

6.3.1.4 Degradation of aflatoxin in non-aflatoxin producers with antioxidant (Se)

Antioxidant did not lead to significant change in extrinsic B1 or G1 reduction levels. (Wilcoxon rank sum exact test, “Spp” v “Spp+Afla” *P* > 0.05). As expected, treatment of the isolates with antioxidant did not change the AF levels (remained < LOQ) as the isolates were non-producers.

6.3.1.5 Change in aflatoxin in low-aflatoxin producers with antioxidant (Se)

Antioxidant in the presence of extrinsic B1 led to an increase in the levels of B1 (“Spp+Afla+AntiOx” v “Spp+Afla”, Table 3B; Figure 2 [B]). However, when antioxidant was introduced to the species without B1 (“Spp+AntiOx”), the maximum levels of AF the species could produce did not exceed that produced by the species without treatment (“Spp”). With G1, there was no significant difference in levels with or without treatment with Se as long as G1 had been introduced extrinsically, showing ineffectiveness in the overall breakdown of G1 by the “Spp” with or without Se (Table 3B, Figure 2 [E]). There was an observed reduction in maximum levels of G1 produced when “Spp” had been treated with Se (“Spp+AntiOx”) although the overall difference in G1 between “Spp” and “Spp+AntiOx” was not significant.

6.3.1.6 Change in aflatoxin in high-aflatoxin producers with antioxidant (Se)

There was no effect of antioxidant in the presence of extrinsic B1 on overall levels of B1. However, the maximum levels of B1 increased in the presence of Se from 192 ng/ml to 9034 ng/ml, showing a reduction in the modulation of the maximum levels of B1 in the presence of extrinsic B1.

When antioxidant was introduced to the species without B1 (“Spp+AntiOx”), the maximum levels of AF the species could produce did not exceed that produced by the species without treatment (Table 3B, Figure 2 [C]). The overall difference in generated B1 between treatment of species with antioxidant and non-treatment was, however, not significant. With G1, there was no significant difference in levels of G1 with or without treatment with Se as long as G1 had been introduced extrinsically (Figure 2

[F]). This showed that G1 was not effectively broken down by the "Spp" with or without Se. However, there was an observed increase in maximum levels of the G1 produced in the presence of Se, which increased from 982.8 ng/ml to 1320.5 ng/ml. As regards antioxidant treatment on species, there was no significant effect of Se on G1 levels ("Spp" v "Spp+AntiOx", $P > 0.05$).

Considering that high producer strains did not significantly change levels of AF produced due to low antioxidant dose (Se = 0.40 µg/g), we investigated the gene expression patterns of structural gene *aflD* and regulatory gene *aflR* as well as AF response in two selected high AF producer strains (MLV14F and ELG33C).

6.3.1.6.1 Gene expression in aflatoxin pathway genes in high producer isolates due to antioxidant

It was observed from the two randomly tested isolates that in both, the up-regulation of the regulatory gene *aflR* led to the up-regulation of structural gene *aflD* (which is involved in AF precursor molecule decoration) and vice-versa (Figure 3; Spearman correlation: rho = 0.90; *P* value < 0.001).

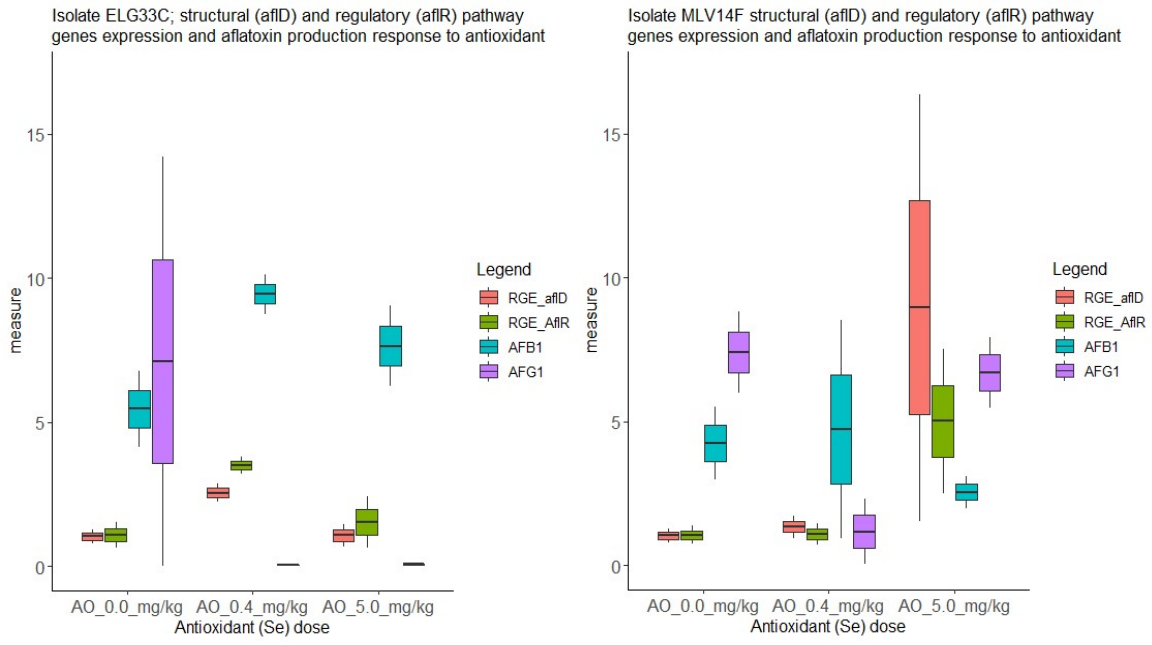

Figure 3. Relative gene expression change at different selenium (antioxidant, Se) treatment levels and change in B1 and G1 production in *Flavi* lab culture isolates. AFB1 = Aflatoxin-B1; AFG1 = aflatoxin-G1; Abbreviations: AO = Antioxidant; RGE = relative gene expression; Se = Selenium.

However, the two isolates responded differently to levels of antioxidant introduced. Isolate MLV14F showed an upregulation of genes at Se dose 0.4 µg/g and a downregulation and at the much higher (8-fold) antioxidant dose. However, the isolate ELG33C upregulated genes at a much higher AO level (8-fold) with no effect at 0.4 µg/g Se dose. There was no clear correlation between gene expression in the two pathway genes and AF levels. However, it was exceptionally observed that B1 in isolate ELG33C, whose gene upregulation responded to lower Se levels, increased with upregulation of *aflR* and *aflD*, and reduced with the downregulation of the two genes.

6.3.2 Fitness of isolates due to antioxidant treatment

Without antioxidant (Se) treatment, both atoxigenic and toxigenic fungi were of equal fitness (Wilcoxon rank sum exact test, P = 0.739). However, a difference in fitness was detected at treatment level at 0.86 µg/g (Figure 4, P = 0.023). The non-producer isolates were fitter than aflatoxin-producing counterparts (geometric mean: atoxigenic = 655 spores/ml, toxigenic = 209 spores/ml).

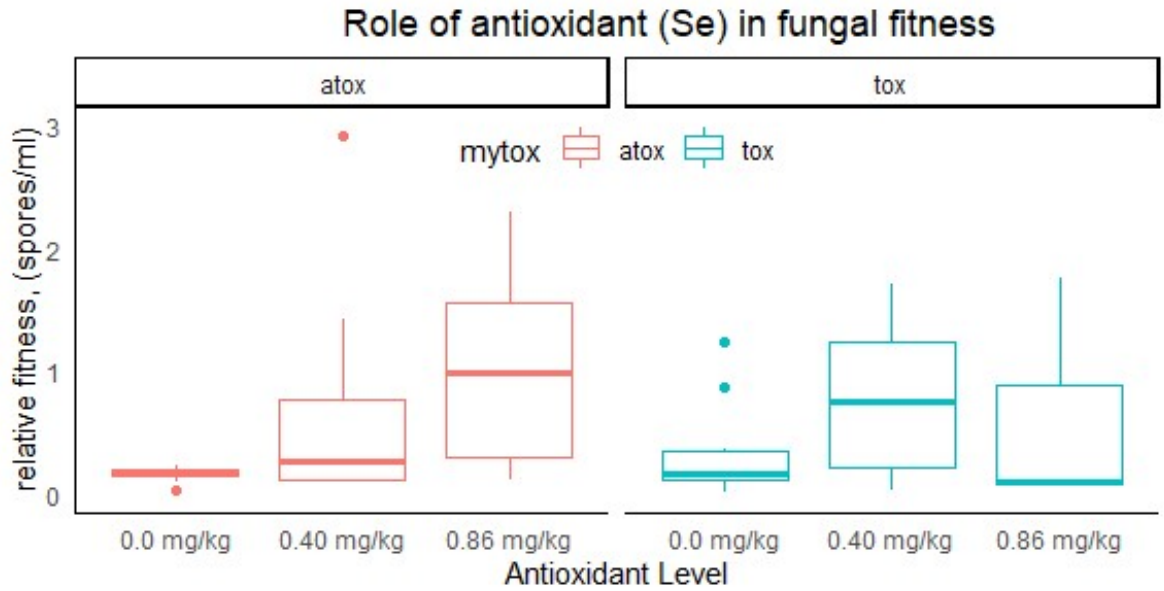

Figure 4. Boxplot of the change in *Flavi* fitness as a result of Se antioxidant treatment levels in mg/kg 3%-sucrose CMA. The difference in the relative fitness between toxigenic (tox) and atoxigenic (atox) *Flavi* at Se treatment level 0.86 mg/kg was significant (P = 0.023).

6.4 Discussion

6.4.1 Degradation of aflatoxin levels in *flavi* through antioxidative mechanism

In the investigated strains, we demonstrate that toxigenic *Flavi* in fact modulate levels of B1 in their environment to within levels they produce it. To do so, they partly degrade the B1 and also maintain the B1 to within a certain level. The modulation is evidenced by the reduction in the added amount of B1 to medium, to levels within what the species produced ("Afla" v "Spp+Afla" in Figure 2 [B], [C]). Furthermore we demonstrate that this is done through an antioxidative mechanism such that B1 plays an antioxidative role. The antioxidative role is evidenced by the increase in levels of AF in the medium in which both AF and selenium (Se) had simultaneously been added compared to AF alone without addition of Se ("Spp+Afla" v "Spp+Afla+AntiOx" in Figure 2 [B], [C]). The mechanism in non-producers may however not be the same as for producers as seen by the no effect of Se on breakdown of extrinsically introduced AF (Figure 2 [A], Table 3B). The observed increase in levels of B1 suggests that the antioxidative role of B1 as a free-radical scavenger was demoted by presence of the antioxidant Se, with Se presumably taking the more active role of the wipe out of oxidative elements. Examples of such oxidative elements includes reactive oxygen species (ROS) as previously described (Doyle & Marth, 1979; Jake C. Fountain *et al.*, 2016; Narasaiah *et al.*, 2006). Our results also show that B1 is the preferred natural antioxidative biochemical in *Flavi* compared to G1. This is evidenced by the fact that the addition of B1 and G1 to medium resulted in the breakdown of B1 for all types of isolates (except MKA01K), whereas G1 was not digested in most isolates (Figure 2 [D], [E], [F]). In this regard, overall, extrinsic introduction of B1 and G1 resulted in higher maximum levels of G1 for low and high producers in contrast to B1 whose levels reduced. The proposed preference of B1 to G1 in the antioxidative AF modulation in the *Flavi* environment may also be indicative of why B1 is usually found in significant proportion of total AF in a number of *Flavi* strains (Cotty & Cardwell, 1999; de Oliveira Rocha *et al.*, 2012; Donner *et al.*, 2009; Kachapulula *et al.*, 2017a; S. Mohale *et al.*, 2013); Supplemental Table 1). B1 is also seen to be produced in higher amounts in a good number of strains studied compared to rest of AF types (Cotty & Cardwell, 1999; Kachapulula *et al.*, 2017a). The modulation of AF levels can also be noted from a previous investigation that while *A. parasiticus*, for example, is a producer of AF in medium, it also simultaneously degrades the toxin through peroxidase enzyme (Doyle & Marth, 1979).

As regards degradation of AF by non-producer strains, in their previous study (Maxwell *et al.*, 2021) showed that atoxigenic strains of *Flavi* digest B1. In our study, we further show that low producers of B1 similarly do digest B1. Furthermore we demonstrate that the mechanism of the digestion may not be the same between atoxigenic and toxigenic *Flavi*. While the low- and high-producers degraded the B1 in an antioxidative mechanism, the non-producers did not seem to degrade it in such a mechanism. This is explained by the fact that a significant increase in B1 is observed for low and high B1 producers when antioxidant (Se) is introduced to the environment alongside extrinsic B1. This is in comparison to introduction of extrinsic B1 without Se. The increase is however not observed for non-producers between the two treatments ("Spp+Afla" v "Spp+Afla+AntiOx", $P > 0.05$; Table 3B). Contrary, Se had no effect of the digestion of the B1 in non-producers which sustained its digestion to similar levels as that without addition of Se (Figure 2 [A]).

From a practical perspective, our findings also suggest the presence of antioxidant in kernel before *Flavi* infection may deter increase in AF levels in the kernel above certain limits. This is in comparison to the introduction of the antioxidant when AF and toxigenic *Flavi* are already present in the kernel (model "Spp" : "Spp+AntiOx"). This would imply that alteration of the abiotic environment of kernel at preharvest by introduction of non-toxic levels of mineral Se antioxidant may be useful in deterring spikes in AF contamination. The approach would be problematic if the introduction of Se is at postharvest such that a product that already has AF is biofortified with Se, and gets contaminated with toxigenic *Flavi*. This would equate the model "Spp" : "Spp+Afla+AntiOx" in our investigation, potentially leading to spike in B1 and G1. Furthermore, our findings may also partly explain why a study on Brazilian nuts showed a higher accumulation of AF in nuts with higher levels of Se than those with lower levels of the antioxidant (Pacheco & Scussel, 2007). Considering that nuts are soil borne, they are likely to get contaminated with aflatoxigenic *Flavi* and subsequently AF in early stages of seed growth. This then renders the AF produced by *Flavi* unutilised in an antioxidative mechanism, due to the Se in the seed, as seen by higher levels of B1 and G1 in our study under the combination "Spp+Afla+AntiOx."

It should be noted that for the complete impediment of *Flavi*, higher levels of the antioxidant Se would have to be administered such that the *Flavi* fitness is reduced through non-sporulation (Asghari-Paskiabi *et al.*, 2019; Hassan *et al.*, 2022) and

subsequently prevent the AF production by the *Flavi*. The increase of the antioxidative capacity of a plant has been suggested previously as means of reducing the oxidative stress in *Flavi* and subsequently AF production (Jake C. Fountain *et al.*, 2016). The antioxidative capacity of a plant by using elements like Se would have to be high enough. This is as shown by our findings that the non-lethal dose (0.86 µg/g) did not deter AF production (on YES medium) in *Flavi*, although it reduced the maximum B1 levels produced. Higher Se antioxidative capacity can be achieved through use of nano particle format of Se. Se nanoparticles in higher levels are less toxic to organism than mineral Se (Bhattacharjee *et al.*, 2019).

6.4.1.1 Gene expression in high aflatoxin B1/G1 producer isolates in response to antioxidant (Se)

From the results of two randomly selected high producer strains, (soil isolate ELG33C; maize isolate MLV14F) we demonstrated that the two biosynthetic genes *aflR* (regulatory) and *aflD* (structural) were consistently either both upregulated or downregulated in response to the abiotic stimulus Se-antioxidant. Furthermore, we observed, a positive correlation in isolate ELG33C between expression levels of both *aflD* and *aflR* and the levels of B1 produced. The consistence was, however, not observed in isolate MLV14F as well as the G1 levels for both isolates (Figure 3).

Although the transcription factor *aflR* and structural gene *aflD* are involved in AF production in *Flavi*, most studies have shown no correlations between the gene expression levels in the two genes with levels of AF produced (Accinelli *et al.*, 2008; Al-Saad *et al.*, 2016; Bernáldez *et al.*, 2017; Rodrigues *et al.*, 2009; Yunes *et al.*, 2020). While some few studies have shown a level of correlation with AF levels produced, the studies have either been inconsistent with each other or the expression inconsistent with expected highest production conditions for AF (Chang *et al.*, 2007; Gallo *et al.*, 2016; Obrian *et al.*, 2007); for example high expression of *aflD* at both lowest B1 levels and lowest *aflR* expression and vice versa (Gallo *et al.*, 2016). This may show that response to stimuli may be strain dependent, for example the observed strain differences in response to CO_2 (Baazeem *et al.*, 2021) or temperature (Yunes *et al.*, 2020). The possible strain dependence is also seen in our study on expression patterns of *aflD* / *aflR* to Se stimulus (Figure 3). In our study, the levels of available mRNA from *aflD* and *aflR* differed from the specific induced levels of Se as antioxidant. Our preliminary findings show that soil isolate ELG33C downregulated the two pathway

genes at elevated control concentration (5 µg/g) after the preliminary upregulation at 0.40 µg/g. On the other hand, maize isolate MLV14F only upregulated the two pathway genes at elevated control concentration of 5 µg/g with no significant change at 0.40 µg/g. This would suggest that the downregulation of the genes in isolate MLV14F may probably occur at a higher Se level than the control elevated concentration of 5 µg/g. A higher isolate sample size in our study would be required in order to further explore the gene-expression-aflatoxin-production correlation with antioxidant. A further factor most investigators would have to consider is the prevention of oxidative elements that could degrade the AFs in the medium in the course of experiment, leading to poor or inconsistent correlations.

6.4.2 Fitness response of atoxigenic and toxigenic isolates under antioxidant (Se) treatment

At maximum non-lethal dose of mineral Se administered (0.86 µg/g), we demonstrate the increase in fitness of the tested atoxigenic strains of *Flavi* compared to toxigenic counterparts, rejecting our initial hypothesis. Different fungi can thrive differently under the same abiotic environment, or indeed under different abiotic environments. Considering the alteration of the abiotic environment for *Flavi* in our study, such a comparative increase in fitness of atoxigenic compared to toxigenic counterparts could be an important attribute in promoting population of toxigenic over atoxigenic strains. This provides the need to further investigate the inhibitory concentration of Se nano particles that would trigger a growth inhibition of toxigenic strains, while at the same time allowing some atoxigenic counterparts to grow without complete inhibition. This is considering that biocontrol of AF premised on competitive exclusion relies on atoxigenic *Flavi* to outgrow toxigenic counterparts and colonise host crop (Alaniz Zanon *et al.*, 2018; Cotty & Bhatnagar, 1994). From a practical perspective, integrating of preharvest Se-biofortification of maize can have prospects to play a useful role as part of a broader AF biocontrol strategy.

6.5 Conclusion

While atoxigenic *Flavi* are known to degrade B1 in their environment and as seen in this study, toxigenic *Flavi* of this investigation modulated B1 in an antioxidative role, by so doing to levels less than what they produce. We further see that B1 happened to be a preferred antioxidative biochemical for the *Flavi* compared to G1. Furthermore, we do observe that atoxigenic *Flavi* used in this study were fitter than toxigenic

counterparts under antioxidant treatment contrary to expectations, but a positive attribute. Hence, work may need to be done on the prospective use of antioxidative elements such as selenium (Se) in nano-particle form, to drive a better fitness of atoxigenic fungi over toxigenic strains. This would be in form of a broader biocontrol strategy in the competitive exclusion of *Flavi*. Furthermore, it should also be noted that when considering the use of antioxidants such as Se in substrate such as maize, consideration should be made on the AF contamination status of the substrate. From a practical perspective, Se biofortification may preferably be done at preharvest on maize, or if at post-harvest when product is without *Flavi* and AF contamination. This is because, while Se could deter potential spike in B1 and G1 as observed in this study, its application to environment already harbouring B1 and *Flavi* can conversely lead to spike in the dreaded B1 when conditions are conducive for *Flavi* to proliferate.

6.6 Supplemental materials

Supplemental Table 1. Percentage (%) aflatoxin producing ability of isolates

Isolate	Clade	Toxigenicity	G2	G1	B2	B1
EKW40A†	*parasiticus*	High	0.3	28.2	0.0	71.5
EKW36B	*parasiticus*	High	2.1	64.3	0.9	32.7
MKA01K†	*flavus*	High	0.6	19.6	0.0	79.9
ELG33C†	*parasiticus*	High	2.1	0.1	12.9	84.9
		Mean	**1.3**	**28.0**	**3.5**	**67.2**
EKZ10A	*parasiticus*	Low	2.2	72.2	0.0	25.7
MKZ06B†	*minisclerotigenes*	Low	0.0	0.0	0.0	100.0
ELV13C	*parasiticus*	Low	0.0	71.3	0.0	28.7
		Mean	**0.7**	**47.8**	**0.0**	**51.4**

† Depicts isolates that produced more B1 than G1. A significant proportion of total aflatoxin is B1 with 4 out of the 7 isolates producing B1 > 50%.

Supplemental procedure 1.

Procedure for quantification of isolated RNA by Qubit fluorometer

The Qubit working solution was prepared by adding, per sample equivalent, 1 µl of RNA reagent to 199 µl Qubit buffer, and vortexed. Next, the lower calibration standard was prepared by adding 10 µL of Qubit standard number #1 to 190 µL of Qubit working solution into a 2 ml microcentrifuge tube and vortexed. The standard was inserted into the fluorometer (Qubit, Invitrogen, MA, USA) after exactly 2 min and read. The same calibration procedure was then repeated but with high calibration standard #2. Calibration was done using programme “RNA BR.”

RNA sample concentrations were analysed on the calibrated fluorometer by adding 1 µL sample to 199 µL Qubit Working Solution, followed by a vortex and a 2 min wait. Sample was read immediately after elapse of wait time.

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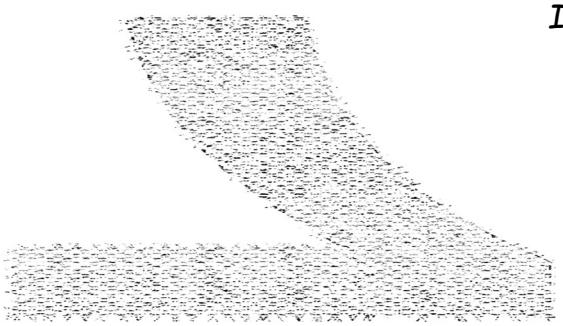
7

"Laughter is brightest
in the place where
food is good " –

Irish proverb



Chapter VII

General discussion

7.0 Introduction

In this thesis, I explored the eco-dynamics of fungi infecting preharvest maize, from the perspective that maize is an ecological niche in which they thrive. I investigated biotic (fungal correlations) and abiotic (rainfall, antioxidant) factors influencing the fungal contamination with special attention to *Aspergillus* and its aflatoxin. In addition, I investigated *Fusarium* and its fumonisin-B1. Some species belonging to the genus *Aspergillus* are known to produce aflatoxins (AFs), which are mycotoxins of specific concern. Similarly, some species of *Fusarium* are known to produce Fumonisins (FBs).

For this general discussion, I focus on two main discussion points that emerged from my thesis work:

1) the spectrum of fungi naturally thriving on maize as their ecological niche with a focus on *Sarocladium* and *Fusarium*, and the prospect for control of *Fusarium's* FB1 in maize.

2) The role of antioxidant in toxigenic and atoxigenic *Aspergilli* section *Flavi* (*Flavi* for short) and the prospects for control of aflatoxin using antioxidant.

In light of these two discussion points, as illustrated in Figure 1 under 'Prospects,' the main findings of my thesis work are summarized in Table 1 below. The two discussion points are elaborated in section 7.1.1 and 7.1.2.

Table 1 Specific objectives of the thesis outline including the hypotheses/predictions

Objective	Finding	Hypothesis and Prediction
a) To describe the spectrum of fungi infecting maize and the effect of rainfall patterns on the fungal microbiome	- *Fusarium* and *Sarocladium* were the most prevalent fungi in Zambian preharvest maize and their relative abundance was not influenced by rainfall pattern. - Although largely stable, extreme rainfall pattern (dry conditions) altered the composition of the preharvest maize mycobiome.	- The prediction that the structure of the preharvest mycobiome is largely composed of the commonly reported *Fusarium* and *Stenocarpella* in Zambia was not true. On the contrary it was largely composed of *Fusarium* and *Sarocladium*. - The hypothesis that rainfall pattern influences the preharvest maize mycobiome composition was not rejected.
b) Elucidate evidence of influence of niche partitioning on fungal genera abundances and subsequently FB and AF in maize	- *Fusarium* was negatively correlated with *Sarocladium* irrespective of weather pattern. - Higher levels of *Sarocladium* resonated with lower levels of FB1	- *The* prediction that fungi with negative correlation with *Fusarium* or *Aspergillus* irrespective of weather pattern exist was true. - The hypothesis that fungal genera with a negative correlation with *Fusarium* (or *Aspergillus*) lead to lower levels of FB1 (or AF), when *Fusarium* (or *Aspergillus*) are in low abundance, was not rejected.
c) To investigate the risk for natural infection of preharvest maize with *Flavi* and AF	- Although *Flavi* were always detected in soil (*Flavi* reservoir), loose husk-cover condition of the maize ear did not lead to *Flavi* ingress in maize ears. Rather dry weather condition (dry spell) was likely predisposing for the ear infection with *Flavi* and AF. - There was a high selection frequency for aflatoxigenicity in maize (83 to 97%).	- The hypothesis that loose-husk does not lead to higher infection of preharvest maize with *Flavi* was not rejected - The prediction that *Flavi* from the drier agroecological zone 1 in Zambia, have a higher selection for aflatoxigenicity than those from the wetter AEZ 3 was not true.

(*Table 1 cont'd to next page*)

Objective	Finding	Hypothesis and Prediction
d) To investigate the *Flavi* community structure between soil and maize, and how maize *Flavi* diversity may influence aflatoxin in maize.	- Soil *Flavi* community was different from maize, and was largely dominated by A. *parasiticus*. On the contrary, the maize *Flavi* community was more diverse and largely dominated by *A. parasiticus* and *A. minisclerotigenes*. - The two most aflatoxigenic species detected on maize namely *A. minisclerotigenes* and *A. parasiticus* drove the contamination levels of maize with AFs.	- The prediction that the *Flavi* community structure between maize and soil is different was true. However, on the contrary, maize had a higher species diversity than the soil. - The hypothesis that the diversity of aflatoxigenic *Flavi* in Zambia influences the AF levels in preharvest maize was not rejected.
e) Determine the role in which aflatoxin is utilised in toxigenic and atoxigenic *Flavi*, and the effect of antioxidant on fitness of atoxigenic and atoxigenic *Flavi*.	- Toxigenic isolates utilise B1 through an antioxidative mechanism, but not the atoxigenic isolates. - Atoxigenic isolates were fitter than toxigenic isolates in presence of the antioxidant selenium.	- The hypothesis that atoxigenic *Flavi* degrade AF in an antioxidative mechanism was rejected. - The prediction that toxigenic *Flavi* are fitter than atoxigenic counterparts in presence of an antioxidant was not true.

(*Table 1 cont'd*)

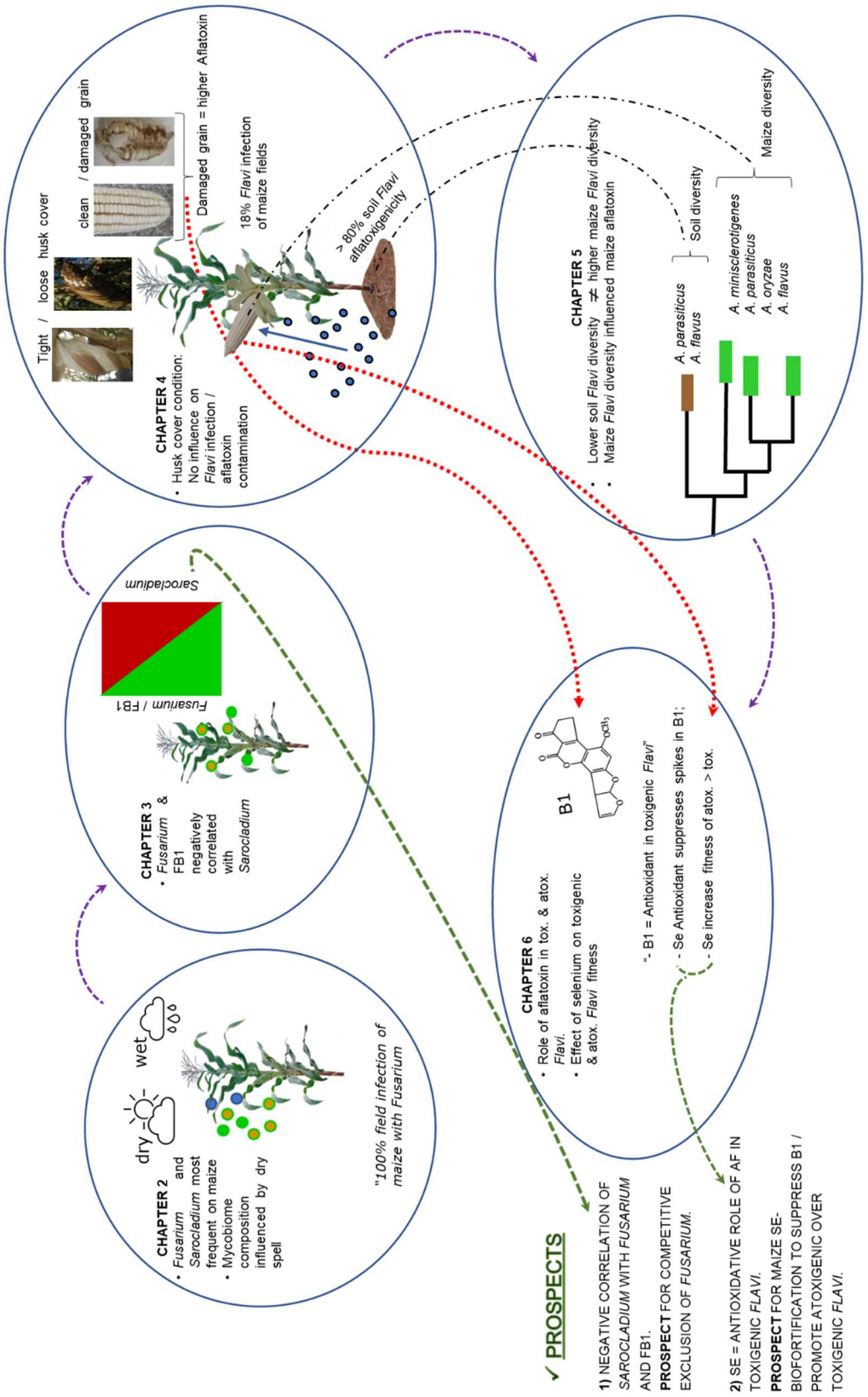


Figure 1. Overview of the findings laying down the two main discussion points, on prospects of competitive exclusion of *Fusarium* and the antioxidative suppression of AF and toxigenic *Flavi*. The prospects are elaborated in the discussion section of this chapter.

Abbreviations: Atox – non-aflatoxin producing *Flavi*; B1 – Aflatoxin-B1; FB1 – Fumonisin-B1; Tox – Aflatoxin producing *Flavi*.

7.1 Discussion and recommendations

7.1.1 Contamination dynamics of fungi infecting maize and influence on mycotoxins

Fungi contaminating maize are ubiquitous in nature and grow as diverse communities on the maize host. Fungal microbiome composition can be dictated by abiotic factors such as rainfall, temperature, carbon source of host, *et cetera* (Arroyo *et al.*, 2008; Giorni *et al.*, 2009; Medina *et al.*, 2017; Mellon *et al.*, 2000; Mellon *et al.*, 2005; Sejakhosi Mohale *et al.*, 2013; Perrone *et al.*, 2020; Samsudin *et al.*, 2016). In my thesis, I detected a diverse fungal community on preharvest Zambian maize comprising 61 fungal-genera (Chapter 2). The mycobiome was dominated by *Fusarium* and *Sarocladium* by ASV relative abundance representation and field frequency of infection. This was irrespective of the prevailing rainfall pattern. The high frequency of *Fusarium* on the maize mycobiome (100% frequency of field detection, 45% Amplicon Sequence Variant, ASV, relative abundance representation), may explain the observed perpetual contamination of the maize with FBs, a mycotoxin mainly produced by some species belonging to the genus *Fusarium*. This is also demonstrated by the positive correlation between levels of *Fusarium* and FB1 (Chapter 3). FB is a perpetual contaminant of preharvest maize in Zambia (Chapter 3; (Schjoth *et al.*, 2009)) as well as other regions (Akello *et al.*, 2021; Chen *et al.*, 2018; WHO, 2018c). It should be noted that *Sarocladium* is a fungus that has not been reported in regional studies of Southern Africa including previous Zambian studies. Considering the high ASV relative abundance of *Fusarium* and *Sarocladium*, these were the fungi of interest in this investigation with respect to FB in the maize. To better understand and explore this relation between *Sarocladium* and *Fusarium*, their correlation with respect to their relative abundances across fields was investigated, revealing a strong negative correlation between the two fungi. Furthermore, higher levels of *Sarocladium* were associated with lower levels of FB1 (Chapter 3).

I attribute the high frequency of detection of *Fusarium* and *Sarocladium* on the preharvest maize to it being their ecological niche, as also seen by their high ASV relative abundance representation (Chapter 3). This is consistent with their relative abundance not being influenced by prevailing weather conditions (Chapter 2). However, we also reported a strong negative correlation between the abundance of both species (Chapter 3). Under good niche partitioning, fungi will successfully coexist

through utilisation of different niche resources (Chesson, 2000). In contrast, under poor niche partitioning, competitive exclusion arises with the possible outcome, one species or genus excluding the other. As observed in this study, the strong negative correlation between *Fusarium* and *Sarocladium* could be seen as a signature for poor niche partitioning between the two genera. The strong negative correlation between these two fungi offers prospects for use of *Sarocladium* in the competitive exclusion of *Fusarium*. To further support this, one of the key prerequisites for a genus or species to be deployed as a biocontrol agent is that the competing microorganism (fungus in this study) should be able to effectively and widely populate the crop and thrive in the environment in which the target pathogen thrives. This was indeed observed for *Sarocladium* on maize kernels also populated by *Fusarium*. Moreover, higher levels of *Sarocladium* were associated with lower levels of FB1 (Chapter 3).

7.1.1.1 Prospects for competitive exclusion of *Fusarium* for the combat of fumonisins (FBs)

The importance of using such a prospective genus as *Sarocladium* for the competitive exclusion of *Fusarium* for the combat of FBs is that *Sarocladium* is largely a rice pathogen (Sakthivel *et al.*, 2002). Furthermore, the secondary metabolites beauvericin and enniatin-B1 that *Sarocladium* produces (Blaszczyk *et al.*, 2021) are currently of no or little concern in the food safety landscape compared to FBs. However, it should be noted that some species of the clade *Sarocladium* may be human opportunistic pathogens (Perez-Cantero & Guarro, 2020; Tabliago *et al.*, 2022). This hence warrants the need for further investigations into such strains of *Sarocladium*, non-pathogenic to both maize and human, for purposes of competitive exclusion of *Fusarium* for the biocontrol of FBs in maize. For example, *Sarocladium zeae* has recently been described for the biocontrol of the mycotoxin deoxynivalenol in wheat as it is able to naturally contaminated the crop and compete against *Fusarium* (Kemp *et al.*, 2020). In a similar approach, the ubiquitous soil dweller *Trichoderma* (Bacon *et al.*, 2001; Egidi *et al.*, 2019), has been used as the prospective fungus for the biocontrol of *Fusarium spp*. and its trichothecenes (Błaszczyk *et al.*, 2017; Busko *et al.*, 2008; Filizola *et al.*, 2019; Tian *et al.*, 2016). Although *Trichoderma* has been demonstrated as a candidate antagonist in the biocontrol of *Fusarium* in wheat (Filizola *et al.*, 2019; Juan Palazzini *et al.*, 2018), its poor maize colonisation in Zambia's climatic environment was visible by its absence on the maize mycobiome (Chapter 2). This finding is further supported

by the results of a previous study of maize in which *Trichoderma* was detected in only one out of 11 districts of Zambia (Mukanga *et al.*, 2010). This demonstrates that *Trichoderma*, although a ubiquitous soil dweller, may not be the most prospective candidate in the competitive exclusion of maize *Fusarium*, compared to *Sarocladium*, for instance. Although experimental successes have been scored in preventing *Fusarium* proliferation by use of Biocontrol Agents (BCA) such as *Bacillus subtilis* (Bacon *et al.*, 2001; Khan *et al.*, 2018; Palazzini *et al.*, 2016), this has not translated into the successful use of *Fusarium* BCA at commercial level (Kagot *et al.*, 2019), hence the need for other prospective fungi.

With respect to *Aspergillus*, I demonstrated that, unlike *Fusarium,* the abundance of *Aspergillus* in the preharvest mycobiome was rainfall pattern dependent. It had a stronger inclination of grain colonisation towards the southerly low rainfall AEZ1 with frequency range of detection 40 to 100%, whereas for the northerly high rainfall AEZ3, the frequency of field detection was 0 to 40% (Chapter 2). Of all the genera identified, it was demonstrated that *Hirsutella* had a negative correlation with *Aspergillus* (Chapter 3). From a practical perspective, this suggests that *Hirsutella* could be used for competitive exclusion of *Aspergillus*. However the correlation between *Hirsutella* abundance and AF levels in corresponding field maize samples could not be ascertained, assuming that higher abundances of *Hirsutella* would correspond with lower levels of AF. This was due to the low number of the samples that were positive for AF above quantitation limit under field conditions (n = 11/80). The advantage in the prospective use of *Hirsutella* in competitive exclusion of *Aspergillus* would be that as *Hirsutella* is unrelated to *Aspergillus*, this abates fears of the potential risk of genetic recombination between toxigenic and atoxigenic *Flavi*, such that more virulent strains are generated (Alberts *et al.*, 2017). This is considering that the current biocontrol of *Aspergillus* uses atoxigenic strain as BCA to outcompete the toxigenic counterparts (Abbas, 2005; Atehnkeng *et al.*, 2022; Cotty, 2006; Cotty & Bhatnagar, 1994; Dorner JW *et al.*, 1999) which may be envisaged as a potential platform for genetic recombination between atoxigenic and toxigenic strains of the *Aspergilli* (Damann Jr, 2015; K. Ehrlich *et al.*, 2015; Horn *et al.*, 2009; Moore, 2015; Olarte *et al.*, 2012; Varga *et al.*, 2014). However, previous studies have counter-argued that chances of such genetic recombination in natural ecosystems are very low (Grubisha & Cotty, 2015) and that chances of creating more virulent strains are very minimal (Bandyopadhyay

et al., 2016). Furthermore, *Hirsutella* is non-pathogenic to maize, has a restricted host range and appears harmless to non-target organisms. However, some early warning limitations observed in this study for the strong candidature of *Hirsutella* in the competitive exclusion of *Aspergillus* was its weak kernel infection as observed by its strong reduction in its abundance from external to internal mycobiome (Chapter 3), coupled with the lack of an overall strong negative correlation with *Aspergillus* (< -0.25). The additional limitation is that *Hirsutella* can suffer short shelf life as a biopesticide (Reddy *et al.*, 2020) should it be used as BCA for the competitive exclusion of *Aspergillus*. Considering that I could not ascertain presence of any genera including *Hirsutella* with negative effect on levels of AFs, I explored within *Aspergillus* additional prospects for reduction of AF contamination in maize. This is also considering that the correlation between *Hirsutella* and *Aspergillus* was not very strong and that the relative abundance of *Hirsutella* from external to internal mycobiome significantly reduced (Chapter 3). I initially explored the diversity of *Flavi* and understand how this may influence AF levels in maize. I furthermore, explored the mechanism aggravating the production of AF by *Flavi* and how its production could subsequently be countered.

7.1.2 Diversity of *Aspergillus* section *Flavi* (*Flavi*) and aflatoxin production in relation with to oxidative stress

Beyond the genus level of *Aspergillus* identification, the *Flavi* are a diverse community of fungi which include species known to produce aflatoxins (AFs). In this study, we detected both toxigenic (aflatoxin producers) and atoxigenic (non-aflatoxin producers) *Flavi*. Furthermore, we detected a higher diversity of *Flavi* members than previous country studies (Kachapulula *et al.*, 2017b; Mukanga *et al.*, 2010) such as the previously unreported notoriously aflatoxigenic *A. minisclerotigenes* (Chapter 5). A higher selection frequency for toxigenicity was detected compared to atoxigenicity, with soil members having a higher selection for toxigenicity (> 80% frequency) than maize isolates. Atoxigenic isolates are a source of Biocontrol Agents (BCA) for the competitive exclusion of toxigenic *Flavi* to combat AFs in crops like maize, cotton and groundnuts (Bock & Cotty, 1999; Medina *et al.*, 2017; Raksha Rao *et al.*, 2020). The approach relies on broadcasting the atoxigenic biocontrol agent (BCA) to the soil, allowing the BCA to readily sporulate and subsequently infect the maize through silk (Bandyopadhyay *et al.*, 2016; Cotty & Bhatnagar, 1994; Senghor *et al.*, 2020). This then leads to the infection of the maize with the non-aflatoxigenic biocontrol strains. It

should also be noted that the infection of *Flavi* in maize will also depend on climatic conditions. In this study, I showed that the drier AEZ1 of Zambia was more prone to *Flavi* infection of maize in field compared to the wetter AEZ3 over the two sampled seasons (Chapter 4). A similar propensity is seen in the drier areas of the US South Texas prone to AF contamination (Jaime-Garcia & Cotty, 2010). Considering the importance of dry conditions on *Flavi* infection of maize ears (Chapter 4), it should be noted that competitive exclusion of *Flavi* for the biocontrol of AF can suffer setbacks under very dry conditions (Bandyopadhyay *et al.*, 2016). This is due to failure of the biocontrol agent broadcast to soil to sporulate as the biocontrol mechanism depends on moisture for the atoxigenic strains immobilised on nutrient matrix to sporulate and infect the crop. This therefore calls for the integration of additional measures aiding the success in competition of atoxigenic strains against toxigenic counterparts, or better still utilising such measures that would suppress AF accumulation in crop by *Flavi* altogether.

Aflatoxin has been linked to, among evolutionary roles, a response to oxidative stress in *Flavi* (J. C. Fountain *et al.*, 2016; Jayashree & Subramanyam, 2000; Narasaiah *et al.*, 2006). In this case, the aflatoxin produced by the *Flavi* has been suggested to aid the wipe out of reactive oxygen species in the cells to alleviate the oxidative stress. My thesis research suggests that altering the abiotic conditions of the seed by introducing an antioxidative element (e.g. through biofortification) could be such suppressive measure of AF production in *Flavi*. However, the preliminary risk would also be in that the suppression of AF production in the toxigenic strains would increase their fitness against atoxigenic counterparts. This is because toxigenic *Flavi* spend energy and resources on producing AFs. This signifies that the addition of an antioxidant that reduces AF production should therefore enable wildtype toxigenic *Flavi* to expend the energy to other needs, including spore production.

7.1.2.1 Prospect of antioxidant biofortification of maize to control aflatoxin

Due to niche partitioning, driven by abiotic environment, the alteration of an abiotic environment can promote the abundance of one species over another. In my thesis, I explored this by promoting a higher fitness of the atoxigenic over the toxigenic *Flavi* by utilising an antioxidant on the *Flavi* in medium. In the investigation, it became clear in Chapter 6 that B1, but not G1, is utilised by toxigenic isolates in an antioxidative

mechanism. On the contrary, the atoxigenic strains, which are known to digest AF, do not utilise B1 in such a role. In addition, both toxigenic and atoxigenic *Flavi* did not utilise G1. It should be noted that of the AF variants in crop (B1, B2, G1 and G2), B1 is the most carcinogenic and most feared of the AF variants (IARC, 2012; Li *et al.*, 2022; Rushing & Selim, 2019). Alteration of the abiotic environment by increasing the antioxidative capacity of medium in which *Flavi* is present, could have the potential to reduce the B1 production by *Flavi*. This is considering that in *Flavi*, AF has been demonstrated to alleviate oxidative stress, such that introducing an exogenous antioxidant would render the function of AF redundant, making the production of the AF not needed in the *Flavi*. The antioxidant used in my thesis was selenium (Se), which in non-lethal dose for plants could also trigger an increase in fungal fitness in terms of sporulation. For example, while antioxidant is a source of oxidative stress alleviation, the first inducement of the antioxidant (Se at 0.4 µg/g) lead to an increase in fitness of both toxigenic and atoxigenic *Flavi* (Figure 4 in Chapter 6), suggesting a link of the stress-relieving Se to better the sporulation of such wildtype *Flavi* used in the investigation. Nonetheless, although I hypothesised that toxigenic strains would be fitter than atoxigenic strains in presence of Se as antioxidant, the hypothesis was rejected as, rather, atoxigenic isolates were eventually significantly fitter (Se at 0.86 µg/g) than the toxigenic counterparts. I attribute my findings in the fitness difference between toxigenic and atoxigenic isolates to the fact that in the toxigenic isolates, the Se (a micronutrient and antioxidant) may have been channelled towards oxidative stress alleviation and not fully towards the trigger of higher spore production. In contrast, the atoxigenic isolates may have channelled the Se towards triggering higher spore production without channelling it for oxidative stress alleviation. Such would, hence, result in a higher spore production in atoxigenic than toxigenic *Flavi* as illustrated in Figure 2. This scenario was confirmed by the observation that the toxigenic isolates significantly reduced the utilisation of extrinsically introduced AF in the presence of Se, indicating that Se was also used in an antioxidative role. In contrast, addition of Se and AF to medium of the atoxigenic showed that, the Se had no effect on the breakdown of AF, indicating that Se triggered higher spore production without being co-utilised as an antioxidant. From a practical perspective, it shows that an element such as Se can potentially be used to alter the population dynamics in favour or atoxigenic strains over toxigenic counterparts, and thus suppress B1 production in toxigenic *Flavi*. This would be a useful augmentation to the current

biocontrol efforts based on competitive exclusion. It could for instance be in the form of Se-biofortification of the maize, increasing the antioxidant capacity of the maize seed.

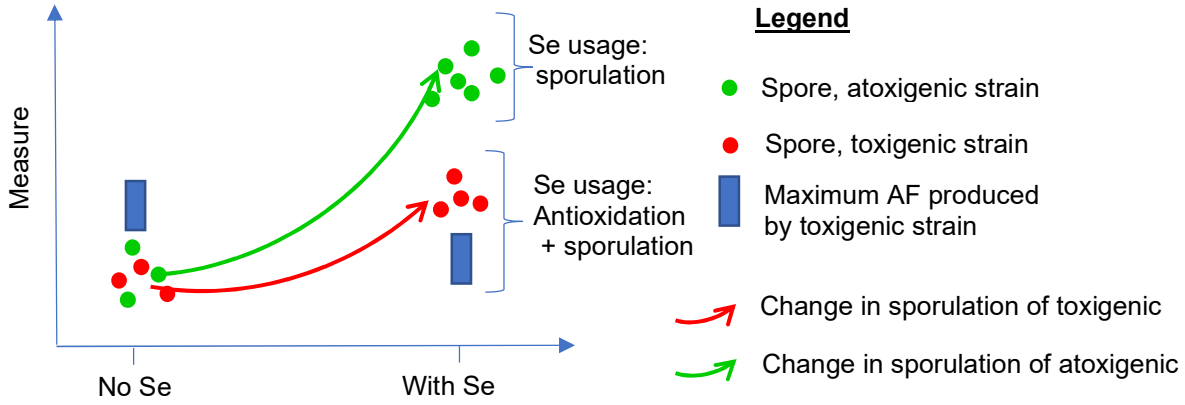


Figure 2. Illustration of the idealistic channelling of selenium (Se) as antioxidant in wildtype *Flavi* influencing levels of aflatoxin and fitness of the fungi.

However, presence of the antioxidant Se and AF in an environment of active *Flavi* would lead to an increase in AF levels as observed in our experimental scenario (Chapter 6). This is due to the non-utilisation of the B1 as a form of an antioxidative biochemical but rather the utilisation of Se as the preferred antioxidant. This would be translated as a spike in B1 levels due to the antioxidant (Se). This could occur for example when grains that contains AF and active *Flavi* is biofortified with the antioxidant at post-harvest. Therefore, enrolment of Se-biofortification to reduce B1 spikes while promoting atoxigenic over toxigenic *Flavi* would have to be done preharvest and not post-harvest. For the complete inhibition of both AF production and the sporulation of *Flavi* on kernels, the use of selenium nanoparticles (SeNPs) is suggested rather than mineral Se. This is recommended as higher levels of Se could stop AF production by *Flavi* as well as their sporulation altogether (Asghari-Paskiabi *et al.*, 2019; Hassan *et al.*, 2022), but high Se levels would be toxic to the plant (Kaur *et al.*, 2022; Naseem *et al.*, 2021). SeNPs at the same concentration level of mineral selenium are several-fold more effective than mineral Se (Bhattacharjee *et al.*, 2019). Preharvest biofortification is therefore suggested without the risk of spikes in levels of AF while increasing the micronutrient value of the maize grain with Se.

7.2 Concluding remarks

My thesis has provided insights into the fungal contamination dynamics in maize revealing a diverse fungal genera conglomerate from which genera such as *Sarocladium* could be tapped into for the prospective competitive exclusion of *Fusarium* in maize for the combat of FBs. Further *in vitro* investigations would be needed for the identification of non-pathogenic strains of *Sarocladium* for this purpose. Considering that the abiotic environment of the maize grain can be altered through biofortification with an antioxidant such as Se, this study provides cues on how Se fortification could potentially influence a better fitness of atoxigenic *Flavi* over toxigenic counterparts, or prevent spikes in AF at preharvest. Further investigations with additional toxigenic and atoxigenic strains would be needed to create further evidence on change in fungal fitness in further of atoxigenic *Flavi*.

Finally, it should be noted that the part of the investigation involving field maize fungal contamination dynamics was carried out in only one region of the world - Zambia, which has a tropical climate. It could not be carried out simultaneously in different climatic regions, but this is highly recommended as the results of the present work may not be directly applicable to other climatic regions. In addition, since only two growth seasons were included, the study was limited to the influence of weather variables and not climate as a whole. However, considering the ubiquity of *Fusarium* and *Aspergillus*, the findings give potentially widely applicable clues on additional prospects towards competitive exclusion for the combat of FBs and AFs.

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Summary

Fungi are ubiquitous in nature. Through niche partitioning various fungi can infect a single host and co-exist by occupying different niches resulting in a diverse fungal community. Besides being plant disease agents, fungi are producers of harmful metabolites termed mycotoxins. In **Chapter 1** we provided a background on fungi infecting preharvest maize and mycotoxins contamination thereof. Special attention was drawn towards *Aspergillus* and its aflatoxin (AF). In addition, *Fusarium* and its fumonisin-B1 (FB1) was also investigated. In **Chapter 2**, we detailed the fungal microbiome on the preharvest maize, revealing *Fusarium* and *Sarocladium* to be the most dominant members irrespective of rainfall pattern, out of the 61 fungal genera detected in the mycobiome. In **Chapter 3** we explored the fungal contamination dynamics of preharvest maize from a niche partitioning perspective, in order to understand how the fungal relative abundance correlations may influence levels of FB1 or AF. We demonstrated a strong negative correlation between presence of *Sarocladium* and *Fusarium*, with higher abundance of *Sarocladium* resonating with lower abundance of *Fusarium* and levels of FB1. In **Chapter 4** we focused on *Aspergillus* and investigated the natural infection of *Flavi* in maize, with consideration that soil is the reservoir for *Flavi*. Findings showed that *Flavi* were present throughout the environment (soil) but their field maize infection was 18%, with infection largely corresponding to the weather variable under a dry spell. Considering the observed high selection frequency of soil *Flavi* for aflatoxigenicity, we hence studied in **Chapter 5** the community structure of *Flavi* between soil and maize. Findings showed a difference in *Flavi* diversity between the soil and maize communities with maize having a higher diversity than soil. The finding suggests that extrapolation of the risk of AF contamination in maize should not be done on basis of soil *Flavi* aflatoxigenicity as has been done in some studies. In **Chapter 6**, we investigated the degradation of aflatoxin-

B1 (B1) by toxigenic and atoxigenic wildtype *Flavi* in an antioxidative mechanism. In conclusion, we demonstrated that toxigenic *Flavi* degrade B1 in an antioxidative mechanism, whereas atoxigenic *Flavi* do not degrade it in such a role. We further found that the antioxidant selenium can prevent spikes in AF from *Flavi*, while promoting better fitness (sporulation) of atoxigenic when compared with toxigenic isolates. In **Chapter 7**, I discussed the findings and recommendations from two prospective: a) on the competitive exclusion of *Fusarium* by *Sarocladium* for the combat of FBs in preharvest maize; b) on the use of antioxidant such as selenium to prevent B1 spikes in preharvest maize, while promoting the fitness of atoxigenic over toxigenic *Flavi*.

Acknowledgements

On 17th September 2018, I arrived in the Netherlands from Zambia to start the journey on my PhD trajectory. With such a sandwich PhD, the first thing that strikes you before you settle down is cultural shock: from as many bicycles as there probably are humans with the thought of accidentally bumping into one, to a student calling a professor simply by their first name without title. The last one in my country… taboo! only to learn that its culturally normal in the Netherlands. I should admit that I struggled a bit to adapt to it.

Commencing the PhD track, I had very little idea first and foremost how the PhD project would unfold. Time seems to have flown faster than blinking. It's already done. Completing this thesis, still feels like I started the PhD journey just yesterday. How would I describe completing the PhD track? I would say "*The problem with success is that it looks simple*" – NASA 2017; or that "*It wasn't easy, but it was worth it*" - a phrase from Cece Winans Song. "Not easy" due to the high detail of the work, from the planning, the experiment execution (partly executed during a pandemic), to processing and aligning the narrative of the dissertation from a tsunami of information built over the years. "Worth it" in that it has brought about a lot of learning experiences and interactions. One of the key lessons to learn from my PhD track is that a PhD cannot be done in isolation. Single-handed, I would not have executed and completed this thesis. Goes the saying in my native language that "*one finger cannot pick a louse*;" now glorified by this PhD trajectory.

First and foremost, I want to give my special thanks to Sijmen Schoustra who was my daily supervisor. You have been so approachable, so accommodating, helpful in the course of this PhD research, patient, ability to apply social intelligence,… continue being such a supervisor. My sincere gratitude for your unstinting support during this PhD track. The acknowledgements I can give cannot fit in this section. I want to say thank you for having showed confidence in me at the beginning to take me as your student. The confidence equivalent to overtaking at a curve on a single lane, as the PhD track can have many unknowns. By the way you once surprised me that you were able to cook our local dish '*nshima*' during one of those social events at Genetics. Thumbs up!

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mystery to me hidden inside an enigma how you manage to split yourself to lecture, do university administrative work to direct traffic, be a promotor to so many PhD students, supervise them and even attend their PhD defences. I would wrongly try to convince myself that you probably have a spitting image of yourself somewhere. Mmmh, I leave the skill to you. Through your team, let the cordial and relaxed environment at Genetics, continue. To my team of supervisors thank you so much for helping while executing my thesis and shaping my dissertation. Sincere gratitude Anne van Diepeningen for being so helpful out of your vast knowledge and taking the timeout to attend to me. Your strokes of pen into my thesis have been invaluable. Thank you Dr Njapau for the support to have this sandwich PhD take off during the pre-enrolment, and thank you for taking time to attend to my thesis. Very grateful for having made the field works while in Zambia to be smoothly executed. And thanks to you too Dr Paul for the assistance during the field works and for agreeing to be thesis advisor when you were approached.

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accommodating. I was able to cordially interact with a couple of you: Anna, Ben, Eric, Eveline, Frank, Gabriella, Hylke, Jianhua, Mariska, Suzette,… cannot name everyone. Thanks too to my colleagues from Zambia at Wageningen University as we could practice our native language during those come-together dinners.

Outside Laboratory of Genetics, I wish to extend my acknowledgements to Claudius vande Vijver, for being so inspiring to PhD students through their tracks, including myself. Anyone who attends your sessions during those PhD weekends cannot come out dry. Imagine falling into a river and coming out dry. Not permissible ;-). I, furthermore, extend my gratitude to my spouse and friend Sylvia for the patience while away from time to time during my sandwich PhD tenure. One of the key lessons I learn is that the PhD track requires two time zones: one for academics and one for social life, as the two time zones are as miscible as is oil and water or iron and clay.

At global level, my sincere gratitude to the Wageningen University and Research for having provided the funding to make this PhD research possible, co-financed with the National Institute for Scientific and Industrial Research (NISIR). I further extend my gratitude to the International Atomic Energy Agency for the mycotoxins testing capability built at NISIR, which was very useful in the analysis of mycotoxins on this thesis while in Zambia. Below that, thank you too to the people at Mycotoxicology laboratory at NISIR for a helping hand in some bulk sample preparations, – Matildah, Mike, Temwani. It could have been too exhausting to do that work single handed.

Finally, I dedicate this thesis in loving memory of mum and dad, lost at my young age. Many are the heroes but you mum forever remains the greatest of them all to me. Allow me to thank the omnipotent Creator, who pours rain on everyone, for his grace to overcome odds.

Biography
& Bibliography

Bwalya, " Who is he? What is he? Where does he come from?" In the double-inverted commas, a phrase from the once famous detective fiction movie "Robocop."

Bwalya was born on 31st October 1979 in Lusaka, Zambia. He started his education career from primary school, aged five. The knowledge about one day becoming a scientist was as little as he was then. His elder sister used to jokingly say "you will become a scientist" for being curious about the peripheral world. After completing final 12th grade secondary school in 1996, he was awarded a Russia-Zambia government full scholarship for undergraduate study. He therefore proceeded to study at the Mendeleyev University of Chemical Technology of Russia, in Moscow. He studied from 2000 to 2007, braving the harsh winters, and turned bilingual in English and Russian. He graduated and obtained his MEng in Biotechnology under the University's faculty of Ecology. His thesis was on the reduction of chemical oxygen demand of effluent using a symbiosis of anaerobic microorganisms coupled to an aerobic stage (aerobic biofilm). Upon graduation, Bwalya returned to Zambia and worked for the mines on Zambia's Copperbelt province, from 2007 – 2009, under the Analytical Services Department as Senior Chemist. He resigned from the mines in late 2009 to explore something different, returning to Lusaka to immediately join the National Institute for Scientific and Industrial Research (NISIR). He joined as a Scientific Officer under the Mycotoxicology section. Under NISIR, he also partly worked under the Biotechnology section, as additional duties, between 2011 and 2012. At Biotechnology section, he worked as part of laboratory technical staff on a project on determination of similarities between different cattle breeds in Zambia using microsatellite-based Randomly Amplified Polymorphic DNA PCR. At Mycotoxicology section, he worked under Dr Henry Njapau on projects including the National Mycotoxins Monitoring Programme, auspices of the International Atomic Energy Agency, as laboratory technical staff; an IITA Biocontrol programme for Aflatoxin in maize and groundnuts, similarly in the role of laboratory technical staff working on mycotoxins. In 2014, Bwalya was appointed as Head of Centre of NISIR hub in Chilanga then called the Animal Science Research Centre, a position which he held until 2018, before taking up a PhD position at Wageningen University and

Research (WUR), in the Netherlands. It was in 2017 along his career when he applied for a sandwich PhD position at Laboratory of Genetics, at WUR. This was on a project initiated by Dr Sijmen Schoustra entitled “Elimination of toxin producing fungi by competitive exclusion: the case of biocontrol in the maize production chain in Zambia.” Securing co-financing in 2018 through the WUR sandwich PhD programme and NISIR, Bwalya took up the PhD Candidate position on sandwich basis at Laboratory of Genetics, the Laboratory of Prof. Bas Zwaan. This was under the supervision of Dr Sijmen Schoustra, Associate Professor at Laboratory of Genetics. In his research, Bwalya explored the spectrum of fungi infecting maize, paying particular attention to *Aspergillus* and *Fusarium* and their mycotoxins, aflatoxin and fumonisin. This is the project that leads to this dissertation following the successful completion of the project described in this thesis titled “*On the fungal contamination dynamics in maize: towards competitive exclusion to control mycotoxins*.” As part of the work has been presented at local and international conferences, and partly published, the research output of this dissertation adds to the global scientific knowledge pool for the betterment of the understanding of the fungal contamination dynamics in maize at harvest. It is envisaged that this augments current efforts aimed at generating the intelligence to combat aflatoxins and fumonisins in maize.

Publications

Full papers

- **Bwalya Katati**, Pierre Schoenmakers, Henry Njapau, Paul Kachapulula, Bas. J. Zwaan, Anne D. van Diepeningen, and Sijmen. E. Schoustra. (*Accepted 14th April 2023)*. Preharvest maize fungal microbiome and mycotoxin contamination: case of Zambia’s different rainfall patterns. ***Journal of Applied and Environmental Microbiology****, in press*.

- Juliet Akello, Alejandro Ortega-Beltran, **Bwalya Katati**, Joseph Atehnkeng, Joao Augusto, Mwila Chama, George Mahuku, David Chikoye, and Ranajit Bandyopadhyay. (2021). Prevalence of aflatoxin- and fumonisin-producing fungi associated with cereal crops grown in Zimbabwe and their associated risks in a climate change scenario. ***Foods***, 10(2), 287. https://doi.org/10.3390/foods10020287.

Submitted:

- **Bwalya Katati**, Stan Kovacs, Henry Njapau, Paul Kachapulula, Bas J. Zwaan, Anne D. van Diepeningen, and Sijmen E. Schoustra. Aflatoxigenic *Aspergillus* modulates aflatoxin-B1 levels through an antioxidative mechanism.

Conference presentations

- **Bwalya Katati**, Henry Njapau, Paul Schoenmakers, Paul Kachapulula, Bas J. Zwaan, Anne D. van Diepeningen, and Sijmen E. Schoustra. The Maize Mycobiome and Mycotoxin Contamination in Relation to Climatic Patterns. *Presented during the 3rd African symposium on mycotoxicology, 4 – 7th September, 2022. Stellenbosch, Capetown, South Africa.*

- **Bwalya Katati**, Bas J. Zwaan, Anne van Diepeningen, Pierre Schoenmarkers, Paul Kachapulula, Henry Njapau, and Sijmen E. Schoustra. Prospecting Mycotoxin Biocontrol from Preharvest Maize Mycobiome. *Poster presented during the 2022 Gordon Research Conference on Cellular and Molecular Fungal Biology, 26th June – 1st July, 2022. Plymouth, New Hampshire, United States of America.*

- **Bwalya Katati**, Bas J. Zwaan, Anne van Diepeningen, Pierre Schoenmarkers, Paul Kachapulula, Henry Njapau, and Sijmen E. Schoustra. The Maize Mycobiome and Implication on Mycotoxin Contamination in Relation to Climatic Patterns. *Presented during the 31st Fungal Genetics conference, 15 – 20th March, 2022. Online*.

- **Bwalya Katati**, Anne D. van Diepeningen, Henry Njapau, Paul Kachapulula, Bas J. Zwaan, Stan Kovacs, and Sijmen E. Schoustra. A triphasic characterisation of *Aspergillus* section *Flavi* reveals potential influence of diversity on aflatoxin in maize. *Presented during the 2021 KNVM (Royal Dutch Society for Microbiology) Annual meeting of the section mycology, 26th November, 2021. Online*.

- **Bwalya Katati**, Bas J. Zwaan, Anne van Diepeningen, Pierre Schoenmarkers, Paul Kachapulula, Henry Njapau, and Sijmen E. Schoustra. Maize fungal microbiome dynamics and aflatoxin contamination. *Poster presented during the 2021 Netherlands Society for Evolutionary Biology conference, 19 – 20th April. Online*.

Training and Education Statement of the Graduate School of Production Ecology and Resource Conservation

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. De Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review/project proposal (4.5 ECTS)

- On the fungal contamination dynamics in maize: prospects for competitive exclusion to control aflatoxin and fumonisin

Post-graduate courses (5.7 ECTS)

- Linking community and ecosystem dynamics; University of Groningen (2018)
- Bayesian statistics; PE&RC (2018)
- Basic statistics; PE&RC/SENSE (2018)
- Design of experiments; WIAS/PE&RC (2018)

Deficiency, refresh, brush-up courses (3 ECTS)

- Genomics; WUR (2020)

Laboratory training and working visits (0.9 ECTS)

- Food and indoor fungi; Westerdijk Fungal Biodiversity Institute (2018)

Competence strengthening/skills courses (2.05 ECTS)

- Scientific publishing; PE&RC (2018)
- Reviewing a scientific paper; WGS (2018)
- Research data management; WUR Library (2020)
- Supervising BSc & MSc thesis students; WUR/ESP (2020)
- Start to teach; WUR/ESP (2021)

Scientific integrity/ethics in science activities (0.6 ECTS)

- Scientific integrity; WGS (2020)
- Ethics in animal science; WGS (2021)

PE&RC Annual meetings, seminars and the PE&RC weekend (2.8 ECTS)

- PE&RC First years weekend (2018)
- PE&RC Day (2018)
- PE&RC Midterm weekend (2021)
- PhD Last stretch and writing propositions (2021)

- PE&RC Last years' weekend (2022)
- WGS PhD carousel (2022)

Discussion groups/local seminars or scientific meetings (5.8 ECTS)

- Towards healthy and sustainable food systems in an urbanising world (2018)
- KNVM Section mycology annual meetings (2018-2022)
- Traditional fermented foods to promote food and nutrition security in Africa (2019)
- Wageningen evolution and ecology seminars (2019-2021)
- Food systems scientific meetings; African food safety network (2019-2021)
- Science communication interest group (2020-2022)
- Mycotoxins and post-harvest losses in sub-Saharan Africa (2021)
- 3rd All Africa post-harvest congress and exhibition (2021)
- Traditional fermented foods to promote One Health in Africa (2022)

International symposia, workshops and conferences (5.4 ECTS)

- Netherlands society for evolutionary biology; poster presentation; online, 19 – 20th April, 2021.
- KNVM (Royal Dutch Society for Microbiology) Annual meeting of the section mycology; oral presentation; online, 26th November, 2021.
- 31st Fungal genetics conference; oral presentation; online, 15 – 20th March.
- Gordon research conference on cellular and molecular fungal biology; poster presentation; Holderness, New Hampshire, United States of America, 26th June – 1st July, 2022.
- 3rd African symposium on mycotoxicology; oral presentation; Stellenbosch, Capetown, South Africa, 4 – 7th September, 2022.

Societally relevant exposure (0.3 ECTS)

- Women in Farming: Mycotoxins awareness talk (2021)

BSc/MSc thesis supervision (3 ECTS)

- Pierre Schoenmakers, MSc thesis: aflatoxins in maize from North and South Zambia
- Stan Kovacs, BSc thesis: genetic diversity of *Aspergillus section Flavi*

The research described in this thesis was carried out at the Laboratory of Genetics at Wageningen University, Wageningen, The Netherlands, and partly at Mycotoxicology Laboratory at National Institute for Scientific and Industrial Research, Lusaka, Zambia. The research was financed through the Wageningen University and Research sandwich PhD programme, and through staff development facility of National Institute for Scientific and Industrial Research.

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