CRANFIELD UNIVERSITY

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Impact of Ecophysiological Factors on Biocontrol of Aflatoxin Contamination of Maize by Atoxigenic *Aspergillus flavus* Strains

Cranfield Health (Applied Mycology Group)

PhD
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ABSTRACT

This study has (a) evaluated the biodiversity in toxigenic mycobiota associated with maize from subsistence farmers' stores in five climatic regions of Lesotho in two seasons, (b) compared the effect of ecophysiological factors on interactions between atoxigenic (AFL^-) and toxigenic (AFL^+) *Aspergillus* strains and control of aflatoxin B_1 (AFB_1) contamination of maize, (c) examined the mechanism of action of AFL^- strains in relation to mycelial growth rate, sporulation, germination rate, germ tube extension, C-source utilisation patterns and hydrolytic enzymes and (d) examined ecophysiological approaches to enhance competitiveness of the atoxigenic strains.

The total fungal populations of the maize from different regions in the two seasons was not significantly different ($p>0.05$). *Fusarium verticillioides*, *F. proliferatum* and *F. subglutinans* predominated in different regions in both seasons based on molecular analyses. In the 2009/10 season the isolates of these species all produced fumonisin B_1 (FB_1) while in the 2010/11 season very few produced FB_1. *Aspergillus flavus* isolates (2009/10) were recovered from the mountains and Senqu river valley samples while the 2010/11 isolates predominantly from the foothills and northern lowlands. The mountain isolates of *A. flavus* produced highest levels of AFB_1 (20 mg kg^{-1}). *Aspergillus parasiticus* was only isolated from the foothills, Senqu river valley and southern lowlands samples and the AFB_1 levels produced ranged from 0-3.5 mg kg^{-1}. *Aspergillus* section *Circumdati* isolates were least frequently encountered in both seasons. In the 2009/10 season, the isolates from the northern lowlands produced ochratoxin A (OTA) in culture. All isolates of *A. niger* from different regions in both seasons did not produce OTA. An analysis of the maize samples for a range of mycotoxins was done using a targeted multi-mycotoxin analysis. At least one sample from each region in both seasons was FB_1 positive. FB_1 levels for 2010/11 samples were higher than in the 2009/10 season range (7–936 µg kg^{-1}; 2–3 µg kg^{-1} respectively). In both seasons the maize from the mountain regions had the highest contamination levels with FB_1. Deoxynivalenol (DON) was recovered from all the samples analysed with the

highest mean contamination of 1469 μg kg^{-1} in samples from the northern lowlands. Moniliformin (MON) was detected from all agroecological zones in the two seasons (5–320 μg kg^{-1} in 2009/10; 15–1205 μg kg^{-1} in 2010/11). Emerging toxins such as fusaproliferin (FUS) and beauvericin (BEA) were also detected. OTA was not detected in any of the samples analysed. Only one 2009/10 sample in the Senqu river valley was positive for AFB_1.

The potential of three AFL^- *A. flavus* strains from different geographical origins in Africa were examined for *in vitro* and *in situ* competitiveness against two toxigenic AFL^+ strains of *A. flavus* and subsequent inhibition of AFB_1 production under different environmental conditions. Temperature, a_w and substrate influenced the types of interaction between the three AFL^- and two AFL^+ strains. The competitiveness and AFB_1 reduction ability of the three AFL^- strains when challenged with the two AFL^+ strains was evaluated by inoculation of 100, 25:75, 50:50, 75:25 and 100% ratios of mixed spore suspensions *in vitro* on milled maize agar (MMA) over 28 days and *in situ* on stored maize grain for 14 days respectively at 0.99, 0.96 and 0.90 water activity (a_w). For all the treatments, effect of a_w and inoculum ratio and their interaction was highly significant ($p<0.05$). Toxin inhibition was >80% *in vitro* at both 0.99 and 0.96 a_w. Similarly, toxin reduction *in situ* was influenced by the AFL^+ strain assayed, a_w and the inoculum ratio. Where control was achieved, it was greatest with more available water (0.96 a_w) than under water stress (0.90 a_w) and the toxin reduction was between 1.45 – 77.18%.

The possible mechanisms of action were examined to better understand the similarities and differences between AFL^- and AFL^+ strains of *A. flavus*. This showed that temperature and a_w and their interactions had a significant influence on the germination, growth, sporulation, extracellular enzyme production capacity and nutritional utilisation patterns ($p<0.05$). There were no differences between AFL^- and AFL^+ strains in lag phases prior to growth, growth rates, and sporulation. The rates of germination rate, germ tube extension and extracellular enzyme production were variable between the two groups of strains and therefore no clear distinctions could be drawn between AFL^- and

AFL^+ strains in this respect. Based on carbon source (CS) utilisation patterns, the niche size of both AFL^- and AFL^+ strains in the current study was greater at higher temperatures and under wetter conditions. Additionally, based on the computed niche overlap indices (NOIs), regardless of temperature, when water was freely available, AFL^- and AFL^+ strains were able to coexist. However, under moisture stress, the nutritional competitiveness of the strains was variable. Temporal carbon source utilisation sequences (TCUS) of AFL^- and AFL^+ strains were compared. At 0.99 a_w most CS sources were utilised by the strains and the time to detection (TTD) of each strain was shortest on monosaccharides at the same level of a_w. Conversely, under moisture stress the least number of CS was utilised and the CS sources used included proline, glutamine and alanine in addition to monosaccharides.

Attempts were made to improve the ecophysiological quality of conidial inocula of the $AFL2^-$ strain by modifying maize-based media with different solutes and on stored sorghum grain. The conidia produced on 0.92 a_w Proline modified media germinated fastest when tested on water agar at 0.92 and 0.96 a_w. However, the rate of germ tube elongation was fastest for conidia from the 0.92 a_w Glucose modified medium.

Spore productivity on sorghum solid substrate adjusted to 0.96 a_w was highest (9.2 log spores g^{-1} dry wt sorghum) when compared to the other two treatments. Use of spores harvested from the 0.92 a_w sorghum solid substrate gave complete inhibition of toxin production on maize grain at 0.90 a_w while condia from the 0.92 a_w Glycerol treatment inhibited AFB_1 production better at 0.96 a_w in maize grain in 50:50 ($AFL2^-$:$AFL1^+$) mixed maize cultures.

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DEDICATION

This work is dedicated to my late grandmother ‘Makao Mookho Mohale.

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CHAPTER 1: General Introduction and Literature Review

1.1 Introduction

Maize is the most preferred dietary staple after rice and wheat for 900 million poor consumers in low- and middle-income countries, 71% of which live on <US$2 a day (CCIMYT and IITA, 2011). Nevertheless, most communities in the developing world which characteristically constitute the vast majority of resource poor, smallholder farmers who do not have physical and economic access to nourishment they need on a sustained basis (FAO and EU, 2010). In order to provide physical access to food, sound land husbandry practices and mitigation of adverse effects of climate change for sustenance of agricultural ecosystems are mandatory. Efficient distribution systems that include transportation, storage and processing are also requisites. On the other hand, economic access is attainable through creation of employment opportunities in order to afford means of production and efficient marketing strategies (CCIMYT and IITA, 2011).

In Lesotho, subsistence agriculture employs 80% of the rural population. There is a concerted effort by the government with the assistance of regional organisations and International Development Agencies (IDA) to increase agricultural productivity. However, little or no effort has been made to ensure quality produce from ‘farm to fork’. This is in vast contrast to the situation in the developed world where better institutional and technical aptitude as well as sound infrastructure make enforcement of food quality standards an integral part of the food chain management systems.

The resultant therefore, is a myriad of problems in the developing world chief of which include impediments to trade and public health concerns (Wu, 2004; Lewis et al., 2005). The food safety situation in these developing regions is so dire that, in 2011, the African Union Commission (AUC) spearheaded the creation of a Partnership for Aflatoxin Control in Africa (PACA) which seeks to address agriculture and food security, health and trade issues associated with aflatoxin prevalence and exposure in Africa. In the case of Lesotho, food quality as it relates to trade in the short and medium term may not be a key issue as the country is yet to produce enough food to meet its national requirements (FAO and EU, 2010). However, recent reports on stunting (39%) in children <5 yrs of age in Lesotho particularly in rural areas and the high prevalence rate of HIV (23.6%) are a cause for great concern (Owusu-Ampomah et al., 2009). Indeed the deleterious effects of food contaminants including children's growth impairment, immunosuppression and death by mycotoxins have been reported (Gong et al., 2004; Egal et al., 2005). This situation is relatively unknown for maize produced in Lesotho.

Thus, information on the mycotoxigenic fungi and mycotoxin levels in maize would serve as a basis for tailoring appropriate control interventions if need be. Currently, through similar studies, the use of atoxigenic (AFL^{-}) strains to control aflatoxin contamination has proven efficacious in different regions of the world (Dorner, 2004). This method uses autochthonous AFL^{-} strains to competitively exclude their toxigenic (AFL^{+}) counterparts. It is environmentally benign, cheaper than the use of fungicides and may have carry over effects into storage. Potentially, this strategy can be used with success in Lesotho too once

there is preliminary information on the prevalence and population dynamics of *A. flavus* in the different regions of the country.

1.2 Spoilage fungi and mycotoxin contamination of maize

Although several fungi are associated with maize production, some members of the genus *Aspergillus* and *Fusarium* are the most economically important (Wu, 2004). Infection of maize by these fungi can occur over a wide range of environmental conditions. Abiotic and biotic stress conditions predispose the crop to infection. These fungi can continue to be a problem in storage if the grain is not stored appropriately. This section summarises information on toxigenic fungi commonly associated with maize. An overview of *A. flavus* and aflatoxins is given in Section **1.2.1**.

Fusarium species are pathogens of maize (Logrieco et al., 2007). They are causative agents of both stalk and ear rot of maize (**Figure 1. 1**). Species belonging to this genus are also producers of hazardous mycotoxins. *Fusarium* mycotoxins of economic importance include fumonisins (produced mainly by *F. verticillioides* and related species), trichothecenes including deoxynivalenol (DON) produced by members of *Fusarium graminearum* species complex. DON (**Figure 1. 2**) is associated with growth impairment in experimental animals through feed refusal. It can potentially lead to anorexia in young children (Flannery et al., 2012). Emerging mycotoxins such as fusaproliferin (FUS), beauvericin (BEA) and moniliformin (MON) are also produced by *F. subglutinans* and other related species (Shephard et al., 1996; Reynoso et al., 2004; Boutigny et al., 2012). Of the *Fusarium* mycotoxins referred to above, fumonisin B_1 (FB_1: **Figure 1. 2**) is the most toxic. Ingestion of fumonisin

contaminated maize has been associated with high incidence of oesophageal cancer in Transkei, South Africa. The International Agency for Research on Cancer (IARC) classifies FB_1 as Group 2B i.e. possibly carcinogenic to humans (IARC, 2002).

Figure 1. 1 White to pink coloured mycelial mass characteristic of *Fusarium* ear rot of maize caused by *Fusarium verticillioides*. Source: (Anonymous, 2013a).

(A)

(B)

(C)

Figure 1. 2 Chemical structures of selected *Fusarium* mycotoxins (A) fumonisin B_1, (B) deoxynivalenol, (C) moniliformin . Source: (Anonymous, 2013b)

Co-infection of maize with toxigenic fungi belonging to *Aspergillus section Nigri* and *Circumdati* has previously been reported (Magnoli et al., 2007). Recently Saleemi et al. (2012) reported co-occurrence of toxigenic *A. ochraceus* and *A. carbonarius* on Pakistani maize. These fungi produce immunosuppressive and teratogenic Ochratoxin A (OTA). OTA (**Figure 1. 3**) too is classified as Group 2B carcinogen by IARC.

Figure 1. 3 Chemical structure of Ochratoxin A. Source: (Anonymous, 2013b).

1.2.1 *Aspergillus flavus* and aflatoxins on maize

Pathogenicicity and toxigenicity: Within the genus *Aspergillus*, *A. flavus* is the most common aflatoxigenic species associated with maize. **Figure 1. 4** below shows the life cycle of *A. flavus* on maize. *Aspergillus flavus* overwinters as mycelium or as sclerotia. Sclerotia germinate to produce conidia. The conidia (asexual spores) are dispersed by insects or wind to the maize ears where they infect the maize kernels. Although *A. flavus* is predominantly saprophytic, it is pathogenic to maize and causes ear rot (**Figure 1. 5**). Disease is more severe when weather is warm and dry (Azaizeh et al., 1989; Gqaleni et al., 1997). Hail or insect damaged kernels are also more susceptible to *Aspergillus* ear rot (Windham et al., 1999). Post-harvest contamination of food commodities with aflatoxigenic fungi is a result of elevated ambient temperature and relative humidity during storage, inadequate drying of produce, mechanical damage during harvesting and threshing, as well as damage due to stored product insect-pests (Hell et al., 2000; Mutegi et al., 2009; Hell and Mutegi, 2011).

Colonisation of ripening maize cobs by *A. flavus* can result in contamination with aflatoxins (AFs). Aflatoxins are toxic secondary metabolites of *A. flavus* and related species. There are four naturally occurring aflatoxins namely: aflatoxin B_1, B_2, G_1 and G_2 (AFB_1, AFB_2, AFG_1 and AFG_2). These are distinguished by the colour of their fluorescence under long-wave ultraviolet illumination (B = blue; G = green). The subscripts relate to their relative chromatographic mobility. The chemical structure of AFB_1, the most toxic of the aflatoxins, is shown in **Figure 1. 6** below. The International Agency for Research on Cancer

(IARC) classifies AFB_1 as a Group 1 carcinogen i.e. carcinogenic to humans (IARC, 2002).

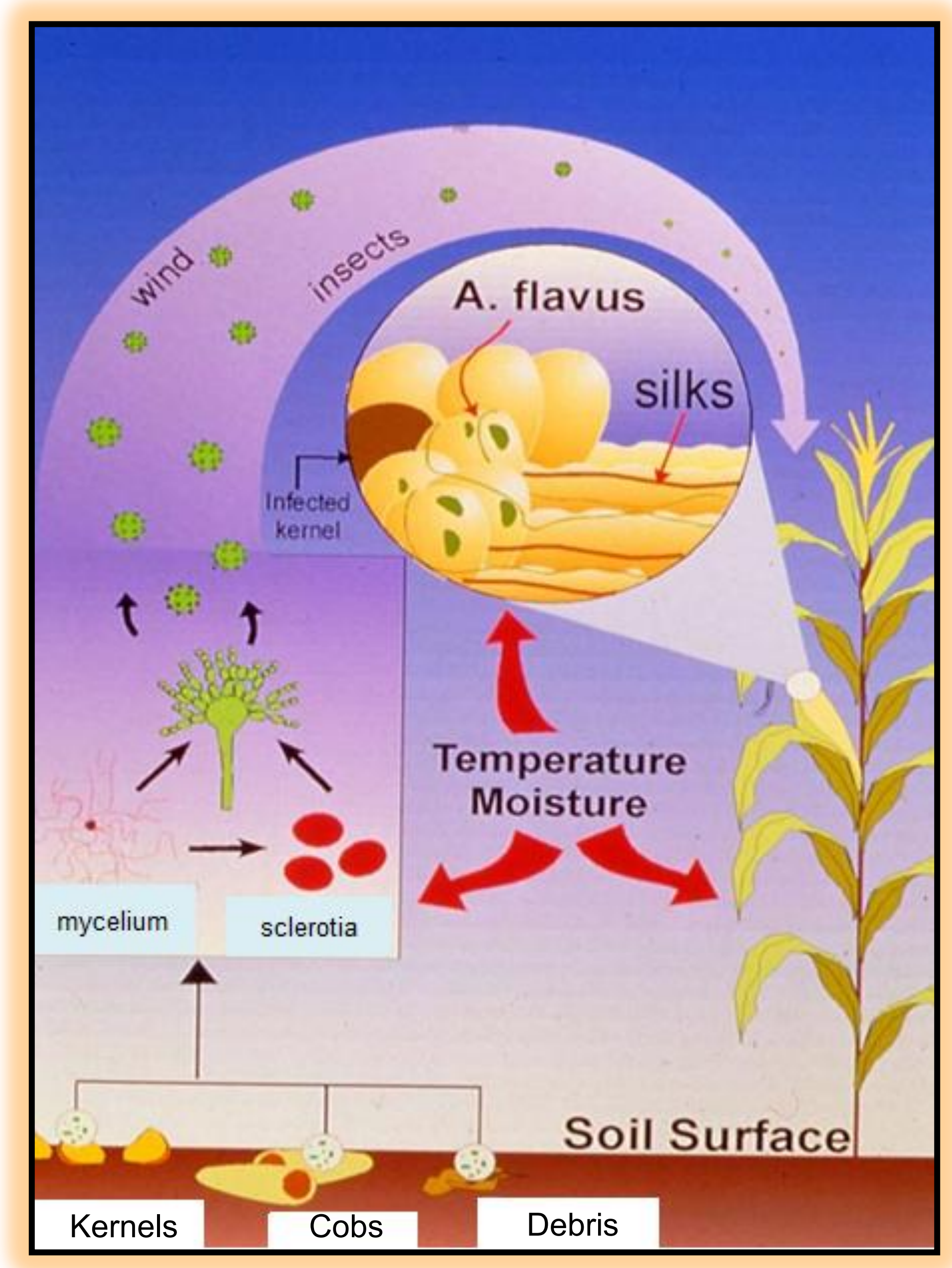

Figure 1. 4 Schematic presentation of the life cycle of *Aspergillus flavus* on maize. Source: (Anonymous, 2005)

Figure 1. 5 Characteristic green conidia of *Aspergillus flavus* sporulating on maize. Source: (Anonymous, 2013a)

Figure 1. 6 Chemical structure of AFB_1. Source: (Anonymous, 2013b)

1.2.2 Impact of aflatoxins on human health and trade

Health: Contamination of food with AFs makes the food unfit for consumption as the toxins are injurious to health (Lewis et al., 2005). Aflatoxin B_1 ingestion has been positively correlated with the incidence of liver cancer and incidence of malnutrition in children (Peers et al., 1987; de Vries et al., 1990). Ingestion of maize contaminated with AFB_1 may also lead to death (Lewis et al., 2005).

Trade: Marketability of aflatoxin contaminated produce is reduced due to stringent standards of permissible limits on aflatoxin contamination set by the importing countries. Thus, economic viability of farmers' enterprises is compromised because commodities containing above the regulatory limit of aflatoxins cannot be sold for food use; their use must be diverted; often with lower profit margins (Wu and Khlangwiset, 2010).

1.2.3 Variability in *Aspergillus flavus*

Of the mycotoxigenic fungi, *A. flavus* is the most studied. Previous studies have shown that *A. flavus* population structure within and between different regions of the world varies morphologically, genetically and in the ability to produce secondary metabolites (Cotty et al., 1994; Cotty and Cardwell, 1999; Vaamonde et al., 2003; Hua et al., 2012).

Morphology: Based on sclerotial size, *A. flavus* strains are divided into two groups. Strains that produce large sclerotia (average diameter > 400 µm) are L-strains while small sclerotia producing ones are S-strains (average diameter < 400 µm: (Cotty and Cardwell, 1999). Typically, the AFL^+ isolates of these two groups (L- and S-strains) produce aflatoxin B_1 and B_2 only. S-strains although not frequently isolated produce numerous sclerotia and are potent producers of AFs and they are more stable in aflatoxin production compared to L-strains (Horn and Dorner, 1999). Strains that do not produce sclerotia at all have also been isolated (Abbas et al., 2005).

Genetic variability: Previous studies have also shown that the anamorphic state of *A. flavus* comprises different vegetative compatibility groups (VCGs) (Bayman and Cotty, 1991; Bayman and Cotty, 1993). Genetic diversity between such individuals is achieved through the parasexual cycle. In parasexuality, two vegetatively compatible somatic hyphae of different genetic combinations fuse together (anastomose) leading to the formation of a heterokarytic mycelium (during which nuclei and other cytoplasmic components occupy the same cell). The unlike nuclei fuse (karyogamy) to form a diploid (zygote) nucleus (**Figure 1. 7**). Genetic recombination takes place without the formation and fusion of

gametes. The progeny produced during the cycle will be genetically different from the original parent mycelium owing to mitotic crossing over that would have occurred (Glass et al., 2000).

In *A. flavus*, hyphal anastomosis is difficult to perceive. Therefore, complementary nitrate-non-utilising (*nit*) mutants have been used to identify vegetatively compatible isolates (Bayman and Cotty, 1991). Firstly, *nit* mutants are generated on potassium chlorate medium. The basis for this is that cells that are able to reduce nitrate to nitrite usually reduce chlorate to chlorite which is toxic (Cove, 1976). Therefore cells that are unable to reduce and assimilate nitrate will not be poisoned by nitrite and will outgrow wild-type hyphae (Bayman and Cotty, 1991). According to Bayman and Cotty (1991) there are three classes of *nit* mutants namely, *niaD* mutants which lack the structural gene product for nitrate reductase and these are able to utilise nitrite and hypoxanthine; *nirA*$^-$ mutants lack a regulatory protein needed for synthesis of nitrate and nitrite reductase and cannot utilise nitrite and lastly, *cnx* mutants that lack a molybdenum-containing co-factor necessary for nitrate reductase and xanthine dehydrogenase and are unable to utilize hypoxanthine. Once generated, the mutants are phenotyped based on their ability to utilise different nitrogen sources. Complemetarity is tested by pairing the phenotyped mutants on medium containing $NaNO_3$ as the only nitrogen source. Wild type growth and profuse sporulation at the intersection of two complementary mutant colonies as a result of hyphal anastomosis and nutritional complementation in the heterokaryon is indicative of complementation (Novas and Cabral, 2002). A heterokaryon will only form in mutant pairs of vegetatively compatible isolates.

Indeed phenotypic differences have been reported as a result of parasexuality. According to Wicklow and Horn (2007) if two parent hyphae that are vegetatively compatible anastamose culminating in heterokaryon, the resultant progeny contributes to increase in aflatoxin production. Mehl and Cotty (2010) have also shown that *A. flavus* isolates belonging to different VCGs vary in their competitive abilities.

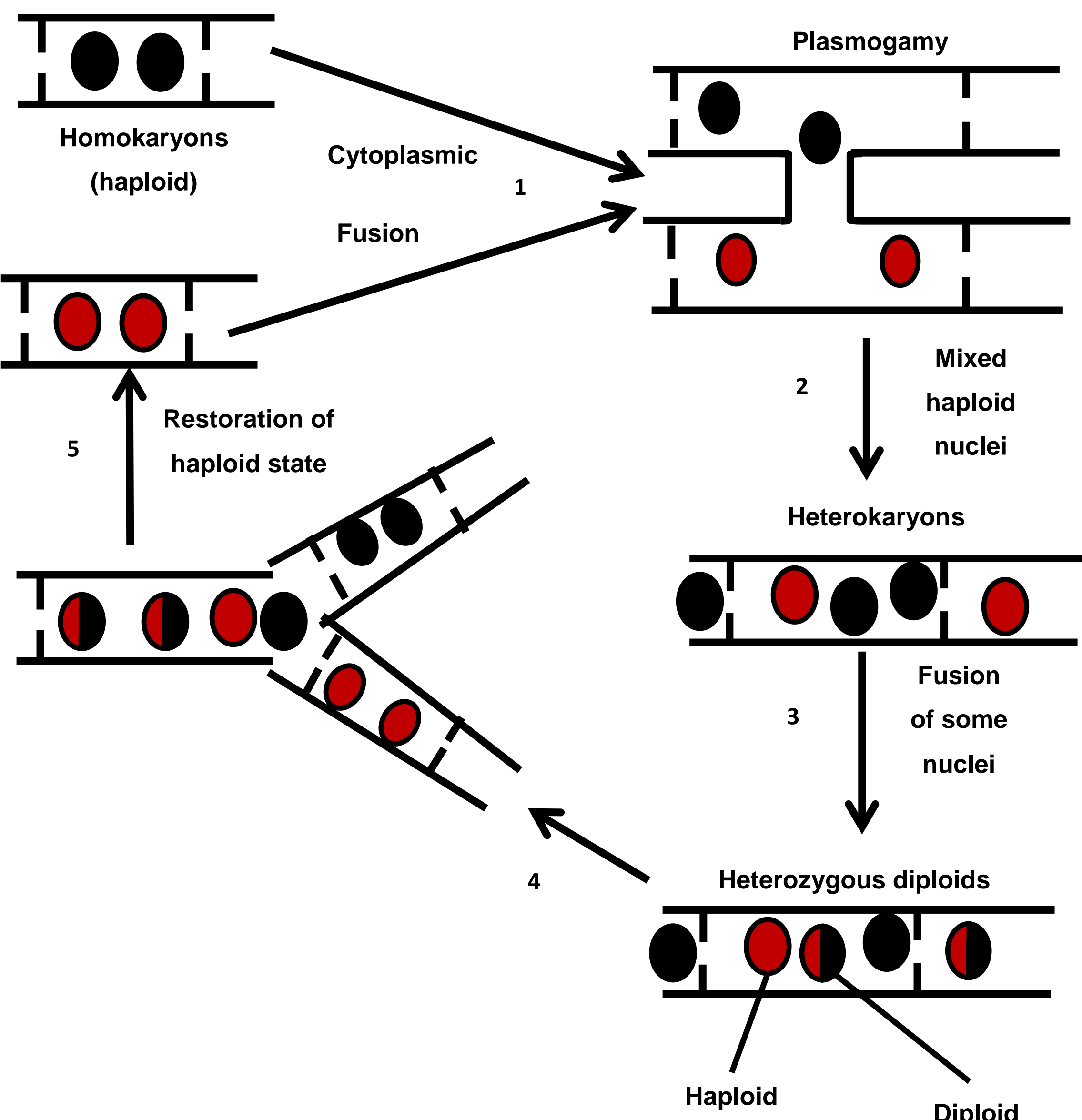

Figure 1. 7 Schematic presentation of the processes involved during parasexual cycle. Adopted from Glass et al. (2000).

Molecular differences: Previous studies have attributed atoxigenicity of some *A. flavus* strains to mutations in genes or deletions of genes responsible for aflatoxin biosynthesis (Ehrlich and Cotty, 2004; Chang et al., 2012). Ehrlich and Cotty (2004) have reported that strain AF-36 used to control aflatoxin contamination in cottonseed has a defective polyketide synthase gene. According to Chang et al. (2005) the entire aflatoxin gene cluster from the hexA coding region in an AFL^- NRRL21882 (active ingredient of biofungicide Afla-Guard®) is deleted. Strikingly, it has also been observed that another subset of AFL^- strains is constituted by organisms with complete sets of genes responsible for aflatoxin biosynthesis (Criseo et al., 2008). Perhaps in these individuals, aflatoxin production has no adaptive value (Chang et al., 2005). This explanation concurs with Cotty et al. (1994) and their description of the variation in niche occupied by L- and S-trains. The authors reported that L-strains of *A. flavus* are not potent producers of aflatoxins and they are mainly found on the aerial parts of the crop compared to S-strains which are mostly rhizosphere inhabitants. They speculate that unlike degradative enzymes, aflatoxins have no role in virulence and thus, do not have any adaptive value to the L-strains which occupy the aerial parts of crops. The ability of the S-strains to produce copious amounts of aflatoxins may be due to an important role that these toxins play for them to survive in the soil (Cotty et al., 1994).

Virulence: *Aspergillus flavus* is primarily a saprophyte and can utilize a broad spectra of nutrient sources (Mellon et al., 2007). It is therefore not surprising that it has the capacity to produce different enzymes which assist in the breakdown of complex substrates (Mellon et al., 2007) The fungus uses these

hydrolases as virulence factors (Cotty, 1989). Different *A. flavus* isolates vary in their ability to produce hydrolytic enzymes (Cleveland and Cotty, 1991; Brown et al., 2001). However, few studies have examined the effect of environmental factors on the ability of these strains to produce hydrolytic enzymes (Alam et al., 2009).

1.3 Control of aflatoxin contamination

Although different strategies are used in the management of aflatoxin contamination of crops only a few have shown potential. While an integrated disease management approach (IDM) may be the most effective way to control aflatoxin contamination of crops, concerns about the use of chemicals in agriculture and associated cost of some of the strategies outlined in **Table 1. 1** are prohibitive. The use of biocontrol control agents (BCAs) has thus been identified as the most promising alternative. It is a low-cost strategy and makes use of indigenous, naturally occurring antagonists or competitors of toxigenic strains of *A. flavus*.

1.3.1 Biological control of aflatoxin contamination of crops

Biological control of aflatoxin contamination of crops is based on interactions, antagonism and/or competitive exclusion of AFL^+ strains by an AFL^- strain or other BCA used.

Table 1. 1 Crop and insect management practices for control of aflatoxin contamination.

Strategy	Rationale	Citation
Irrigation	Heat and moisture stress	(Magan and Aldred, 2007b)
Fertilisation	N-deficient corn more susceptible	(Magan and Aldred, 2007b)
Insecticide application	Insect-damaged kernels more susceptible	(Magan and Aldred, 2007b)
Bt hybrids	Insects responsible for penetration into grain	(Brown et al., 1999)
Natural resistance to insects	Insects responsible for penetration into grain	(Betran et al., 2004)
Biological control	Competitive displacement of toxigenic isolates	(Dorner, 2004)
Fungicides	Reduce inoculum density	(Magan and Aldred, 2007b)

Adopted from Abbas et al. (2009) with modofications.

***Antagonism*:** In antagonistic interactions, microorganisms produce metabolites that impede spore germination (fungistasis), kill the cells (antibiosis) or modify the environment, e.g., acidification of the environment so that pathogens cannot grow, or promoting plant growth and plant defensive mechanisms.

Antagonistic bacteria and yeasts have been shown to inhibit the growth of *A. flavus* and subsequent toxin production. For example, Reddy et al. (2009), *Bacillus subtilis* and *Pseudomonas fluorescens* showed 72 and 74% inhibition of *A. flavus* growth and a 54 and 63% reduction of AFB_1, respectively. Palumbo et al. (2007) evaluated bacterial isolates from Mississippi maize field soil and maize rhizosphere samples for use as BCAs against *A. flavus* and *F. verticillioides*. They reported that *P. chlororaphis* and *P. fluorescens* consistently inhibited the growth of *A. flavus* and F. verticillioides. *Bacillus megaterium* was efficacious in reducing postharvest decay of peanut kernels caused by *A. flavus in vivo* (Kong et al., 2010). Under laboratory conditions, Hua et al. (1999) have shown the ability of *Pichia anomala* to inhibit *A. flavus* growth. Anjaiah et al. (2006) reported >50% reductions in *A. flavus* population when groundnuts were inoculated with antagonistic strains of *Pseudomonas*, *Bacillus* and *Trichoderma* in greenhouse and field experiments.

The Archilles' heel in the use of yeasts and most bacteria however, is that the two have higher moisture requirements than the filamentous fungi they are intended to control. Additionally, preharvest aflatoxin contamination takes place under hot and dry conditions, and most bacteria and yeasts become inactive under these conditions (Yin et al., 2008). Notwithstanding this, Glare et al.

(2012) posit that certain formulations of yeasts or bacteria can prolong shelf-life without any loss in viability.

***Competition**:* Competition occurs when two populations use the same resource whether space or a limiting nutrient. The rationale behind the use of competitors in biocontrol programs is in accordance with Gause's Law of competitive exclusion, i.e., two species that compete for the exact same resources cannot stably coexist (Atlas and Bartha, 1998). It is generally believed that this is the basis for use of autochthonous AFL^- strains, which are physiologically compatible with their physical and chemical environment to control aflatoxin contamination of peanuts, cotton and maize. This strategy has been examined with varying levels of success in different parts of the world (**Table 1. 2**).

1.3.2 Factors affecting the efficacy of BCAs

Determinants of efficacy of BCAs can broadly be classified into those factors associated with agronomic practices, abiotic, biotic factors and method of formulation of the BCA.

Agronomic practices: Inoculum concentration of the BCA and the time at which it is applied during the different stages of the development of the maize crop is crucial for the effective control of aflatoxin contamination. Dorner et al. (1998) demonstrated that increasing AFL^- inocula from 2 to 50 g m^{-1} row of peanuts, leads a reduction in aflatoxin concentration in peanuts. Pitt and Hocking (2006) also showed that increasing spores of AFL^- from 10^2 to 10^6 spores g^{-1} of soil leads to an increase in infection of mature peanuts at harvest by AFL^- strains. They suggested that this increase in AFL^- spore numbers gave

the strains a competitive edge over the AFL^+ strains. Chang and Hua (2007) used strain TX9-8 to prevent aflatoxin accumulation by CA28.

Table 1. 2 Reduction of aflatoxin contamination by atoxigenic strains.

Crop	**Results**	**Atoxigenic strain used**	**Citation**
Cotton	90% inhibition of AFB_1	AF-36.	(Brown et al., 1991)
Peanuts	98% inhibition of aflatoxin .	NRRL21882, active ingredient afla-Guard®	(Dorner, 2004)
Maize	86% Reduction of aflatoxin	CT3 and K49	(Abbas et al., 2006)
Peanut	Greatly reduced *A. flavus* populations by up to 99% in the soil of peanut fields	AF051	(Yin et al., 2008)
Maize	Greatly reduced toxin amount produced by the highly toxigenic S-strain in maize	BN30	(Cardwell and Henry, 2004)
Maize	Greatly reduced toxin amount produced by the strain La3228 (70.1-99.9% reduction)	Eleven atoxigenic *A. flavus* strains evaluated	(Atehnkeng et al., 2008)
Maize	Reduction of aflatoxin by up to 88% respectively	NRRL21882, active ingredient of afla-Guard®	(Dorner, 2010)

They reported that co-inoculation of the two in the ratio 1:1 prevented aflatoxin accumulation by *A. flavus* CA28. In contrast, Dorner (2010) reported that there was no significant difference between the effect of two application rates (11.2

and 22.4 kg ha^{-1}) of Afla-Guard® on the aflatoxin contamination and incidence of toxigenic *A. flavus* isolates on maize.

The timing of the application of the BCA is important for its efficacy. Cotty and Bhatnagar (1994) showed that applying AFL^- strains prior to the toxigenic strain reduced contamination of cotton significantly. According to Chang and Hua (2007) inoculation of AFL^- strain TX9-8 and AFL^+ L- and S-strains of *A. flavus* at the same time lead to inhibition of toxin production whereas, when an AFL^- strain TX9-8 was inoculated 24 hrs after toxigenic L- and S-strains of *A. flavus* started growing, there was no reduction in AF accumulation.

Abiotic factors: Temperature and water activity (a_w), both have a direct and indirect effect on the efficacy of BCAs. Both temperature and a_w affect the germination, mycelial growth and sporulation of BCAs (Marín et al., 1998; Köhl and Molhoek, 2001). All microorganisms have a characteristic optimal growth temperature at which they exhibit their highest growth and reproduction rates. Microorganisms also have minimal growth temperatures below which they are metabolically inactive and upper temperature limits beyond which they fail to grow (Sanchis and Magan, 2004). In many ecosystems, temperature fluctuates on a daily and seasonal basis. For example, Pitt and Hocking (2006) evaluated the growth of both AFL^- *A. flavus* and *A. parasiticus* strains inoculated on barley (as a carrier) in the soil and compared the results with growth of the same strains under laboratory conditions. This study revealed that *A. parasiticus* grew readily at 15°C in the laboratory but did not grow at this soil temperature value. Instead, growth in soil occurred when the temperatures were 17.5 and 20°C.

The authors concluded that application of biocontrol inoculum to the soil should be delayed until soil temperature reaches 20°C.

Liquid water is essential for all biochemical processes. For microorganisms, the critical factor is the available liquid water rather than the total amount of water present in a substrate. The amount of water actually available for microbial use is given by the concept of water activity (a_w). Water activity can be decreased not only by solutes (osmotic forces) but also by being absorbed to solid surfaces (matric forces). By definition, a_w of free distilled water is 1.0. Osmotic and matric forces usually lower a_w to some fraction of this value. The amount of water available in a substrate for microbial growth is related to the total moisture content (wet weight basis) by a moisture sorption curve. This helps to determine the actual moisture content and whether microorganisms can grow in it or not. Most microorganisms require a_w values >0.96 for active metabolism, but some filamentous fungi are capable of growth at even lower a_w levels (Magan, 1997). The a_w of a substrate is also linked by an equation to the water potential of the soil, which is another method for assessing the amount of water in soil for microbial growth. Overall, at standard temperature and pressure a change in 0.1 a_w is equivalent to change in water potential of -1.4 MPa.

Biotic factors: Ecosytems are characterised by interactions between diverse microbial populations. The interactions can be positive (additive, synergistic and mutualistic) or negative (competitive and antagonistic). When BCAs are delivered into the field for control of pathogens, they are forced to interact with the native microflora. If the BCA is foreign to the field it is being applied to, it is likely to encounter severe negative interactions with indigenous populations,

particularly if those communities are well established in that niche (Atlas and Bartha, 1998).

These interactions in turn are mediated by abiotic stress factors (temperature and a_w) resulting in dominance of one species or co-existence of different species (Magan and Aldred, 2007a). Thus, a BCA well adapted to the field conditions to which it is applied is likely to give better control of the target pathogen.

Carriers of the BCA: The method of delivering AFL^- spores safely to the soils or to ripening maize or application post-harvest is very important (Pitt and Hocking, 2006; Lyn et al., 2009). Lyn et al. (2009) reported a 97% reduction in aflatoxin from use of the AFL^- *A. flavus* strain K49 formulated into a sprayable, clay-based water dispersible granule compared to 65% reduction when K49 was unformulated. According to Pitt and Hocking (2006) aflatoxin formation was reduced by more than 95% when they used AFL^- formulated strains of *A. flavus* and *A. parasiticus* to control aflatoxin contamination in peanuts in Australia. Formulating a BCA assists in the uniform distribution of conidia in the soil, and some materials such as molasses results in strong adherence of the spores to the grain and also increases the level of nutrients available to the biocontrol agent resulting in good establishment in soil (Pitt and Hocking, 2006).

1.4 Physiological manipulation of BCAs

Many of the niches that fungi occupy are not constant; their characteristics fluctuate frequently and suddenly, presenting stressful situations for their inhabitants (Magan, 2006; Gasch, 2007). Fungi that are xerophilic/xerotolerant

survive large changes in environment because of their well established mechanism of environmentral stress response (Magan, 2006). These fungi are able to synthesise low molecular weight polyols which enable their enzyme systems to function effectively (Magan, 1997). Specifically, *A. flavus* accumulates glycerol and erythritol in its conidia when subjected to osmotic stress (Nesci et al., 2004).

By the same token, for fungal propagules used as BCAs against some pathogens and insect-pests of crops to be effective, they should be able to withstand environmental stress conditions (Köhl and Molhoek, 2001). Indeed, studies have shown that physiological manipulation can enhance stress tolerance and performance of BCAs (Magan, 2006). Improvement is achieved through accumulation of sugars & polyols and these in turn enhance germinability of such spores even at marginal environmental conditions. For example, Hallsworth and Magan (1995) demonstrated that conidia of entomopathogenic fungi: *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces farinosus* that had higher intracellular concentrations of glycerol and trehalose germinated faster and were able to germinate at lower a_w compared to those from other treatments. Abadias et al. (2001) showed that *Candida sake* with higher intracellular concentration of glycerol and erythritol was more resistant to water stress. Indeed, Ruijter et al. (2004) was able to correlate the accumulation of both glycerol and erythritol with the production of glycerol dehydrogenase and erythritol dehydrogenase respectively in *A. oryzae* on wheat grain solid-state culture. They suggested that production of these

enzymes is induced by osmotic stress typical of solid-state fermentations (SSFs).

Gasch et al. (2007) has also suggested that there is a direct correlation between the magnitude of the environmental shift (as a result of modification) and the duration of the adaptation phase, suggesting that more time is required for cells or conidia to adapt to a severe environmental transition. Thus a robust modification characterized by accumulation of high amounts of compatible solutes is likely to shorten the time for adaptation and therefore render a BCA more competitive under unfavourable, fluctuating environmental conditions.

1.4.1 Factors affecting the concentration and types of compatible solutes accumulated in stressed propagules

The concentration and types of compatible solutes accumulated in respose to stress have a profound bearing on the final efficacy of improved inocula of BCAs. It has been demonstrated that these are in turn influenced by a number of factors. These factors are outlined below.

Substrate/medium: Previous studies have shown that a build-up of a particular type of polyol or sugar and concentrations thereof is dependent on the substrate and not the a_w used to effect the modification. For example, in determining the effect of solutes in sugar and polyols accumulation in *C. sake*, Abadias et al. (2001) demonstrated that the highest concentration of total sugars was obtained from the molasses medium amended with glucose regardless of the a_w sought. The highest concentration of polyols were found in cells grown in medium modified with glycerol. Ruijter et al. (2004) also suggested that the mixture of

polyols they obtained in their study should have been a result of substrate (wheat grains) they used in SSF.

Age of modified cells/conidia: It has also been suggested that the concentrations of the different solutes (polyols and sugars) accumulated vary with the age of the modified cells. For example, Abadias et al. (2000) demonstrated that in 24-h-old *C. sake* cells, mannitol was the predominant polyol present while glycerol and arabitol were the most abundant polyols in 48-h-old cells.

Organism being modified: Different organisms exhibit different physiological changes to stress conditions. Hallsworth and Magan (1994) demonstrated that *M. anisopliae* accumulated the highest concentration of total polyol of the three entomopathogenic fungi they grew on glucose media. They also showed that under all conditions tested, compared to *B. bassiana* and *P. farinosus*, conidia of *M. anisopliae* contained the least amount of trehalose.

1.5 Justification of the study

The current investigation has been developed because of the lack of knowledge in some key areas:

1. Dearth of information on the mycotoxicological quality of maize, a major dietary staple in Lesotho;
2. The influence of atoxigenic *A. flavus* strains for control of aflatoxin contamination of crops that have neglected the importance of abiotic factors such as temperature and water stress and their interactions on the competitiveness of biocontrol agents against fungal pathogens.

1.6 Aim and objectives

The present study was primarily aimed at enumerating toxigenic mycobiota associated with stored maize in Lesotho and examining efficacy of atoxigenic *A. flavus* strains for control of aflatoxin production by toxigenic strains on maize. These have been addressed as self-contained Chapters on each component of the work.

Specific objectives:

Chapter 2:

1. To screen and identify mycotoxigenic fungi in stored maize kernels from five main agro-ecological zones of Lesotho in two cropping seasons (2009/2010 and 2010/2011) using molecular methods;
2. To examine fungal isolates for ability to produce mycotoxins;
3. To quantify levels of mycotoxins in maize samples from different regions of Lesotho using a multi-mycotoxin analyses method;

Chapter 3:

4. Examine the sclerotial characteristics of the AFL^{+} and AFL^{-} isolates on three different media;
5. Assess the toxigenicity profiles of the isolates on maize;
6. Determine the genetic relatedness of the isolates ;
7. Test isolates for presence of three structural genes (*aflD, aflP, aflM*) and one regulatory gene *aflR*.

Chapter 4:

8. To evaluate the effect of a_w and temperature and their interactions on:

(a) *in vitro* colony interactions and relative growth rate of two AFL^- local strains from Lesotho when interacting with one AFL^+ strain;

(b) types of interactions between AFL^- and AFL^+ strains and overall competitiveness by computing Index of Dominance (I_D) from their interaction scores on milled maize agar (MMA);

(c) the efficacy of AFL^- strains to inhibit toxin production when grown together in dififerent ratios (100 AFL^+, 25:75, 50:50, 75:25 and 100 AFL^-) with AFL^+ on MMA and on maize grain.

Chapter 5:

9. Compare the relative lag phases prior to growth and mycelial growth rates between AFL^- and AFL^+ strains on MMA;

10. Compare the relative CS utilization patterns of these two groups of strains under different temperatures and a_w regimes;

11. Compare the niche overlap indices (NOIs) to determine whether AFL^- and AFL^+ strains can occupy the same or different niches;

12. Use a rapid Bioscreen C method for quantifying temporal carbon utilisation patterns between AFL^- and AFL^+ strains under interacting temperature x a_w conditions.

Chapter 6:

13. To examine effect of a_w x temperature interactions on:

(a) relative spore germination;

(b) germ tube extension rates;

(c) rates of sporulation;

(d) the key extracellular enzyme production profiles by the two groups of strains of *A. flavus* on Czapek yeast extract agar (CYEA).

Chapter 7:

14. To examine the production of inocula of atoxigenic strain $AFL2^-$ spore propagules *in vitro* by growing it on MMA of different a_w (amended with different solutes such as sorbitol, proline, glycerol, glucose and NaCl) and *in situ* on sorghum;

15. Evaluate germinability and germ tube extension of modified spores on tap water agar (TWA) amended to 0.92 and 0.96 a_w using polyethylene glycol 200 (PEG);

16. Examine efficacy of aflatoxin B_1 control by such spore inocula of atoxigenic strain $AFL2^-$ in mixed cultures (50:50) with toxigenic strain $AFL1^+$ on maize grain.

Figure 1. 8 shows the relationship between the different components of the research and the links between them.

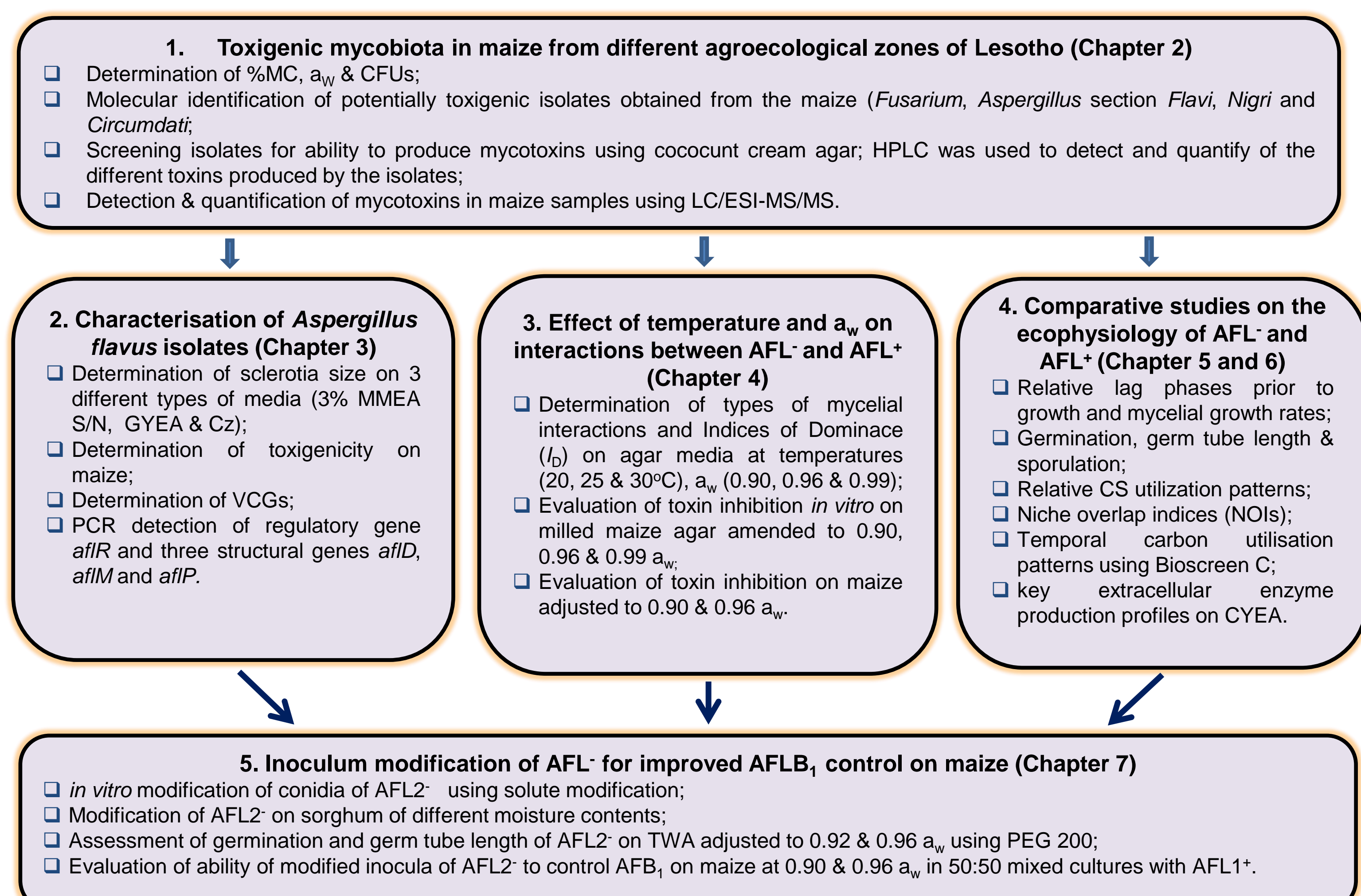

Figure 1. 8 Flow diagram of the different phases of the project.

CHAPTER 2: Mycotoxigenic fungi and mycotoxins associated with stored maize from different regions of Lesotho

2.1 Introduction

Mycotoxigenic fungal genera of economic importance in maize (*Zea mays* L.) growing regions include species belonging to *Aspergillus* and *Fusarium* (Fandohan et al., 2003; Kimanya et al., 2008; Chilaka et al., 2012). These fungi can infect the crop both pre- and post-harvest (Cotty and Jaime-Garcia, 2007; Klich, 2007). Although a developing maize plant is resistant to fungal infection and subsequent contamination with mycotoxins, environmental stress predisposes the crop to contamination. Wounds on the crop caused by birds, insects, hail and/or hot, dry conditions lead to significant infections by these fungi (Windham et al., 1999). Poor drying at harvest and warm, moist storage conditions can result in an increase in infection levels and toxin production (Magan and Aldred, 2007b).

In Africa, with the exception of South Africa, only a few surveys of mycotoxigenic fungi have been carried out in relation to maize (Hell and Mutegi, 2011). Indeed, no surveys have been carried out in Lesotho. This is surprising as maize is the most important dietary staple accounting for about 72% of total cereal production and it constitutes over 80% of the rural diet where 75% of the population live in Lesotho (FAO and EU, 2010). Characteristically, most farmers in Lesotho are resource poor and therefore unable to afford improved methods of production and storage of their produce. Their produce is therefore, susceptible to contamination with mycotoxigenic fungi (Hell and Mutegi, 2011).

Onyike and Nelson (1993) isolated *Fusarium* species including potentially toxigenic isolates (*F. verticillioides*, *F. nygamai*, *F. graminearum* and *F. subglutinans*) from arable soils from the southern lowland regions of Lesotho (**Figure 2. 1**) that had previously been planted to sorghum and millet. However, that study did not determine the toxigenicity profiles of the isolates obtained therein. There are also limited data on the mycobiota of processed cereal products imported into Lesotho (Mohale and Allotey, 2011). This is in contrast to the wealth of studies in neighbouring South Africa that have shown high incidence of *Fusarium* species in maize produced by small holder farmers (Waalwijk et al., 2008; Ncube et al., 2011). Occurrence of *Fusarium* spp. has been positively correlated with fumonisins (FBs) contamination of maize produced by subsistence farmers in South Africa (Waalwijk et al., 2008). Additionally, no information is available on the isolation of *Aspergillus* section *Flavi* or section *Circumdati* species in food crops from Lesotho and whether these fungi are toxigenic or not.

The objectives of this study were: (1) to screen and identify mycotoxigenic fungi in stored maize kernels from five main agro-ecological zones of Lesotho in two cropping seasons (2009/2010 and 2010/2011) using molecular methods; (2) to examine fungal isolates for ability to produce mycotoxins and (3) to quantify levels of mycotoxins in maize samples from different regions of Lesotho using a multi-mycotoxin analyses method.

2.2 Materials and Methods

2.2.1 Survey sites

A country-wide survey was conducted in Lesotho during 2009/10 and 2010/11 cropping seasons. The country is land locked and entirely surrounded by the Republic of South Africa (**Figure 2. 1**). At 1400 metres, Lesotho has the highest base altitude of any country. The climate is temperate with dry, cold winters and hot wet summers. Winter period precipitation is mainly in the form of annual snow fall over the mountainous region and sometimes over the lowlands. Monthly mean winter minimum temperatures of –10.7°C can be reached, and daily winter minimum temperatures can drop as low as -21°C in the mountainous areas. Based on elevation and climate, the country is divided into five agro-ecological zones (**Table 2. 1**).

Table 2. 1 Characteristics of agro-ecological zones in Lesotho.

AEZ	**Characteristics**			
	Area (km^2)	**Altitude (m)**	**Range mean annual rainfall (mm)**	**Range mean annual temperature (°C)**
SRVLS	2690	1400–2000	300-600	12-30
LSLS	3350	<1800	500-800	20-32
LNLS	2550	<1800	800-1000	15-32
FLS	4530	1 800-2000	900–1000	7-20
MLS	18040	2000–3500	1 000-1200	7-20

Key: AEZ = Agroecological zone, SRVLS=Senqu River Valley, LSLS=Southern lowlands, LNLS=Northern lowlands, FLS=Foothills, MLS=Mountains.

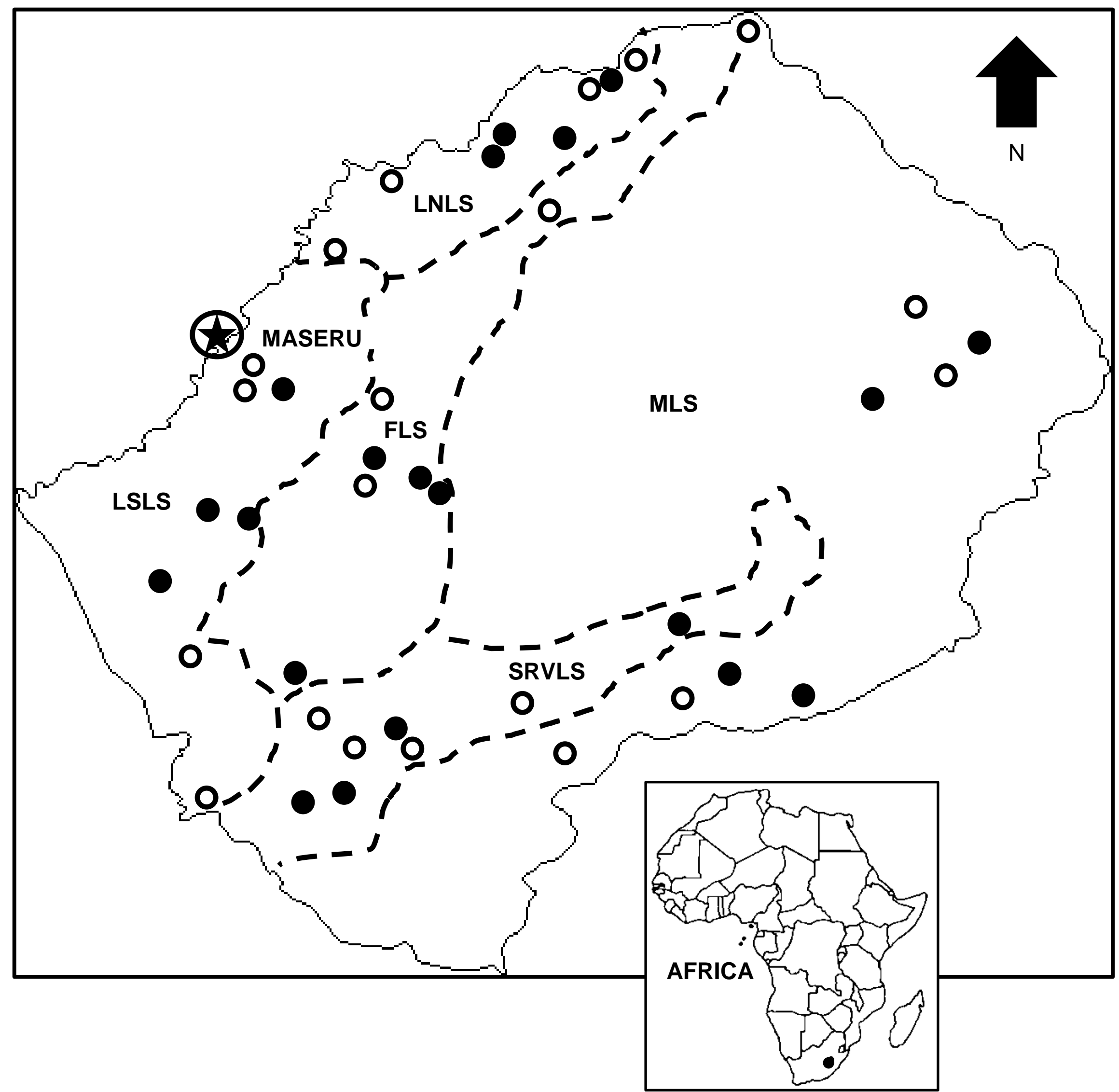

Figure 2. 1 Map of Lesotho showing relative position of agro-ecological zones (AEZ) and sampling locations in 2009/2010 (black circles) and 2010/2011 (white circles) seasons. SRVLS=Senqu River Valley, LSLS=Southern lowlands, LNLS=Northern lowlands, FLS=Foothills, MLS=Mountains.

2.2.2 Sampling

In each season (2009/10 and 2010/11) four villages in each agro-ecological zone were selected (**Figure 2. 1**). Selected villages in each agro-ecological zone were at least 15 km apart.

In each village, a farmer who had grown maize during the 2009/10 and 2010/11 cropping seasons and had it in storage for 4–6 months (October-March) was identified. A representative sample, was obtained by randomly sampling from the farmer's store and combining the subsamples into one lot from which 1 kg was drawn. A total of 40 maize grain samples were collected during the two seasons. The collected samples were sent by courier to the Applied Mycology Group laboratory, Cranfield University, England and kept at 4°C until analysis.

2.2.3 Determination of moisture content and measurement of water activity

A portion of each sample collected, was divided into three 10 g sub-samples which were independently weighed and the mass of each recorded as 'wet weight of sample'. The sub-samples were dried in an oven at 110°C for 24 hrs. Thereafter, they were placed in a desiccator jar containing silica gel and left to cool and weight recorded. The mass was recorded as 'dry weight of sample'. Percentage moisture content (%MC) was then calculated on a dry weight basis.

Before drying a subsample of maize, 5 g of it was placed in a water activity meter container and placed in the chamber of a AquaLab® 3 TE machine (Decagon Devices, Inc., Pullman, Washington, USA). This brought the sample to equilibrium at 25°C and enabled the water activity of the samples to be measured.

2.2.4 Mycological analyses

Enumeration of fungi: Sub-samples (10 g) were soaked for 3 hrs in 90 ml of sterile distilled water supplemented with 0.05% (w/v) technical agar (LP001359; Oxoid LTD, Basingstoke, UK) and 0.025% (w/v) Tween 80 (Razzaghi-Abyaneh

et al., 2006). The samples were then homogenised for 2 min in a stomacher (Lab-Blender 400, Seward Medical, London, UK) in batches of four samples each. Serial dilutions (10^2-10^4) were performed. Aliquots (0.1 ml) from each dilution for each sample were spread plated in triplicate on Dichloran 18% glycerol agar (DG18; CM0729, Oxoid LTD, Basingstoke, UK) in 9 cm Petri plates and incubated at 25°C for 7 days. The colonies growing on the plates were counted and their numbers expressed as colony forming units per gram sample (CFU/g dry weight sample).

Fungal isolation and identification: From each sample, 100 maize kernels were sub-sampled. Fifty (50) kernels from the sub-sample were first surface-disinfected with NaOCl (0.4%) for 2 min and left to dry on sterile filter paper. Subsequently, the dry kernels were directly plated (five kernels per plate) on DG18 (Pitt and Hocking, 2009). The DG18 plates were incubated at 25°C for 7 days. The plates were then inspected visually for fungal growth with the aid of a stereo microscope. Fungal occurrence, i.e., number of maize samples from which *Aspergillus* section *Flavi*, section *Nigri*, section *Circumdati* and *Fusarium* were isolated in each agroecological zone per season (Fandohan et al., 2005) was determined. Subsequently, these fungal genera were sub-cultured on Malt Extract agar (MEA; CM59, Oxoid LTD, Basingstoke, UK) slants and kept at 4°C until identified using molecular methods to species level and tested for toxin production capacity.

2.2.5 Molecular analysis

DNA extraction: The DNA was extracted from mycelia obtained from single spore cultures of the *Aspergillus* section *Flavi* (14), *Nigri* (5), *Circumdati* (6) and

Fusarium (25) grown for 7 days on MMA plates overlaid with cellophane according to the method described by Rodríguez et al. (2012). Mycelia were ground to fine powder with a mortar and pestle in liquid nitrogen. 100 mg of the powder was transferred into a 2 ml Eppendorf tube and mixed with 500 µl of CTAB lysis buffer (140 mM D-sorbitol, 40 mM N-lauroylsarcosine, 20 mM Cetyl Trimethyl Ammonium Bromide (CTAB), 1.4 M NaCl, 20 mM Na_2EDTA, 4.0 M Polyvinylpyrrolidone (PVPP), 0.1 M Tris-HCl, pH 8.0) containing 5 µl of 2-mercaptoethanol. Ten microlitre (10 µl) proteinase K solution (10 mg ml^{-1}) was added to the tubes followed by vortexing for 30 sec. The tubes were incubated at 65°C for 1 h. After, centrifugation at 13000 rpm for 5 min, the supernatant was transferred to a new tube to which 500 µl of chloroform (HPLC grade) was added followed by vortexing for 30 sec. After centrifugation at 13000 rpm for 20 min, the upper layer was transferred into a new tube and 10 µl RNase A solution (10 mg ml^{-1}) added before incubation at 37°C for 1 h. Five-hundred (500 µl) of chloroform was then added, vortexed for 30 sec and centrifuged at 13000 rpm for 5 min at 4 °C. The supernatant was transferred into a new tube and mixed with 500 µl of cold isopropanol to precipitate the DNA. This was followed by centrifugation at 10000 rpm for 2 min and the supernatant was removed. After adding 500 µl of 75% ethanol, and centrifugation at 13000 rpm for 2 min, the supernatant was discarded and DNA was resuspended in 100 µl of Tris-EDTA (TE) buffer pH 8.0 and kept at -20ºC until use as template for PCR amplification.

PCR amplification: PCR reactions for the ITS1-5.8S-ITS2 region were performed using two primer pairs ITS1-ITS2 and ITS3-ITS4 (Bellemain et al.,

2010). The PCR reaction mixture consisted of buffer 10X (5 µl), 50 mM $MgCl_2$ (3 µl), 10 mM dNTPs (1 µl) and 2 µl of 10 µM each primer in a total reaction volume of 50 µl. The amplification program used was: 1 cycle of 5 min at 94ºC, 40 cycles of 1 min at 94 ºC, 1 min at 50ºC and 2 min at 72ºC and finally 1 cycle of 5 min at 72ºC. PCR products were separated on a 100 ml 1.5% agarose in-gel-stained with 10 µl Safeview nucleic acid stain (NBS Biologicals, Cambridgeshire, UK). A DNA molecular size marker of 2.1 – 0.15 kbp (Promega BioSciences, CA, USA) was used to determine the size of the PCR product with the aid of Genesnap version 7.09.02 image acquisition software (Syngene, Cambridge, UK). PCR products amplified with ITS1-ITS2 and ITS3-ITS4 primer pairs ranged between 169-180 and 246-298 bp, respectively. These were purified and sequenced at Macrogen (Netherlands). The sequences obtained were analysed using the ChromasPro software (v.1.5) and compared with other sequences previously deposited in the NCBI database (http://www.ncbi.nlm.nih.gov). The identification of fungal species was based on the BLAST tool of the NCBI database. When assigning an isolate a species name, only BLAST search results showing >99% similarity with an isolate's ITS sequence were considered.

2.2.6 Screening for the ability to produce mycotoxins

Fluorescence on Coconut Cream agar (CAM): Spores from 7 day old growing cultures of *Aspergillus* section *Flavi*, *Nigri* and *Circumdati* isolated from maize kernels were suspended in sterile media consisting of 0.05% (w/v) technical agar and 0.025% (w/v) Tween 80. This was done so as to prevent formation of stray colonies on plates. The suspensions were centrally inoculated in triplicate

with the aid of a loop on Petri plates of 50% coconut cream agar (CAM: (Davis et al., 1987) supplemented with 0.05% chloramphenicol. Two known strains, i.e., aflatoxin-positive strain (*A. flavus* NRRL 3357) obtained from Prof. D. Bhatnagar of the Southern Regional Research Centre, New Orleans, LA, USA and an OTA-positive *A. steynii* strain Collection No. IBT 23096 obtained from Prof. J. C. Frisvad of the Technical University of Denmark, Lynby, Denmark were used as positive controls for aflatoxigenicity and ochratoxigenicity, respectively. The plates were incubated at 25°C in the dark for 10 days after which the reverse of the plates was observed under UV light (365 nm) for formation of blue or greenish blue fluorescence, indicators of ability to produce aflatoxins (AFs) and OTA respectively.

2.2.7 HPLC analysis

Extraction of aflatoxins from cultures: Aflatoxins were extracted from agar plugs cut out with the help of a 3 mm diam. cork borer across the diameter of 10-day old *Aspergillus* section *Flavi* cultures growing on Yeast Extract Sucrose agar (YES: 2% yeast extract, 15% sucrose, 0.05% $MgSO_4 \cdot 7H_20$) at 25°C. The plugs were subsequently placed in pre-weighed 2 ml Eppendorf tubes (Sigma-Aldrich, Chemie GmbH, Steinheim, Germany) and weighed. Five-hundred microlitres (500 µl) of HPLC grade chloroform was added to the tubes and the mixture shaken on a rotary shaker for 30 min. The chloroform portion of the mixture was pipetted into fresh tubes and evaporated to dryness overnight.

Preparation of standards: Two-hundred microlitres (200 µl) of aflatoxin (R-Biopharm Rhône LTD, Darmstadt, Germany) dissolved in methanol comprising of 200 ng each of aflatoxin B_1 (AFB_1) & aflatoxin G_1 (AFG_1) and 60 ng each of

aflatoxin B_2 (AFB_2) & aflatoxin G_2 (AFG_2) was prepared. The stock solution was pipetted into 2 ml Eppendorf tubes and left to evaporate to dryness overnight inside a fume cupboard and thereafter, derivatized as described subsequently.

Derivatization, detection and quantification of aflatoxins by HPLC: Firstly, 200 µl hexane was added to the residue followed by the addition of 50 µl triflouroacetic acid (TFA). The mixture was then vortexed for 30 sec and then left for 5 min. Thereafter, 950 µl of 10% acetonitrile was added and the entire contents of the tube were vortexed for 30 sec after which the mixture was left for 10 min to allow for thorough separation of layers. The hexane layer was discarded and the aqueous layer filtered through syringe nylon filters (13 mm x 0.22 µm, Jaytee Biosciences LTD, UK) directly into amber salinized 2 ml HPLC vials (Agilent Technologies Inc., Palo Alto, CA, USA) for HPLC analysis. All analytical reagents used were of HPLC grade.

A reversed-phase HPLC with fluorescence detection was used to confirm the identity and also to quantify AFB_1, AFB_2, AFG_1 and AFG_2. The HPLC system used constituted a pump, Agilent 1200 series (Model) and a fluorescence detector (excitation and emission wavelength of 360 and 440 nm, respectively). Separation was achieved through the use of C_{18} column (Phenomenex® Gemini; 150 x 4.6, 3 µm particle size: Phenomenex, CA, USA) preceded by a Phenomenex® Gemini C_{18} column; 3 mm, 3 µm guard cartridge. Isocratic elution with methanol:water:acetonitrile (30:60:10, v/v/v) as mobile phase was performed at a flow rate of 1.0 ml min^{-1}. Injection volume was 20 µl. A set of working standards was injected (1 to 5 ng of aflatoxins per injection) and standard curves were generated by plotting the area underneath the peaks

against the amounts of AFB_1, AFB_2, AFG_1 and AFG_2 standards injected. Linear regression was performed in order to establish a correlation relationship (Correlation coefficient, R^2=0.99).

Extraction of fumonisins from cultures: Fumonisins were extracted from agar plugs from 10-day old cultures of *Fusarium* growing on fumonisin-inducing medium containing: 0.05% malt extract, 0.1% yeast extract, 0.1% mycological peptone, 0.1% KH_2PO_4, 0.03% $MgSO_4 \cdot 7H_2O$, 0.03% KCl, 0.005% $ZnSO_4 \cdot 7H_2O$, 0.001% , $CuSO_4 \cdot 5H_2O$, 2% fructose and 1.5% agar (w/v). The plugs were then placed in pre-weighed 2 ml Eppendorf tubes and weighed. Fumonisins were extracted by adding 1000 µl acetonitrile:water (50:50, v/v) and after shaking the mixture for 60 min, the solution was filtered through nylon filters direct into fresh 2 ml Eppendorf tubes and stored at 4°C until analysis.

Derivatization, detection and quantification of FB_1 by HPLC: Sample extract (50 µl) was derivatized by mixing with 200 µl of *o*-phthaldehyde (OPA) solution prepared by dissolving 40 mg OPA in 1 ml of methanol followed by addition of 5 ml of 0.1 M Sodium tetraborate solution and 50 µl of 2-mercaptoethanol (Gnonlonfin et al., 2008). Several, different concentrations of FB_1 standard (50 µg ml^{-1}: Sigma-Aldrich, Chemie GmbH, Steinheim, Germany) were similarly derivatized prior to injection.

The derivatized sample (25 µl) was analyzed by HPLC with a fluorescence detector set at excitation and emission wavelengths of 355 and 440 nm, respectively. Separation was achieved through the use of C_{18} column (Phenomenex® Gemini; 150 x 4.6, 3 µm particle size: Phenomenex, CA, USA).

The mobile phases used were (A) methanol:0.05 M aqueous Sodium dihydrogen phosphate (1:1, v/v) and (B) acetonitrile:water (80:20, v/v) and were pumped at a flow rate of 1.0 ml min^{-1}. A gradient elution program was 100% A during first 5 min followed by rapid increase to 50% B and then holding for 6 min. Thereafter, B was increased to 75% and then 100% a minute later and the system held at this concentration of B for 2 min. Injection volume was 20 µl. A set of working standards was injected (0.025 to 0.0625 ng of fumonisins per injection) and standard curves were generated by plotting the area underneath the peaks against the amounts of FB_1 and FB_2. Linear regression was performed in order to establish a correlation relationship (Correlation coefficient, R^2 = 0.97).

Extraction, detection and quantification of ochratoxin A (OTA) in cultures by HPLC: OTA production was tested in 14 strains belonging to *Aspergillus* section *Nigri* and 6 strains of *Aspergillus* section *Circumdati*. Briefly, OTA was extracted from YES agar plugs obtained from 10 day-old cultures by addition of 1 ml of methanol, followed by shaking for 1 hr. The methanol phase was filtered into HPLC vials and analyzed by reversed-phase HPLC with fluorescence detection. The HPLC conditions were similar as described for AFB_1 except that separation was achieved through the use of C_{18} column (Phenomenex® Luna; 150 x 4.6, 5 µm particle size: Phenomenex, CA, USA). Isocratic elution with acetonitrile:water:glacial acetic acid (57:41:2, v/v/v) was used at a flow rate of 1.0 ml min^{-1}. Injection volume was 20 µl. A set of working standards was injected 1.25 to 6.25 of OTA per injection) and standard curves were generated by plotting the area underneath the peaks against the amounts of OTA. Linear

regression was performed in order to establish a correlation relationship (Correlation coefficient, R^2=0.98).

2.2.9 Extraction and analysis of toxins from maize samples by LC/MS/MS

A targeted metabolomics approach was used to quantify contamination levels in maize grain samples from different geographical regions for the following mycotoxins: DON, AFB_1, FB_1, FUS, BEA, MON and OTA using the multiple mycotoxin method of Sulyok et al. (2006). Briefly, 2 ml of acetonitrile:water:glacial acetic acid (97:2:1, v/v/v) was added to 0.5 g of ground maize kernels followed by shaking for 90 min. The mixture was subsequently centrifuged for 2 min at 3000 rpm supernatant transferred into glass vials and aliquots of 350 µl diluted with the same amount of acetonitrile:water:glacial acetic acid (20:79:1, v/v/v). Five microlitres (5 µl) of diluted extract were injected into the LC/MS/MS system for analysis. For more details on the methodology followed, kindly refer to Sulyok et al. (2006). The LODs (ug/kg) and percentage recoveries in parentheses were: FB_1, 3 (81.5%); FB_2, 1.5 (100%); FB_3, 2 (103.3%), AFB_1 (91.3%); AFB2, 1 (91.7%); Fusaproliferin, 3 (85.2%); Fusaric acid, 5 (95.4%); Moniliformin, 0.5 (71.8%); Beauvericin, 0.02 (154.8%); Zearalenone, 0.3 (96.8%). Analysis of all samples was done in Austria in the Christian Doppler Laboratory for Mycotoxin Research.

2.2.10 Statistical analysis

All data were subjected to Shapiro-Wilk test to determine normality and Levene's test to assess variance homogeneity. Percentage moisture content and water activity data satisfied the two assumptions. Subsequently, one-way analysis of variance (ANOVA) was performed. Conversely, the colony forming

units data violated the two assumptions of ANOVA even after transformations and therefore non-parametric analysis (Wilcoxon /Kruskal-Wallis test: (p=0.05) was used for analysis (Chan and Walmsley, 1997). Where there was significance after Kruskal-Wallis test, median comparisons for each pair of the different agro-ecological zones were achieved through the use of Wilcoxon - Each Pair test (p=0.05) while significance in ANOVA was followed by comparisons of the means using Tukey HSD (p=0.05). Statistical package JMP®10 (SAS Institute Inc., 2008, Cary NC, USA) was used to perform the analysis.

2.3 Results

2.3.1 Moisture content, water activity and mould load of the maize samples

A two-way between-groups analysis of variance was conducted to explore the possible effect of season and agro-ecological zones on the moisture content and water activity of maize samples from Lesotho in 2009/10 and 2010/11 cropping seasons. The means and standard deviations are presented in **Table 2. 2**. There was statistically significant main effect for season, agro-ecological and their interactions on both percentage moisture content (% MC) and a_w. For both responses the size of the main effect of agro-ecological zones was the greatest (**Table 2. 3**). Post-hoc comparisons (Tukey HSD test; p=0.05) indicated that the mean % MC (14.7%) in 2009/10 season, of samples originating from LNLS were significantly higher than any of the other samples collected from all the other regions in the same season. There were no differences in the (% MC)

between regions in 2010/11. In 2009/10, samples from LNLS and FLS had significantly higher a_w (0.50 a_w) than samples from the other regions.

The number of colony forming units between samples collected in the two seasons (**Table 2. 2**) were not significantly different (H(1) = 0.80, *p* = 0.37), with a mean rank of 22 and 19 for 2009/10 and 2010/11 seasons respectively. Similarly, there was no significant difference in CFUs between different ecological zones in both seasons (H(4) = 3.58, p = 0.47) for 2009/10 and (H(4) = 8.6, p = 0.07) for 2010/11.

Table 2. 2 Mean percentage moisture content ,water activity and colony forming units values of the maize samples collected from different agro-ecological zones of Lesotho in 2009/10 and 2010/11 cropping seasons.

Season	AEZ	% MC±SD	a_w±SD[2]	$Log_{10}(2*cfu +1)g^{-1}$ dry sample ±SD[3]
2009/10	SRVLS	11.5±1.6[ab1]	0.48±0.02[bc]	4.35±1.11
	LSLS	9.7±0.6[a]	0.45±0.02[c]	4.08±0.81
	LNLS	14.7±0.3[c]	0.54±0.04[a]	4.87±0.90
	FLS	11.9±0.4[b]	0.53±0.04[ab]	2.92±2.30
	MLS	11.6±0.5[b]	0.48±0.02[bc]	3.33±2.43
2010/11	SRVLS	10.2±1.0	0.42±0.03b	ng[4]
	LSLS	10.8±1.0	0.46±0.04[ab]	4.45±0.72
	LNLS	11.0±0.7	0.50±0.02[a]	3.85±1.10
	FLS	11.3±0.9	0.50±0.04[a]	4.37±1.36
	MLS	9.9±1.1	0.45±0.05[ab]	3.54±2.37

[1]Means in a column followed by the same letter are not significantly different. [2]SD = standard deviation, [3]CFUs were not significantly different between seasons based on Mann-Whitney U test and agroecological zone based on Wilcoxon/Kruskal-Wallis. [4]ng = there was no fungal growth from SRVLS samples.

Table 2. 3 Two-way analysis of variance (ANOVA) for percentage moisture content and water activity of maize samples as a function of cropping season and agro-ecological zone (AEZ).

Response	Factor	*df*	*MS*	*F*	η^2
% MC	Season	1	0.003	13.4*	0.3
	AEZ	4	0.001	6.7*	0.5
	Season x AEZ	4	0.001	3.0*	0.3
a_w	Season	1	15.6	19.7*	0.4
	AEZ	4	8.3	10.5*	0.6
	Season x AEZ	4	5.9	7.5*	0.5

*$p<0.05$

2.3.2 Isolation Frequency and toxigenicity

Colonies and microscopic features of potentially toxigenic fungal genera isolated from maize are shown in **Figure 2. 2**. The mycological analysis of maize samples suggests that *Fusarium* was the principal contaminant of maize grain in Lesotho in the two seasons of the survey. Although frequency of isolation of toxigenic fungi was not significantly different between regions in both seasons. Within regions, there were significant differences between isolation frequencies of the toxigenic genera (**Table 2. 4**). In general, *Fusarium* was the most frequently isolated while *Aspergillus* section *Circumdati* was the least prevalent in both seasons.

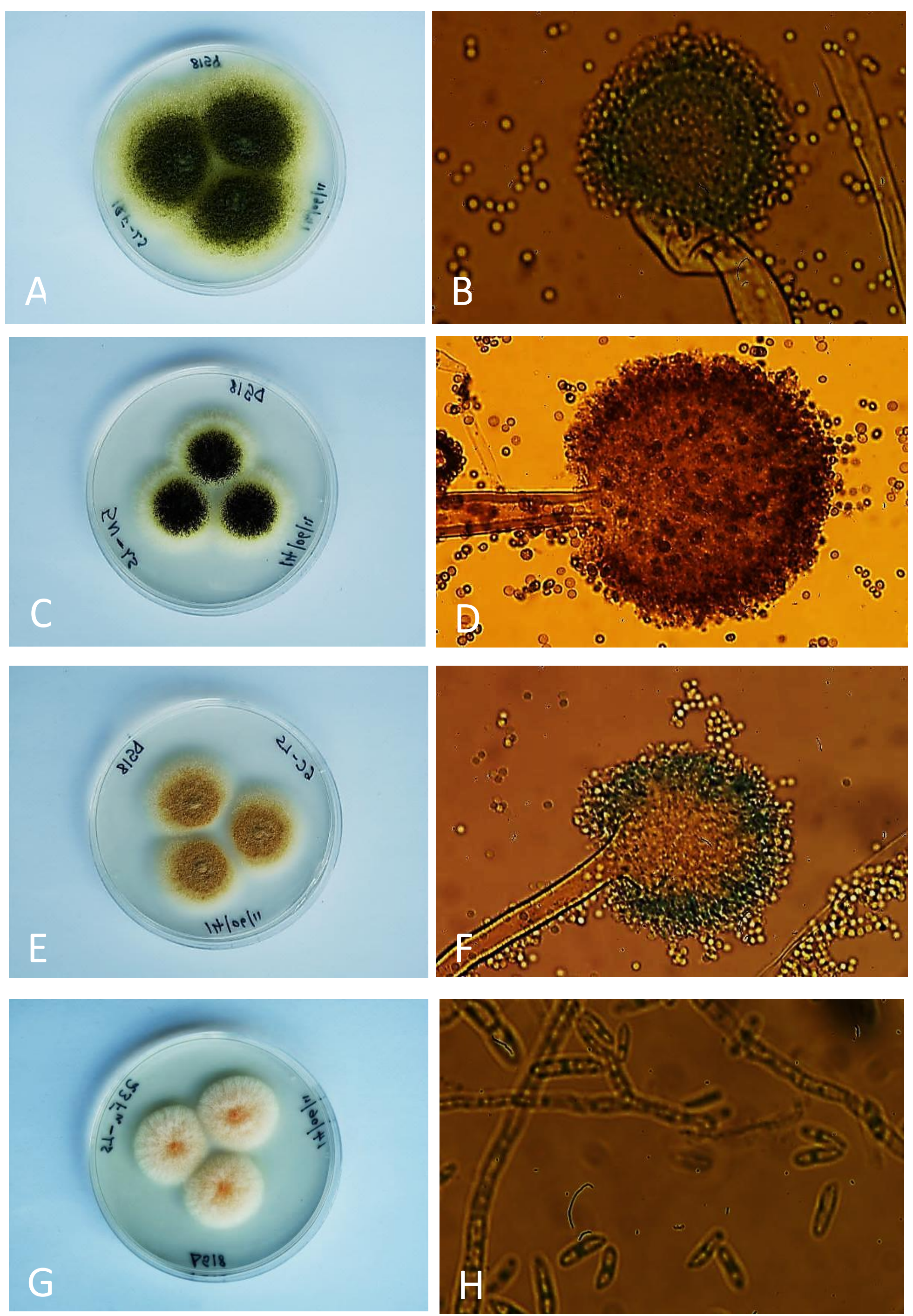

Figure 2. 2 Potentially toxigenic fungal genera isolated from stored maize grain from Lesotho. A, C, E and G: Seven-day-old cultures of *Aspergillus* section *Flavi*, section *Nigri*, section *Circumdati* and *Fusarium* species respectively growing on 9 cm DG18 plates. B, D and F: Conidiophores and conidia 400X of strains belonging to *Aspergillus* section *Flavi*, *Nigri*, *Circumdati* respectively. H. Conidiophores with phialides forming macroconidia and microconidia 400X of *Fusarium*.

Table 2. 4 Isolation frequencies of toxigenic fungal genera recovered from stored maize across Lesotho.

	Mean % Isolation Frequency±SE (range)[a¶]									
	2009/10					**2010/11**				
Genus	**SRVLS[b]**	**MLS**	**FLS**	**LSLS**	**LNLS**	**SRVLS**	**MLS**	**FLS**	**LSLS**	**LNLS**
Fusarium	29±21a (4-90)	39±17a (14-90)	19±6a (4-30)	31±20a (6-90)	10±8a (0-32)	57±22a (4-100)	19±18 (0-74)	52±23a (0-98)	44±19 (0-92)	43±18a (0-76)
Aspergillus section *Flavi*	2±1c (0-4)	2±2b (0-6)	1±1b (0-2)	2±2b (0-6)	nd[c]	nd	nd	1±1b (0-4)	nd	11±8b (0-32)
Aspergillus section *Nigri*	11±11b (0-42)	1.00±1.00b (0-4)	2±1b (0-4)	15±1b (0-44)	1±1b (0-2)	1.50±0.96b (0-4)	nd	nd	nd	1±1c (0-2)
Aspergillus section *Circumdati*	1±1c (0-4)	nd	nd	nd	1±1b (0-2)	nd	nd	nd	nd	1±1c (0-2)

[a]SE = standard error of the mean. [b]Means in each column followed by the same letter are not significantly (p>0.05). [c]nd = No isolates of the particular section were recovered from the maize samples.

Gene sequence analysis of isolates (*Fusarium*, 25; *Aspergillus* section *Flavi*, 14; *Aspergillus* section *Circumdati*, 3; and *Aspergillus* section *Nigri*, 2) was carried out to look at fungal diversity in stored maize from Lesotho. The results revealed dominance of members of *Fusarium* section *Liseola* including *F. proliferatum*, *F. subglutinans*, *F. sacchari* and *F. verticillioides* (**Table 2. 5**) and the majority were able to produce both FB_1 and FB_2 albeit in small quantities. All *F. subglutinans* and *F. sacchari* isolates screened for ability to produce FB_1 were toxigenic.

Of the 14 sequenced *Aspergillus* section *Flavi* isolates, 7 were *A. parasiticus* and they were all isolated from maize from the 2009/10 season (**Table 2. 6**). Six of these isolates were isolated from samples from the warmer, drier regions of LSLS and SRVLS. Only one *A. parasiticus* isolate came from the FLS which is relatively cooler. Three *A. flavus* isolates came from samples from the south-eartern MLS. In 2010/11 season *A. flavus* isolates were recovered from samples that came from FLS and LNLS regions only.

The results of the preliminary screening on CAM of the ability of the sequenced isolates of *A. flavus* and *A. parasiticus* to produce AFs are presented in **Table 2. 6**. The reverse of the CAM plates of 7 (3 from MLS, 2 from SRVLS and 2 from FLS) out of 14 isolates fluoresced blue when observed under UV, an indication of the ability to produce aflatoxins (**Figure 2. 3**). None of the isolates recovered from LSLS and LNLS regions produced toxins on CAM. Eight isolates (4 *A. flavus* and 4 *A. parasiticus*) tested positive for AFB_1 when analysed by HPLC-FLD. Some isolates from the MLS region were able to produce the highest quantities of AFB1 (>100 mg kg^{-1}).

Table 2. 5 Production of fumonisin B_1 and B_2 by *Fusarium* species isolated from subsistence maize from different regions of Lesotho.

Season	**AEZ**	**Isolate ID**	**Species**	**Fumonisin (mg kg^{-1})**	
				FB_1	**FB_2**
2009/10	MLS	*Fu*3LS	*F. sacchari*	0.84±0.43	nd
	MLS	*Fu*4LS	*F. subglutinans*	5.22±0.59	nd
	MLS	*Fu*8LS	*F. verticillioides*	1.38±0.35	nd
	MLS	*Fu*14LS	*F. proliferatum*	0.90±0.02	3.63±0.63
	MLS	*Fu*15LS	*F. proliferatum*	6.59±0.74	nd
	SRVLS	*Fu*2LS	*F. verticillioides*	4.27±0.20	1.22±0.44
	FLS	*Fu*16LS	*F. subglutinans*	1.79±0.37	nd
	FLS	*Fu*22LS	*F. proliferatum*	5.70±0.5	nd
	LSLS	*Fu*5LS	*F. subglutinans*	4.49±0.07	1.54±0.69
	LSLS	*Fu*6LS	*F. proliferatum*	4.24±0.33	nd
	LSLS	*Fu*7LS	*F. subglutinans*	2.70±0.20	1.13±0.34
	LNLS	*Fu*17LS	*F. subglutinans*	6.72±0.68	1.68±0.50
	LNLS	*Fu*23LS	*F. proliferatum*	6.63±0.92	nd
2010/11	MLS	Fu37LS	*F. proliferatum*	nd	1.45±0.37
	MLS	Fu40LS	*F. verticillioides*	4.65±0.60	12.0±1.66
	SRVLS	*Fu*30LS	*F. proliferatum*	0.58±0.10	0.38±0.23
	SRVLS	Fu41LS	*F. proliferatum*	nd	0.74±0.09
	SRVLS	Fu43LS	*F. proliferatum*	nd	0.20±0.04
	FLS	Fu32LS	*F. proliferatum*	nd	0.42±0.02
	FLS	Fu33LS	*F. subglutinans*	nd	0.57±0.13
	FLS	Fu35LS	*F. proliferatum*	nd	0.17±0.10
	LNLS	*Fu*31LS	*F. verticillioides*	nd	0.92±0.17
	LNLS	Fu34LS	*F. verticilloides*	nd	nd
	LNLS	Fu39LS	*F. verticillioides*	0.10±0.04	nd
	LNLS	Fu42LS	*F. verticillioides*	nd	nd

AEZ=Agroecological zone, nd=not detected, MLS=Mountains, LSLS=Southern lowlands, LNLS=Northern lowlands, SRVLS=Senqu river valley, FLS=Foothills.

Table 2. 6 Production of aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) by *Aspergillus* section *Flavi* isolates from subsistence maize collected from different regions of Lesotho.

Season	**AEZ**	**Isolate ID**	**Species**	**Fluorescence on CAM**	**Aflatoxin conc. (µg kg^{-1})**			
					AFB_1	**AFB_2**	**AFG_1**	**AFG_2**
2009/10	MLS	*Af*18LS	*A flavus*	+	22752.9±3729.9	872.2±92.5	nd	nd
	MLS	*Af*23LS	*A flavus*	+	130407.2±91590.1	6083.8±4201.8	nd	nd
	MLS	*Af*25LS	*A flavus*	+	66856.5±9872.4	2396.3±389.4	nd	nd
	SRVLS	*Af*20LS	*A. parasiticus*	+	7674.4±1745.1	424.4±78.8	18685.1±2617.9	1467.5±169.5
	SRVLS	*Af*24LS	*A flavus*	-	nd	nd	nd	nd
	SRVLS	*Af*27LS	*A. parasiticus*	+	3552.1±219.9	nd	7702.8±906.2	342.7±25.7
	SRVLS	*Af*28LS	*A. parasiticus*	-	281.8±65.1	nd	nd	nd
	LSLS	*Af*19LS	*A. parasiticus*	-	nd	nd	nd	nd
	LSLS	*Af*22LS	*A. parasiticus*	-	nd	nd	nd	nd
	LSLS	*Af*26LS	*A. parasiticus*	-	nd	nd	nd	nd
	FLS	*Af*31LS	*A. parasiticus*	+	1627.2±143.5	169.8±=11.5	8050.3±719.6	794.5±32.6
20010/11	FLS	*Af*39LS	*A flavus*)	-	821.3±40.9	nd	nd	nd
	LNLS	*Af*40LS	*A flavus*	-	nd	nd	nd	nd
	LNLS	*Af*42LS	*A flavus*	-	nd	nd	nd	nd
		NRRL3357*	*A flavus*	+	23641.5±1911.8	863.9±47.4	nd	nd

AEZ=Agroecological zone, nd=not detected, i.e. less than limit of detection: < 0.04, 0.016, 0.025, 0.5 and 0.013, 0.3 0.02 ng injectiong^{-1} for AFG1, AFB1, AFG2 and AFB2; MLS=Mountains, LSLS=Southern lowlands, LNLS=Northern lowlands, SRVLS=Senqu river valley, FLS=Foothills, *= Control.

Out of the 6 isolates of *A. ochraceus* recovered from maize samples during the two seasons, 2 which originated from the LNLS region were toxigenic (data not shown). The other four isolates that originated from the SRVLS (2) and LNLS (2) region tested negative for OTA production on CAM. None of the *Aspergillus niger* isolates was positive for OTA. All strains that tested positive for OTA on CAM were confirmed producers when analysed using HPLC-FLD (data not shown).

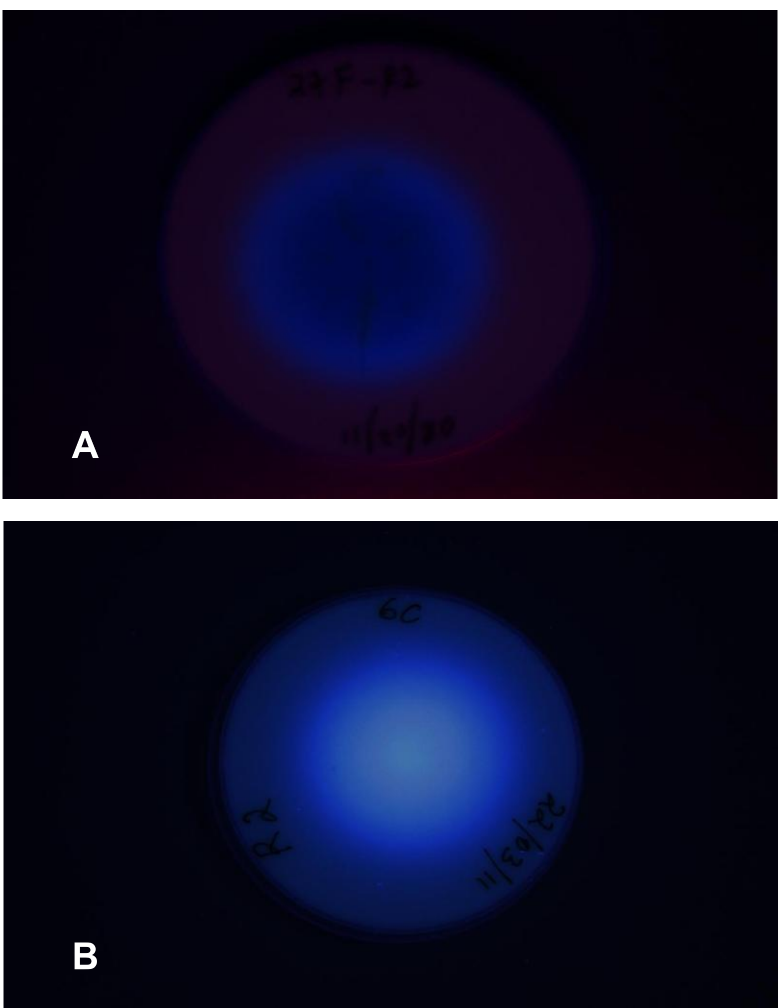

Figure 2. 3 Reverse of CAM cultures of (A) *Aspergillus parasiticus* (strain *Af*27LS) and (B) *Aspergillus ochraceus* (strain *Ac*6LS) fluorescing blue and greenish blue respectively under UV 365 nm after 10 days of incubation at 25°C.

2.3.4 Mycotoxin contamination of maize

Table 2. 7 shows the results of analysis of mycotoxins on maize samples from the different climatic regions during the two seasons using a targeted metabolomic approach. Most of the samples from the different ecological zones were contaminated with FB_1 levels ranging from 2 to 3 µg kg^{-1} in 2009/10 while higher FB_1 contamination levels (6.74 – 935.7 µg kg^{-1}) were recorded in 2010/11. Similarly, MON was also detected from all regions in the two seasons and the amounts in 2009/10 were lower than those for the 2010/11 season. FUS and BEA were also detected although the latter was encountered in 2010/11 samples only. DON was detected from all samples and the levels were much higher in 2010/11 (79–1469 µg kg^{-1}) samples than in the 2009/10 samples (1-2 µg kg^{-1}).

2.4 Discussion

The recommended moisture content level for safe storage of maize is <13%. Generally, all of the samples in both seasons had MC levels <13% except 2009/10 samples from the LNLS region. This corresponded with a_w levels of <0.70 in both seasons. These conditions would not allow growth of most filamentous fungi including *Fusarium* and *Aspergillus* species and subsequent production of mycotoxins (Gnonlonfin et al., 2008).

Thus any occurrence and quantities of fungal propagules on the maize are likely to be a result of initial infection of the crop before harvest, during harvesting or post-harvest drying (Magan and Aldred, 2007b).

Table 2. 7 Mycotoxin levels in maize samples from the different geographical regions of Lesotho in 2009/10 and 2010/11 seasons.

		Mycotoxin levels (μg kg^{-1})±SE									
Season	AEZ	FB_1	FB_2	FB_3	AFB_1	FUS	Fusaric acid	MON	DON	BEA	ZON
2009/10	MLS	3.12±0.45	2.89±0.38	1.88±0.77	nd	nd	nd	4.79*	1.60±0.15	nd	nd
	SRVLS	2.24±0.04	2.54±0.12	1.87±0.95	0.43*	524.8*	nd	320.1*	1.47±0.07	nd	nd
	FLS	2.52±0.22	2.43±0.02	2.02±0.04	nd	nd	nd	213.5±199.6	1.77±0.31	nd	nd
	LSLS	2.63±0.09	2.20±0.17	2.00±0.40	nd	nd	nd	14.6*	1.30±0.03	nd	nd
	LNLS	3.05±0.24	2.15±0.10	1.36±0.40	nd	390.1±162.5	nd	11.8±9.73	1.59±0.24	nd	nd
2010/11	MLS	935.7*	1905.0*	67.2*	nd	nd	1360.2*	484.2±481.9	228.6±76.7	1.39±1.18	nd
	SRVLS	6.74*	nd	nd	nd	145.4*	410.8±355.6	14.6±12.05	82.2±26.4	1.61±0.02	nd
	FLS	6.30±0.11	nd	nd	nd	nd	147.9±54.6	849.4±1167.0	93.0±39.8	1.01±0.76	nd
	LSLS	7.65±0.07	3.63±0.33	1.22*	nd	1007.3*	209.03*	1205.04±1184.3	78.9±34.4	205.4±202.2	11.04*
	LNLS	23.8*	10.5*	nd	nd	128.5*	56.8±15.8	35.1±31.0	1469.4±1432.2	1.16±0.73	10.2*

* Toxin was detected from only one sample. nd=not detected. aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2) and ochratoxin A (OTA) were not detected in any of the samples (data not shown). AEZ=Agroecological zone, MLS=Mountains, LSLS=Southern lowlands, LNLS=Northern lowlands, SRVLS=Senqu river valley, FLS=Foothills, FB_1=fumonisin B_1, FB_2=fumonisin B_2 FB_3=fumonisin B3, AFB_1 =aflatoxin B_1, FUS=fusaproliferin, MON=moniliformin, DON=deoxyninalenol, BEA=beauvericin, ZON=zearalenone.

Samples from northern lowlands (LNLS) consistently had the highest a_w values in the two seasons of the survey when compared to other regions. Northern lowlands, mountains and foothills annually receive greater rainfall than the southern lowlands and the Senqu River Valley (**Table 2. 1**). Since farmers depend on natural sunlight to dry their produce it is common practice to leave the crop in the field up to May in the mountains and mid-winter (June) in the lowlands. Thus, the prevailing climatic conditions during this period (drying period) will determine the final moisture content of the maize crop at harvest. The ambient conditions during storage also affect the moisture content levels of produce. Storage methods in Lesotho are variable and depend on an individual farmer's financial status. Affluent farmers have proper silo structures for storage of their produce while the majority who are rural farmers store their produce in multi-purpose huts. Although this was not investigated, it would appear that the method of storage had no influence on the final moisture content of maize from the different regions of the country.

The mycological analysis suggests that *Fusarium* was the principal, potentially toxigenic contaminant of maize grain followed by *Aspergillus* in Lesotho during the two seasons of the survey. Previously, Fandohan et al. (2005) reported a similar pattern of *Fusarium* > *Aspergillus* in maize from Benin. The low occurrence of *Aspergillus* section *Circumdati* on maize, as shown in the current study has previously been reported in other regions of the world (Magnoli et al., 2006). These suggests that maize might not be the preferred substrate for members of *Aspergillus* section *Cicumdati*. Previous interaction studies on fungi isolated from maize by Lee and Magan (2000) showed low niche overlap index

values of *A. ochraceus* compared to *A. flavus* and *A. niger* on maize-relevant carbon sources over a range of environmental conditions.

The *Fusarium* species isolated were *F. proliferatum*, *F. subglutinans*, *F. sacchari* and *F. verticillioides* and these belong to *Fusarium* section *Liseola*. Most of them produced small quantities of FB_1 and FB_2. Although *F. verticillioides* isolates are known to produce copious amounts of FB_1 (Alberts et al., 1990), isolates of this species from maize which produced amounts of FB_1 comparable to those produced by the isolates in the current study have been reported (Fandohan et al., 2005). Additionally, some *F. verticillioides* isolates were unable to produce FB_1 in the present study. This has previously been reported for isolates of this species (Nelson et al., 1991).

Contrary to reports that *F. subglutinans* and closely related species such as *F. sacchari* (Desjardins et al., 2000) do not produce fumonisins, in the current study, all *F. subglutinans* and *F. sacchari* isolates screened for ability to produce FB_1 were toxigenic as reported previously by Leslie et al. (1992). Co-occurrence of these species on maize has previously been reported in the USA (Munkvold et al., 1998) even though *F. verticillioides* and *P. proliferatum* prefer warmer temperatures than *F. subglutinans* which predominates in cooler temperate areas (Marasas et al., 1979). The impact of climate change factors may explain the occurrence of these fungi in those areas which are known to be unfavourbale (Magan et al., 2011) for example, isolation of *F. subglutinans* from samples that came from drier, warmer LSLS and SRVLS areas as opposed to cooler and wetter MLS and FLS (see **Table 2. 1**). The same is true for the isolation of *F. verticillioides* or *F. proliferatum* in cooler areas.

The isolation of *A. parasiticus* and *A. flavus* from the drier areas of the country in 2009/10 may be due to conditions predisposing colonisation of maize by these species because of their known xerotolerance and growth at 0.80-0.90 a_w. The results of the preliminary screening on CAM have shown that although a qualitative method, CAM is a useful indicator when determining the ability of isolates to produce AFs mycotoxins (Sultan and Magan, 2010). Based on the number of toxigenic isolates and the amounts of AFB_1 (>100 mg kg^{-1}) produced, it is evident that environmental factors in these areas favour *A. flavus* (south-eastern MLS) and *A. parasiticus* (SRVLS). The current findings are a good basis for a bigger study on toxigenic members of *Aspergillus* section *Flavi* particularly in these areas.

In the present study, most samples were contaminated with *Fusarium* mycotoxins. This information reaffirms the observation that different *Fusarium* species, especially members of section *Liseola*, are principal contaminants of maize in different regions of Lesotho. For example, *Fusarium verticillioides* produces FBs only, while *F. proliferatum* and *F. subglutinans* co-produce FBs, FUS, MON and BEA (Ritieni et al., 1997; Shephard et al., 1999). We also believe that the trace amounts of some of these mycotoxins in maize could be an influence of environmental conditions prevailing at the time. Indeed, production of secondary metabolites by *Fusarium* is affected by temperature and relative humidity (Marín et al., 2004). DON was detected from all samples and the levels were much higher in 2010/11 (79–1469 µg kg^{-1}) samples than in the 2009/10 samples (1-2 µg kg^{-1}). Paradoxically, none of the fungal isolates tested produced DON in culture. The current observation concur with previous

reports that members of *Fusarium* section *Liseola* do not produce DON (Thiel et al., 1982). Thus, contamination of maize with DON could have occurred pre-harvest on ripening maize, most probably by *Fusarium graminearum* species complex. Because the maize was stored for up to 6 months before sampling the species was not isolated from the maize samples directly. They may have been succeeded by members of *Fusarium* section *Liseola* during ripening and harvesting. Previous studies have shown that during competition, *F. verticillioides* suppresses the growth of *F. graminearum* (Velluti et al., 2000). Additionally, *F. verticillioides* tends to colonise maize previously infected with *F. graminearum* more rapidly (Picot et al., 2012).

In summary, this is the first detailed study on the toxigenic fungi associated with stored maize from different agroclimatic regions of Lesotho. It suggests predominance of fumonisin producing *F. proliferatum*, *F. verticillioides* and *F. subglutinans* across the different regions of the country and prevalence of highly toxigenic *A. flavus* and *A. parasiticus* in the south-eastern and south-western regions respectivley. Exposure of consumers to both AFB_1 and FB_1 in Lesotho is low when compared to some areas in South Africa where it has been linked to oesophageal cancer (Shephard et al., 2007). However, the relatively high concentrations of DON, MON and FUS suggests that rural populations in Lesotho may be exposed to a mixture of mycotoxins. Understanding the potential implications of such exposure requires more detailed toxicological investigations.

CHAPTER 3: Characterisation of toxigenic and atoxigenic *Aspergillus flavus* strains from stored maize from different regions of Lesotho

3.1 Introduction

A description of *in vitro* toxigenicity profiles of *A. flavus* isolates from maize in different regions of Lesotho was given in **Chapter 2**. Most toxigenic (AFL^+) strains of *A. flavus* together with their atoxigenic (AFL^-) counterparts were recovered from maize samples originating from the southern and south eastern regions of the country. The levels of aflatoxin B_1 (AFB_1) produced by some strains were >100 mg kg^{-1} while for some strains, even on a conducive YES medium no aflatoxins (AFs) were detected.

Indeed *A. fla*vus populations are genetically diverse and co-occurrence of phylogenetically related AFL^+ and AFL^- strains in agricultural commodities is quite common (Hua et al., 2012). Genetic variation in *A. flavus* is reflected in the ability to produce AFs, sclerotial morphology, vegetative compatibility and at a molecular level. Based on sclerotial size, there are L-strains which produce large sclerotia (average diameter >400 µm) and variable amounts of aflatoxins, while S-strain isolates produce small sclerotia (average diameter <400 µm). S-strains consistently produce higher levels of aflatoxins than L-strain isolates (Cotty, 1989). Some strains of *A. flavus* do not form sclerotia and according to Abbas et al. (2005) they are unlikely to produce AFs.

In vegetative compatibility (see Section **1.2.3**), hyphae of individuals that are vegetatively compatible fuse to form a heterokaryon. Parasexual recombination can occur between nuclei of the two individuals through mitotic recombination resulting in a progeny that has traits of either parent hyphae (Glass et al., 2000). Although this has been used to study genetic diversity of *A. flavus* in various agricultural fields (Hua et al., 2012), hyphal anastomosis in *A. flavus* is difficult to perceive. Therefore, complementary nitrate-non-utilising (*nit*) mutants are used to identify vegetatively compatible isolates (Bayman and Cotty, 1991). First, *nit* mutants are generated on potassium chlorate medium and then phenotyped using different nitrogen sources. A mutant unable to utilise a particular nitrogen source is classified as shown in **Table 3. 1**. For a more detailed description of the *nit* mutants (see Section **1.2.3**).

Previous studies have also shown molecular variation in *A. flavus* populations. While the presence of a complete aflatoxin gene cluster in some AFL^+ and AFL^- isolates of *A. flavus* (Criseo et al., 2008) has been reported, complete deletions of some genes and mutations in AFL^- strains have also been observed (Ehrlich and Cotty, 2004; Chang et al., 2005). According to Pitt and Hocking (2006), isolates that have deletions or mutations in the aflatoxin gene are good candidates for screening for potential use as biocontrol agents (BCAs) because it is unlikely they can revert to toxigenicity.

There was interest in understanding the nature of 3 strains isolated from stored maize from Lesotho (2 atoxigenic and 1 toxigenic) as there could be among them, potential candidates for use in biocontrol of aflatoxin contamination in Lesotho.

Table 3. 1 Phenotypic classification of *nit* mutants

***nit* mutant**	**Utilisation of Nitrogen source**				**Deficiency**
	NH_4^+	NO_3^-	NO_2^-	Hypoxanthine	
niaD	√	X	√	√	Structural gene product for nitrate reductase
nirA⁻	√	X	X	√	Regulatory protein for synthesis of nitrate and nitrite reductase
cnx	√	X	√	X	molybdenum-containing co-factor for nitrate reductase and xanthine dehydrogenase
CRUN	√	√	√	√	-

√ = able to utilise the N source

X = cannot utilise the N source

CRUN = chlorate resistant sectors which are able to utilise nitrate

The objectives of this study were to (a) examine the sclerotium characteristics *A. flavus* strains on three different media, (b) assess the toxigenicity profiles of the strains on maize, (c) determine the genetic relatedness of the isolates and (d) test isolates for presence of three structural genes (*aflD*, *aflP*, *aflM*) and one regulatory gene *aflR* in the biosynthetic gene cluster for aflatoxin biosynthesis.

3.2 Materials and Methods

3.2.1 Fungal Strains

Three isolates of *A. flavus* isolated from farmer maize stores in Lesotho were used in this study. The isolates were designated with the code *Af*IDLS, where *Af* refers to *A. flavus*, ID is the isolate identity number and LS stands for Lesotho. These isolates were *Af*18LS, *Af*19LS and *Af*24LS. A type strain NRRL3357 provided by Prof. D. Bhatnagar of the Southern Regional Research Centre, New Orleans, LA, USA was included as a positive control for AF production. Strain EGP15, isolated from Egyptian peanuts, was included as a negative control for AF production. The two strains were also used as positive controls in the molecular studies. EGP15 was previously shown to be positive for the genes that were being studied (Abdel-Hadi, 2011).

3.2.2 Morphological characterisation

Media: The following media were used in this study. Czapek Solution Agar (Cz): 30 g sucrose, 3 g $NaNO_3$, 0.5 g $MgSO_4 \cdot 7H_2O$, 1 g K_2HPO_4, 15 g agar, 1000 ml distilled water; Yeast Extract Glucose Agar (GYEA: 0.5% yeast extract, 2% glucose and 1 ml of trace element solution, 1000 ml distilled water) and 3% maize meal extract agar with 30 g sucrose and 3 g $NaNO_3$ (3% MMEA S/N:

(Nesci et al., 2007). The medium was prepared by mixing 15 g of coarsely ground maize flour with 500 ml distilled water. The mixture was brought to a boil by heating, followed by holding at that temperature for 30 min and then left to cool before being sieved through four layers of muslin cloth. Ten grams (10 g) of agar were added to 500 ml of the maize extract and this was thoroughly mixed before autoclaving at 121°C for 15 min. Both Cz and GYEA have been shown to result in profuse production of sclerotia (Cotty, 1989). MMEA S/N was used because the Lesotho *A. flavus* strains were isolated from stored maize.

Sclerotial production by *Aspergillus flavus* group isolates: For each isolate, a spore suspension in 0.05% (w/v) technical agar and 0.025% (w/v) Tween 80 was prepared from 7 days old malt extrat agar (MEA) cultures. With the aid of a loop a suspension for each isolate was centrally inoculated on Petri plates containing the three media described above. Inoculation was done in triplicates on each medium. The isolates were incubated at 30°C in the dark for 14 days. Conidia were washed from the plates with 70% isopropanol (IPA). This was followed by dislodging of sclerotia with a spatula onto a filter paper. The dislodged sclerotia were cleaned with water in a beaker with repeated rinsing and decanting and then air dried. The number and diameter of the dried sclerotia were estimated. Diameters of the sclerotia (15 sclerotia per each replicate plate per media) were measured with the help of Axiovision image Analysis on the assumption that the sclerotia were spherical. Measurements were done in two directions perpendicular to each other. The number of sclerotia produced on each replicate plate were also counted.

3.2.3 Toxigenicity of isolates

Maize mycoflora: The original mycoflora of the maize was assessed for contamination with *Aspergillus* section *Flavi* by sampling 100 kernels from the bag of maize and plating them on DG18 (5 kernels per plate) and incubating for 7 days at 25°C. The kernels were found to be free of contamination of members of *Aspergillus* section *Flavi*. On the basis of this information, it was decided to use the maize grain to assess the effect of *in situ* production of AFs by strains from Lesotho. Testing isolates on different media for ability to produce AFs is important because capacity by isolates to produce toxins is medium dependent (Mehl and Cotty, 2013).

Treatment application: Maize grains (10 g) were placed in glass culture vessels with a microporous lid (Magenta, Sigma Ltd, UK). Subsequently, two-hundred microlitres (200 µl) of spore suspension (approx. 10^{6} spores ml^{-1}) of each of the following strains: 2 AFL^{-} strains (*Af*19LS and *Af*24LS) and AFL^{+} strain *Af*18LS was inoculated onto the maize in each glass vessels. The containers were gently shaken to ensure even distribution of spores on the maize. The vessels were placed in a plastic chamber and incubated at 25°C for 14 days. After 14 days, samples were ground and kept at -20°C until extraction and clean-up prior to HPLC analysis. Treatments were done in triplicate. Strain EGP15 and *A. flavus* NRRL3357 were also inoculated in separate vessels and they served as AFL^{-} and AFL^{+} controls respectively.

Extraction of aflatoxins: For *in vitro* studies, AFs were extracted from agar plugs cut out with a 3 mm diam. cork borer across the diameter of 10 days old *A. flavus* cultures grown on Yeast Extract Sucrose agar (YES: 2% yeast extract,

15% sucrose, 0.05% $MgSO_4$ $7H_2O$) at 25°C. The plugs were subsequently placed in pre-weighed 2 ml Eppendorf tubes (Sigma-Aldrich, Chemie GmbH, Steinheim, Germany) and weighed. Five-hundred microliters (500 µl) of HPLC grade chloroform was added to the tubes and the mixture shaken in a rotary shaker for 30 min. The chloroform portion of the mixture was pipetted into fresh tubes and evaporated to dryness overnight. Although the Lesotho strains had already been screened for the ability to produce AFs (see Section **2.3.2**) on YES, it was important to re-evaluate their toxigenicity because secondary metabolite production is not a stable trait, i.e., some fungal cultures loose ability to produce toxins particularly if not preserved properly.

Aflatoxins were extracted from ground maize (10 g) with 100 ml 80% methanol followed by shaking for 2 h and filtering through Whatman No.1. The filtrate was diluted 5 fold with phosphate buffered saline (PBS). 20 ml diluted filtrate was passed through an Neogen immunoaffinity (Neogen, Ayr, Scotland) followed by washing with with 20 ml 25% methanol and AFB_1 was eluted with 1.5 ml of 100% methanol. The eluate was dried under a stream of nitrogen gas before derivatisation and subsequent HPLC analysis.

Derivatization, detection and quantification of AFB_1: Firstly, 200 µl hexane was added to the residue followed by the addition of 50 µl triflouroacetic acid (TFA). The mixture was then vortexed for 30 sec and left for 5 min. Thereafter, 10% acetonitrile solution was added and the entire contents of the tube vortexed for 30 sec after which the mixture was left for 10 min to allow for thorough separation of layers. The hexane layer was discarded and the aqueous layer filtered through syringe nylon filters nylon (13 mm x 0.22 µm,

Jaytee Biosciences LTD, UK) directly into amber salinized 2 ml High Performance Liquid Chromatography (HPLC) vials (Agilent Technologies Inc., Palo Alto, CA, USA) for HPLC analysis. All analytical reagents used were of HPLC grade.

Reversed-phase HPLC with fluorescence detection was used to confirm the identity and also quantify AFB_1. The HPLC system used constituted a pump, Agilent 1200 series and an Agilent fluorescence detector (excitation and emission wavelength of 360 and 440 nm, respectively). Separation was achieved through the use of a C_{18} column (Phenomenex® Gemini; 150 x 4.6 mm, 3 µm particle size: Phenomenex®, CA, USA) preceded by a Phenomenex® Gemini C_{18} column; 3 mm, 3 µm guard cartridge. Isocratic elution with methanol:water:acetonitrile (30:60:10, v/v/v) as mobile phase was performed at a flow rate of 1.0 ml min^{-1}. Injection volume was 20 µl. A set of working standards was injected (1 to 5 ng of AFB_1 per injection) and standard curves were generated by plotting the area underneath the peaks against the amounts of AFB_1. Linear regression was performed in order to establish a correlation relationship (Correlation coefficient, R^2=0.99).

3.2.4 Vegetative compatibility groups

Media: One-thousand millilitres (1000 ml) of basal medium comprising: 30 g sucrose; 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_20$; 0.5 g KCl; 10 mg $FeSO_4 \cdot 7H_2O$; 15 g agar and trace element solution 0.2 ml was prepared. Minimal medium (MM) was made by adding 3 g of $NaNO_3$ to 1 litre of basal medium. *Nit* mutants were generated on MM amended with 4% potassium chlorate (MMC).

Generation of nitrate non-utilising mutants: Agar plugs of mycelia of 3 *A. flavus* isolates from Lesotho maize along with the two controls were transferred from potato dextrose agar (PDA) to 4% MMC. Culture plates were incubated at 30°C and the margins of the colonies with restricted growth were examined regularly for fast growing sectors, consisting of sparse mycelium (Bayman and Cotty, 1991). These chlorate resistant sectors were then transferred to MM with ammonium tartrate replacing $NaNO_3$ as the sole nitrogen source. Only *nit* mutants that were resistant to chlorate grow as wild-type isolate on MM containing ammonium. A minimum of two *nit* mutants from each strain were obtained.

Nit *mutants phenotypes*: The phenotypes of the *nit* mutants were determined by growing the mutants on four media amended with different nitrogen sources: sodium nitrite (0.5 g/litre), ammonium tartrate (1 g/litre), sodium nitrate (3 g/litre) and hypoxanthine (0.2 g/litre).

Complementation tests: A heterokaryon is formed by vegetatively compatible *nit* mutants when grown on MM, i.e., a line of wild-type growth at the point where the mutants touch. Pairings comprised atoxigenic mutants against toxigenic ones only. No self-compatibility was determined.

3.2.5 PCR amplification

DNA extraction: DNA was extracted from mycelia obtained from single spore cultures of strains *Af*18LS, *Af*19LS, *Af*24LS and two control strains: NRRL3357 and EGP15 grown for 7 days on MMA plates overlaid with cellophane at 25°C. For DNA extraction, the method previously optimised by Rodríguez et al. (2012)

was carried out. Mycelia were ground to a fine powder with a mortar and a pestle in liquid nitrogen. Hundred milligram (100 mg) of the powder was transferred into a 2 ml Eppendorf tube and mixed with 500 µl of CTAB lysis buffer (140 mM D-sorbitol, 40 mM N-lauroylsarcosine, 20 mM Cetyl Trimethyl Ammonium Bromide (CTAB), 1.4 M NaCl, 20 mM Na_2EDTA, 4.0 M Polyvinylpyrrolidone (PVPP), 0.1 M Tris-HCl, pH 8.0) containing 5 µl of 2-mercaptoethanol. Ten microlitre (10 µl) proteinase K solution (10 mg ml^{-1}) was added to the tubes followed by vortexing for 30 sec. The tubes were incubated at 65°C for 1 h. After, centrifugation at 13000 rpm for 5 min, the supernatant was transferred to a new tube to which 500 µl of chloroform (HPLC grade) was added followed by vortexing for 30 sec. After centrifugation at 13000 rpm for 20 min, the upper layer was transferred into a new tube and 10 µl RNase A solution (10 mg ml^{-1}) added before incubation at 37°C for 1 h. Five-hundred (500 µl) of chloroform was then added, vortexed for 30 sec and centrifuged at 13000 rpm for 5 min at 4 °C. The supernatant was transferred into a new tube and mixed with 500 µl of cold isopropanol to precipitate the DNA. This was followed by centrifugation at 10000 rpm for 2 min and the supernatant was removed. After adding 500 µl of 75% ethanol in water, and centrifuging at 13000 rpm for 2 min, the supernatant was discarded and DNA was resuspended in 100 µl of Tris-EDTA (TE) buffer pH 8.0. DNA yield was estimated spectrophotometrically with the Picodrop (Picodrop Ltd, Cambridgeshire, UK) and kept at -20 °C until being used as template for PCR amplification.

PCR amplification: PCR amplifications were performed in 25 µL containing $MgCl_2$-free reaction buffer, 1.5 mM $MgCl_2$, 1.25 U of Taq polymerase, 200 µM of

each dNTP, 0.2 μM of each primer (see **Table 3.2** for primers used) and 1 ng/μL of template DNA. The amplification PCR conditions were: (1) 1 step at 94 °C for 3 min; (2) 30 cycles of the following three steps: 1 min at 94 °C, 1 min at 55 °C, 1 min 72 °C; and (3) one final 10 min step at 72 °C. For each gene assayed, a non-template control PCR reaction was used to confirm that the constituents of the reaction mixture were not contaminated.

PCR products were separated on a 100 ml 1.5% agarose in-gel-stained with 10 μl Safeview nucleic acid stain (NBS Biologicals, Cambridgeshire, UK) and separated by submerged electrophoresis using 1XTAE buffer at 80V for 1h. Next, amplification products were visualized using G:Box iChemi (Syngene, Cambridge, UK). The products were photographed using the integrated camera and the software GeneSnap (Syngene, Cambridge, UK). A DNA molecular size marker of 2.1 – 0.15 kbp (Promega BioSciences, CA, USA) was used to determine the size of the PCR product.

Table 3.2 Details of the target genes, primer sequences and expected PCR product length in base pairs (bp).

Primer pair	**Gene**	**Primer sequence (5' - 3')**	**Optimal annealing temperature**	**PCR product length (bp)**	**Reference**
aflR-F	*aflR*	CGAGTTGTGCCAGTTCAAAA	55	400	(Geisen, 1996)
aflR-R		AATCCTCGCCCACCATACTA			
omtA-F	*aflP*	GTGGACGGACCTAGTCCGACATCAC	65	537	(Geisen, 1996)
omtA-R		GTCGGCGCCACGCACTGGGTTGGGG			
nor1-F	*aflD*	ACCGCTACGCCGGCACTCTCGGCAC	65	797	(Geisen, 1996)
nor1-R		GTTGGCCGCCAGCTTCGACACTCCG			
ver1-F	*aflM*,	GCCGCAGGCCGCGGAGAAAGTGGT	65	999	(Geisen, 1996)
ver1-R		GGGGATATACTCCCGCGACACAGCC			

3.3 Results

3.3.1 Morphology and AFB_1 production profiles

Two *A. flavus* strains (*Af*18LS and *Af*19LS) from Lesotho maize along with the controls NRRL3357 and EGP15 assayed produced sclerotia on GYEA (**Figure 3. 1**). Only *Af*19LS produced sclerotia when grown on all the 3 different media. *Af*24LS did not produce sclerotia on any of the media used. Average sclerotial diameter of all the sclerotia-producing strains was >400 µl. *Af*19LS produced the greatest number of sclerotia on all media compared to the other strains. *Af*18LS and the toxigenic control (NRRL3357) produced fewer sclerotia. Only *Af*18LS and toxigenic strain NRRL3357 produced AFB_1 on both YES and viable maize kernels. The quantities of AFB_1 produced by *Af*18LS and strain NRRL3357 were significantly different on viable maize (Table 3. 3).

3.3.2 Genetic and molecular analysis

Twelve chlorate resistant sectors (2 each for atoxigenic strains *Af*19LS, *Af*24LS and control EGP15; and 3 each for toxigenic strain *Af*18LS and control NRRL3357) were obtained and phenotyped on four different nitrogen sources. Sectors that were chlorate resistant but able to utilise nitrate (CRUN) were generated from all the 3 strains isolated from the Lesotho maize and control strain EGP15 (**Table 3. 4**). Sixty percent (60%) of the total (12) chlorate resistant sectors that were phenotyped were *nia*D mutants (**Figure 3. 2**). *cnx* was generated from *Af*19LS only. *nirA*$^{-}$ mutants were generated from the two control strains (NRRL3357 and EGP15). Mutants from toxigenic and atoxigenic strains were paired and compatibility was identified by a line of luxuriant growth at the zone of interaction. All the pairings failed to generate the heterokaryon.

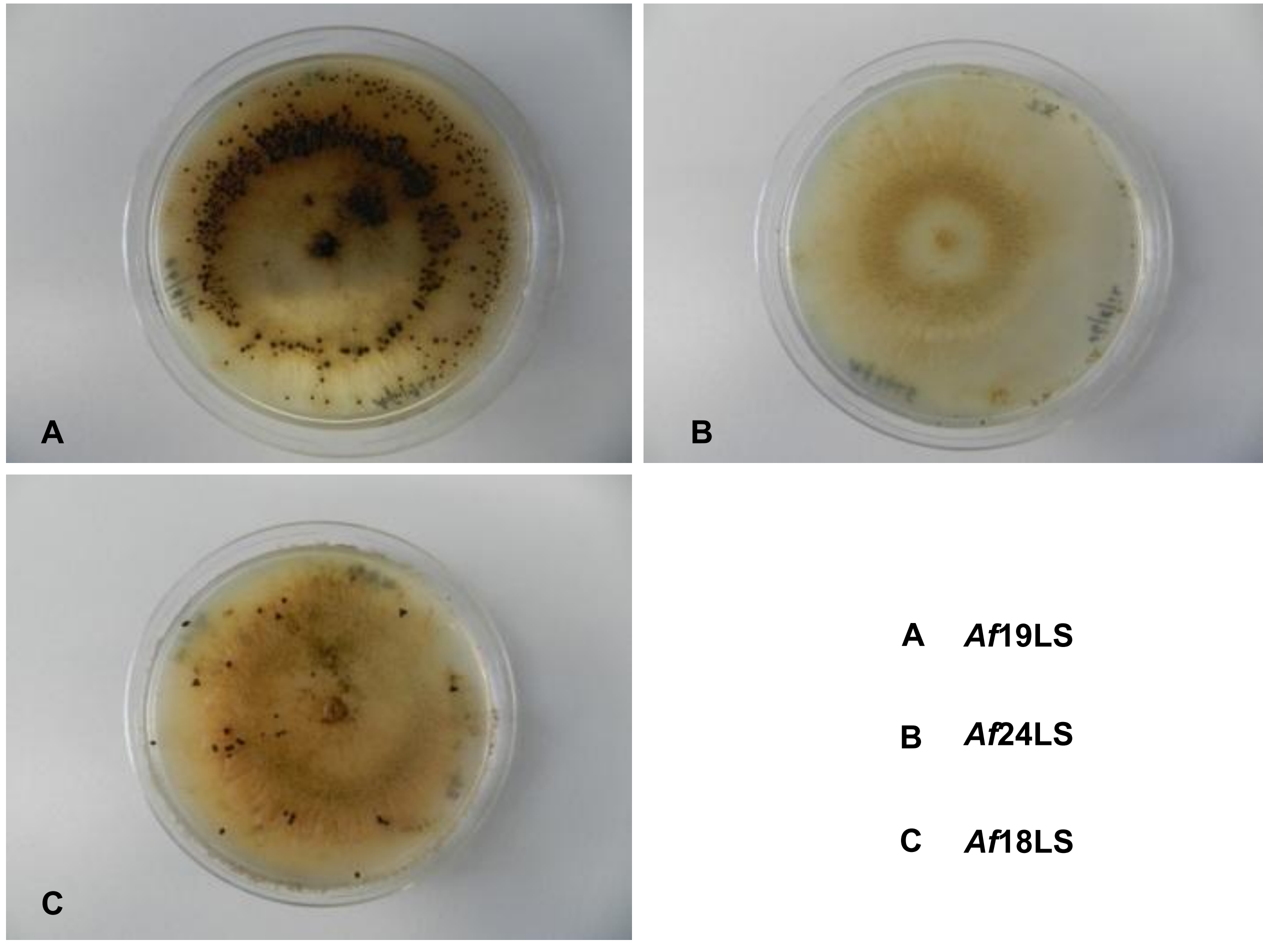

Figure 3. 1 Sclerotial production by *Aspergillus flavus* strains (*Af*19LS, *Af*24LS and *Af*18LS) on glucose yeast extract agar (GYEA) after 14 days of incubation at 30°C in the dark.

Table 3. 3 Sclerotial size, number and AFB_1 production by *A. flavus* strains.

Strain	**Sclerotia size (μm)±SD**[a]			**Sclerotia numbers per plate ±SD**[b]			**AFB_1 ng g^{-1}±SD**[cd]	
	Cz	**GYEA**	**MMEA S/N**	**Cz**	**GYEA**	**MMEA S/N**	**YES**	**Viable Maize**
*Af*18LS	-	768.9±108.6	-	-	23.7±5.0	-	2678.3±3.2a	2906.2±2.2a
*Af*19LS	548.3±91.9	919.4±115.6	799.4±254.0	>1000	556.3±17.6	15.3±5.5	ND	ND
*Af*24LS	-	-	-	-	-	-	ND	ND
Controls								
EGP19	444.0±58.11	515.6±77.2		99.7±17.8	98.7±10.3	-	ND	ND
NRRL3357	883.5±216.1	666.22±35.1		53.7±9.6	11.3±2.1		2725.2±3.1a	2474.58±2.1b

[a]For sclerotial size, results are means of 15 sclerotia for each medium type while sclerotia numbers are averages of three replicates per strain. [b]For sclerotia forming strains, differences in sclerotia number and diameter were significant between media types ($p<0.05$). [c]Similarly, AFB_1 values are means of three replicates. The a_w of viable maize kernels was amended to 0.96 a_w prior to inoculation with fungal spores. [d]Differences in quantities of AFB_1 produced by toxin producing strains between media were significant ($p<0.05$). For AFB_1 production potential, values for a variable within each column followed by the same letter are not significant ($p>0.05$) based on t-test. Only AFB_1 producing strains were compared. Standard deviations (SD) were calculated based on log transformed AFB_1 (ng g^{-1} medium) amounts. ND = Not detected.

Table 3. 4 Classification of mutants based on nitrogen utilisation patterns

	Chlorate resistant	**Nitrogen source**				
Wild type	**sector ID**	**NH_4^+**	**NO_3^-**	**NO_2^-**	**Hypoxanthine**	**Mutant**
*Af*18LS	*Af*18-1	√	X	√	√	*niaD*
*Af*18LS	*Af*18-2	√	√	√	√	CRUN
*Af*18LS	*Af*18-3	√	X	√	√	*niaD*
*Af*19LS	*Af*19-1	√	√	√	√	CRUN
*Af*19LS	*Af*19-2	√	X	√	X	*cnx*
*Af*24LS	*Af*24-1	√	√	√	√	CRUN
*Af*24LS	*Af*24-2	√	X	√	√	*niaD*
NRRL3357	NR-1	√	X	X	√	*nirA*$^-$
NRRL3357	NR-2	√	X	√	√	*niaD*
NRRL3357	NR-3	√	X	√	√	*niaD*
EGP15	EGP-1	√	X	X	√	*nirA*$^-$
EGP15	EGP-2	√	√	√	√	CRUN

√ = nitrogen source utilised, X = unable to utilise nitrogen source, CRUN = chlorate resistant mutants able to utilise nitrate as nitrogen source.

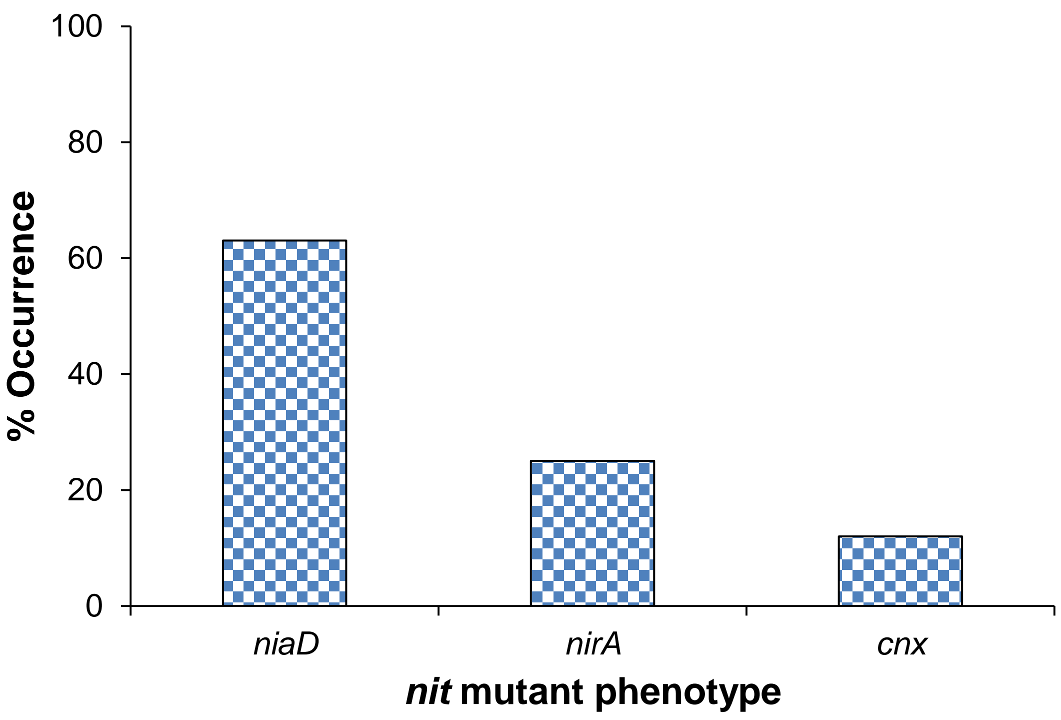

Figure 3. 2 Relative numbers of three classes of mutants (*niaD*, *nirA* and *cnx*) obtained from the 5 strains of *Aspergillus flavus* assayed. At least one type of mutant from each strain was recovered.

Figure 3. 3 shows the band patterns of electrophoresis after PCR amplification of some genes involved in the aflatoxin biosynthesis pathway for both toxigenic and atoxigenic *A. flavus* strains. Strains *Af*18LS and *Af*19LS together with controls: NRRL3357 and EGP15 were positive for the amplicons of three structural genes (*aflM, aflD*, and *aflP*) and the regulatory gene *aflR*. Non-sclerotia producing, atoxigenic strain *Af*24LS was positive for *afl*D only.

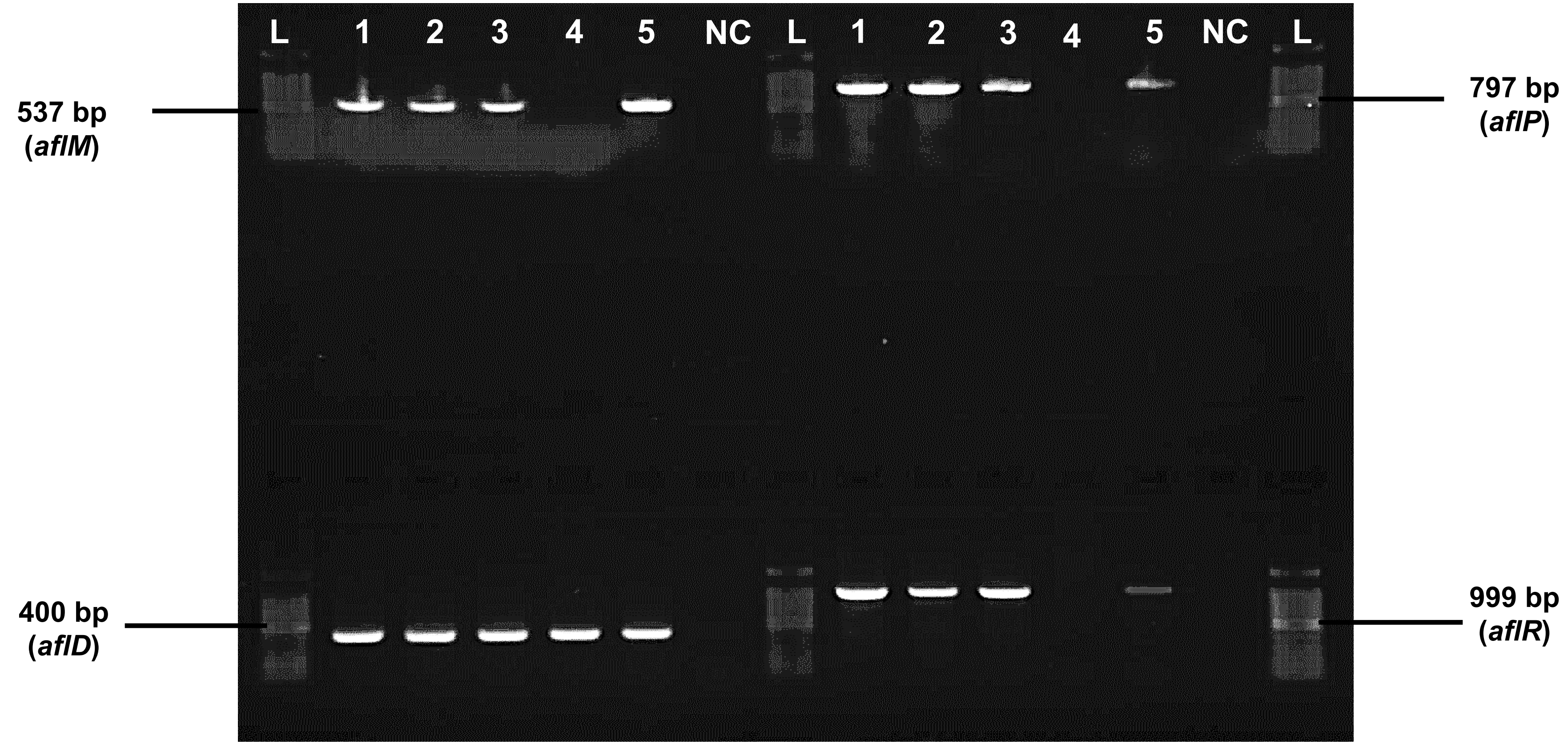

Figure 3. 3 Electrophoresis patterns of PCR amplicons *aflM, alfP*, *aflD* and *aflR* on agarose gel, Lane L- DNA molecular size marker of 2.1-0.15 kbp; Lane 1- *Af*18LS; Lane 2 - *f*19LS; Lane 3 - EGP19; Lane 4 – *Af*24LS; Lane 5 – NRRL3357 and Lane NC – negative control (reaction mixture with no templates).

3.4 Discussion

This is the first study on variability in *A. flavus* associated with stored maize from different regions of Lesotho. Although 2 of the 3 strains from Lesotho maize produced large sclerotia, the numbers of sclerotia they produced was different. Cotty (1989) suggested a basis for differentiating between *A. flavus* isolates using sclerotial size and number. According to the criterion, *A. flavus* comprises L-strains that produce few and large sclerotia (average scletorial diameter >400 μm) and S-strains with numerous, small sclerotia (average scletorial diameter <400 μm). The atoxigenic strain *Af*19LS on average produced large sclerotia, especially on Cz medium. This strain could be closely related to the S-strain even though it did not produce any aflatoxins. According to Cotty et al. (1994), although S-strain morphotypes are relatively rare in nature, they consistently produce high quantities of aflatoxins and numerous, small sized sclerotia.

There was a striking similarity between toxigenic strain *Af*18LS and the type strain NRRL3357 in the number of sclerotia produced. The two produced a very small number of sclerotia. Strain *Af*24LS did not produce any sclerotia or aflatoxins on any of the media used. Previously, Calvo et al. (2004) reported that in mutants of *A. parasiticus* in which *veA* was deleted, *afl*R and *afl*J were not expressed and that sclerotia and AF intermediate versicolorin A were not produced. This is a possible explanation for the inability of *Af*24LS to neither produce AFs nor form sclerotia. Indeed, our results are in agreement with Abbas et al. (2005) suggestion that sclerotia non-producers are unlikely to produce aflatoxins.

The AFL^+ and AFL^- strains were vegetatively incompatible, i.e., they did not form a heterokaryon when paired, suggesting that the two groups are genetically isolated. Vegetative compatibility is an indicator of genetic relatedness. Mutants of strains belonging to the same VCG are able to exchange genetic material through the formation of a heterokaryon (Novas and Cabral, 2002). Indeed, the stored maize ecosystem harbours diverse populations of *A. flavus*. Based on *nit* mutants generated from such strains and subsequent complementation tests, strains are classified into vegetative compatibility groups (VCGs: (Cove, 1976). In the current study, the *nit* mutants recovered were in the following proportions *niaD* (63%), *nirA* (25%) and *cnx* (13%). Previously, Novas and Cabral (2002) reported a similar trend. The relative numbers of *nit* mutants recovered in their study were *niaD* > *nirA* > *cnx*.

Huang et al. (2011) suggested that toxin inhibition during interaction between AFL^- and AFL^+ is independent of VCGs. In contrast, Wicklow and Horn (2007) found the opposite effect. Based on these observations, AFL^- strains from Lesotho could potentially be used to control aflatoxin production by *Af*18LS without reservations as they would not form a heterokaryon resulting in greater toxin accumulation.

Strains *Af*18LS and *Af*19LS were positive for the amplicons of the four genes assayed. *Af*18LS produced AFB_1 *in vitro* on YES and *in situ* on maize while *Af*19LS did not. Abdel-Hadi et al. (2011) demonstrated that the presence of a gene that encodes for aflatoxin production does not *per se* imply that the strains they examined produce aflatoxins. They also showed that production of toxins was dependent on the media. Some of the isolates they examined did not

produced toxins *in vitro* but did *in situ*. *Af*24LS was only positive for *afl*D. These results support the suggestion that if a strain is a non-sclerotia former, which is the case with strain *Af*24LS, it is likely to be atoxigenic.

Several studies have recently shown very low but positive correlation between expression of some genes and toxin production. Depending on how well it competes with AFL^-, strain *Af*24LS is good candidate for screening as biocontrol control agent. Provided it is not used to control toxin production by a member of its same VCG, it is less likely to revert to toxigenicity.

CHAPTER 4: Effect of environmental factors on *in vitro* and *in situ* interactions between atoxigenic and toxigenic *A. flavus* strains and control of aflatoxin contamination of maize

4.1 Introduction

Most growing crops are colonised by a wide range of microorganisms in both the rhizosphere and phyllosphere. The dominance of individual or groups of species may be due to abiotic factors or due to the ability to compete effectively in a particular ecological niche. This is sometimes promoted by the ability to produce secondary metabolites (Magan and Aldred, 2007a). There are significant problems in peanuts and other nuts, maize and cotton with aflatoxin (AF) contamination due to infection by *Aspergillus* section *Flavi*, especially *A. flavus* (Wu, 2004; Lewis et al., 2005).

There has thus been an interest on the use of biological control strategies to minimise aflatoxin (AF) contamination of these economically important commodities. These approaches include the use of atoxigenic strains (AFL^-) of *A. flavus* which can compete effectively with the toxigenic strains (AFL^+) in soil or the application of bacteria or yeasts, especially to maize, for control of AF contamination from silking to harvest of the maize cobs (Abbas et al., 2006). The former approach has been now applied effectively in cotton in the USA and maize in West Africa by using individual or mixtures of local AFL^- strains (Dorner, 2004; Dorner, 2010).

There has been interest in the mechanism of action of such AFL^- strains and their ability to control AFL^+ strains and reduce aflatoxin production under

different environmental conditions. Indeed Magan and Lacey (1984) showed that intra-strain or inter-species interactions are influenced by both water availability, temperature and the nutritional nature of the substrate niche. They used an Index of Dominance (I_D) approach to examine such colony based interactions by quantitative scores of different species based on whether they mutually intermingled, were antagonistic either on contact or at a distance, or one species/strain able to dominate others under different environmental conditions.

Indeed, Magan and Aldred (2007a) suggested that interactions between different species or strains of fungi are in a state of flux because of the strong influence that environmental conditions play on their competitiveness. This approach has been successfully used to evaluate the competitiveness of different fungi in different agro-ecosystems (Magan and Aldred, 2007a; Marín et al., 1998). Interestingly, for AFL$^-$ strains this approach has not been previously used to assist in the selection of potentially effective candidates for biocontrol of AFL$^+$ strains. It is critical to have knowledge of the relative ability of an AFL$^-$ strain to control an AFL$^+$ strain and reduce AF production over a defined range of environmental conditions. It has been suggested that environment affects the interaction between AFL$^+$ *A. flavus* and *Fusarium verticillioides* strains as well as between AFL$^-$ and AFL$^+$ strains based on relative growth rates and carbon utilisation patterns when examining niche overlap between them (Giorni et al., 2009).

It has also been suggested that AFL$^-$ strains of *A. flavus* may outcompete toxigenic strains for space and also for nutrients. Reduced sporulation of AFL$^+$

strains in the presence of AFL^- strains is testimony to the ability of AFL^- strains to compete effectively (Mehl and Cotty, 2010). Recently, Huang et al. (2011) suggested that inhibition of toxin production was not due to competitive exclusion, but elicited by direct hyphal contact, a phenomenon referred to as "touch inhibition". This suggests that when the two strains come into contact, the aflatoxin biosynthetic pathway is some how affected. Again, no account of environmental stress on such interactions was considered.

The objectives of this study were to evaluate the effect of a_w and temperature on (a) *in vitro* mycelial interactions between two AFL^- strains from Lesotho when interacting with their toxigenic counterparts, (b) the relative efficacy of the AFL^- in mixed cultures with AFL^+ in the following ratios: 100% AFL^+, 25:75, 50:50, 75:25 and 100% AFL^- on maize-based media and on maize grain to inhibit toxin production by the latter.

4.2 Materials and Methods

4.2.1 Fungal strains

Three isolates of *A. flavus* isolated from farmer maize stores in Lesotho were used in this study. The isolates were designated with the code *Af*IDLS, where *Af* refers to *A. flavus*, ID is the isolate identity number and LS stands for Lesotho. These isolates were *Af*18LS, *Af*19LS and *Af*24LS. A type strain NRRL3357 provided by Prof. D. Bhatnagar of the Southern Regional Research Centre, New Orleans, LA, USA was included as a positive control for AF production. Strain EGP15, isolated from Egyptian peanuts, was included as a negative control for AF production.

4.2.2 Media and inoculum preparation

Malt extract agar (MEA, Oxoid Ltd, Basingstoke, UK) and milled maize agar (MMA; 3% maize meal, 2% agar) was used to quantitatively evaluate the effect of a_w on interaction between the atoxigenic and toxigenic strains. The a_w of both media was amended with glycerol to achieve 0.99, 0.96 and 0.90 a_w values by addition of appropriate amounts of glycerol. Media was autoclaved for 20 min at 121°C and the molten cooled media poured into 9 cm Petri plates. Coconut cream agar (CAM: 50% coconut cream, 0.05% Chloramphenicol, 2% agar) was adjusted to the same a_w values using glycerol.

Spore suspensions were prepared from 7-10 day old MEA cultures of the different strains by suspending conidia from the plates in 10 ml sterile water in Universal bottles containing 0.05% (w/v) agar and 0.025% (w/v) Tween 80.

4.2.3 Types of interactions between strains, estimation of Index of Dominance (I_D) and relative colony area

Spore suspensions of each of the paired strains, i.e., AFL^+ and AFL^- prepared as described above, were inoculated 40 mm apart on 9 cm Petri plates containing MEA or 3% MMA. After inoculation, the treatment and replicate plates of the same a_w were sealed in polyethylene bags and incubated at the chosen temperatures 20, 25 and 30°C for 12 days. The treatments were examined regularly and the type of interaction identified and each strain given a score according to Magan and Lacey (1984), i.e., mutual intermingling (1); mutual antagonism when the two colonies meet i.e. on contact (2); mutual antagonism at a distance i.e. the colonies are clearly separate and mycelial fronts do not meet (3); dominance of one species on contact (4 dominant strain;

0 for the inhibited strain); dominance of one species at a distance (5 dominant strain, 0 for the inhibited strain). Numerical scores for each strain in these interactions were added and relative comparisons of competitiveness of individual AFL^- versus AFL^+ strains compared under different environmental conditions to obtain a total Index of Dominance (I_D). All experiments were carried out with three replicates per treatment and repeated twice. Controls consisted of three replicates of each AFL^- and AFL^+ strain centrally inoculated on the same treatment plates at each a_w x temperature condition.

4.2.4 The effect of environmental factors and different ratios of mixed populations of atoxigenic and toxigenic strains on temporal AFB_1 production

The spore concentration for all strains prepared as described above was adjusted to approx. 10^6 spore ml^{-1} with the aid of a haemocytometer and a compound microscope. Subsequently, spores of the AFL^+ and AFL^- strains were mixed in the following ratios: 100% AFL^+; 75:25; 50:50; 25:75 and AFL^- for each combination. The relative ratios made up to 1 ml were combined and 0.1 ml was centrally inoculated on CAM while 0.2 ml was spread plated on MMA media plates. Toxigenic and atoxigenic strains growing alone (100%) on both CAM and MMA served positive and negative controls respectively. CAM plates with the same a_w were sealed in polyethylene bags and incubated at 25°C in the dark for 7 days after which the reverse of the plates was observed under UV light (365 nm) for the interference of a blue fluorescence halo (Degola et al., 2011). MMA plates with the same a_w were also sealed in polyethylene bags and incubated at 25°C for 28 days. Periodically (7, 14, 21 & 28 day of incubation), 5 agar discs (3 mm diam.) were removed with a sterile cork borer randomly

across the diameter of the Petri plate. The agar plugs were placed in pre-weighed (≈1.0774±0.006 g) 2 ml Eppendorf tubes (Sigma-Aldrich, Chemie GmbH, Steinheim, Germany) and kept at -20°C until HPLC analysis. All experiments were conducted with at least 12 replicates per treatment to enable destructive sampling to be done at each time.

4.2.5 Maize grain studies

Moisture adsorption curve: Undamaged sound French maize kernels were used in this study. The initial a_w of the maize kernels was 0.64±0.02. Sub-samples of 10 g of maize grain were placed in Universal bottles. Different amounts of water were added to the maize grain. These were allowed to equilibrate at 4°C overnight. They were then equilibrated at 20-25°C and the a_w and moisture content measured. The a_w was measured using an AquaLab® 3 TE (Decagon Devices, Inc., Pullman, Washington, USA). The moisture content was determined by drying in a drying oven at 117°C for 17 h. The adsorption curve of amount of added water against a_w was plotted and used to determine the exact amount required to modify the maize grain to 0.90 and 0.96 a_w (see **Appendix 1**).

Maize mycoflora: The original mycoflora of the maize was assessed for contamination with *Aspergillus* section *Flavi* by sampling 100 kernels from the bag of maize and plating them on DG18 (5 kernels per plate) and incubating for 7 days at 25°C. The kernels were free of contamination of members of *Aspergillus* section *Flavi*. On the basis of this information, it was decided to use the maize grain to assess the effect of *in situ* co-inoculation of AFL^- and AFL^+ strains on aflatoxin contamination by the latter strains.

Treatment application: All the three AFL^- strains ($AFL1^-$, $AFL2^-$ and $AFL3^-$) were evaluated against the AFL^+ strains ($AFL1^+$ and $AFL2^+$) using the same approach as for the *in vitro* trials. Thus, 100% of each AFL^- or AFL^+ strain and ratios of 75:25; 50:50 and 25:75% spore inoculum were used on the maize grain. The a_w of the maize was modified by addition of the required water from the moisture adsorption curve minus 200 µl and equilibrated at 4°C for 48 h in sealed plastic chambers. The maize grains (10 g) were placed in glass culture vessels containing a microporous lid which allows humidity and air exchange (Magenta, Sigma Ltd, UK). Subsequently, 200 µl of each treatment spore suspension (approx. 10^6 spores ml^{-1}) was added to make up the pre-determined water amounts required and thoroughly mixed. The treatment vessels together with untreated controls were placed in plastic chambers and closed with the lid. In each plastic chamber 2 glass jars (500 ml) containing glycerol-water solutions appropriate to maintaining the equilibrium relative humidity in the chamber at the target treatment a_w were also placed in the chambers. The chambers were incubated at 25°C for 14 days. The glycerol-water solutions were replaced with fresh solutions after 7 days incubation. Three replicates per treatment were used and the experiment repeated twice. After 14 days, samples were ground and kept at -20°C until extraction and clean-up prior to HPLC analysis.

4.2.6 Aflatoxin analyses

Extraction of aflatoxins: For *in vitro* studies, aflatoxins were extracted from MMA agar plugs with 500 µl chloroform followed by shaking for 30 min. The chloroform portion of the mixture was pippeted into fresh tubes and the

chloroform evaporated to dryness overnight before derivatisation and HPLC analysis.

Aflatoxins were extracted from ground maize (10 g) with 100 ml 80% methanol followed by shaking for 2 h and filtering through Whatman No.1 filter paper. The filtrate was diluted 5 fold with phosphate buffered saline (PBS). 20 ml diluted filtrate was passed through a Neogen immunoaffinity column (Neogen, Ayr, Scotland) followed by washing with 20 ml 25% methanol and AFB_1 was eluted with 1.5 ml of 100% methanol. The eluate was dried under a stream of nitrogen gas before derivatisation and subsequent HPLC analysis.

Derivatization, detection and quantification of AFB_1: Firstly, 200 µl hexane was added to the residue followed by the addition of 50 µl triflouroacetic acid (TFA). The mixture was then vortexed for 30 sec and then left for 5 min. Thereafter, 950 µl of 10% acetonitrile solution was added and the entire contents of the tube were vortexed for 30 sec after which the mixture were left for 10 min to allow for thorough separation of layers. The hexane layer was discarded and the aqueous layer filtered through syringe nylon filters nylon (13 mm x 0.22 µm, Jaytee Biosciences LTD, UK) directly into amber salinized 2 ml HPLC vials (Agilent Technologies Inc., Palo Alto, CA, USA) for HPLC analysis. All analytical reagents used were of HPLC grade.

A reversed-phase HPLC with fluorescence detection was used to confirm the identity and also quantify AFB_1. The HPLC system used constituted a pump, Agilent 1200 series (Model) and a fluorescence detector (excitation and emission wavelength of 360 and 440 nm, respectively). Separation was

achieved through the use of C_{18} column (Phenomenex® Gemini; 150 x 4.6, 3 µm particle size: Phenomenex, CA, USA) preceded by a Phenomenex® Gemini C_{18} column; 3 mm, 3 µm guard cartridge. Isocratic elution with methanol:water:acetonitrile (30:60:10, v/v/v) as mobile phase was performed at a flow rate of 1.0 ml $min^{-}1$. Injection volume was 20 µl. A set of working standards was injected (1 to 5 ng of aflatoxins per injection) and standard curves were generated by plotting the area underneath the peaks against the amounts of AFB_1. Linear regression was performed in order to establish a correlation relationship.

4.2.7 Statistical analysis

Shapiro-Wilk test (p=0.05) was used to establish whether data was from a normally distributed population followed by Levene's test (p=0.05) to determine variance homogeneity and when these assumptions were met (p>0.05), data were subjected to two-way ANOVA: p=0.05) with inoculum ratio (5 levels), toxigenic strain (2 levels), and water activity (2 levels) as the main effects and amount of AFB_1 produced as a response. Mean comparisons for each pair of the different independent variables was done using Tukey's HSD. The statistical package JMP®8 (SAS Institute Inc., 2008, Cary NC, USA) was used in the analysis.

4.3 Results

4.3.1 Effect of temperature, substrate and a_w on Index of Dominance (I_D) and types of interaction between atoxigenic and toxigenic strains

The effect of temperature, substrate (medium) and a_w on interaction type and I_D on MEA and MMA are shown in **Table 4. 1**. Generally, temperature, a_w and

substrate influenced the types of interaction between the three AFL^- and two AFL^+ strains. On MEA under warmest and wettest conditions (30°C; 0.96 and 0.99 a_w) AFL^+ dominated over AFL^- strains. However, the AFL^- strains were more dominant under the driest conditions at the same temperature. Overall, at lower temperatures (20, 25°C), regardless of a_w, AFL^+ and AFL^- strains were mutually antagonistic. On the nutritionally poorer MMA (see **Table 4. 1** and **Figure 4. 1**) neither of the two groups of *A. flavus* dominated each other, i.e., they were mutually antagonistic either on contact or at a distance. The AFL^+ strains were more competitive at 30°C on MEA and had greater total I_D values.

Table 4. 1 Effect of water activity (a_w) and temperature on numerical interaction scores and Index of Dominance (I_D) of of each three AFL$^-$ strains when paired with each of the two AFL$^+$ strains on MMA and MEA after 12 days incubation. The underlined and bold scores indicate the changes in competitiveness which can occur (see Section 4.2.3 for a description of the scores).

Media	Interacting strains	**Temperature (°C)**											
		20				**25**				**30**			
		0.99	**0.96**	**0.90**	***I_D***	**0.99**	**0.96**	**0.90**	***I_D***	**0.99**	**0.96**	**0.90**	***I_D***
MEA	AFL1$^+$/1$^-$	2/2	2/2	3/3	**7/7**	2/2	2/2	2/2	**6/6**	0/4	0/4	4/0	**4/8**
	AFL2$^+$/1$^-$	2/2	2/2	3/3	**7/7**	2/2	2/2	2/2	**6/6**	0/4	0/4	4/0	**4/8**
	I_D score	**4/4**	**4/4**	**6/6**	**14/14**	**4/4**	**4/4**	**4/4**	**12/12**	**0/8**	**0/8**	**8/0**	**8/16**
	AFL1$^+$/2$^-$	2/2	2/2	3/3	**7/7**	2/2	2/2	2/2	**6/6**	0/4	0/4	4/0	**4/8**
	AFL2$^+$/2$^-$	2/2	2/2	3/3	**7/7**	2/2	2/2	2/2	**6/6**	0/4	0/4	4/0	**4/8**
	I_D score	**4/4**	**4/4**	**6/6**	**14/14**	**4/4**	**4/4**	**4/4**	**12/12**	**0/8**	**0/8**	**8/0**	**8/16**
	AFL1$^+$/3$^-$	2/2	2/2	3/3	**7/7**	2/2	2/2	2/2	**6/6**	**<u>0/4</u>**	**<u>0/4</u>**	2/2	**2/10**
	AFL2$^+$/3$^-$	2/2	2/2	3/3	**7/7**	2/2	2/2	2/2	**6/6**	0/4	0/4	2/2	**2/10**
	I_D score	**4/4**	**4/4**	**6/6**	**14/14**	**4/4**	**4/4**	**4/4**	**12/12**	**0/8**	**0/8**	**4/4**	**4/20**
MMA	AFL1$^+$/1$^-$	**<u>3/3</u>**	3/3	3/3	**9/9**	2/2	3/3	3/3	**8/8**	2/2	3/3	3/3	**8/8**
	AFL2$^+$/1$^-$	3/3	3/3	3/3	**9/9**	2/2	3/3	3/3	**8/8**	2/2	3/3	3/3	**8/8**
	I_D score	**6/6**	**6/6**	**6/6**	**18/18**	**4/4**	**6/6**	**6/6**	**16/16**	**4/4**	**6/6**	**6/6**	**16/16**
	AFL1$^+$/2$^-$	3/3	3/3	3/3	**9/9**	2/2	3/3	3/3	**8/8**	2/2	3/3	3/3	**8/8**
	AFL2$^+$/2$^-$	3/3	3/3	3/3	**9/9**	2/2	3/3	3/3	**8/8**	2/2	3/3	3/3	**8/8**
	I_D score	**6/6**	**6/6**	**6/6**	**18/18**	**4/4**	**6/6**	**6/6**	**16/16**	**4/4**	**6/6**	**6/6**	**16/16**
	AFL1$^+$/3$^-$	3/3	3/3	3/3	**9/9**	2/2	3/3	3/3	**8/8**	2/2	3/3	3/3	**8/8**
	AFL2$^+$/3$^-$	3/3	3/3	3/3	**9/9**	2/2	3/3	3/3	**8/8**	2/2	3/3	3/3	**8/8**
	I_D score	**6/6**	**6/6**	**6/6**	**18/18**	**4/4**	**6/6**	**6/6**	**16/16**	**4/4**	**6/6**	**6/6**	**16/16**

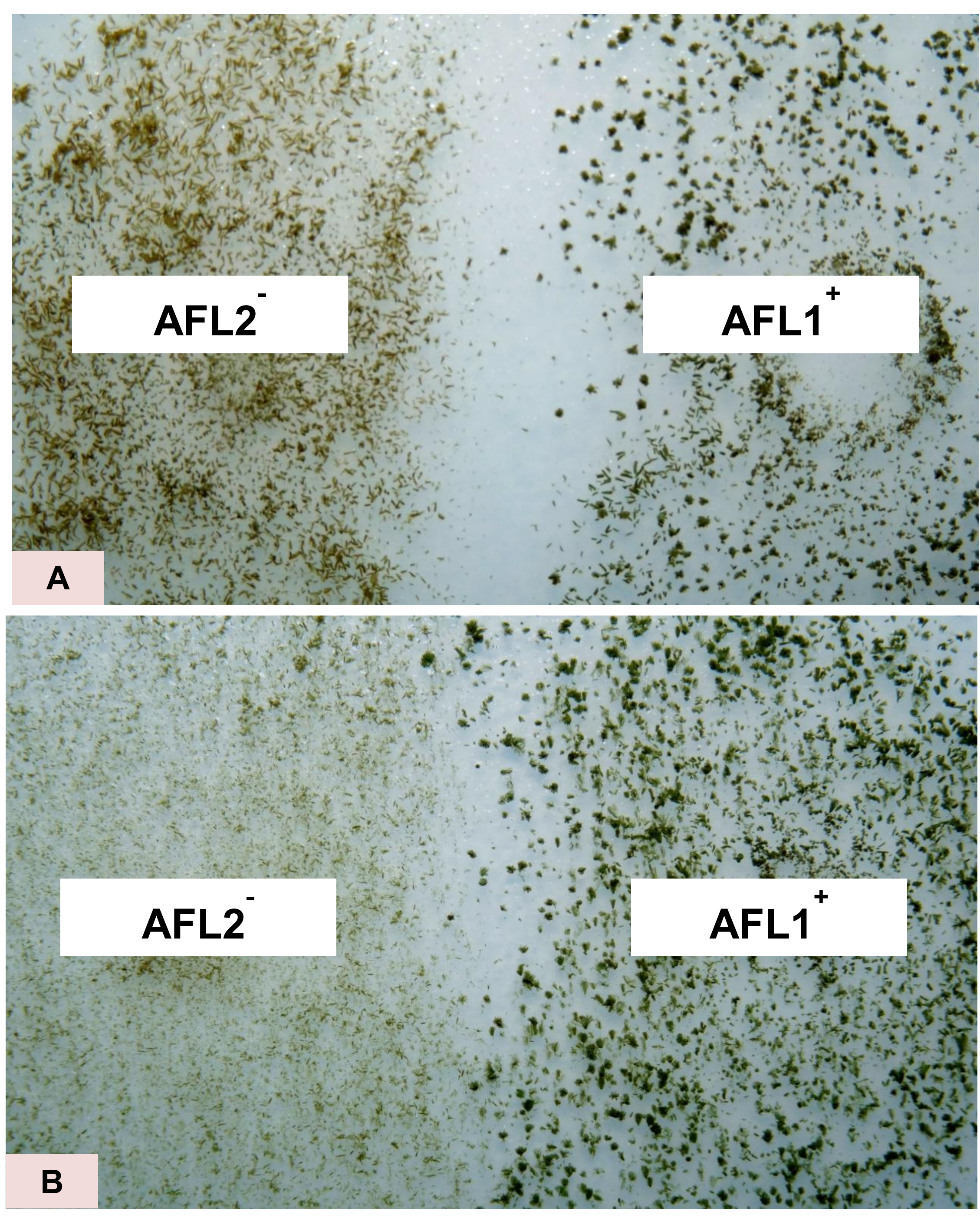

Figure 4. 1 Types of interaction between atoxigenic and toxigenic *A. flavus* strains on nutritionally poor MMA (A) antagonism at a distance when water activity of the medium was 0.96 a_w and (B) antagonism on contact at 0.99 a_w. Plates were incubated at 30°C and interactions were scored after 10 days.

4.3.2 Effect of interactions between atoxigenic and toxigenic strains of *A. flavus* on AFB_1 production *in vitro* and *in situ*

In vitro *mixtures of atoxigenic and toxigenic strains on MMA:* Figure 4. 2 and **Figure 4. 3** show the relative and temporal production of AFB_1 by AFL^+ strains in the presence of different initial ratios of the three AFL^- strains on MMA at 0.99 and 0.96 a_w levels. Since aflatoxin production is reported to be significantly greater at temperatures between 20 - 30°C than at higher and lower temperatures, toxin inhibition was evaluated at 25°C. The overall pattern at 0.99 a_w shows the consistent high production of AFB_1 by the $AFL1^+$ strain over the incubation period. For both toxigenic controls alone, toxin levels increased between the 7-14th day of incubation followed by a steady decrease between the 14th and the 21st day of incubation. At 0.90 a_w AFB_1 was not detected (results not shown). At 0.96 a_w AFB_1 production was highest in the control after 7 days storage and then decreased with an increase in incubation period. In all cases the AFL^- strains significantly decreased the AFB_1 production especially at 25:75 and 50:50% ratios of AFL^+:AFL^- ratios.

These results were similar when using the $AFL2^+$ strain. AFB_1 inhibitory capability of AFL^- strains differed with the different partner AFL^+ strains in mixed cultures. **Table 4. 2** shows the results of analysis of variance to compare the effect of a_w, strain type for both AFL^+ and the three AFL^- strains and their interactions on AFB_1 production *in vitro.*

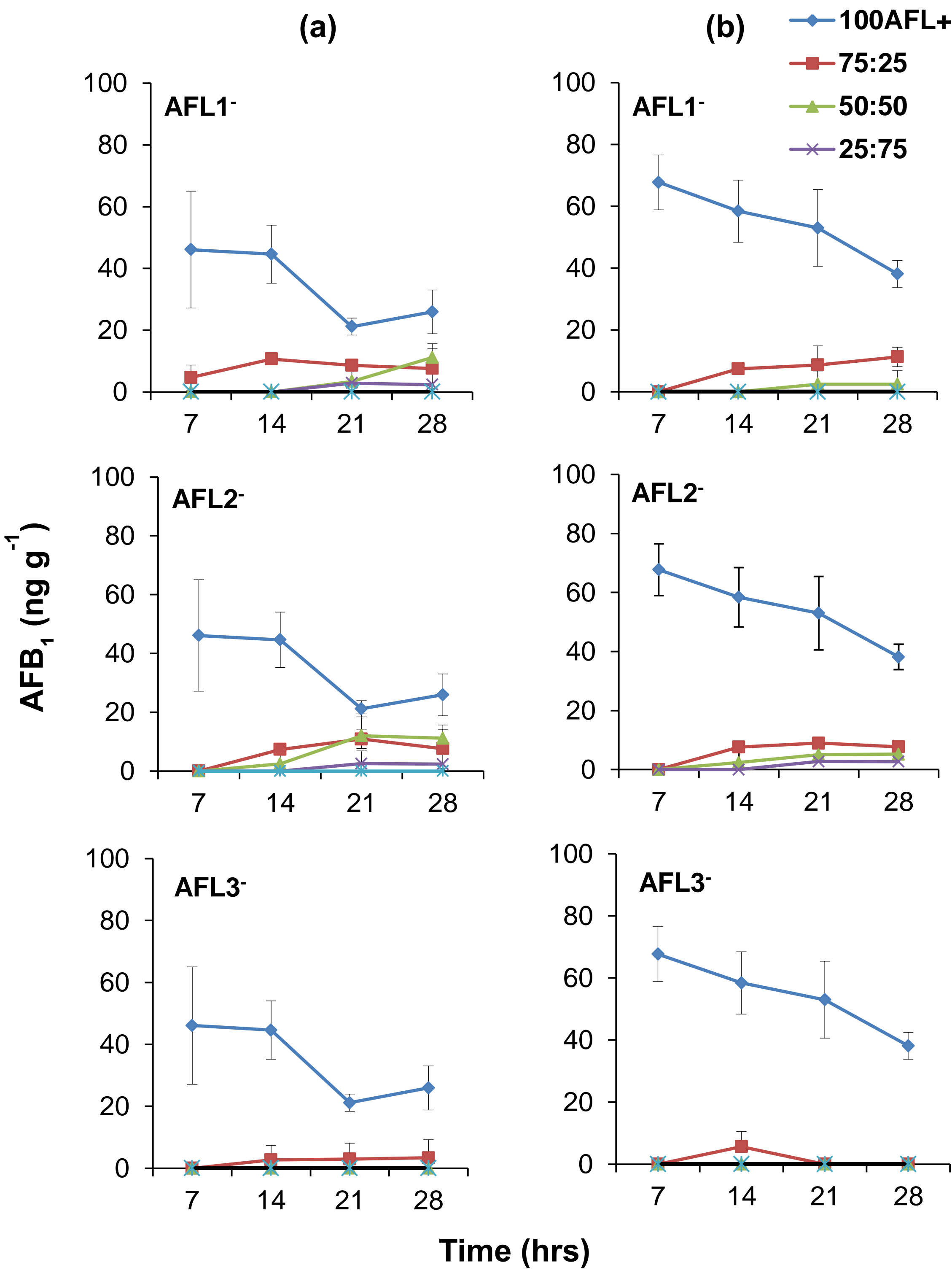

Figure 4. 2 Temporal differences in AFB_1 levels AFL^- strains grown in mixed cultures with (a) $AFL1^+$ and (b) $AFL2^+$ for 28 days at 25°C on MMA amended to 0.96 a_w.

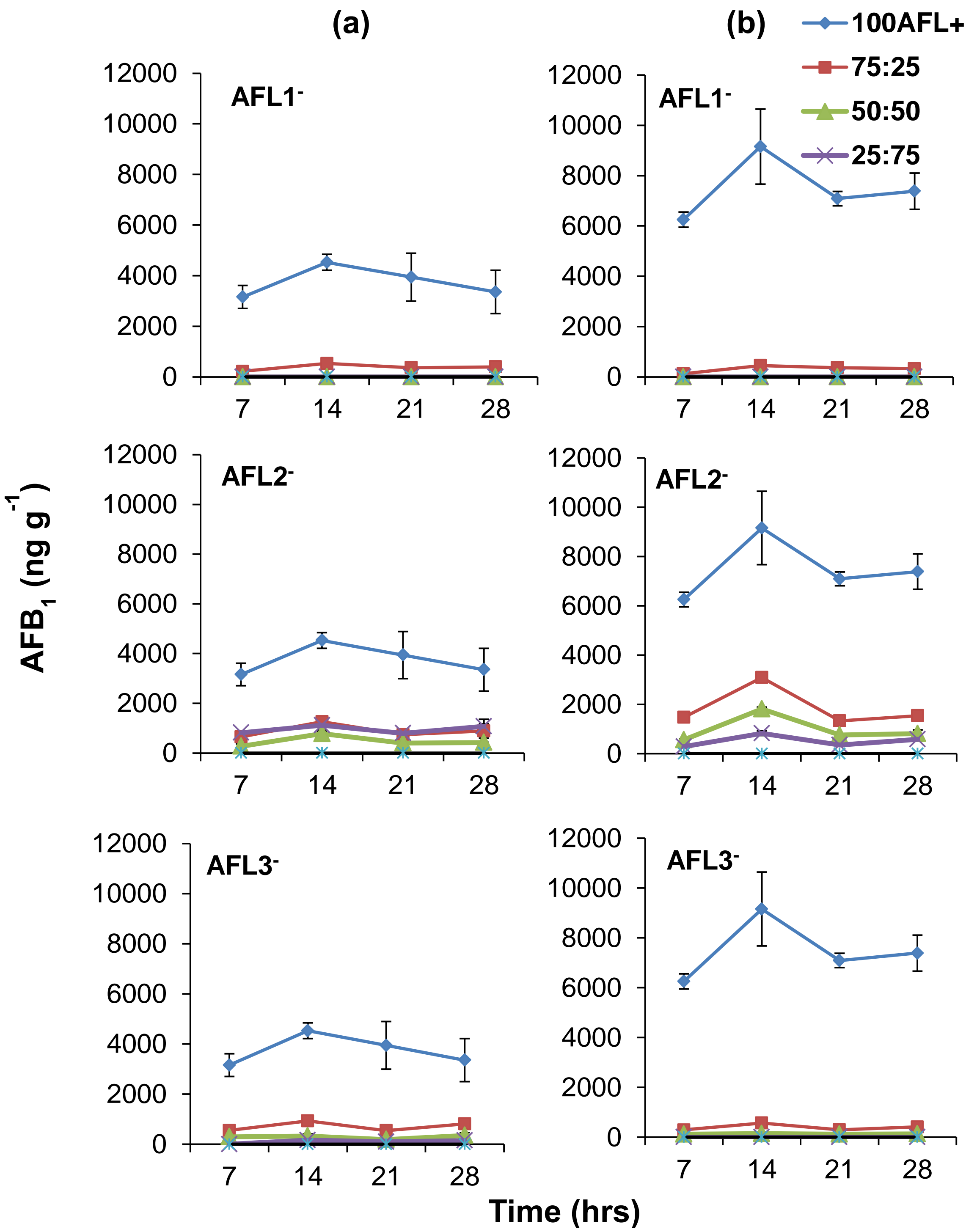

Figure 4. 3 Temporal differences in AFB_1 levels AFL^- strains grown in mixed cultures with (a) $AFL1^+$ and (b) $AFL2^+$ for 28 days at 25°C on MMA amended to 0.99 a_w.

Table 4. 2 ANOVA for relative AFB_1 production as a function of inoculum ratio and water activity amended to 0.96 and 0.99 a_w after 7, 14, 21 and 28 days of incubation at 25°C.

Atoxigenic strain	**Source of variation[ab]**	***df***	***MS***	***F***
$AFL1^-$	a_w	1	22.3	8027.0**
	$AFL^-:AFL^+$	4	62.1	22392.8**
	AFL^+ strain	1	0.02	5.65*
	a_w x $AFL^-:AFL^+$	4	8.48	3058.6**
	a_w x AFL^+ strain	1	0.05	19.0**
	$AFL^+:AFL^+$ x AFL^+ strain	4	0.28	100.2**
	a_w x $AFL^-:AFL^+$ x AFL^+ strain	4	0.02	7.87**
$AFL2^-$	a_w	1	159.7	1623.0**
	$AFL^-:AFL^+$	4	34.6	351.6*
	AFL^+ strain	1	0.44	4.45*
	a_w x $AFL^-:AFL^+$	4	8.61	87.5**
	a_w x AFL^+ strain	1	0.12	1.24
	$AFL^+:AFL^+$ x AFL^+ strain	4	0.45	4.58*
	a_w x $AFL^-:AFL^+$ x AFL^+ strain	4	0.35	3.55*
$AFL3^-$	a_w	1	118.8	1181.1**
	$AFL^-:AFL^+$	4	39.4	391.6**
	AFL^+ strain	1	1.53	15.2**
	a_w x $AFL^-:AFL^+$	4	9.88	98.2**
	a_w x AFL^+ strain	1	3.76	37.4**
	$AFL^+:AFL^+$ x AFL^+ strain	4	1.63	16.2**
	a_w x $AFL^-:AFL^+$ x AFL^+ strain	4	1.05	10.4**

** $p<0.01$, *$p<0.05$. [a]$AFL^-:AFL^+$ = concentration of atoxigenic strain: concentration of toxigenic strain. [b]AFL^+ strain = toxigenic strains

***In vitro* mixtures of atoxigenic and toxigenic strains on CAM:** On CAM, no fluorescent halo, an indicator of ability to produce aflatoxins was observed when the reverse of CAM plates (0.90 & 0.96 a_w) were observed under UV even on aflatoxin positive controls (AFL^+ grown alone) after 7 days. On 0.99 a_w CAM plates, a complete fluorescent halo when AFL^+ grew alone and its interference when grown with AFL^- was observed under UV. The interference increased with increasing concentration of AFL^- relative to AFl^+. There was complete interference of the fluorescent halo (toxin inhibition) at 75% AFL^-:25% AFL^+ mixed inoculum (Figure 4. 4).

***In situ* effects in maize grain**: **Table 4. 3** shows the results of toxin inhibition during interaction between $AFL1^+$ and $AFL2^+$ and the three AFL^- strains at 0.96 and 0.90 a_w on stored maize grain. Atoxigenic strains controlled toxin production more efficiently at higher a_w than under moisture stress. Their efficacy depended on the AFL^+ strain targeted. Increasing the concentration of AFL^- strains relative to the targeted producer strains led to significant inhibition of toxin production by the latter. Water activity, AFL^+ strain assayed and inoculum ratio and their interactions had significant effect on AFB_1 inhibition (**Table 4. 4**).

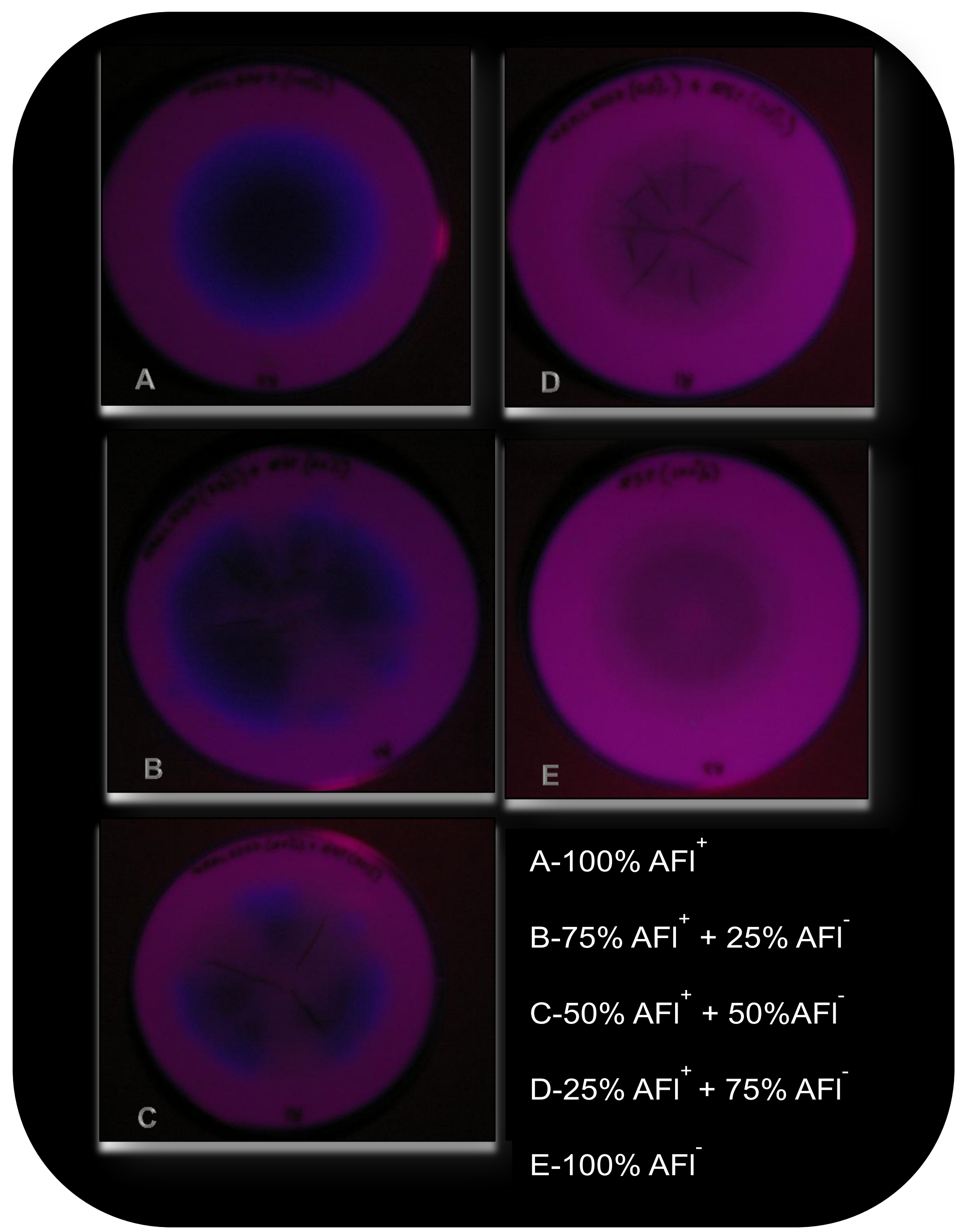

Figure 4. 4 The reverse of CAM (0.99 a_w) plate on which mixed cultures of AFL2$^-$ and AFL1$^+$ strains was grown for after 7 days at 25°C when oberseved under UV (365 nm).

Table 4. 3 Mean quantities of AFB_1 recovered from viable maize kernels maintained at 0.90 and 0.96 a_w after 14 days of incubation at 25°C.

a_w	Interacting strains	Amount AFB_1 (ng g^{-1}) ±STD[a]				
		100[b]	25:75	50:50	75:25	100[c]
0.90	$AFL1^-/1^+$	1022±0.40	1482±167.5	827±71.0	401±92.0	ND
	$AFL1^-/2^+$	860±192.8	1234±38.2	848±71.9	264±2.10	ND
	$AFL2^-/1^+$	1022±0.40	1074±83.3	670±35.7	698±63.8	ND
	$AFL2^-/2^+$	860±192.8	602±55.1	879±85.8	1020±68.6	ND
	$AFL3^-/1^+$	1022±0.40	1376±10.7	1193±5.7	1432±13.1	ND
	$AFL3^-/2^+$	860±192.8	1168±27.6	1264±69.5	1134±48.7	ND
0.96	$AFL1^-/ 1^+$	2849±185.9	1482±167.5	1122±4.2	720±12.6	ND
	$AFL1^-/ 2^+$	2544±73.9	2218±4.8	2065±137.8	1482±167.5	ND
	$AFL2^-/ 1^+$	2849±185.9	697±129.0	877±119.8	875±10.2	ND
	$AFL2^-/ 2^+$	2544±73.9	1244±77.4	729±85.3	581±85.9	ND
	$AFL3^-/ 1^+$	2849±185.9	1693±120.4	1172±248.9	1816±96.0	ND
	$AFL3^-/ 2^+$	2544±73.9	2231±214.5	1159±10.1	1557±123.6	ND

[a] AFB_1 values are means of three replicates. [b] Positive controls ($AFL1^+$ and $AFL2^+$) alone. [c] Negative controls ($AFL1^-$, $AFL2^-$ and $AFL3^-$) alone.

Table 4. 4 ANOVA for relative AFB_1 production as a function of inoculum ratio of mixed culture of toxigenic strains and atoxigenic strains when grown together in different ratios (100%, 25:75, 50:50, 75:25 and 100) on viable maize kernels maintained at 0.90 and 0.96 a_w after 14 days of incubation at 25°C.

Atoxigenic strain	**Source of variation^{ab}**	***df***	***MS***	***F***
AFL1$^-$	a_w	1	0.69	485.2**
	AFL$^-$:AFL$^+$	4	18.2	12722.3**
	AFL$^+$ strain	1	0.02	14.0**
	a_w x AFL$^-$:AFL$^+$	4	0.10	67.9**
	a_w x AFL$^+$ strain	1	0.12	83.7**
	AFL$^+$:AFL$^+$ x AFL$^+$ strain	4	0.01	8.58**
	a_w x AFL$^-$:AFL$^+$ x AFL$^+$ strain	4	0.02	14.6**
AFL2$^-$	a_w	1	0.09	48.7**
	AFL$^-$:AFL$^+$	4	17.4	9747.0**
	AFL$^+$ strain	1	0.001	0.61
	a_w x AFL$^-$:AFL$^+$	4	0.09	50.05**
	a_w x AFL$^+$ strain	1	2.04E-006	0.001
	AFL$^+$:AFL$^+$ x AFL$^+$ strain	4	0.002	1.13
	a_w x AFL$^-$:AFL$^+$ x AFL$^+$ strain	4	0.05	29.2**
AFL3$^-$	a_w	1	0.22	177.8**
	AFL$^-$:AFL$^+$	4	19.2	15542.4**
	AFL$^+$ strain	1	0.005	4.10
	a_w x AFL$^-$:AFL$^+$	4	0.08	61.4**
	a_w x AFL$^+$ strain	1	0.005	4.33
	AFL$^+$:AFL$^+$ x AFL$^+$ strain	4	0.005	3.83
	a_w x AFL$^-$:AFL$^+$ x AFL$^+$ strain	4	0.004	2.88*

** $p<0.01$, *$p<0.05$. ^{a}AFL$^-$:AFL$^+$ = concentration of atoxigenic strain : concentration of toxigenic strain. ^{b}AFL$^+$ strain = toxigenic strains

4.4 Discussion

Based on the hyphal interactions, the present study has shown that AFL^- and AFL^+ strains are generally mutually antagonistic to each. The 'deadlock' nature of the mycelial interaction between these two fungi was further confirmed by the inability of either of them to increase their colony area in the presence of the other. Depending on temperature and water availability, mutual antagonism occurred either on contact or at a distance. When temperatures were higher (30°C), regardless of a_w, AFL^+ strains had greater I_D values than their AFL^- counterparts. At the other temperatures the two groups of fungi were equally competitive.

Magan and Lacey (1984) reported that fungi demonstrate a wide range of interactions on malt and wheat extract agars and that the numerical Index of Dominance for individual fungi in their study varied with a_w, temperature and substrate. The I_D scores are a good guide to the competitiveness of an individual strain or species. Magan and Aldred (2007a) suggested that the interactions between strains/species will be in a state of flux and can change with interacting environmental conditions or changing nutritional matrices. Previous interaction studies between AFL^- and AFL^+ strains of *A. flavus* have not included a_w x temperature effects. Studies by Magan et al. (2010) showed that interactions between mycotoxigenic fungi (*Fusarium culmorum*, *Aspergillus carbonarius*) and other mycobiota on wheat- and grape-based media was affected by a_w x temperature interactions and influenced deoxynivalenol and ochratoxin A respectively. Cotty and Bayman (1993) suggested that the main mechanism of action is competitive exclusion. Huang et al. (2011) however,

suggested toxin inhibition is thigmo-regulated. This observation requires further investigation under a range of realistic environmental conditions.

In the present study the use of different ratios of AFL^- and AFL^+ strains provided good information on the relative ability to control AFB_1 production. Overall, regardless of a_w level used the production of AFB_1 by both $AFL1^+$ and $AFL2^+$ was significantly reduced by the AFL^- strains at both 0.99 and 0.96 a_w. This is important as the latter a_w level equates to significant plant water stress conditions which are almost 4-5 x the wilting point of plants (0.99 a_w = -1.4 MPa water potential). Thus the AFL^- and AFL^+ strains are adapted to moisture stress and able to effectively compete for the resources under a wide range of environmental stresses. Although under water stress conditions the relative control of AFB_1 was not as effective as was obtained with freely available water, these conditions, *in vitro* and *in situ* on stored maize, are a more realistic test of the efficacy of such AFL^- strains.

The temporal effects were interesting as they showed that over a 28 day period it was possible *in vitro* to significantly inhibit AFB_1 production by over 85%. The effective reduction in AFB_1 obtained in both test systems under different a_w levels suggest that these are effective candidate AFL^- strains. While the amounts of AFB_1 produced are much higher than the EU legislative limits (4 µg kg^{-1}) even with 80% control, these conditions are a good method for effective screening of candidates for more practical assays.

The observation that AFL^- and AFL^+ strains are mutually antagonistic either on contact or at an distance suggests that more combative criteria may be

important as has been suggested by Pitt and Hocking (2006). However, the rate of utilization of C-sources and ability to produce extracellular enzymes may also be important characteristics (Alam et al., 2009). It may be that germination rates, germ tube extension and growth rates contribute to competitiveness as these parameters were correlated positively for efficacy of other biocontrol agent in relation to a_w (Pascual et al., 1997). This will of course critically influence the relative inhibition of AFB_1 production which is the most important criteria in this approach.

An understanding of the relative threshold inoculum levels required is a critical factor in the delivery of biocontrol agents. By using a matrix approach using different ratios of AFL^- and AFL^+ strains it was possible to obtain some useful data on the effect of having a greater inoculum of either strain type on AFB_1 production. This suggested that where 50:50 or 25:75 AFL^+ to AFL^- inoculum was used the results were very effective in controlling toxin production. This provides useful information on the relative inoculum levels which may be required to control AFB_1 contamination of maize under different water availability conditions. Studies by Pitt and Hocking (2006) and Degola et al. (2011) suggested that increasing spore inoculum of AFL^- strains relative to AFL^+ ones would give better AF control. However, the economics of the production and formulation of the system may also need to be considered. Recent studies by Probst and Cotty (2012) showed that there was a better correlation between *in vivo* and *in vitro* production of AF on viable and autoclaved maize grain when comparing strains than that in defined liquid culture medium. They suggested that this should be considered when examining efficacy of AFL^- strains for

control of AF production. However, their studies did not include the possible influence of water stress.

Studies of interactions between AFL^- and AFL^+ strains of *A. flavus* on AF production on cotton seed bolls showed that pre-inoculation of wounded cotton bolls with the AFL^- strains was more effective in controlling AFB_1 production than when inoculation was 24 h post-inoculation with the AFL^+ strain (Cotty, 1990). He also showed that inoculum concentration had an significant impact on the relative potential for control of AFB_1. Other studies on ripening maize have found AFL^- strains which can inhibit AFL^+ strains but have not examined the relative thresholds required for effective control (Abbas et al., 2006). Of course control needs to be considered relative to the prevailing legislative limits in different commodities.

We have found some differences in the efficacy of the different AFL^- strains in AFB_1 control. Generally, $AFL1^-$ and $AFL3^-$ were more effective than $AFL2^-$ in our *in vitro* tests. However, on maize grain $AFL2^-$ performed better than the other two atoxigenic strains especially with regard to control of toxin production from strain $AFL1^+$. Previous studies by Degola et al. (2011) in laboratory reported variation in the ability of the atoxigenic strains to reduce the fluorescence intensity when challenged with the toxigenic *A. flavus* strain. In their study, atoxigenic BS07 was not as effective as atoxigenic strains T0ϕ, 696A and MN1 in reducing AFB_1 accumulation when co-inoculated with the AFL^+ strain. Similarly, Abbas et al. (2011) reported variability in the ability of candidate AFL^- strains to inhibit aflatoxin production by AFL^+ strains. However, their tests did

not take account of environmental factors, especially water availability, a critical parameter when considering control of aflatoxin contamination.

In summary this study has shown that three AFL^- strains are mutually antagonistic to two AFL^+ strains under different a_w x temperature conditions. *In vitro* and *in situ* temporal studies of ratios of AFL^- and AFL^+ strains demonstrated that effective reduction of up to 77.2% can be obtained depending on the a_w level and AFL^- strain used.

CHAPTER 5: Comparison of growth, nutritional utilisation patterns and niche overlap indices of toxigenic and atoxigenic *Aspergillus flavus* strains

5.1 Introduction

Interactions between different fungi are inevitable in terrestrial ecosystems (Magan and Aldred, 2007a). This has been utilised for the development of antagonistic fungi to develop biocontrol agents (BCAs) for controlling important plant pathogens pre- and post-harvest. One such pathosystem is the use of atoxigenic (AFL^-) strains of *Aspergillus flavus* and *A. parasiticus* to competitively exclude toxigenic (AFL^+) strains and thus reduce the potential for aflatoxin (AF) contamination of economically important commodities such as maize, groundnuts and cotton (Dorner, 2004). It has also been suggested that the use of indigenous strains on a regional basis may be important to obtain effective control of aflatoxin contamination (Probst et al., 2011). There has thus been interest in understanding the mechanisms of such competitive exclusion of AFL^+ strains by single or mixtures of AFL^- strains.

It was previously suggested by Wilson and Lindow (1994) that the co-existence of microorganism, and potential for niche exclusion particularly on plant surfaces, may be mediated by nutritional-resource partitioning. They suggested that *in vitro* carbon source (CS) utilisation patterns could be used to develop a niche overlap index (NOI) which would help explain the co-existence or niche exclusion by different microbial species or strains and help in the choice of appropriate BCAs. They used the range of similar CSs utilised, and those

uniquely utilised by a strain or species to help determine whether co-existence or nutritional exclusion occurred between them. Thus, NOI values of >0.90 for a competing strain or species was indicative of co-existence between pathogen and antagonist in an ecological niche and a score of <0.90 represented occupation of separate niches.

This was subsequently shown to be more complex, as when environmental parameters and perhaps anti-fungal compounds were also included in a multifactorial approach, utilization patterns were further modified (Magan and Aldred, 2007a). For example, it was demonstrated that, based on utilization of maize CSs, the NOIs for mycotoxigenic fungi such as *Fusarium verticillioides* were >0.90 at water availabilities >0.98 water activity (a_w) at 25-30^oC, indicative of co-existence with *Penicillium* spp., *A. flavus* and *A. ochraceus* (*A. westerdijkaie*). However, with some pairings this decreased to <0.80 NOI indicating occupation of different niches. The niche size and NOI was also shown to be significantly modified by the presence of anti-fungal compounds when examining *Penicillium verrucosum* and other food spoilage moulds and ochratoxin A production (Arroyo et al., 2008).

Mehl and Cotty (2011) showed that the success of AFL^- strains during competition with AFL^+ strains is better when the former is the first to come into contact with the host. However, the effect of environmental factors on the competitive abilities of the isolates was not evaluated in their studies. Lee and Magan (2000) showed that it was important to examine the key CSs in a matrix, e.g. maize, wheat, groundnuts, to obtain useful and relevant results with this approach. Recently Giorni et al. (2009) examined the CS utilization patterns of

strains of *F. verticillioides* and *A. flavus* in relation to temperature and a_w and found large differences suggesting that at 30°C *A. flavus* utilized more CSs regardless of the a_w levels examined (0.87-0.98 a_w) while at 20°C *F. verticillioides* utilized more CSs. The NOIs confirmed the nutritional dominance of *A. flavus* at 30°C and that of *F. verticillioides* at 20°C and 0.98-0.95 a_w. Thus complex relationships between mycotoxigenic fungi can occur relative to the environmental conditions and CS range in an ecological niche.

In a survey of mycotoxigenic fungi associated with stored maize from Lesotho, AFL^- strains of *A. flavus* were recovered (see Section **2.3.2**). Some of these strains were subsequently shown to be able to significantly reduce the aflatoxin B_1 (AFB_1) production by AFL^+ strains both *in vitro* and *in situ* on maize (see Section **4.3.2**). The focus was to examine AFL^- strains of *A. flavus* which originated from Lesotho. There was interest in understanding the similarities and differences between these groups of AFL^- and AFL^+ strains in terms of CS utilisation patterns, NOIs and nutritional dominance or exclusion.

Thus the objectives of this study were to (a) compare the relative lag phases prior to growth and mycelial growth rates between the chosen two groups of strains on maize-based media; (b) the relative CS utilization patterns under different temperatures and a_w regimes; (c) compare the NOIs to determine whether they occupied the same or different niches; and (d) use a rapid Bioscreen C method for quantifying temporal carbon utilisation patterns between the AFL^- and AFL^+ strains under interacting temperature x a_w conditions for the first time.

5.2 Materials and Methods

5.2.1 Fungal strains

A total of five strains were used in this study. Three strains: toxigenic strain *Af*18LS (AFL1^{+}) and atoxigenic strains: *Af*19LS (AFL1^{-}) and *Af*24LS (AFL2^{-}) were isolated from stored maize collected from different agroecological zones of Lesotho: mountainous region, southern lowlands, foothills and Senqu River Valley respectively. One atoxigenic strain EGP15 (AFL3^{-}) was isolated from Egyptian peanuts (Abdel-Hadi, 2011) while a toxigenic *A. flavus* type strain (NRRL3357; AFL2^{+}) was kindly provided by Prof D. Bhatnagar of the Southern Regional Research Centre, New Orleans, LA, USA. Molecular analyses for the biosynthetic aflatoxin pathway structural genes (*aflD*, *aflM*, *aflP*) and a regulatory gene (*aflR*) were carried out (see Section **3.3.2**). For the AFL1^{-} strain all four genes were present; for AFL2^{-} *aflM*, *aflP* and *aflR* were not present; for AFL3^{-} these four genes were all present but no expression on a conducive Yeast Extract Sucrose medium occurred (Abdel-Hadi, 2011). The strains are being deposited in the recognised culture collection of the Technical University of Denmark (Lyngby) and will be available to other scientists. The vegetative compatibility groups (VGCs) of the strains were compared and it was found out that the two AFL^{+} and three AFL^{-} strains were from different VCG groups (Bayman and Cotty, 1991). Both, the AFL^{-} and AFL^{+} strains were all “L” type and produced varying numbers of sclerotia; average diameter >400 µm; (Cotty, 1989). Cultures were maintained on 3% Maize Meal Extract Agar (MMEA; (Marín et al., 1995).

5.2.2 Comparison of lag phase duration and mycelial growth of the toxigenic and atoxigenic strains

Media preparation: For growth studies, 3% MMEA was used. The medium was prepared by mixing 15 g of coarsely ground maize flour with 500 ml distilled water whose water availability was adjusted by the addition of appropriate amounts of glycerol to achieve 0.99, 0.96 and 0.90 a_w. The mixture was brought to a boiling temperature by heating followed by holding at that temperature for 30 min and then left to cool before being sieved through four layers of muslin cloth. Ten grams (10 g) of agar were added to 500 ml of the maize extract and this was thoroughly mixed before autoclaving at 121°C for 15 min. The sterile medium was left to cool before being poured into 9 cm diameter Petri plates.

Computing the lag phase duration and mycelial growth rates: Spore suspensions of test strains were spread plated on MEA plates and incubated at 25°C for 48 h. These plates were used to provide inoculum for the experiments. Agar plugs (diam. 3 mm) were taken from these inoculation plates with a sterile cork borer and transferred by centrally inoculating the MMEA plates at each treatment a_w level. The plates with the same a_w were sealed in polyethylene bags and incubated at 20, 25 and 30°C in the dark for 10 days. The diameter of the colonies was measured in two directions perpendicular to each other daily for this period. The growth rate (mm day^{-1}) of each strain under the different set of environmental factors was computed by plotting the diameter of the colonies against time. Regression lines were made of the time points which represented the linear phase of the growth curves using Microsoft Excel®. The gradient of the linear equation with an associated correlation coefficient of ≥0.98 was

considered the maximum growth rate. The duration of the lag phase was calculated using the same equation to determine the length of time for which there was no perceptible mycelial growth from the agar plugs, i.e., the length of time for which the diameter remained 3 mm (diameter of agar plug before incubation). Experiments were carried out with three replicates per treatment. The experiment was repeated twice.

Media preparation: The media for NOIs studies were prepared according to Giorni et al. (2009) with some modifications. Briefly, a total of 24 carbon sources which are the principal constituents of the maize kernel (Giorni et al., 2009) were incorporated into a minimal medium comprising 0.23% $NaNO_3$, 0.06% $MgSO_4 \cdot 7H_2O$, 0.17% K_2HPO_4 and KH_2PO_4 at a final concentration of 7.99 mg C ml^{-1} (**Table 5. 1**). These media are referred to here as CS solutions. The a_w of each CS solution was amended to 3 values: 0.90; 0.96 and 0.99 by addition of different amounts of NaCl before autoclaving at 121°C for 15 min. The CS solutions of the same a_w were each pipetted (700 µl) into a sterile 24 well microtitre plate with lid (128 x 86 mm, 1.8 cm^2; Nunc, Denmark). For these studies glycerol could not be used to amend the a_w as this would represent an additional carbon source.

Table 5. 1 Amounts of different carbon sources containing 7.99 mg C ml^{-1}.

Carbon source	Amt. of carbon source containing 7.99 mg C ml^{-1} (g)
Amino acids	
L-Leucine	1.46
L-Alanine	1.98
D-Alanine	1.98
D-L-Threonine	2
L-Serine	2.33
D-Serine	2.33
L-Histidine	1.72
L-Proline	1.53
L-Phenylalanine	1.22
L-Aspartic acid	2.22
L-Glutamic acid	1.96
Carbohydrates	
D-Galactose	2
D-Raffinose	1.87
D-Maltose	1.90
D-Fructose	2
Sucrose	1.90
D-Melibiose	1.90
Dextrin ‡	2
Amylopectin ‡	2
Amylose‡	2
D-Glucose	2
Fatty acids	
Oleic acid	1.05
Linoleic acid	1.04
Palmitic acid	1.07

‡ Molar mass variable and the amount of the CS was fixed at 2 g.

Spore preparation and inoculation: Spores from 7 day-old cultures of the two AFL^+ and three AFL^- strains grown on 3% MMEA were harvested with sterile water and separately placed into sterile Universal bottles containing 20 ml of sterile distilled water. The bottles were then vigorously shaken for 5 min followed by centrifuging on a bench top centrifuge for 15 min at 3000 rpm. The supernatant was discarded and thereafter, the spores were washed three times by shaking vigorously with 20 ml of sterile water. After the third washing, spores were re-suspended with the buffer-NaCl sterile solution and the concentration adjusted to 10^6 spores ml^{-1} with the aid of a haemocytometer. The wells containing CS solutions were then inoculated with 100 µl of the spore suspension. Controls comprised microtitre plates with uninoculated CS solutions. Triplicate treatment 24 well microtitre plates at each a_w for each strain were sealed in polyethylene bags and incubated at 20, 25 and 30°C. The initiation of mycelial growth was monitored daily with the aid of a stereomicroscope for 14 days.

Calculation of niche overlap index (NOI): The total number of different carbon sources used by each strain was used to calculate Niche Overlap Index (Wilson and Lindow, 1994) for both the pathogen AFL^+ (both strains) and the antagonists (three AFL^- strains) individually using the equation below.

$$\mathrm{NOI} = \frac{\text{no. of carbon sources in common between pathogen and antagonist strain}}{\text{Total no. of carbon sources utilised by antagonist strain}}$$

In the present study, the NOIs of both AFL^- and AFL^+ were determined under the different sets of environmental conditions (temperature and a_w).

5.2.4 Calculation of temporal carbon utilization sequences (TCUS) using the Bioscreen C

The methodology used was adapted from Medina et al. (2012) with some slight modifications described below. The analysis of the turbidity used a Bioscreen C Microbiological Growth Analyser (Labsystems, Helsinki, Finland). The media and spore inocula preparations used were as described above.

Turbidimetric assay: Non-standard 100-well microtitre plates, specifically manufactured for the Bioscreen were loaded with 200 µl of the different media containing the different CS. Each CS was randomly loaded in 4 different plate locations and then inoculated with 10 µl of an *A. flavus* spore suspension. Experiments were conducted at 25°C. Four wells with spore suspension, without the addition of any CS, were included and used as the non-growth controls.

The optical density (OD) was automatically recorded at 20 min intervals using the 600 nm filter over a seven day period (10080 min). No agitation program was set over the incubation time. Data were recorded using the software Easy Bioscreen Experiment (EZExperiment) provided by the manufacturer and then exported to a Microsoft Excel® Professional 2010 (14.0.4756.1000) (Microsoft Corporation, Redmond, Washington, USA) sheet for further analysis.

Calculation of temporal carbon utilization sequences: Raw data obtained from the Bioscreen was subjected to further analysis. Before analyses, the average of the measurements for each well during the first 60 min was calculated and automatically subtracted from all subsequent measurements in order to remove the different signal backgrounds obtained for the different CS

media. Then the Time to Detection (TTD) for an OD of 0.1 were obtained for 25°C at a_w values of 0.90, 0.96 and 0.99 using a Microsoft Excel® template, which used linear interpolation between successive OD readings to predict the TTD at OD = 0.1.

For each replicate of each CS the TTD was recorded. The average of these numbers, expressing the mean time that the fungal strain needed to reach an OD of 0.1 with a particular carbon source was computed. Data for each AFL^{-} and AFL^{+} strain was then organized according to these TTD values and the TCUS produced accordingly.

5.2.5 Statistical analyses

Log-transformed data were subjected to two-way ANOVA: (p=0.05) with strain (2 AFL^{+} and 3 AFL^{-} *A. flavus* strains), temperature (3 levels: 20, 25 and 30°C), and water activity (3 levels: 0.90, 0.96 and 0.99 a_w) as the main effects and duration of the lag phase and growth rate as responses. Mean comparisons for each pair of the different independent variables (temperature and water activity) was done using Tukey's HSD. The statistical package JMP®8 (SAS Institute Inc., 2008, Cary NC, USA) was used in the analysis.

5.3 Results

5.3.1 Effect of temperature and water activity on lag phase duration and mycelial growth rates

Figure 5. 1 and **Figure 5. 2** compare the lag phases prior to growth and the growth rates of the 2 AFL^+ and 3 AFL^- strains of *A. flavus* grown under different sets of a_w x temperature conditions. Interactions between temperature and a_w had a significant effect on the lag phases and growth rate of the two sets of strains. However, there was no statistically significant difference between the AFL^- and AFL^+ strains in terms of both the mycelial growth rate and duration of the lag phase (**Table 5. 2**). Overall, fastest growth rates were associated with warmest (30°C) and wettest (0.99 a_w) conditions. The lag phase duration was longest under driest (0.90 a_w) and coolest conditions (20°C).

5.3.2 Number of carbon sources utilised by *A. flavus* strains

Figure 5. 3 compares the total number of carbon sources utilised by the $AFL1^-$ and $AFL1^+$ strains of *A. flavus* tested. It should be noted that a smaller number of carbon sources were utilised under cooler and drier conditions than under warmer and wetter conditions by all the strains assayed ($p=<0.0036$). At 30°C and 0.99 a_w all strains were able to utilise the 24 CS relevant to maize assayed. At lower temperatures and reduced a_w, the niche sizes of the strains narrowed. There was no statistically significant difference between $AFL1^-$ and $AFL1^+$ strains in relation to the number of CS utilised based on the three temperatures under wettest conditions tested ($p>0.05$).

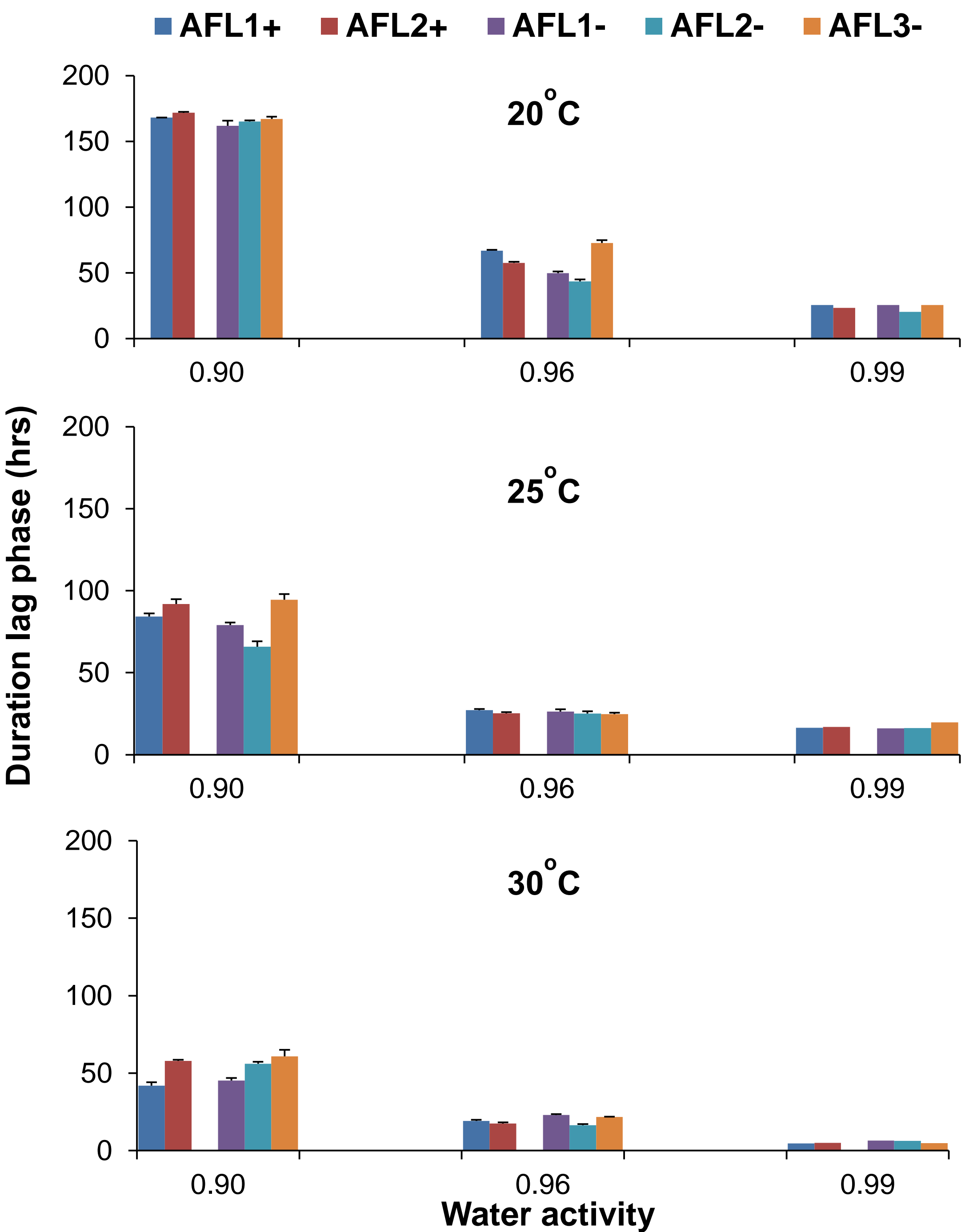

Figure 5. 1 Duration of the lag phase in hrs of *A. flavus* toxigenic strains ($AFL1^+$ & $AFL2^+$) and atoxigenic strains ($AFL1^-$, $AFL2^-$ & $AFL3^-$) grown on MMEA adjusted to three different water activities: (0.90, 0.96 and 0.99 a_w) and incubated at three levels of temperature: 20, 25 and 30°C. For each column, bars represent the standard deviation.

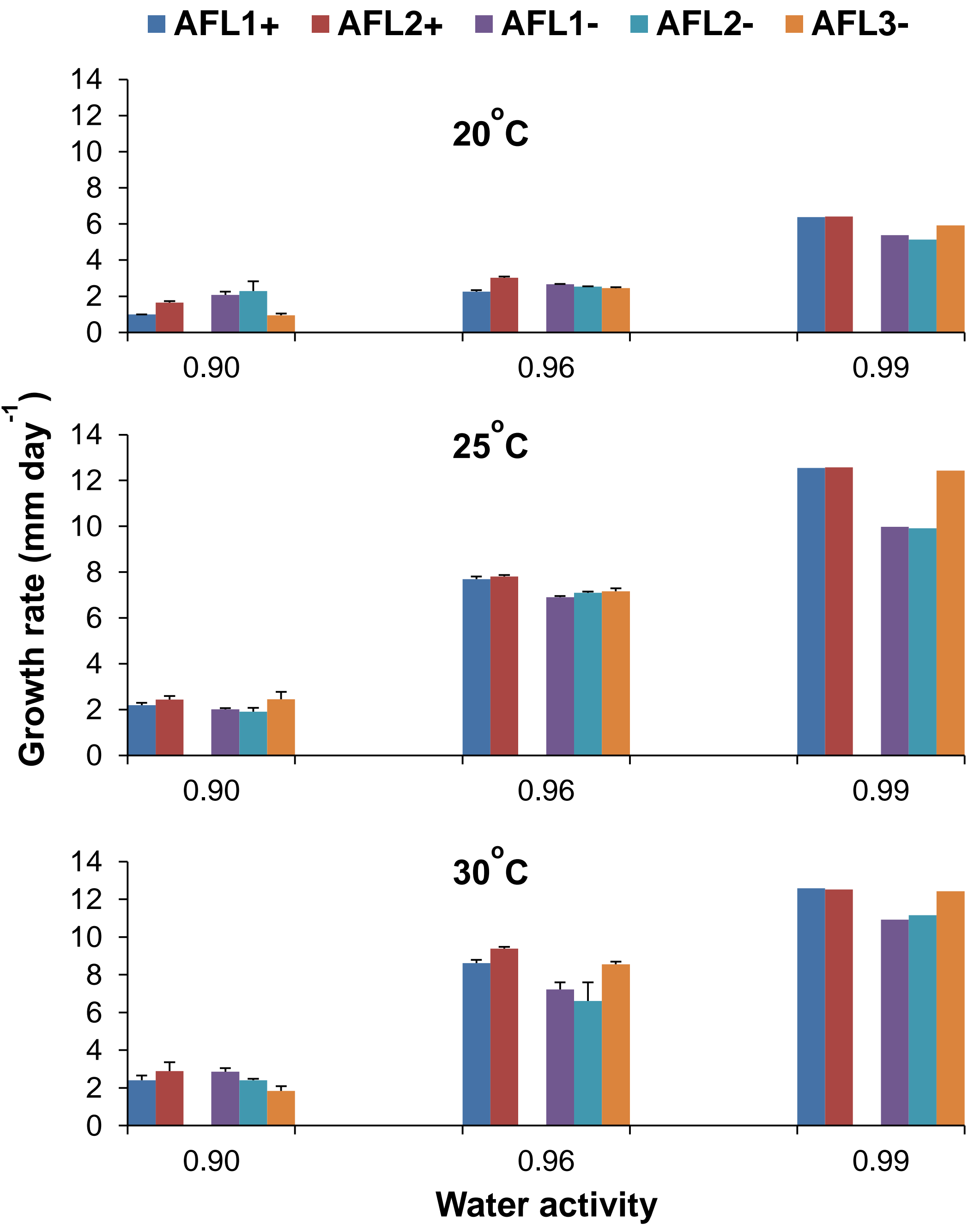

Figure 5. 2 Growth rate values of *A. flavus* toxigenic strains (AFL1$^+$ & AFL2$^+$) and atoxigenic strains (AFL1$^-$, AFL2$^-$ & AFL3$^-$) grown on MMEA adjusted to three different water activities: (0.90, 0.96 and 0.99 a_w) and incubated at three levels of temperature: 20, 25 and 30°C. For each column, bars represent the standard deviation.

Table 5. 2 Two-way analysis of variance (ANOVA) for (a) lag phase duration and (b) growth rate as functions of *A. flavus* strain ($AFL1^+$, $AFL2^+$, $AFL1^-$, $AFL2^-$ & $AFL3^-$), water activity (0.90, 0.96 and 0.99 a_w) and temperature (20, 25 and 30°C).

(a)

Source of variation	***SS***	***df***	***MS***	***F***
strain	0.16	4	0.04	1.29
a_w	14.8	2	7.39	84.8**
temp.	6.39	2	3.19	29.2**
strain x a_w	0.11	8	0.01	0.74
strain x temp	0.29	8	0.04	1.91
a_w x temp.	0.37	4	0.09	4.85**
strain x a_w x temp.	0.30	16	0.02	1.80*
(b)				
strain	0.16	4	0.04	46.4
a_w	14.8	2	4.76	164.7**
temp.	6.39	2	1.62	52.0**
strain x a_w	0.030	8	0.004	0.42
strain x temp	0.05	8	0.006	0.68
a_w x temp.	0.14	4	0.034	3.81*
strain x a_w x temp.	0.14	16	0.009	11.0**

** p<0.01, *p<0.05

5.3.3 Niche overlap indices

Water activity and temperature had an impact on the NOIs of the strains assayed (Table 5. 3). The NOIs are indicative of whether AFL^- and AFL^+ strains co-exist or occupy different niches. Generally, regardless of temperature, at higher a_w levels (0.96 and 0.99 a_w) both AFL^+ and AFL^- strains co-existed (NOIs>0.9). Under conditions of moisture stress (0.90 a_w) the nutritional competitiveness of the atoxigenic and toxigenic strains varied. For example, under drier conditions and warmer temperatures (30°C), the toxigenic strain $AFL1^+$ nutritionally dominated its AFL^- counterparts. At cooler temperatures and moisture stress conditions, toxigenic strain $AFL2^+$ had a NOI = 0.3 while $AFL1^-$ had a NOI = 1 indicating occupation of separate niches. A similar trend was observed between the toxigenic strain $AFL1^+$ when compared to the atoxigenic strains $AFL2^-$ and $AFL3^-$ at the same temperature and 0.90 a_w.

5.3.4 Temporal carbon utilization sequences (TCUS) of the strains

The temporal utilization patterns using the Bioscreen showed that a higher proportion (>92%) of the 24 carbon sources was utilised by all the strains when water was freely available (0.99 a_w; Figure 5. 4 and Figure 5. 5) at 25°C. However, under water stress at 0.90 a_w fewer carbon sources (<38%) were utilised at the same temperature. All the carbohydrates and >90% of amino acids were utilised by all the strains when water was freely available whereas 67% of fatty acids assayed were utilised under the same conditions. Under the driest conditions, none of the fatty acids were used. The proportion of carbohydrates utilised by all the strains at this a_w level was no more than 70% of the total number assayed.

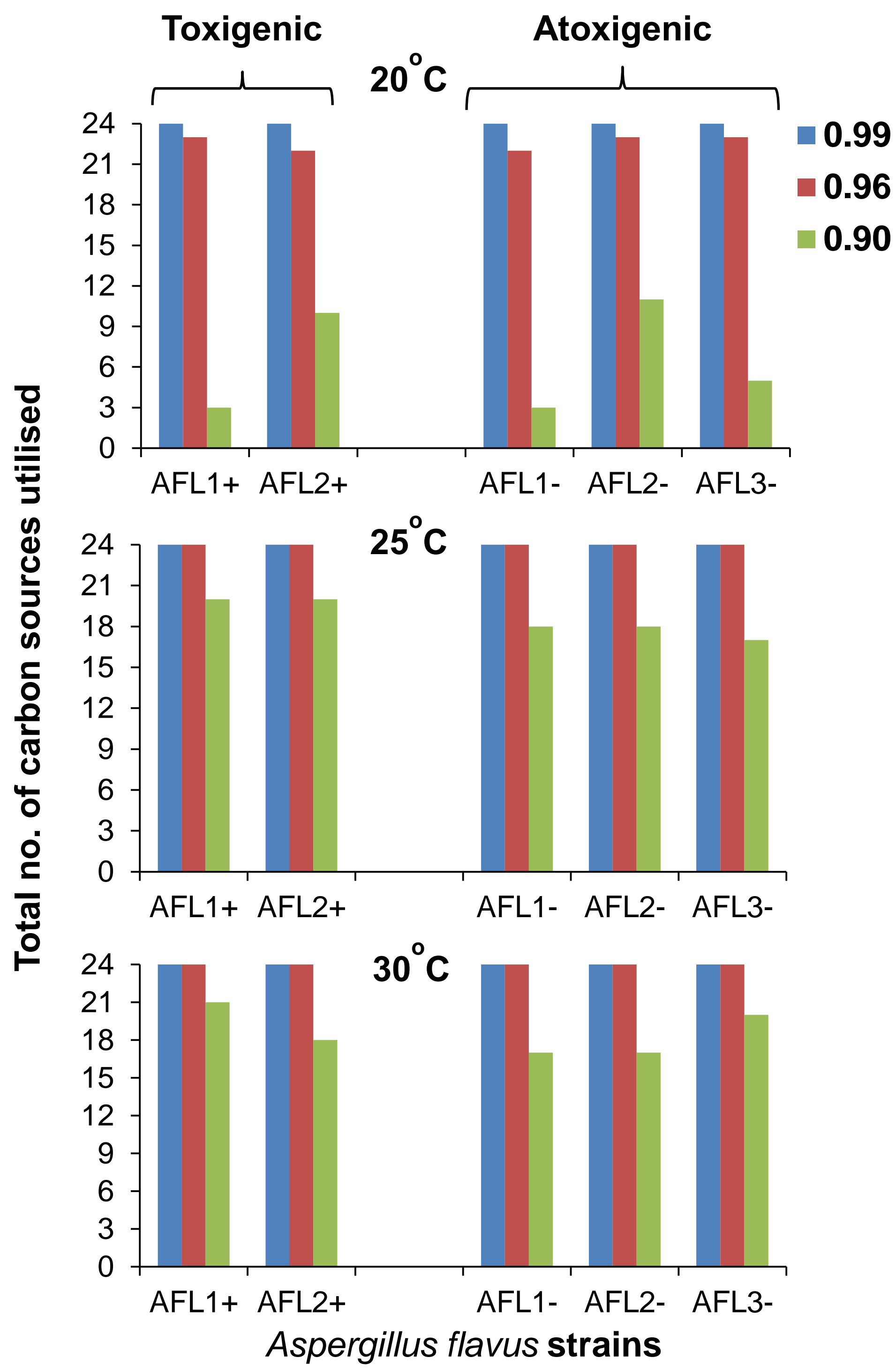

Figure 5. 3 Number of carbon sources utilised by toxigenic strains ($AFL1^+$ & $AFL2^+$) and atoxigenic strains ($AFL1^-$, $AFL2^-$ & $AFL3^-$) at 20, 25 and 30°C and three levels of water activity: 0.90, 0.96 and 0.99 a_w. Differences between temperature and a_w and their interactions were significant ($p<0.05$).

Table 5. 3 Niche Overlap Indices (NOIs) of atoxigenic *A. flavus* strains *Af*19LS ($AFL1^-$), *Af*24LS ($AFL2^-$) and EGP19 ($AFL3^-$) paired with toxigenic strains *Af*18LS ($AFL1^+$) and NRRL3357 ($AFL2^+$), obtained from carbon utilization data at different temperatures (20, 25 and 30°C) and water activity (0.90, 0.96 and 0.99 a_w).

a_w	0.90	0.96	0.99	0.90	0.96	0.99	0.90	0.96	0.99
	$AFL1^-$	$AFL1^-$	$AFL1^-$	$AFL2^-$	$AFL2^-$	$AFL2^-$	$AFL3^-$	$AFL3^-$	$AFL3^-$
20°C									
$AFL1^+$	**0.67/0.67**	0.96/1	1/1	**1/0.27**	1/1	1/1	1/0.60	1/1	1/1
$AFL2^+$	**0.30/1**	0.95/0.95	1/1	**0.80/0.72**	1/0.96	1/1	**0.40/0.80**	1/0.96	1/1
25°C									
$AFL1^+$	0.90/1	1/1	1/1	0.85/1	1/1	1/1	0.85/1	1/1	1/1
$AFL2^+$	0.90/1	1/1	1/1	0.85/0.94	1/1	1/1	0.85/1	1/1	1/1
30°C									
$AFL1^+$	**0.81/1**	1/1	1/1	**0.81/1**	1/1	1/1	0.95/1	1/1	1/1
$AFL2^+$	0.94/1	1/1	1/1	0.94/1	0.96/1	1/1	**0.94/0.85**	1/1	1/1

Key: Values are NOI AFL^+/ NOI AFL^- where:

NOI AFL^+ = ratio of CS utilised in common with AFL^- strains to the number of CS utilised by AFL^+ alone

NOI AFL^- = ratio of CS utilised in common with AFL^+ strains to the number of CS utilised by AFL^- alone

Additionally, there were temporal differences between AFL$^-$ and AFL$^+$ strains in the assimilation of the different types of carbon sources, especially under water stress. For example, AFL1$^+$ and AFL2$^+$ utilised 9 and 8 different CS respectively compared to the AFL$^-$ strains which on average used 6 CS within the experimental timeframe. These differences were attributable to better assimilation of carbohydrate-based CS by AFL$^+$ strains when compared to the AFL$^-$ strains. All the 5 strains assayed utilised glucose, fructose, dextrin, alanine and proline under moisture stress. An even smaller proportion of amino acids (<30% of the total CS assayed) were utilised at 0.90 a_w.

In general, the TTD of the assayed strains was shortest (approx. 24 h) on monosaccharides (D-Glucose, D-Galactose and D-Fructose) and disaccharide (D-Maltose) nutritional regimes. The TTD values for growth on the amino acids L-Histidine, L-Leucine and D-serine were longest (approx. 5 days) when water was freely available. When linoleic acid was used as the sole carbon source at 0.99 a_w, no growth was detected at all for any of the strains assayed, over the duration of the experiment (7 days).

A similar trend was observed at 0.96 a_w albeit with longer TTDs, approximately between 33 h, with simple sugars and 7 days when grown on amino acids. In contrast, the time to detection of the growth of the strains was longest under the driest conditions (>4.5 days) on all the carbon sources assayed. The simple sugars (D-Galactose, D-Fructose and D-Glucose) were still associated with the shortest TTDs, and earlier utilisation of the complex carbohydrate dextrin together with the disaccharide D-Melibiose and amino acids (L-Proline and L-Alanine) by all strains was favoured under conditions of moisture stress.

5.4 Discussion

This study suggests that growth and nutritional utilisation patterns of the AFL^- and AFL^+ strains of *A. flavus* used in this study were relatively similar. Indeed the effect of environmental factors, in this case a_w and temperature and their interactions had a predominant effect on both AFL^- and AFL^+ strains. There was no significant difference between these two groups of strains in terms of lag phase duration or growth rates. In general, the maximum growth rate for all strains assayed was greatest at 30°C and 0.99 a_w.

The question arises as to whether growth rates per se are a key determinant of competitiveness. Mehl and Cotty (2011) suggested that the strains which make contact with the host first, have a competitive edge over slower growers in mixed cultures. In the current study, assessment of growth was done in axenic cultures and this might not be reflective of the actual physiological processes that occur during competition for limited resources or space in mixed cultures. However, the effect of environmental conditions is useful information on the relative ecological fitness of the strains. While previous studies of interactions between cereal fungi by Magan and Lacey (1984) and Magan and Aldred (2007a) suggested that growth rate was not a key attribute alone which determines competitiveness, growth is important in the primary resource capture. However, its role in inhibiting or minimising secondary metabolite production may not be directly related as environmental factors influence the interactions between species and these are in a state of flux spatially and temporally (Magan and Aldred, 2007a).

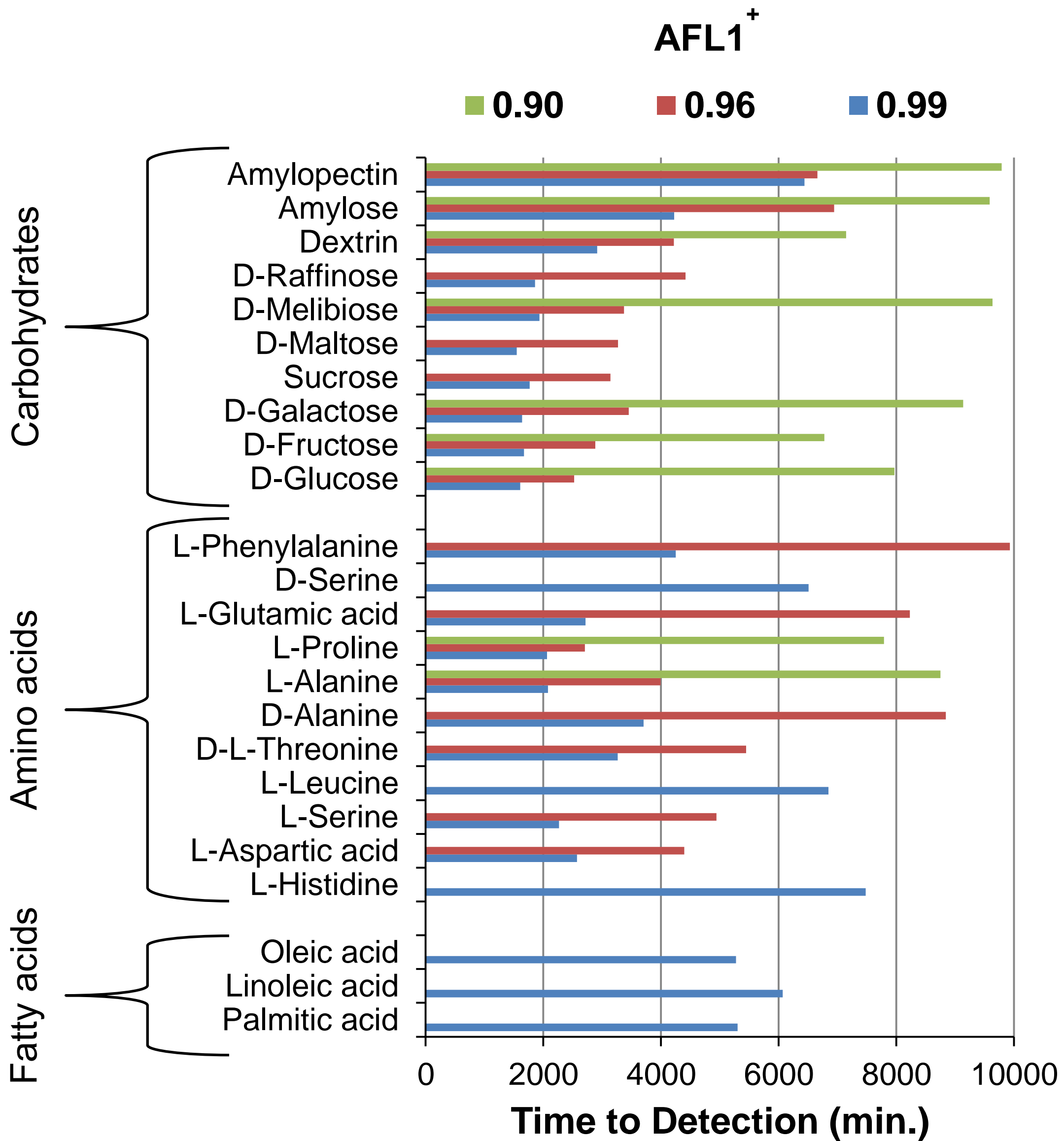

Figure 5. 4 Effect of water activity (0.90, 0.96 and 0.99 a_w) on temporal carbon utilisation patterns of atoxigenic AFL$^+$ (strain *Af*18LS) evaluated by Bioscreen at 25°C.

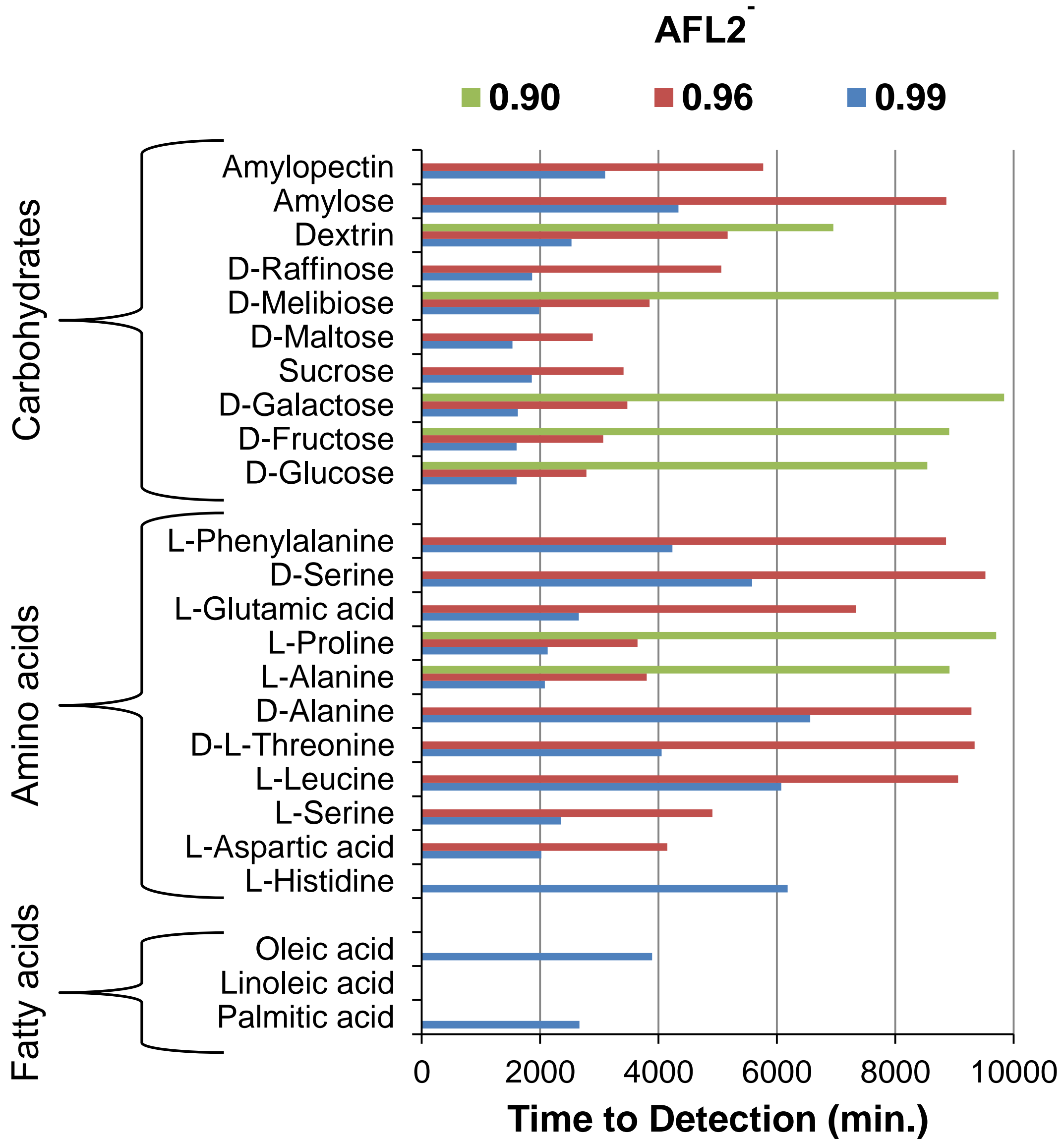

Figure 5. 5 Effect of water activity (0.90, 0.96 and 0.99 a_w) on temporal carbon utilisation patterns of atoxigenic AFL2$^-$ (strain *Af*24LS) evaluated by Bioscreen at 25°C.

Thus, comparative studies on growth rate between AFL^+ and AFL^- stains of *A. flavus* would probably be much more meaningful if designed to include among others, relative times of inoculation (Chang and Hua, 2007) and concentrations of spores of AFL^- relative to AFL^+ strains (Degola et al., 2011) and their interactions when found together in a particular niche.

In the current study, warmer temperatures (25, 30°C) and wetter conditions (0.96, 0.99 a_w) favoured the assimilation of different carbon sources by all the *A. flavus* strains assayed. The number utilised was much lower under water stress and cooler temperatures. These observations are similar to those of Giorni et al. (2009); over the a_w range they examined, niche size of *A. flavus* was greatest at 30°C. Co-existence of fungi is mediated by nutritional resource partitioning. The patterns of carbon utilisation *in vitro* are used to determine niche overlap indices (NOI) and therefore ecological similarity (Arroyo et al., 2008). In the current study, the NOIs are indicative of whether AFL^- and AFL^+ strains co-exist or occupy different niches. Generally, regardless of temperature, at higher a_w levels (0.96, 0.99 a_w) both AFL^- and AFL^+ strains co-existed (NOIs > 0.9). However, the NOIs under drier conditions were more variable for both the AFL^- and AFL^+ strains assayed. From a biological control perspective the ability to occupy the same niche over a wide range of environmental conditions would indicate a good potential for effective competitiveness (Wilson and Lindow, 1994). Giorni et al. (2009) demonstrated that one AFL^- strain they examined did not co-exist with a AFL^+ strain at 0.96 and 0.98 a_w regardless of temperature. In contrast, another AFL^- strain effectively excluded other AFL^+ strains based on the NOIs over a range of a_w x temperature conditions. This suggests variability in ecological competence between AFL^- strains of *A. flavus* when subjected to environmental stress. The present study only used the same

concentrations of each C-source as used by Wilson & Lindow (1994). However, the relative amounts of each C-source in maize grain will differ and this may further influence the utilisation patterns. This has not been previously examined although Lee & Magan (1999) showed that using a common range of C-sources (e.g. in a standard Biolog GN plate) gave very different results from that obtained when the C-sources relevant to the actual cereal matrix is considered.

Although the experimental periods used were one week, the Bioscreen results on niche size of the AFL^- and AFL^+ strains were similar to those obtained using the traditional method. A greater number of carbon sources were utilised under warmer and wettest conditions. Moreover, TCUS demonstrated preferential utilisation of carbohydrates by the strains assayed over amino acids and fatty acids under wettest conditions. The AFL^+ strains assimilated a greater proportion of CS than the atoxigenic strains within the time frame (10080 min) of the experimental set-up under conditions of moisture stress. The TTD values of the strains were shortest on monosaccharides (D-Glucose, D-Galactose and D-Fructose) and a disaccharide (D-Maltose) compared to complex carbohydrates, amino acids and fatty acids when water was freely available. Hexoses support good fungal growth. Indeed being simple sugars the fungi are able to metabolise monosaccarides with ease to produce cellular energy. Conversely, under moisture stress the TTD values of the strains were longest even though utilisation of the simple sugars together with complex carbohydrates such as dextrin and the disaccharide D-Melibiose and amino acids (L-Proline and L-Alanine) by all strains was quite rapid. Fungi accumulate sugar alcohols as osmoprotectants (Schimel et al., 2007). Thus uptake of proline, glutamine and alanine and other amino acid derivatives by fungi for use as

osmolytes may have occurred (Abu-Seidah, 2007). It is possible that the amino acids L-proline and L-alanine are taken up at 0.90 a_w as an adaptation to moisture stress.

The current study suggests an additional basis for selection of a superior AFL^- strain for use in the mitigation of aflatoxin contamination of crops. Timing of application of atoxigenic strains relative to the onset of host tissue colonisation by toxigenic strains, the innate competitive ability of the atoxigenic strains in the presence of their toxigenic counterparts are all important for the successful implementation of control strategies. However, equally important is how well different atoxigenic strains adapt to environmental extremes especially when compared to their AFL^+ counterparts. Thus, characteristics of AFL^- strains which confer superior eco-competence under environmental stress (temperature, a_w) are as important as inherent competitive ability (potency of sporulation, extracellular enzyme production, growth rate) during its development in the host tissues; as the latter are in turn influenced by environmental factors (Alam et al., 2009). Based on CS utilisation patterns the current study suggests suggest ecological similarity between AFL^- and AFL^+ strains particularly under wetter and warmer conditions. Thus, it is possible that competition over available nutrients will definitely occur between the AFL^- and AFL^+ strains although this will be modified by prevailing environmental conditions and host factors.

CHAPTER 6: Relative environmental impacts on spore germination, germ tube extension, sporulation and extracellular enzyme production by toxigenic and atoxigenic strains of *Aspergillus flavus*

6.1 Introduction

There are a number of atoxigenic (AFL^-) strains which are being used to control toxigenic (AFL^+) aflatoxin producing strains of *Aspergillus flavus* in peanuts, maize and cotton ecosystems (Dorner, 2004). Indeed, sometimes mixtures of local strains have been effectively used as an inoculum, often on grain-based substrates to treat soil and to try and out-compete toxin producing strains. However, the mechanism of action of such AFL^- strains has not been completely elucidated. While effective competition may be one characteristic which is important, we have shown that control of aflatoxin production is also influenced by environmental factors such as water availability (water activity, a_w) and temperature. The relative ratio of AFL^- to AFL^+ inoculum *in vitro* and *in situ* on maize grain also significantly influenced the relative amount of control of aflatoxin achieved (see Section **4.3.2**).

The characteristics which could give an AFL^- strain an advantage may be the rate of germination, germ tube extension, hydrolytic enzyme production and perhaps rate of sporulation (Magan and Lacey, 1988). The rate of nutrient utilisation has also been suggested as key criteria to differentiate AFL^- from AFL^+ strains of *A.flavus* (Mehl and Cotty, 2013). However, results of nutritional utilisation patterns between AFL^- from AFL^+ revealed no differences in carbon source (CS) utilisation patterns between AFL^- and AFL^+ strains of *A. flavus* under different environmental conditions. Although

Mehl and Cotty (2011) showed that faster growth response to nutrients conferred a competitive edge in interactions between AFL1$^-$ and AFL1$^+$ strains of *A. flavus* this was not evident for the groups of strains examined in the current study.

Once the fungus has colonised a nutrient source, it must be able to produce the necessary hydrolases to break down the complex nutrient source for colonisation and obtain the nutrients for its growth and establishment. Thus the differential ability to produce extracellular hydrolytic enzyme may be a potential characteristic which could be involved in the competitiveness of AFL1$^-$ strains. Alam et al. (2009) showed that both a_w and temperature had a significant effect on relative production of extracellular enzymes such as esterase, lipase, acid phosphatase, β-glucosidase and N-acetyl-β-D-glucosaminidase by strains of *A. flavus* and *A. parasiticus*. Surprisingly, few studies have tried to examine hydrolytic enzyme production to determine whether they play a role in the competitiveness of AFL1$^-$ strains. Thus studies are required to examine the relative growth characteristics and enzyme production of these two groups of strains under different environmental conditions. The similarities and differences in behaviour could help understand the mechanism of action involved.

The objectives of this study were to examine the effect of a_w, temperature and their interactions on (a) relative spore germination, (b) germ tube extension rates, (c) rates of sporulation and (d) the key extracellular enzyme production capabilities of three atoxigenic and two toxigenic strains of *A. flavus* on a maize-based matrix.

6.2 Materials and Methods

6.2.1 Fungal strains

Five *A. flavus* strains were used in this study. Three strains: toxigenic strain *Af*18LS (AFL1^{+}) and atoxigenic strains *Af*19LS (AFL1^{-}) and *Af*24LS (AFL2^{-}) were isolated from stored maize collected from different agro-ecological zones of Lesotho; mountainous region, southern lowlands, foothills and Senqu River Valley respectively. Atoxigenic strain EGP15 (AFL3^{-}) was used as AFL^{-} control. The strain was isolated from Egyptian peanuts (Abdel-Hadi, 2011) while a toxigenic *A. flavus* type strain (NRRL3357; AFL2^{+}) was used as AFL^{+} control. This strain was kindly provided by Prof D. Bhatnagar of the Southern Regional Research Centre, New Orleans, LA, USA. The three AFL^{-} strains have been demonstrated to not produce aflatoxin B_1 (AFB_1) *in vitro* and on maize grain; while the toxigenic strains are able to produce AFB_1 consistently (see Section **3.3.1**). Cultures of the five strains were maintained on malt extract agar (MEA).

6.2.2 Media and inoculum preparation

The effect of temperature and a_w on percentage germination, germ tube length and sporulation of AFL^{+} and AFL^{-} strains were assessed on 3% milled maize agar (MMA). The medium was prepared by mixing 15 g of finely ground maize flour with 500 ml distilled water whose water availability was adjusted by the addition of appropriate amounts of glycerol to achieve 0.99, 0.96 and 0.90 a_w. Ten grams (10 g) of agar were added to 500 ml of the maize extract and this was thoroughly mixed before autoclaving at 121°C for 20 min. The sterile medium was agitated while cooling to 50°C to obtain a good mixture and then poured into 9 cm diameter Petri plates.

Spore suspensions of the five *A. flavus* strains were prepared by suspending conidia from 7 day old MEA cultures in sterile media comprising 0.05% (w/v) agar and 0.025% (w/v) Tween 80.

6.2.3 Assessment of germination and measurement of germ tube length

For each strain, 100 µl of spore suspension was pipetted and then spread on 3% MMA plates of different a_w treatments. The Petri plates with the same a_w were sealed in polyethylene bags and incubated at 20, 25 and 30°C for 72 h (Magan and Lacey, 1988). Experiments were carried out with three independent replicates per treatment. Periodically (every 12 h for 3 days except for 0.99 a_w plates which were also sampled after the first 6 h), 3 agar plugs were aseptically removed from each replicate plate with the aid of a sterile 10 mm diam. cork borer. The plugs were mounted on a microscope slide, stained with lactophenol cotton blue and examined under the microscope. Fifty single spores per disc were examined. Spores were considered to have germinated when the germ tube was greater than the diameter of the spore. The germ tube length of 10 spores per agar plug for each treatment were also measured every 12 h.

6.2.4 Sporulation studies

3% MMA plates were centrally inoculated with 5 µl aliquot of the spore suspension (approx. 10^6 spore ml^{-1}) in each of the a_w x temperature treatments. In all cases, three independent replicates were used. Plates with the same a_w were sealed in polyethylene bags and incubated at 20, 25 and 30°C for 10 days. After incubation, plates were flooded with 10 ml sterile water containing 0.025% (w/v) Tween 80. Spores were agitated into solution using sterile inoculating loop. The total number of spores was estimated with the aid of a haemocytometer. The number of spores obtained was divided by the area of the colony from which they were obtained.

Number of spores recovered from each treatment were expressed per area (mm^2) per volume diluent (ml) according to Parra et al. (2004).

6.2.5 Semi-quantitative screening for extracellular hydrolytic enzymatic production using API ZYM enzyme strips

***Preparation of enzyme extracts*:** Spore suspensions (approx. 10^6 spores ml^{-1}) of *A. flavus* strains (toxigenic strains AFL1$^+$ & AFL2$^+$ and atoxigenic strains AFL1$^-$, AFL2$^-$ & AFL3$^-$) were each inoculated in triplicate into flasks containing 50 ml Czapek Yeast Extract (CYE) broth (0.3% $NaNO_3$; 0.1% K_2HPO_4; 0.05% $MgSO_4 \cdot 7H_2O$; 0.001% FeSO4 · $7H_2O$; 0.5% yeast extract; 0.003% sucrose) modified with glycerol to 0.96 and 0.90 a_w. The inoculated broth flasks were incubated at 25°C with agitation on an incubator shaker (IKA® KS 4000 i, Krackeler Scientific, Inc., Albany, USA) for 5 days. The mycelium was removed by filtration through Whatman No. 1 filter paper into 50 ml centrifuge tubes and the filtrate centrifuged for 5 min at 10000 rpm. The temperature of the benchtop centrifuge (Heraeus Labofuge 400R, Thermo Scientific, Illinois, USA) was maintained at 5°C. The supernatant was pipetted into 2 ml centrifuge tubes and kept at -20°C until analysis.

The supernatants were then tested for enzymatic activity with the API ZYM generic enzyme strips (bioMérieux SA, Marcy-l'Etoile, France) as specified by the manufacturer with slight modifications. In brief, 65 µl of enzyme extract were pipetted into individual cupules containing buffer and enzyme substrate and the assay mixtures were incubated at 37°C for 4 h in a tray humidified with deionised water. Ten microlitre (10 µl) of ZYM A and 5 µl ZYM B reagents were added into each cupule and the strips were placed under a powerful light source for 5 min so that the negative reactions became colourless. The intensity of the reactions was graded from 0 to 5 with reference to the API ZYM colour reaction chart provided by the

manufacturer (see **Appendix 3A**). Values ranging from 0 (negative reaction) to 5 (reaction of maximum intensity) were assigned to each test reaction corresponding to the colour intensity, using the scale provided by the manufacturer. According to the scale, a reading of 1 to 5 is equivalent to 5, 10, 20, 30 and 40 nanomoles respectively of the API ZYM substrate being hydrolysed.

Based on these assays more detailed quantitative assays were carried out for a smaller number of enzymes which appeared to be produced in high titres extracellularly. These included esterase, lipase, acid phosphatase and N-acetyl-β-glucosaminidase.

6.2.6 Determination of total enzyme activity

Standard curves: Different concentrations of *p*-nitrophenol (*p*NP) solubilised in acetonitrile (ACN) were prepared using 2 M MOPS pH 7.5 and 40 mM Citrate buffer pH 4.8. The prepared solutions of *p*NP (40 μl), and the appropriate buffer (60 μl) were pipetted into Eppendorf tubes and incubated at 37°C in an incubator shaker for 1 h at 200 rpm. Subsequently, solution of Na_2CO_3 (5 μl) was added to each of the tubes and then left for 3 min to allow for the ionisation of *p*NP to form the yellow *p*-nitrophenolate ion. The reaction components of the tubes were added to the wells of the microtitre plates. Absorbance of the *p*-nitrophenolate ion was measured at 405 nm with the aid of a plate reader (Varioskan® Flash spectral scanning Multimode reader, Thermo Fisher Scientific Inc. Pittsburgh, PA, USA) controlled by the SkanIt Software 2.4.3 Research Edition. Calibration curves based on the 2 buffers, with absorbance at 405 nm and concentration of *p*NP (μmol ml^{-1}) as dependent and independent variable respectively were constructed (see **Appendix 3B**).

Total enzyme activity assay: A 200 µl volume of each of the 5 strains of *A. flavus* was spread-plated on Czapek Yeast Extract Agar (CYEA) media modified with glycerol to 0.96 and 0.90 a_w. For each treatment, 12 plates were used. Treatment of the same a_w were kept in sealed polyethylene bags to maintain the equilibrium relative humidity conditions and incubated at 25°C for 5 days. Three (3) replicate plates per treatment were destructively sampled after 2 days of incubation and thereafter every 24 hrs up to the 5th day of incubation. Five agar plugs (8 mm diameter) were removed from each replicate using a surface sterilised cork borer. The plugs were subsequently placed in pre-weighed Universal bottles. The bottles and their contents were weighed and the mass recorded. The bottles were shaken on an orbital shaker for 1 h at 4°C. The washings were decanted into a 1 ml plastic Eppendorf tube and centrifuged for 10 min at 10000 rpm. The temperature of the benchtop centrifuge (Hettich® Mikro 200R, Tuttlingen, Germany) was maintained at 5°C. The supernatant was transferred into fresh Eppendorf tube and total enzyme activity determined.

Hydrolytic activities of the 4 enzymes (esterase, lipase, acid phosphatase and N-acetyl-β-glucosaminidase) were measured spectrophotometically. Firstly, the required molar concentrations of the *p*NP substrates of the enzymes were prepared using appropriate solvents (*p*NP myristate and *p*NP butyrate were solubilised in isopropanol (IPA) while *p*NP N-acetyl-β-D-glucosaminide and (40 µl) *p*NP phosphate were dissolved in 40 mM Citrate buffer (**Table 6. 1**). For each enzyme assay, the reaction mixture comprised 40 µl substrate, 40 µl enzyme extracts and 20 µl buffer in an Eppendorf tube. The contents of the Eppendorf tubes were incubated at 37°C with agitation on an incubator shaker for 1 h at 200 rpm. After incubation, a solution of Na_2CO_3 (5 µl) was added to each of the tubes and then left for 3 min to provide

the basic environment necessary for the ionisation of *p*NP to form the yellow *p*-nitrophenolate ion.

The reaction mixture was then placed in the wells of the 96 well microtitre plates and absorbance read at 405 nm with the aid of a plate reader. Each microtitre plate had a set of wells for controls: a blank (substrate + buffer) which gives an assessment of the extent of non-enzymatic reaction; and the enzyme extract + buffer evaluates any background reaction in the enzyme preparation. Enzyme activity was computed using the following equation:

$$Activity\ = \frac{\Delta A}{\left[\left(\frac{\varepsilon}{\mu mol\ mol^{-1}}\right) \times \frac{0.105\ ml}{assay} \times (60\ min.) \times \left(\frac{Wt_{plugs}}{1\ ml\ sample}\right) \times \frac{0.04\ ml}{assay}\right]}$$

$Where$:

$\Delta A = A_{sample} - A_{blank} = Actual\ absorbance$

$Wt_{plugs} = weight\ of\ agar\ plugs\ (g)$

$\varepsilon = slope\ of\ the\ standard\ curve\ (absorbance\ coefficient)$

$0.105\ ml = total\ volume\ of\ assay$

$0.04\ ml = volume\ ezyme/assay$

$60\ min = time\ of\ incubation$

Table 6. 1 Extracellular enzymes assayed, their *p*-nitrophenol substrate concentrations and buffers used in this study

Enzyme	Substrate	Chemical structure	Buffer	pH
Lipase	10 mM 4-nitrophenyl myristate		200 mM MOPS	7.5
Esterase	10 Mm 4-nitrophenyl butyrate		200 mM MOPS	7.5
N-acetyl-β-glucosamidase	5 mM 4-nitrophenyl N-acetyl-β-d-glucosaminide		40 mM citrate	4.8
Acid phosphatase	8 mM 4-nitrophenyl phosphate		40 mM citrate	4.8

6.2.7 Statistical analyses

All data were subjected to Shapiro-Wilk and Levene's test to assess normality and variance homogeneity respectively. The data violated these assumptions ($p<0.05$) even after transformation and therefore, non-parametric analysis (Wilcoxon /Kruskal-Wallis H test: ($p=0.05$) was conducted to establish whether the main effects for strains, temperature and a_w were significantly different. Where significant differences were observed, post-hoc analysis using Mann-Whitney U ($p=0.05$) was performed to establish which groups for all factors were different. Statistical package JMP®8 (SAS Institute Inc., 2008, Cary NC, USA) was used to perform the analysis.

6.3 Results

6.3.1 Effect of temperature and a_w on spore germination and germ tube extension

In general, conidia of all strains germinated most rapidly under warmest (30°C) and when water was freely available (0.99 a_w). No spores germinated under water stress (0.90 a_w) and at the coolest temperatures (20°C) examined in the time scales of the experiments (**Figure 6. 1** & **Figure 6. 2**). Statistical analysis revealed that the effect of all factors investigated (temperature, a_w, time and strain) were significant ($p<0.05$: **Table 6. 2**). Post-hoc analysis using Mann-Whitney U test indicated that spores of atoxigenic strains AFL1$^-$ and AFL2$^-$ germinated faster than AFL2$^+$ ($p<0.05$). However, they germinated at a similar rate to toxigenic strain AFL1$^+$ under all conditions. For example, when water was freely available (0.99 a_w) and temperatures lower (20°C) it took 24 h for 100% of the conidia of strains AFL1$^+$ and AFL2$^-$ to germinate while it took a longer time for AFL2$^+$ (48 h) to reach the same percentage spore germination. Under warmer and wetter conditions, both AFL1$^+$ and AFL2$^-$ germinated rapidly with

over 90% of the conidia of the two strains having germinated within 6 h. None of AFL2+ had germinated after this period under the same conditions.

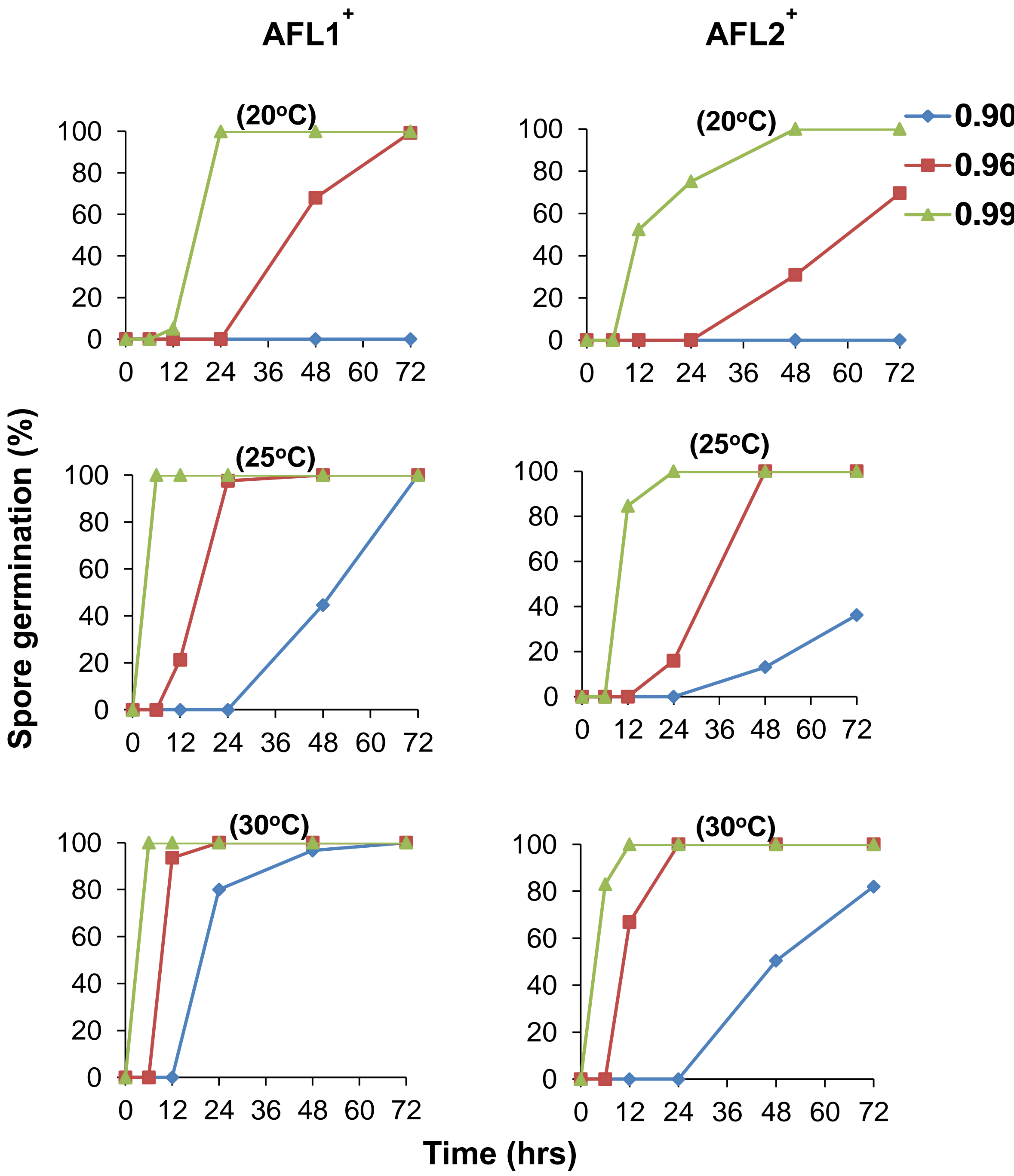

Figure 6. 1 Effect of temperature and water activity on spore germination of AFL1+ and AFL2+ growing on milled maize agar over 72 h period.

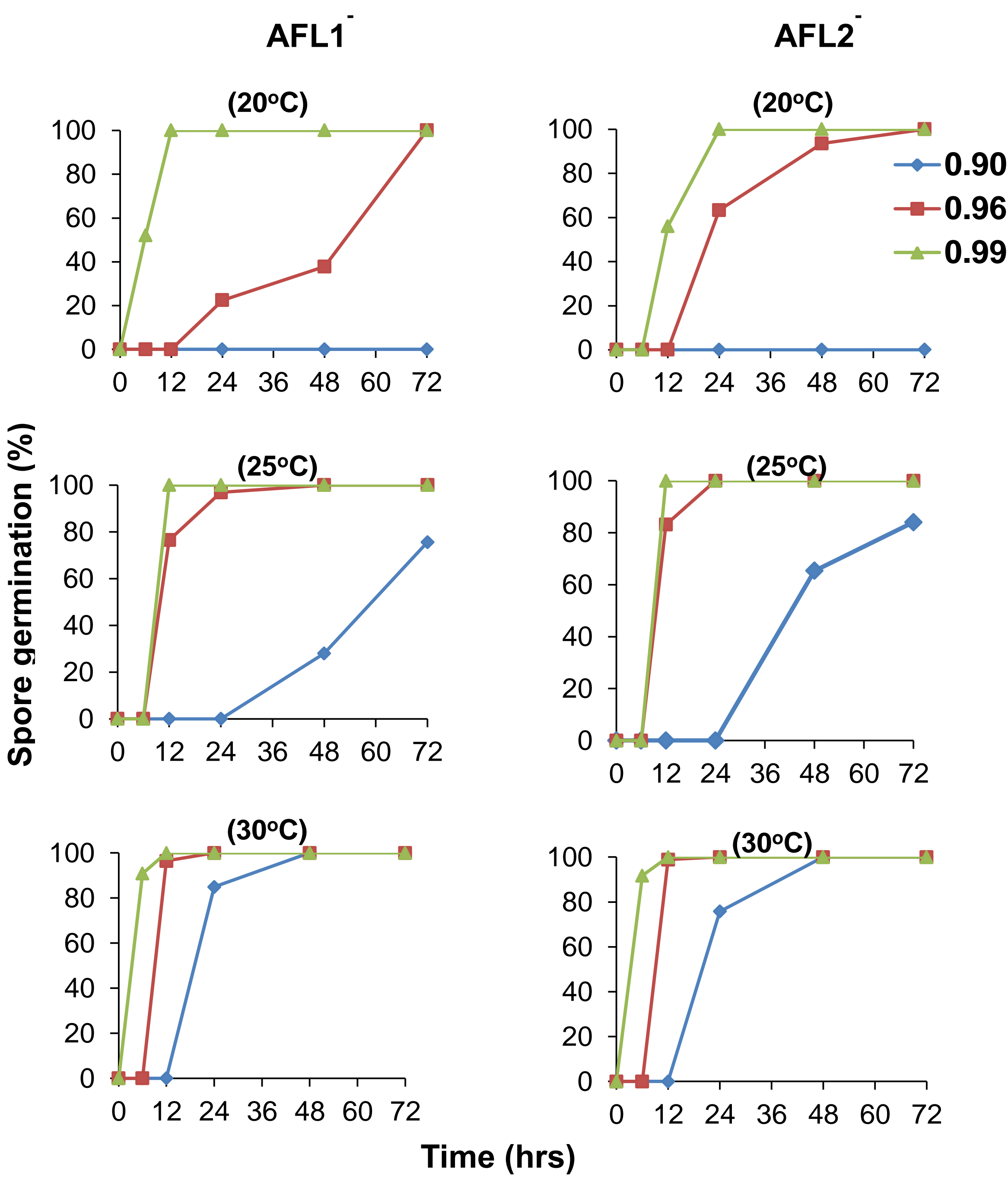

Figure 6. 2 Effect of temperature and water activity on spore germination of AFL1$^-$ and AFL2$^-$ growing on milled maize agar over 72 h period.

Table 6. 2 Kruskal-Wallis One-way analysis of variance (ANOVA) by Rank results for spore germination as a function of *A. flavus* strain (AFL1$^+$, AFL2$^+$, AFL1$^-$, AFL2$^-$ and AFL3$^-$), water activity (0.90, 0.96 and 0.99 a_w), temperature (20, 25 and 30°C) and time (12-72 h).

Factor	**df**	X^2	***p***
Strain	4	29.2	0.00
Temperature	2	224.0	0.00
a_w	2	530.9	0.00
Time	4	780.6	0.00

Figure 6. 3 & **Figure 6. 4** show the changes in germ tube extension in relation to temperature and a_w respectively. Overall, the extension of the germ tube for all strains was fastest at higher temperatures and wettest conditions. However, this was significantly reduced at lower temperatures (20°C) and driest conditions (0.90 a_w: **Figure 6. 5** & **Figure 6. 6**). There were noticeable inter-strain differences in germ tube extension. For example, when tempratures were higher and water freely available AFL2$^-$ had attained an avarage germ tube length of approx. 300 µm after 12 h. This germ tube length was almost twice the length reached by AFL1$^+$ germ tube after the same period under similar conditions. Even when temperatures were lower but water freely available, the germ tube elongation of AFL2$^-$ was still more rapid.

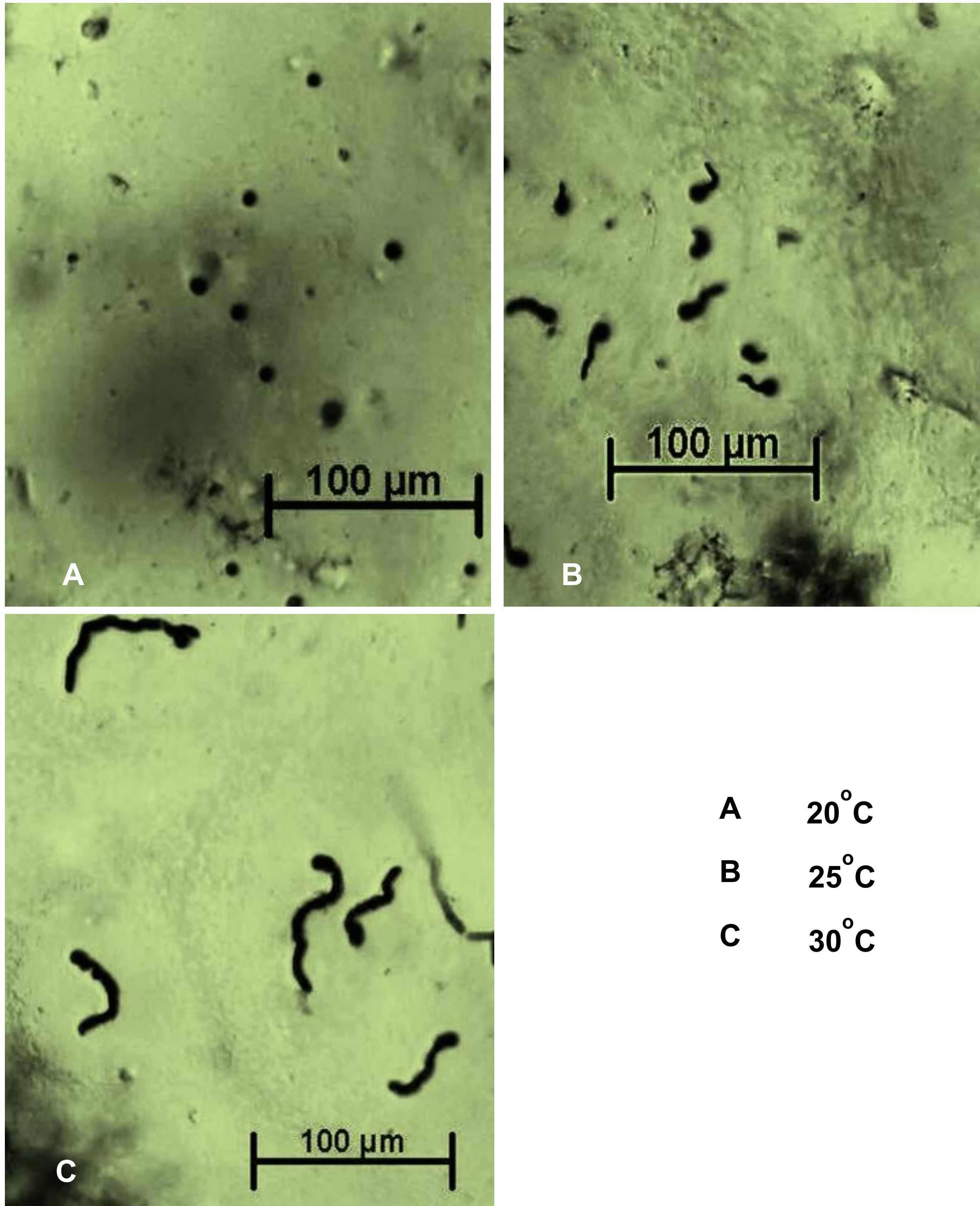

Figure 6. 3 Extension of the germ tube length of strain AFL2- on milled maize agar plates adjusted to 0.96 a_w. The cultures were incubated at three different temperatures for 12 h.

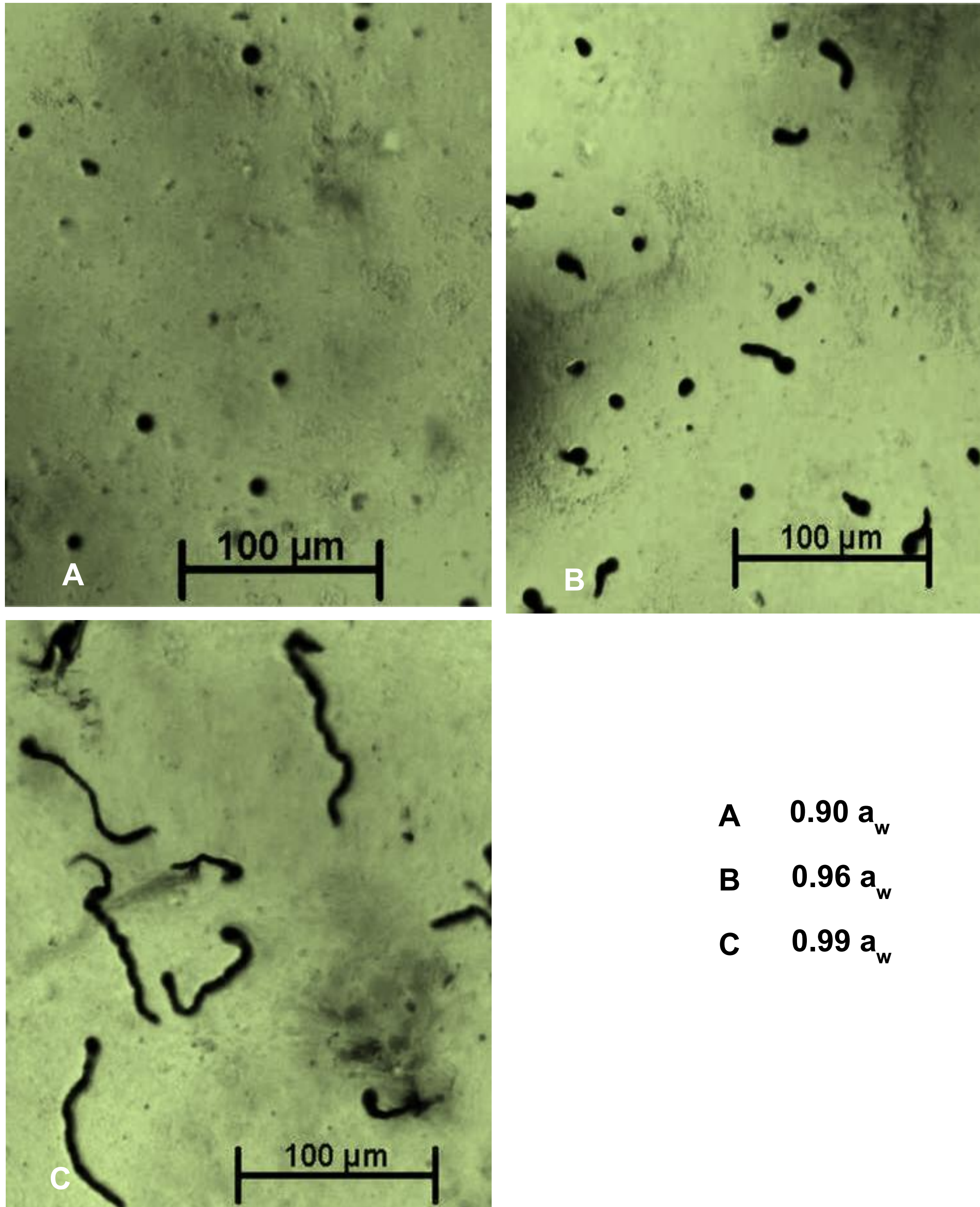

Figure 6. 4 Extension of the germ tube length of strain AFL2$^-$ on milled maize agar plates adjusted to three levels of a_w. The cultures were incubated at 25°C for 12 h.

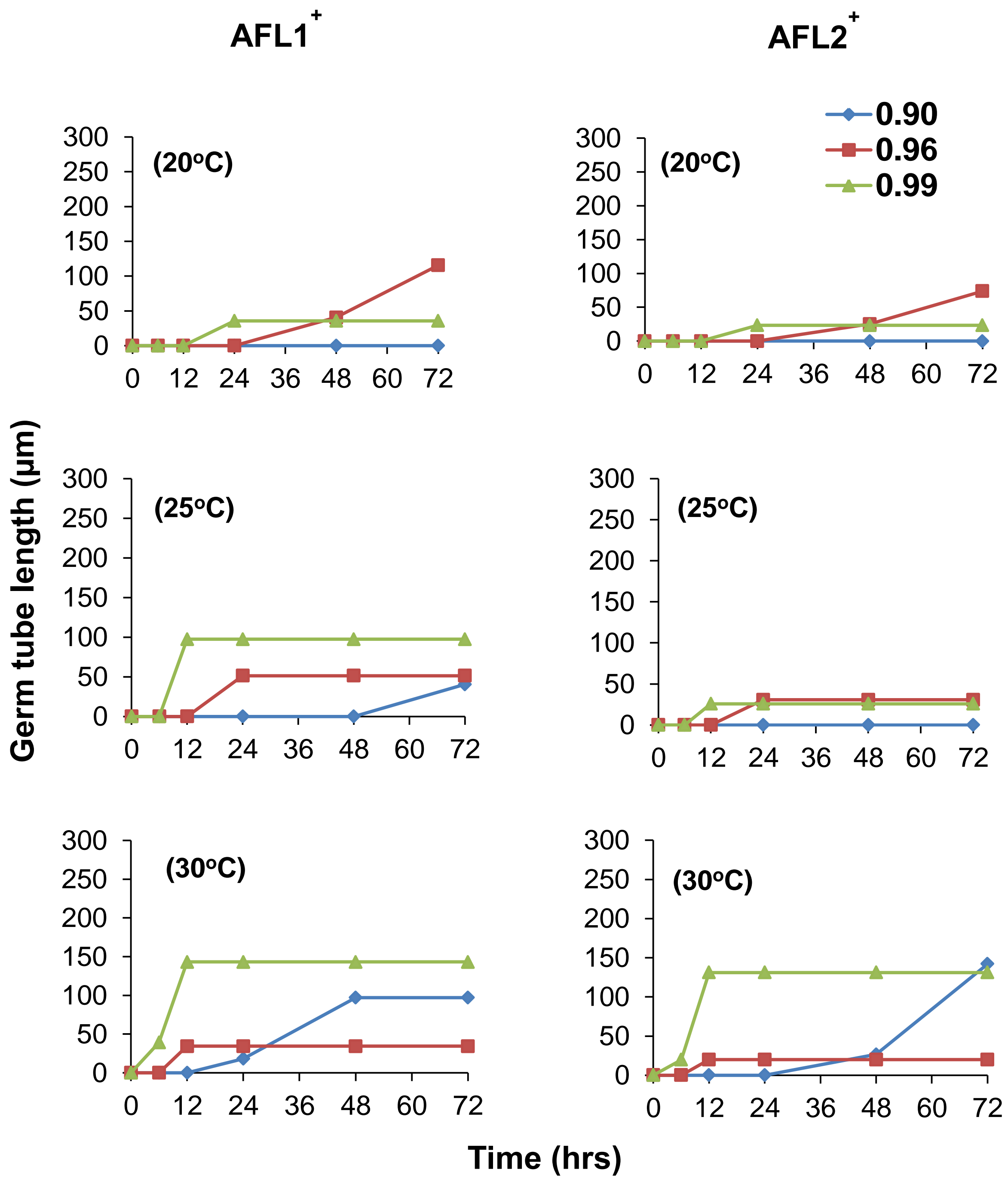

Figure 6. 5 Effect of temperature and water activity on germ tube extension of strains $AFL1^+$ and $AFL2^+$ growing on MMA over 72 h period.

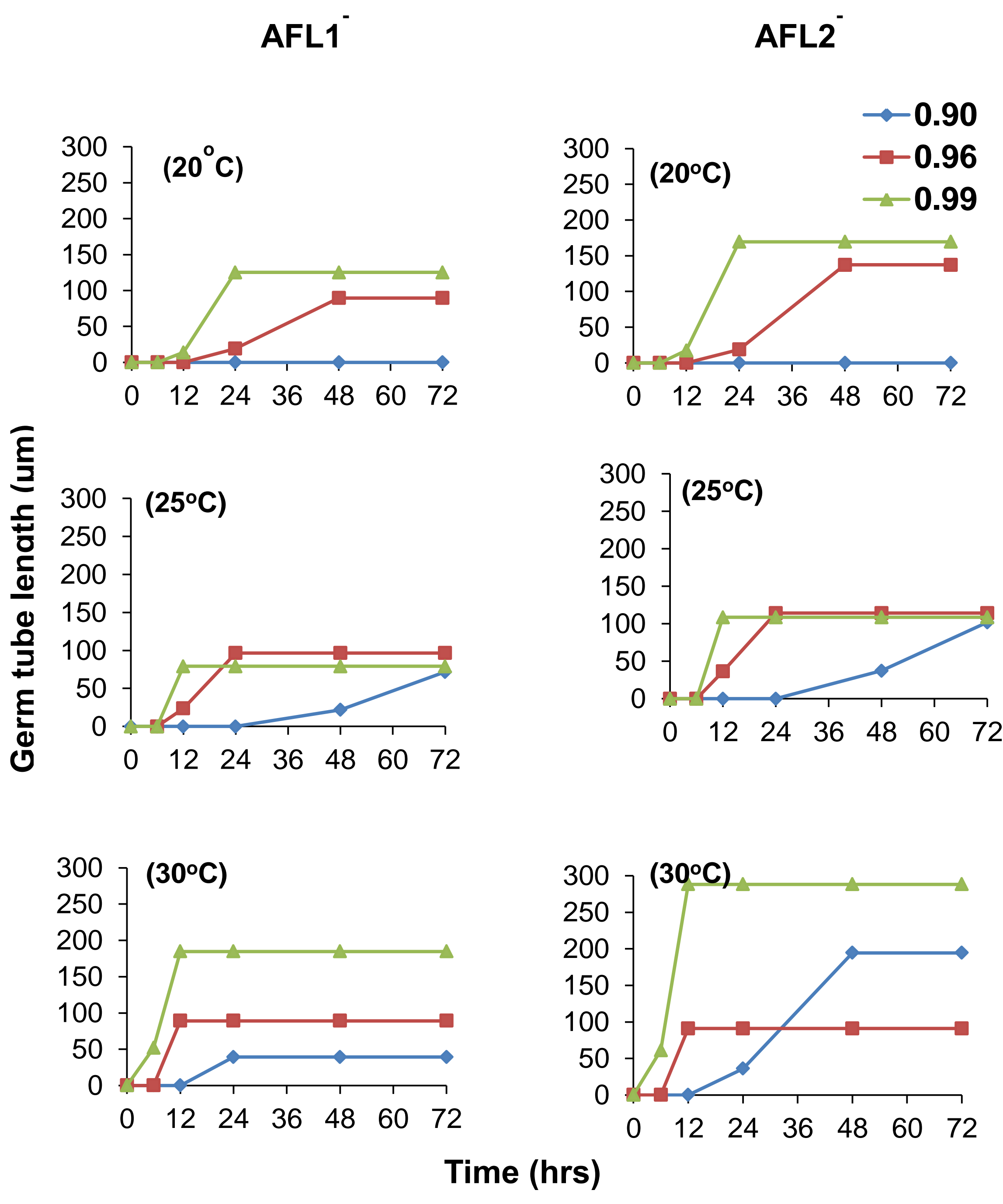

Figure 6. 6 Effect of temperature and water activity on germ tube extension of strains AFL1- and AFL2- growing on MMA over 72 h period.

6.3.2 Effect of temperature and a_w on sporulation

Figure 6. 7 presents the means and standard deviations of number of spores produced by 5 strains of *A. flavus* as log_{10}(conidia mm^{-2} ml^{-1}) under different temperature and a_w conditions. In general, the main effects for both temperature and a_w were significant ($p<0.05$: **Table 6. 3**). Post-hoc comparisons using Mann-Whitney U ($p=0.05$) indicated that sporulation was optimal at 30°C and 0.99 a_w, lowest at 0.90 a_w at all temperatures explored. At 20°C and 0.90 a_w none of the two groups of strains produced asexual spores. However, differences between strains with regard to sporulation were not significant ($H(4) = 6.0$, $p = 0.20$).

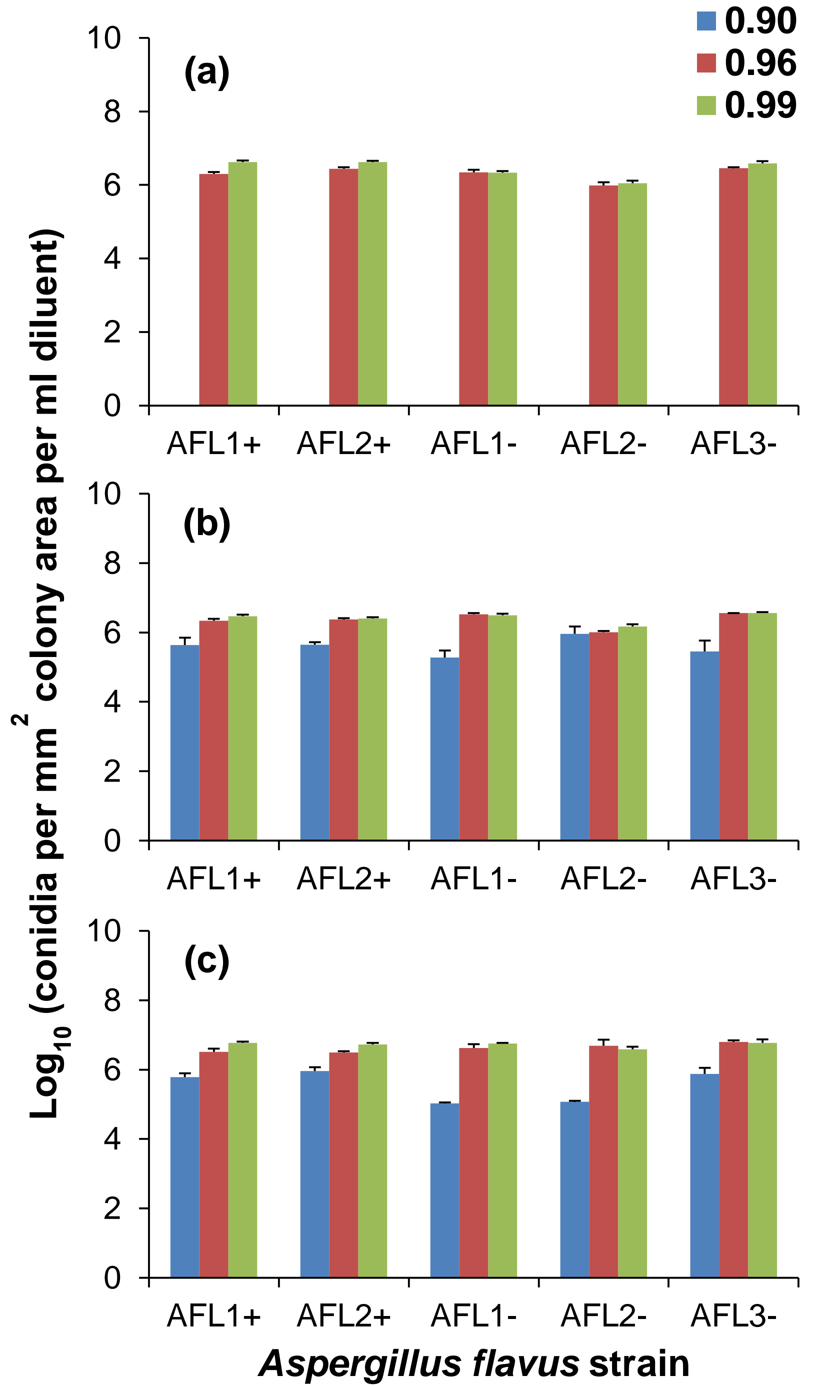

Figure 6. 7 Effect of temperature (a) 20°C, (b) 25°C & (c) 30°C and water activity on sporulation of toxigenic and atoxigenic *A. flavus* strains growing on MMA after 10 days of incubation.

Table 6. 3 Kruskal-Wallis One-way analysis of variance (ANOVA) by Rank results for sporulation as a function of *A. flavus* strain ($AFL1^+$, $AFL2^+$, $AFL1^-$, $AFL2^-$ and $AFL3^-$), water activity (0.90, 0.96 and 0.99 a_w) and temperature (20, 25 and 30°C).

Factor	df	X^2	*p*
Strain	4	6.0	0.20
Temperature	2	16.6	0.00
a_w	2	89.3	0.00

6.3.3 Effect a_w on hydrolytic enzyme production

Figure 6. 8 compares the number and quantities of substrates that enzyme extracts of each of the 5 strains of *A. flavus* were able to hydrolyse. For all strains, a greater number of substrates was hydrolysed by extracts derived from 0.96 than 0.90 a_w treatments from CYE broth cultures. Additionally, a greater degree of substrate hydrolysis was achieved when 0.96 a_w extracts were added to the cupules. In general, enzyme extracts from $AFL1^+$, $AFL2^+$ and $AFL3^-$ hydrolysed more substrates compared to extracts from $AFL1^-$ and $AFL2^-$ (**Figure 6. 9**).

The temporal changes in total activity of acid phosphatase and N-acetyl-β-glucosaminidase produced by each of the five strains of *A. flavus* are compared in (**Figure 6. 10** & **Figure 6. 11**). Significantly greater total activity of all enzymes was observed at higher a_w than under drier conditions (0.90 a_w). The main effect of time of incubation and strain assayed was significant for acid phosphatase and N-acetyl-β-glucosaminidase activity. However, the activity of lipase and esterase was not significantly different (**Table 6. 4**). Overall, N-acetyl-β-glucosaminidase from the two toxigenic strains was greater than atoxigenic strains'. The acid phosphatase activity of the atoxigenic strain $AFL1^-$ had a mean rank of 30.2 which was significantly higher

than that of the toxigenic strain AFL2$^+$ (mean rank = 18.9: data not shown). However, the phosphatase activity of the other atoxigenic strains was not significantly different from the activity of both AFL1$^+$ and AFL2$^+$.

Figure 6. 8 API ZYM strips showing enzymatic activity (colour change and intensity) of extracts of AFL2$^-$ grown on CYE broth adjusted to (a) 0.96 and (b) 0.90 a_w.

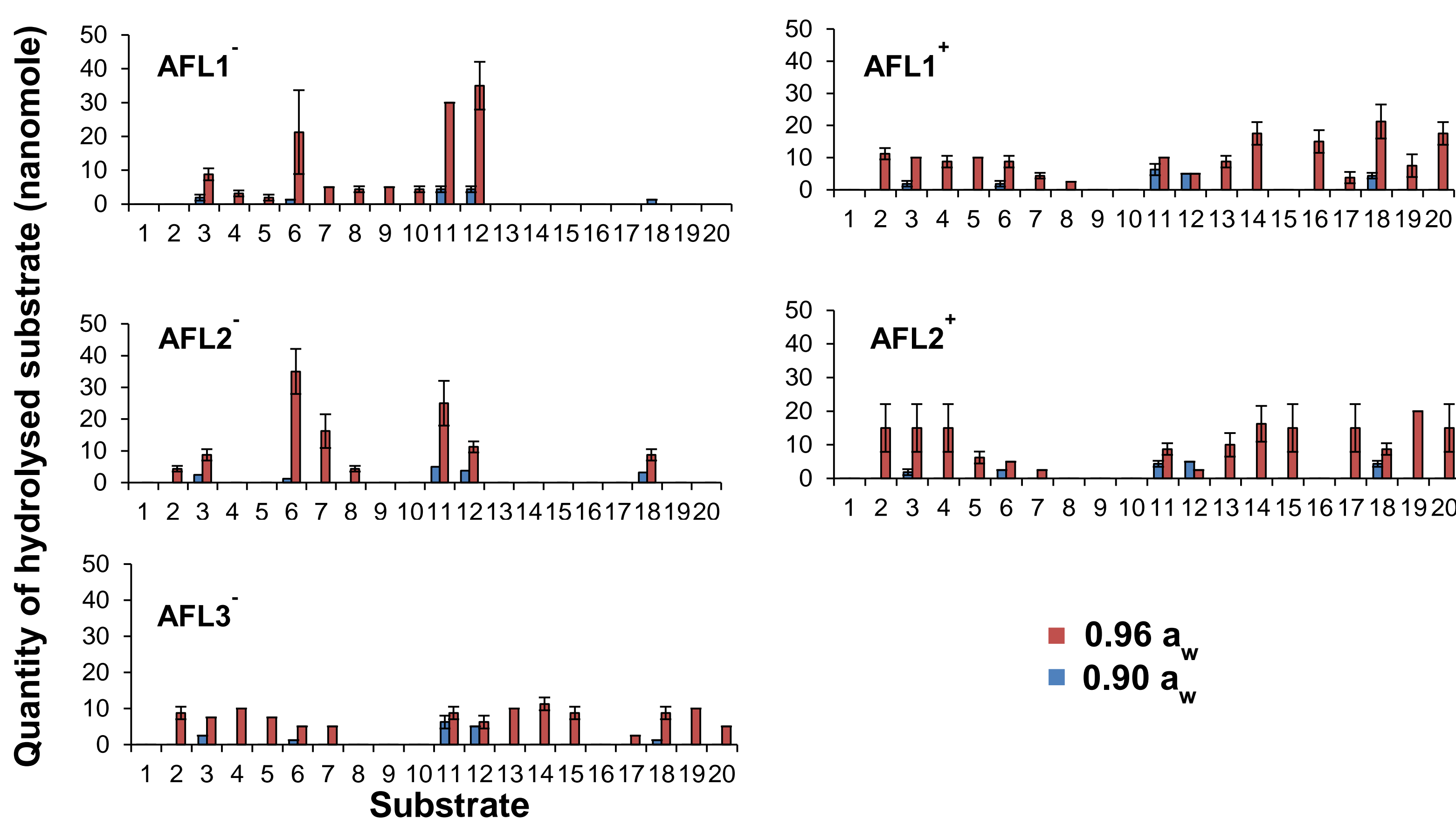

Figure 6. 9 Comparison of quantity of hydrolysed substrate (nanomole) by enzyme extracts of toxigenic and atoxigenic strains grown on 0.96 and 0.90 a_w CYE broth at 25°C for 120 h. See **Appendix 3A** for the names of the different substrates in the cupules

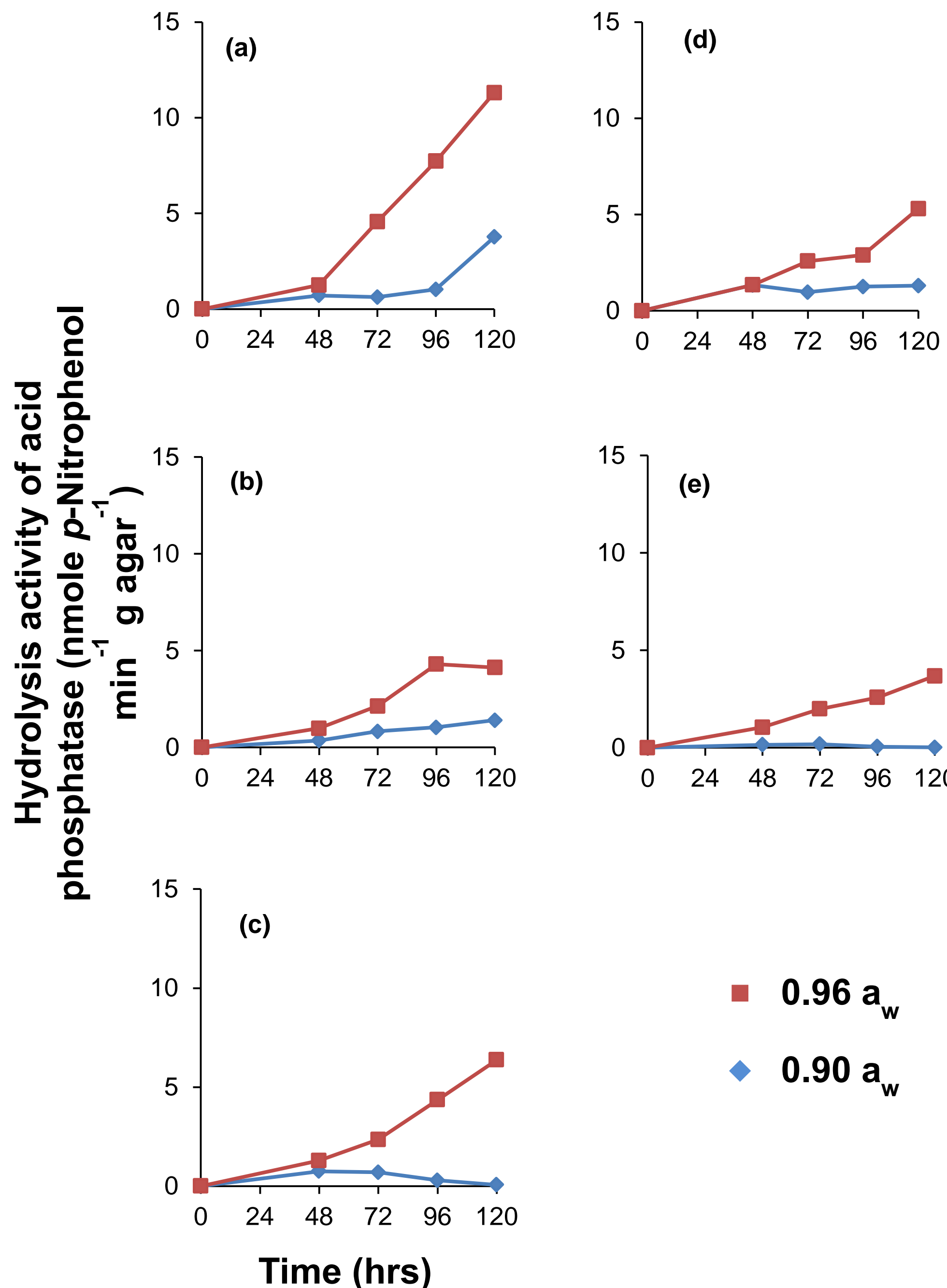

Figure 6. 10 Comparison of acid phosphatase activity from atoxigenic (a) AFL1$^-$, (b) AFL2$^-$, and (c) AFL3$^-$) and toxigenic (d) AFL1$^+$ and (e) AFL2$^+$ strains of *A. flavus* grown on CYEA amended to (0.90, 0.96 a_w) over a period of 120 h.

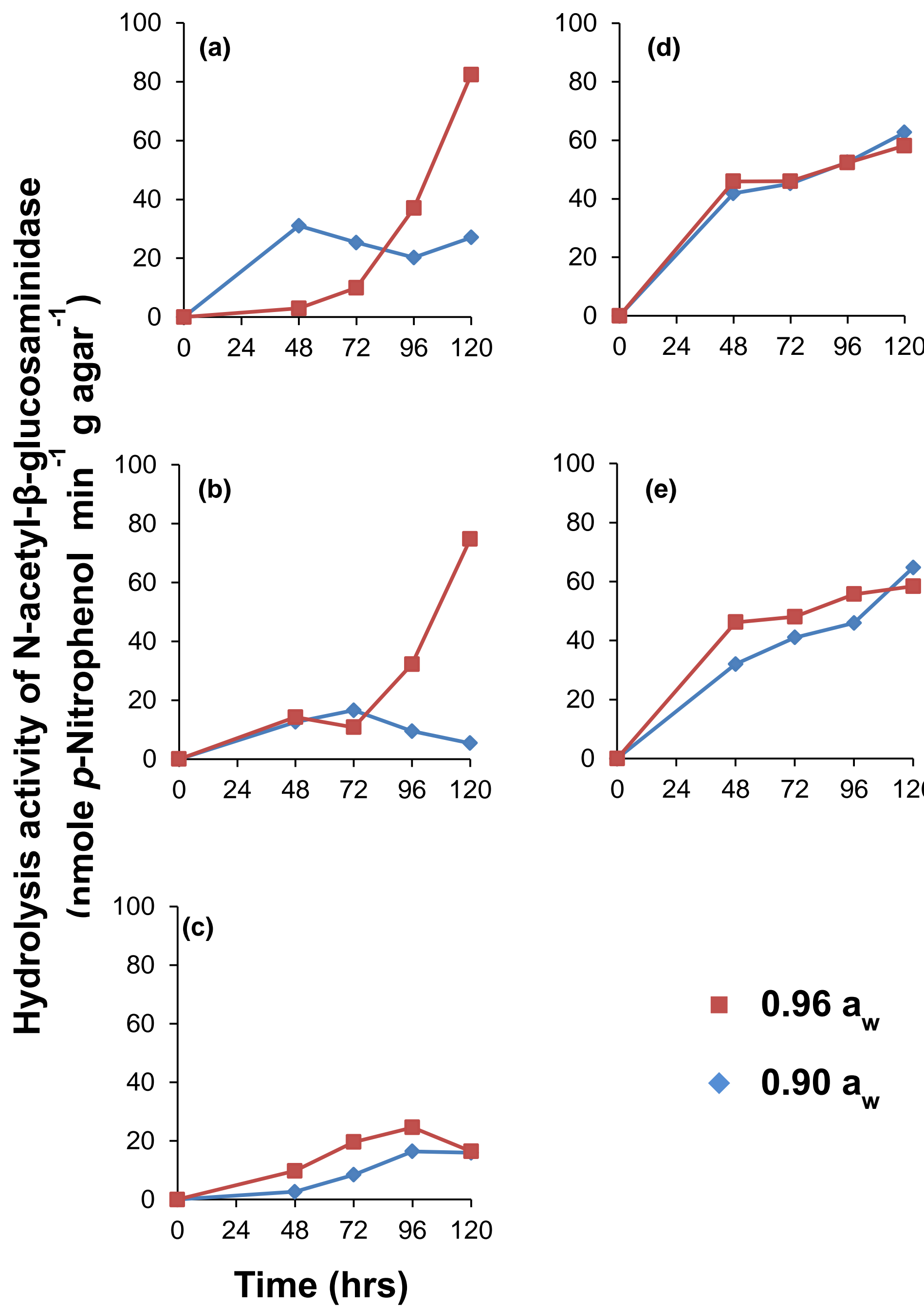

Figure 6. 11 Comparison of *N*-acetyl-β-glucosaminidase activity from atoxigenic (a) $AFL1^-$, (b) $AFL2^-$, and (c) $AFL3^-$) and toxigenic (d) $AFL1^+$ and (e) $AFL2^+$ strains of *Aspergillus flavus* grown on CYEA amended to (0.90, 0.96 a_w) over a period of 120 h.

Table 6. 4 Kruskal-Wallis One-way analysis of variance (ANOVA) by Rank results for total hydrolysis activity of esterase, lipase, acid phosphatase and N-acetyl-β-glucosaminidase as a function of *A. flavus* strain ($AFL1^+$, $AFL2^+$, $AFL1^-$, $AFL2^-$ and $AFL3^-$), incubation time and water activity. The different enzyme extracts were obtained from cultures grown on CYA incubated at 25°C.

Factor		**Esterase**		**Lipase**		**Acid phosphatase**		**N-acetyl-β-glucosaminidase**	
	df	X^2	*p*	X^2	*p*	X^2	*p*	X^2	*p*
Strain	4	3.02	0.56	7.35	0.12	11.12	.025	58.84	0.00
a_w	1	82.75	0.00	36.37	0.00	64.08	0.00	4.65	0.031
Time	3	4.84	0.18	1.63	0.66	13.54	.004	17.69	0.001

6.4 Discussion

This study was aimed at examining whether the rates of germination and germ tube extension or hydrolytic enzyme production could be used to differentiate atoxigenic from toxigenic strains under different environmental conditions and help explain the mechanism of action for controlling AFB_1 production.

Germination was most rapid under warmest and freely available water conditions and no spores germinated at 0.90 a_w and 20°C. Under warmest and wettest conditions, all strains germinated rapidly with 100% of the conidia of all strains having germinated within 12 h of incubation. A similar result has previously been reported by Bluma et al. (2008) on maize meal extract medium. Athough Bluma et al. (2008) investigated the effect of essential oils under varying a_w conditions on germination of *A. flavus*, they reported that under wetter conditions the germination rate of the strains ranged between 92-100% after 12 h of incubation. However, in their study the effect of temperature was

not investigated. Gadgile and Chavan (2010) demonstrated that spore germination of strains of *A. flavus* that cause rot of mango fruit increased with increase in temperature and relative humidity (RH).

In the present study, atoxigenic strains AFL1$^-$ and AFL2$^-$ germinated faster than the toxigenic strain AFL2$^+$ under the different set of environmental conditions but their germination rates were not different from toxigenic strain AFL1$^+$. Although these comparisons were made in axenic cultures, similarities and differences in intrinsic germination rates between the AFL$^-$ and AFL$^+$ strains can occur because quite diverse populations of *A. flavus* exist on crops (Horn and Dorner, 1999). While higher germination rates exhibited by the AFL$^-$ strains might be interpreted as a sign of greater competitiveness, it is common for more competitive strains to co-exist with less competitive strains due to adaptation and differences in ecological life strategies (Mehl and Cotty, 2010). It has also been suggested that where the competing populations (in this case AFL$^-$ and AFL$^+$ strains) have similar environmental requirements, the dominant population may be the one that is able to effectively utilise most efficiently the finite resources (Atlas and Bartha, 1998).

However, in Section **5.3.3** it was shown that the AFL$^-$ and AFL$^+$ strains had similar CS utilisation patterns. It therefore follows that other factors other than efficiency of uptake and utilisation of nutrients should explain the dominance of one group over the other. Indeed where germination cannot be used to explain dominance of one strain/species over the other, stress tolerance becomes the deciding factor (Atlas and Bartha, 1998). Based on environmental factors (temperature and water availability), atoxigenic strains AFL1$^-$ & AFL2$^-$ and

toxigenic strain $AFL1^+$ were equally competitive (based on germination rate) and they are therefore likely to co-exist when it is wetter and warmer. However, when $AFL1^-$ & $AFL2^-$ are found in the same habitat as $AFL2^+$, increasing the temperature and amount of available water may lead to the dominance of the two AFL^- strains. In nature, temperature and available water regimes are in a state of flux, thus the advantage shifts back and forth, leading to seasonal shifts in proportions of the two populations ($AFL1^-$ or $AFL2^-$ and $AFL2^+$ (Atlas and Bartha, 1998; Magan and Aldred, 2007a).

Indeed the strains used in the study came from different geographical areas of Africa and were isolated from different substrates; it is possible that the differences observed are a result of ecological adaption and perhaps differences in ecological life strategies mentioned earlier. The atoxigenic strains $AFL1^-$ & $AFL2^-$ and toxigenic strain $AFL1^+$ were isolated from maize kernels from Lesotho while both the type strain NRRL3357 (strain $AFL2^+$) and $AFL3^-$ were isolated from peanuts. Maize-based artificial medium was used to assess the germination in the present study and this might have conferred an ecological advantage to the three strains (atoxigenic $AFL1^-$ & $AFL2^-$ and toxigenic strain $AFL1^+$) described above resulting in better germination rates than the two control strains ($AFL2^+$ and $AFL3^-$).

Overall, the germ tube extension of spores of all strains was fastest at higher temperatures and wettest conditions. However, this was significantly reduced at lowest temperatures and driest conditions. When temperatures were higher and water freely available germ tube length of $AFL2^-$ was twice that of $AFL1^+$ after 12 h of incubation. Extensive germ tube extension might confer a competitive

advantage during host contact and its eventual colonisation. Indeed, Mehl and Cotty (2011) suggested that the strains which make contact with the host first, have a competitive edge over slower growing ones in mixed cultures. $AFL2^-$ gave good control of aflatoxin B_1 of maize when co-inoculated with $AFL1^+$ on maize matrices (Section **4.3.2**). It is possible that the control could have been a result of faster germ tube elongation rates of this strain when compared to $AFL1^+$.

In the current study sporulation was optimum when water was freely available (0.99 a_w) and temperatures warmest (30°C). However, when the AFL^- and AFL^+ strains were compared no differences in sporulation were observed between them, regardless of temperature when water was readily available. Despite seeding sclerotia of *A. flavus* instead of spore suspension on a defined medium (Czapek Dox agar), Giorni et al. (2012) reported that *A. flavus* sporulated profusely at 0.99 a_w and 30-35°C; and that at ≤0.90 a_w over similar temperature ranges sporulation was reduced. This observation concurs with the findings of the current study. In contrast, Mehl and Cotty (2010) reported that different *A. flavus* strains produced different quantities of conidia on maize. However, their study did not take into account the effect of environmental factors on sporulation. In the current study, sporulation was assessed in axenic cultures and not subjected to the effects of competition. Since reduced sporulation is accompanied by increase in toxin production by toxigenic strains (Cotty, 1989), similarities in sporulation between the AFL^- and AFL^+ strains may suggest a fitness advantage of atoxigenic strains (in respect of sporulation) in mixed

cultures. It is possible that during competition, toxigenic strains channel the limited resources towards production of AFs than for dispersal (sporulation)

The relative production of hydrolytic enzymes by the different strains of *A. flavus* were also examined under different a_w conditions. Initial screening suggested that there were some key extracellular enzymes which could be important in infection of maize under different interacting environmental conditions. Subsequnetly, production of four key enzymes by the different *A. flavus* strains was shown to be significantly affected by changes in a_w. In general, N-acetyl-β-glucosaminidase activity in the current study was greater for the two AFL^+ strains compared to the AFL^- strains. Acid phosphatase production was greater for AFL^- strains than toxigenic strains. There was no significant difference in lipase and esterase production ability between AFL^- and AFL^+ strains. Previous studies by Alam et al. (2009) also compared a strain of *A. flavus* and *A. parasiticus* in relation to production of hydrolases under different a_w and temperature conditions. Production of these enzymes was shown to be affected by incubation period and a_w. Gadgile and Chavan (2010) demonstrated that production of hydrolases by *A. flavus* was reduced at lower temperatures (10^oC) when the environmental relative humidity was 35% and this observation is consistent with the results of the present study. Although temperature was not varied in the present study, it was showed that increasing water activity lead to increase in extracellular enzyme production and vice versa by the different *A. flavus* assayed.

The current study has shown that there are differences in the measures of ecological fitness between atoxigenic and toxigenic strains. It is however

important to note that the differences observed may not necessarily be due to the strains being toxigenic or not but rather due to ecolological adaption because they were isolated from different regions and substrates. The effect of abiotic factors (temperature and a_w) on both germination, germ tube extension and hydrolytic enzyme production have been demonstrated. This study has shown that competitive advantage need not be based solely on ability to utilise substrate rapidly. Tolerance to environmental stress also may be an important factor in determining the outcome of the competition. This is a case of competition for survival under those conditions which do not favour germination. Nevertheless, the information obtained may be useful for screening of atoxigenic strains for control of aflatoxin contamination of agronomically important crops.

Additionally, hydrolytic enzymes do not appear to be a key mechanism of action of AFL^- strains to dominate toxigenic ones, regardless of a_w x temperature conditions. Huang et al. (2011) suggested that toxin inhibition is thigmo-regulated and not per se a result of competitive exclusion. Such studies if conducted over a range of realistic environmental conditions might shed some light as to what exactly mediates AF control during interactions between AFL^- and AFL^+ strains.

CHAPTER 7: Improving water stress tolerance of an atoxigenic *Aspergillus flavus* strain grown on maize-based media and sorghum grain for control of aflatoxin contamination of maize

7.1 Introduction

In recent years, the use of atoxigenic (AFL^-) *Aspergillus flavus* strains to competitively exclude aflatoxin producing strains and hence lead to the control of aflatoxin contamination of nuts, cotton and maize has received a lot of attention. The strategy employs native, locally occurring AFL^- strains. Atoxigenic strains are inoculated on sorghum carrier which also acts as a nutrient source for the fungus. The colonized sorghum is applied to the soil surface of crops in the field.

Since field conditions are characterized by fluctuating abiotic factors including water availability and temperature which affect the efficacy of biocontrol agents (BCAs); (Magan, 2006); improving BCAs for stress tolerance is crucial. Previous studies have shown that modification of BCAs can improve ecological fitness and enhance efficacy (Hallsworth and Magan, 1995; Teixidó et al., 1998; Mokiou and Magan, 2008).

Efficacy of AFL^- strains to inhibit toxin production during interaction with toxigenic strains (AFL^+) is certainly influenced by water availability and was demonstrated in Section **4.3.2**. Water availability was also shown to affect nutrient utilisation by both AFL^- and AFL^+ although no differences were observed between the strains when they were compared (see Section **5.3.2**). It

was also demonstrated that under water stress, the two groups of strains preferentially utilised osmolytes such as proline. Indeed, when fungal cells are subjected to water stress, osmoprotectants are synthesised or accumulated inside the cells to equilibrate the total water potential of the cytoplasm with that of their surrounding environment (Csonka, 1989). The types and amounts of compatible solutes accumulated are governed by substrate, and the amount of stress that the organism is subjected to (Hallsworth and Magan, 1994; Mokiou and Magan, 2008). Microbial water stress response in particular has successfully been exploited to manipulate intracellular osmolyte built-up in BCA inocula with the view to improving their water stress tolerance (Mokiou and Magan, 2008; Andersen et al., 2006).

Despite the dynamic nature of agro-ecosytems in respect of temperature and moisture stress, the performance of physiologically manipulated conidia of AFL^- strains produced on sorghum grain of different moisture contents to examine aflatoxin B_1 control efficacy has not been previously tested.

The objectives of this study were: (a) examine the effect of different solute-modified maize-based media (proline, sorbitol, glucose, glycerol, NaCl) and (b) on sorghum grains of different water availability on production of conidia of the atoxigenic *A.flavus* strain $AFL2^-$. These conidial treatments were examined for germination and germ tube extension in water agar based media modified to 0.96 and 0.92 a_w. The best treatments were tested in stored maize grain of different a_w levels in mixed culture of 50% $AFL2^-$ and 50% $AFL1^+$ for relative aflatoxin B_1 control.

7.2 Materials and Methods

7.2.1 Fungal strain

The *A. flavus* strain AFL2$^-$ used in the current study was isolated from stored maize from Lesotho. It lacked the regulatory gene *aflR* and two structural genes: *aflP* and *aflM* of the aflatoxin biosynthetic pathway. The strain was previously shown to give better control of AFB_1 production during interaction with AFL1+ on maize than any of the other AFL$^-$ tested in mixed concentrations of the lowest and intermediate concentrations, i.e., 25:75 & 50:50 (AFL2$^-$:AFL1$^+$; see Section **4.3.2**).

7.2.2 ***In vitro*** **solute-modified media for conidial inocula production**

The basal medium was 3% milled maize agar (MMA). It had a water activity (a_w) of 0.98 measured with AquaLab® 3 TE, (Decagon Devices, Inc., Pullman, Washington, USA). The a_w of the basal medium was amended using solutions of proline, sorbitol, glucose, glycerol and NaCl to 0.96 and 0.92 a_w. The amounts of each of the 5 solutes added to arrive at the desired a_w are given in **Table 7. 1**.

Table 7. 1 Amount of solute (g) to be added to 100 ml of water.

	Water activity[¶]	
Solute	**0.96**	**0.92**
Proline	20.00	32
Sorbitol	30.75	54.66
Glucose	39.85	77.66
Glycerol	18.40	41.40
NaCl	7.01	13.50

[¶]The water with adjusted water activity was used to prepare 3% milled maize agar.

***Inoculation and growth of* A. flavus *AFL2*$^-$**: The plates of MMA modified with different solutes were centrally inoculated with a 5 µl spore suspension 7 day old conidial suspensions of AFL2$^-$ (approx. 10^6 spores ml^{-1}). The treatments were separately placed in polyethylene bags and incubated at 25^oC for 10 days. There were three replicates per treatment.

Spore harvesting and preparation: Colonies from each treatment and replicate were flooded with 10 ml sterile solutions of polyethylene glycol 200 (PEG) solutions of the same a_w as the treatment. The procedure equilibrates the total water potential of the cytoplasm of the fungal cells with that of their surrounding environment so that any accumulated or synthesised solutes inside the fungal cell do not leach out owing to a diffusion gradient (Ypsilos and Magan, 2004). This would be the case where pure water is used. Control spores were harvested from colonies grown on non-modified MMA medium using sterile distilled water with 0.01% (w/v) Tween 80

The spore suspensions were placed into sterile 50 ml centrifuge tubes. The tubes were then vigorously shaken for 30 min followed by centrifuging on a bench top centrifuge for 5 min at 3000 rpm. The temperature of the bench top centrifuge (Heraeus Labofuge 400R, Thermo Scientific, Illinois, USA) was maintained at 5°C. The supernatant was discarded and the spores resuspended in 5 ml PEG 200 solutions of the appropriate a_w.

7.2.3 Production of conidia on sorghum grain

Moisture adsorption curve: Autoclaved sorghum grain variety *Khunoane* from Lesotho was used in this study. A sorghum moisture adsorption curve was initially constructed to determine the amounts of water required to add to sorghum to amend the a_w to the treatment levels required: 0.98, 0.96 and 0.92. The curve was obtained by adding different quantities of water to 5 g sub-samples of sorghum grain previously autoclaved. The treatments were stored at 4°C for 48 h to equilibrate before equilibration at 25°C and measuring the a_w and the moisture content.

Based on the moisture adsorption curve of sorghum (see **Appendix 4**), water (minus 200 ul for inoculum addition) was added to the sorghum samples (5 g) to achieve the target a_w levels of 0.98, 0.96 and 0.92 $a_{w.}$ The sorghum was stored in culture vessels (Magenta, Sigma Ltd, UK) which were covered with plastic microporous lids and kept at 4°C for 48 h to equilibrate. Subsequently, after equilibration at 25°C, 200 µl of each treatment spore suspension (approx. 10^4 spores ml^{-1}) was added to make up the predetermined water amounts required. The treatment vessels along with untreated controls were placed in plastic chambers. Additionally, 2 glass jars (500 ml) containing glycerol-water solutions

were included to maintain the equilibrium relative humidity the same as the sorghum treatment levels. The chambers were incubated at 25°C for 12 days. The glycerol-water solutions were replaced with fresh solutions after 6 days incubation. Spores from sorghum grain were also harvested with PEG 200 solutions of appropriate a_w followed by filtration through cheese cloth. The filtrate was centrifuged as described above. Spores from different treatments were also counted with a haemocytometer. The spore yield was expressed as number of spores per dry weight of sorghum. All experiments were carried out with three replicates per treatment.

7.2.4 Evaluation of germination

For each spore treatment 100 µl of spore suspension was pipetted and then spread on tap water agar (TWA) plates containing of 22% and 46% (w/v) PEG corresponding to 0.96 and 0.92 a_w. The Petri plates with the same a_w were sealed in polyethylene bags and incubated at 25°C for 24 h. Experiments were done in triplicates per each treatment.

Three (3) agar plugs were aseptically removed from each replicate plate with the aid of a sterile cork borer (10 mm diam.). The plugs were mounted on a microscope slide, stained with lactophenol cotton blue and examined under the microscope. Fifty (50) single spores per disc were examined. Spores were considered to have germinated when the germ tube was greater than the diameter of the spore. The germ tube lengths of 10 spores per agar plug for each treatment were also determined.

7.2.5 Control of aflatoxin production on maize grain by treatments

Treatment application: Treatments that had the best percentage spore germination and germ tube extension rates under water stress were evaluated for the ability to inhibit toxin production *in situ* on maize in 50:50 mixed cultures with $AFL1^{+}$. The amount of water required to achieve 0.96 and 0.90 a_w was derived from the maize moisture adsorption curve (see Section **4.2.5** and **Appendix 1)**. The maize had also previously been assessed for contamination with *A. flavus* (see Section **3.2.3**). As described above, the required amounts of water were added to 10 g maize in culture vessels (Magenta, Sigma Ltd, UK). The vessels were closed with microporous plastic lids and kept at 4^oC for 48 h to equilibrate. Subsequently, after equilibration at 25^oC, 200 µl of each treatment spore suspension (approx. 10^4 spores ml^{-1}) was added to make up the predetermined water amounts required. The treatment vessels along with untreated controls were placed in plastic chambers as described previously. The chambers were maintained at 25^oC for 14 days. Glycerol-water solutions were replaced with fresh solutions after 7 days incubation. There were three replicates per treatment. After 14 days, samples were dried, ground and kept at -20^oC until extraction and clean-up prior to HPLC analysis for AFB_1.

Extraction of aflatoxins: Aflatoxins were extracted from ground maize (10 g) with 100 ml 80% methanol followed by shaking for 2 h and filtering through Whatman No.1 filter paper. The filtrate was diluted 5 fold with phosphate buffered saline (PBS). Twenty millilitres (20 ml) diluted filtrate was passed through an Neogen immunoaffinity (Neogen, Ayr, Scotland) followed by washing with 20 ml 25% methanol and AFB_1 was eluted with 1.5 ml of 100%

methanol. The eluate was dried under a stream of nitrogen gas before derivatisation and subsequent HPLC analysis.

Derivatization, detection and quantification of aflatoxins by HPLC: Firstly, 200 µl hexane was added to the residue followed by the addition of 50 µl triflouroacetic acid (TFA). The mixture was then vortexed for 30 sec and then left for 5 min. Thereafter, a mixture of water:acetonitrile (9:1, v/v) was added and the entire contents of the tube were vortexed for 30 sec after which the mixture was left for 10 min to allow for thorough separation of layers. The hexane layer was discarded and the aqueous layer filtered through syringe nylon filters nylon (13 mm x 0.22 µm, Jaytee Biosciences LTD, UK) directly into amber salinized 2 ml HPLC vials (Agilent Technologies Inc., Palo Alto, CA, USA) for HPLC analysis. All analytical reagents used were of HPLC grade.

A reversed-phase HPLC with fluorescence detection was used to confirm the identity and also quantify AFB_1. The HPLC system used constituted a pump, Agilent 1200 series (Model) and a fluorescence detector (excitation and emission wavelength of 360 and 440 nm, respectively). Separation was achieved through the use of C_{18} column (Phenomenex® Gemini; 150 x 4.6, 3 µm particle size: Phenomenex, CA, USA) preceded by a Phenomenex® Gemini C_{18} column; 3 mm, 3 µm guard cartridge. Isocratic elution with methanol:water:acetonitrile (30:60:10, v/v/v) as mobile phase was performed at a flow rate of 1.0 ml min^{-1}. Injection volume was 20 µl. A set of working standards was injected (1 to 5 ng of AFB_1 per injection) and standard curves were generated by plotting the area underneath the peaks against the amounts

of AFB_1. Linear regression was performed in order to establish a correlation relationship (Correlation coefficient, R^2=0.98).

7.2.6 Statistical analyses

All data were subjected to Shapiro-Wilk and Levene's test to assess normality and variance homogeneity respectively. Where data violated these assumptions ($p<0.05$) even after transformation, non-parametric analysis (Wilcoxon /Kruskal-Wallis H test: ($p=0.05$) was conducted otherwise ANOVA was used to establish whether main effects for solute type and a_w on spore yield, germination, germ tube elongation and toxin inhibition were significantly different. Germination and toxin data were arcsine-transformed and log-transformed respectively before analaysis. The back transformed data are shown in the tables and figures for easier comparison. Where significant differences were observed, post-hoc analysis using Tukey HSD ($p=0.5$) for ANOVA was used to compare the means. Where H test was used to compare medians of the treatments, Mann-Whitney U ($p=0.05$) was used to establish which groups for all factors were different. Statistical package JMP®8 (SAS Institute Inc., 2008, Cary NC, USA) was used to perform the analysis.

7.3 Results

7.3.1 Effect of water activity x solute on relative spore germination and germ tube extension

Germination rate and germ tube extension of unmodified and modified conidia on 0.92 a_w TWA after 24 and 48 hrs are presented in

Table 7. 2. With the exception of conidia obtained from the 0.92-proline, 0.92-glucose and 0.92-glycerol modified MMA treatments, none of the spore treatments germinated after 48 h. Although germination rate of 0.92-proline treatment was the highest under drier conditions after 24 and 48 h, the germ tube length was shorter than that of 0.92-glycerol treatment after 48 h.

Under slightly wetter conditions the germination rate (approx. 100%) of conidia grown on the 0.92-proline, 0.96-proline, 0.92-glucose and 0.92-sorbitol modified media was highest and was almost double that of unmodified conidia (**Table 7. 3**). 0.92-proline and 0.96-glucose treatments had shorter germ tube lengths compared to the e unmodified spores at 0.96 a_w after 24 h incubation. Under these conditions, the 0.92-glucose treatment had significantly faster germ tube extension rates of any treatment ($p<0.05$). Results of the one-way analysis of variance (ANOVA) are shown on (**Table 7. 4**). This shows that solute type was had a siginificant effect on both germination rate and germ tube elongation.

Table 7. 2 Values represent mean percentage spore germination and germ tube length (± Standard Deviation, SD) of modified and non-modified atoxigenic *Aspergillus flavus* strain AFL2$^-$ when grown on tap water agar (TWA) modified to 0.92 a_w using polyethylene glycol 200 and incubated at 25°C for 24 and 48 h.

Modifying solute	**Spore germination±SD (%) after:**		**Germ tube length±SD (µm) after[2]:**	
	24 h	48 h	24 h	48 h
Unmodified	NG[1]	NG	NG	NG
Glycerol 0.96	NG	NG	NG	NG
Glycerol 0.92	2.0±0.82	37±5.61	9±3.14	33±9.71
Glucose 0.96	NG	NG	NG	NG
Glucose 0.92	9.0±1.63	19±3.29	9.0±1.23	11±3.70
Proline 0.96	NG	NG	NG	NG
Proline 0.92	39±5.61	59±5.61	18±5.22	29±10.16
NaCl 0.96	NG	NG	NG	NG
NaCl 0.92	NG	NG	NG	NG
Sorbitol 0.96	NG	NG	NG	NG
Sorbitol 0.92	NG	NG	NG	NG

[1] NG = not germinated. **[2]** Mean number of germinated spores.

Table 7. 3 Values represent mean percentage spore germination and germ tube length (± Standard Deviation, SD) of modified and non-modified atoxigenic *Aspergillus flavus* strain AFL2^{-} when grown on tap water agar (TWA) modified to 0.96 a_w using polyethylene glycol 200 and incubated at 25°C for 24 h.

Modifying solute	**Spore germination±SD (%)**	**Germ tube length±SD (µm)[1]**
Unmodified	54.33±2.94^{a}	120.74±39.25^{b}
Glycerol 0.96	91±2.10^{d}	254.96±82.66^{cd}
Glycerol 0.92	91.33±2.42^{d}	226.33±72.10^{cd}
Glucose 0.96	77.67±6.38^{b}	119.22±42.26^{b}
Glucose 0.92	98±1.79^{e}	305.81±84.82^{d}
Proline 0.96	98.67±1.63^{e}	235.69±66.42^{cd}
Proline 0.92	99±1.10^{e}	65.41±22.51^{a}
NaCl 0.96	81±4.86^{bc}	191.25±62.59^{c}
NaCl 0.92	87.67±4.27^{cd}	281.61±53.05^{d}
Sorbitol0 96	64±4.56^{a}	178.25±65.23^{bc}
Sorbitol 0.92	97.33±2.07^{e}	265.24±91.50^{cd}

[1] Mean number of germinated spores.

Table 7. 4 One-way analysis of variance (ANOVA) for germination and germ tube extension of spores of atoxigenic strain AFL2^{-} obtained from cultures grown on MMA adjusted to 0.96 a_w with different solutes. as a function of modifying solute.

Response	**Source of variation**	***df***	***MS***	F^2	η^2
% Spore germination	Solute[1]	10	0.56	65.84**	0.92
	error	55			
	Total	65			
Germ tube length	Solute	10	0.64	27.92**	0.65
	error	154	0.023		
	Total	164			

[1]Five different solutes (proline, sorbitol, glucose, glycerol and NaCl) were used. Each solute was used to adjust the a_w of basal medium (MMA) to 0.92 and 0.96 a_w. [2]ANOVA was performed only on data emanating from 0.96 a_w TWA treatements.

7.3.2 Production and quality of inoculum obtained from sorghum grain of varying moisture levels

The highest conidial production (9.2 log spores g^{-1} dry wt sorghum) was obtained from growing the atoxigenic strain on sorghum adjusted to intermediate water activity (0.96 a_w) and this was significantly higher than the conidial yield ($p<0.05$) in the wetter 0.98 a_w and the drier 0.92 a_w treatments. The percentage germination and germ tube length of the conidia obtained from these three sorghum substrates (0.98, 0.96 and 0.92 a_w) when plated on TWA at 0.96 a_w is shown in **Table 7. 5**. Overall, the statistical analysis showed that the effects of a_w were highly significant for both spore germination (ANOVA, p $p<0.001$: **Table 7. 6**) and germ tube extension ($H(2) = 29.8$, $p<0.001$, results of analysis not shown).

In the case of spore germination, post-hoc comparisons using Tukey HSD indicated that germination rates for conidia obtained from the 0.92 and 0.96 a_w sorghum treatments were significantly faster than conidia obtained from the wetter 0.98 a_w sorghum treatment when plated on 0.96 a_w TWA after 24 h. No spores germinated on 0.92 a_w TWA plates even after 48 hrs. The germ tube length of conidia modified on 0.92 a_w sorghum, was the longest on 0.96 a_w TWA plates. There were no differences in the germ tube extension between conidia obtained from 0.96 and 0.98 a_w sorghum treatments (Mann-Whitney U, $p=0.05$).

Table 7. 5 Mean percentage spore germination and germ tube length of atoxigenic strain AFL2$^-$ obtained from sorghum grain at different a_w levels and grown on TWA modified to 0.96 a_w with polyethylene glycol 200 after incubation at 25^oC for 24 h.

a_w[1]	Spore yield (log_{10}(no.spores)/g sorghum)[2]	Spore germination±SD (%)[3]	Germ tube length±SD (µm)
0.98	8.8[a]	90.67±1.63[a]	43.62±13.52[a]
0.96	9.2[b]	97.67±2.34[b]	50.55±11.79[a]
0.92	8.5[a]	98.67±4.13[b]	116.62±28.73[b]

[1]Water acitivity of sorghum grain. [2]Values are means of three replicates. [3]Means in a column followed by the same letters are not significantly different ($p>0.05$).

Table 7. 6 One-way analysis of variance (ANOVA) for spore yield of strain AFL2$^-$ grown on sorghum amended to three levels of aw (0.92, 0.96 and 0.98 a_w) and their germination on TWA modified to two levels of aw (0.92 and 0.96 a_w) with PEG 200.

Response	Variation[3]	*df*	*MS*	*F*
Spore yield	Between groups	2	$1.55x10^{18}$	27.5**
	Within groups	6	$5.64x10^{16}$	
	Total	8		
% Spore germination	Between groups	2	114.0	13.6**
	Within groups	15	8.40	
	Total	17		

** $p<0.01$

7.3.3 Comparison of conidial inocula of the atoxigenic strain AFL2- from modified maize-based media and sorghum grain for control of aflatoxin B1 production by the toxigenic strain AFL1+ on stored maize grain

Figure 7. 1 shows the results of toxin inhibition during interactions between modified conidial treatments of $AFL2^-$ and spores of $AFL1^+$ colonising maize grain adjusted to 0.96 and 0.90 a_w. Overall, significantly higher toxin was detected in the moist maize (mean rank 672.0) grain inoculated with mixed cultures of modified AFL^- and AFL^+ compared to drier maize (mean rank 231.0) regardless of the source of $AFL2^-$ spores (Mann-Whitney U, p=0.00). However, under drier conditions, compared to other treatments, conidial inoculum of $AFL2^-$ from the 0.92-sorghum, 0.92-glucose, 0.92-glycerol and the unmodified treatments with mean ranks of 2.0, 5.3, 10.0 and 8.7 respectively, significantly reduced AFB_1 production by strain $AFL1^+$ ($H(6) = 18.9$, $p=0.004$). When the 0.92-proline treatment was used there was an increase in AFB_1 production by the AFL1+ strain colonising maize, even higher than the positive control.

On moist maize grain (0.96 a_w), conidia of $AFL2^-$ modified to 0.92 a_w with glycerol significantly inhibited toxin production better than 0.92-proline and 0.92-glucose treatments ($p<0.05$). Conversely, interactions between spores of $AFL1^+$ and conidia of AFL2- conidia from the 0.92-proline and and glucose-modified treatments resulted in a significant increase in toxin production. Analysis of variance results comparing the effects of different conidial treatments on AFB_1 inhibition on maize adjusted to 0.96 a_w are given in **Table 7. 7**. This shows that conidial treatment type had a significant effect on toxin production by $AFL1^+$.

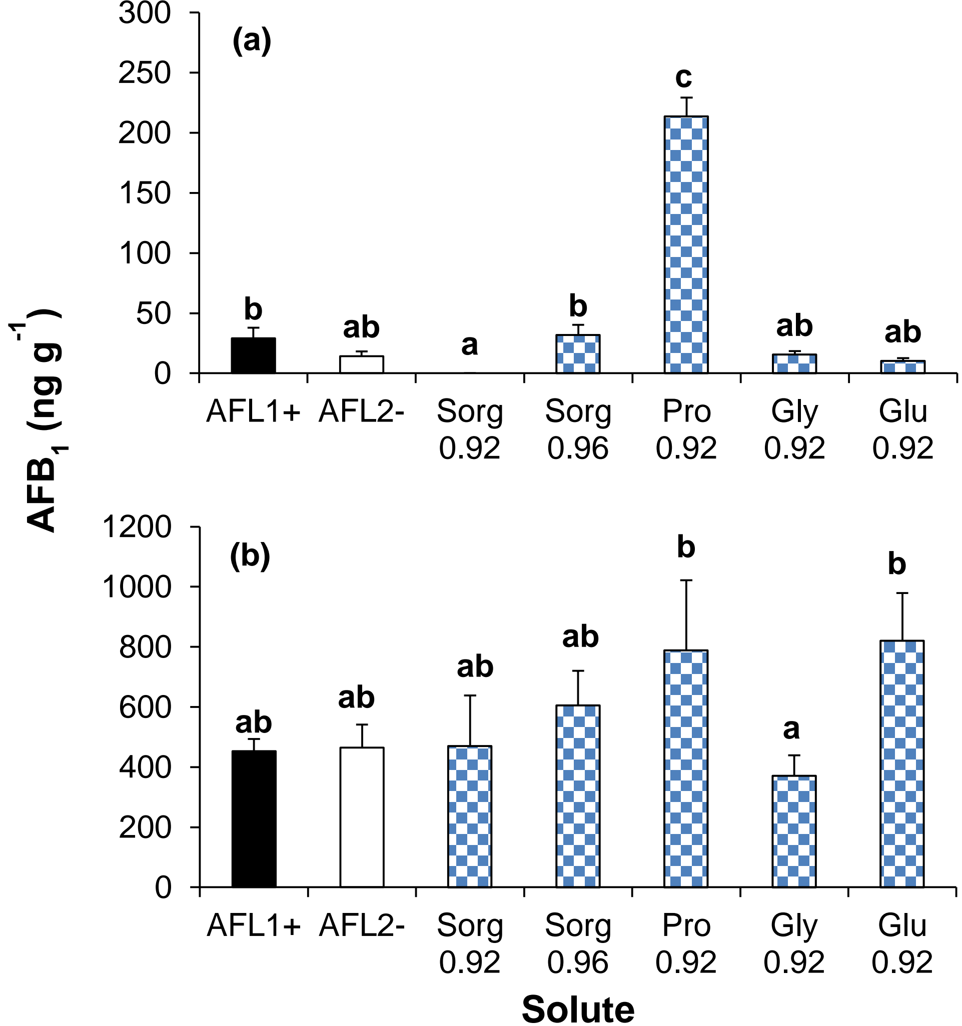

Figure 7. 1 Toxin inhibition by osmotically-amended spores of $AFL2^-$ during interaction with $AFL1^+$ in 50:50 ratio mixed cultures on maize adjusted to (a) 0.90 and (b) 0.96 a_w. Columns represent mean of 3 replicates. Columns headed by the same letter are not significantly different (p>0.05).

***Key**:* $AFL1^+$ (■) = toxigenic strain alone (positive control), All mixed cultures contain $AFL1^+$ in 50:50 ratios with: $AFL2^-$ (□) = unamended spores of atoxigenic strain $AFL2^-$, Sorg 0.92 = $AFL2^-$ spores modified on 0.92 a_w Sorghum; Sorg 0.96 = $AFL2^-$ spores modified on 0.96 a_w Sorghum, Pro 0.92 = $AFL2^-$ spores modified on MMA amended to 0.92 a_w with proline; Gly 0.92 = $AFL2^-$ spores modified on MMA amended to 0.92 a_w with glycerol; Glu 0.92 = $AFL2^-$ spores modified on MMA amended to 0.92 a_w with glucose.

Table 7. 7 One-way analysis of variance (ANOVA) for toxin inhibition by modified AFL2$^-$ during interaction with AFL1$^+$ on maize grain adjusted to 0.96 a_w.

Variation	*df*	*MS*	*F*
Between groups	6	92866.103	4.885**
Within groups	14	19012.253	
Total	20		

7.4 Discussion

The present study is the first to compare the germinative capacity of an atoxigenic *A. flavus* strain modified *in vitro* using different solutes and *in situ* on sorghum grain and its ability to inhibit toxin production on maize. The study has demonstrated that although the proline-modified conidial treatments which germinated fastest under both drier and wetter conditions, their germ tube extension rates were not as good as the other treatments, especially at 0.96 a_w. Under wetter conditions, 0.92- and 0.96-proline, 0.92-glucose and 0.92-sorbitol produced conidia germinated fastest, while the 0.92-glucose-modified treatment had the highest germ tube extension under these conditions. Overall, conidia obtained from the driest sorghum treatment had the fastest germination and germ tube elongation rates on TWA. However, the highest conidial production was obtained from the wetter sorghum a_w treatments.

The first stage of germination is metabolic and is triggered by rehydration. In the absence of adequate osmotica inside the spores especially when the external environment is drier than the interior, spores will not imbibe enough water to

effect initiation of germination. Thus, water influx is achievable if conidia are able to maintain higher osmotic pressure relative to their immediate environment (Magan, 1997; Deacon, 2006). Fungi maintain higher osmotic pressure through the production of a mixture of osmolytes which vary in their level of osmotic pressure that they exert to their surroundings (Davis et al., 2000).

It has previously been shown that concentrations and types of these solutes accumulated in fungal spores can be increased through physiological manipulation (Hallsworth and Magan, 1995; Abadias et al., 2001; Mokiou and Magan, 2008). Hallsworth and Magan (1995) showed that under water stress conidia of *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces farinosus* that had accumulated more glycerol after modification was able to germinate faster when compared to unmodified spores. Abadias et al. (2000) used exactly the same solutes used in the current study (see Section **7.2.2**) to modify the a_w of molasses medium on which they grew *Candida sake* and reported a significant correlation between glycerol and glucose and the cell water potential regardless of the modifying solute used.

In the current study, no attempt was made to detect and quantify the osmolytes accumulated by atoxigenic *A. flavus* strain AFL2$^-$ as a result of stress when grown on osmotically amended media. However, the findings suggest that the 0.92-proline derived conidia may have accumulated compatible solutes with high osmotic pressure, high concentrations of these osmolytes or both. Indeed, Abadias et al. (2001) showed that modifying *C. sake* cells in proline-based media at lowered a_w resulted in the highest amounts of total intracellular solutes

after 24 h compared to most of the solutes they used. The same authors also showed that glucose and glycerol were principal compatible solutes accumulated in *C. sake* cells modified with proline (Abadias et al., 2000). It is thus, possible that glucose accumulation within spores provided the necessary energy for initiation of germination (Deacon, 2006). The prime role of glycerol might be that of effecting movement of water into the spore through provision of a high osmotic gradient between spores and its surrounding resulting in increased imbibition of water by the former.

Additionally, proline is an important amino acid that can be taken up by the fungal spores for eventual synthesis of proteins which is one of the important steps preceding the formation of the germ tube (Pass and Griffin, 1972). Indeed Leighton and Stock (1970) suggested that spore coat proteolysis is a primary event in conidial germination. Proline could be a key amino acid in those proteolytic enzymes. Ho and Miller (1978) also reported that proline significantly decreased during the germination process. They concluded that it was metabolically utilised during germination. Again, this observation supports a possible utilisation of proline during the synthesis of key proteins in the initiation of germination.

In line with the recommendation by Köhl et al. (2011) that suitability for commercial use of BCA is dependent on among other factors, cost-effectiveness of mass production, the current study also assessed spore yield of the biocontrol agent strain AFL2$^-$ on sorghum at different a_w levels. Spore productivity was highest on intermediate a_w sorghum treatments (9.23 log spores g^{-1} dry wt sorghum) while under wetter and drier conditions spore

production was slightly less at 8.5-8.8 log spores g^{-1} dry wt sorghum. This suggests that intermediate moisture contents are best for the production of AFL^- strains on sorghum substrates. This is interesting as the a_w/moisture content of sorghum used as an inoculum for “AflaSafe” strains used in West Africa is not specified.

Studies by Frey (2000) showed the fungal biocontrol *Ulocladium atrum* spore production was significantly increased over the standard fermentation (7.20 log spores g^{-1} fresh oats) by either increasing the culture temperature from 20 to 25°C or by reducing the a_w from 0.998 (control) to 0.985 a_w during a 4 week fermentation period on oats. Studies by Pascual *et al.* (1998) also showed that a decrease in the a_w of wheat grain substrate from 0.996 to 0.98 a_w doubled spore production by *Epiccocum nigrum* a biocontrol agent of *Monilinia* rot in peaches. Gervais et al. (1988) found that spore production by cultures of *Trichoderma viride* and *Penicillium roqueforti* was stimulated when substrate (agar) a_w levels were reduced from 0.98 to 0.96 a_w. Further studies by Frey (2000) gave better results on bulgar wheat (9.15 log spores g^{-1} fresh substrate) with 10 days incubation at 25°C. Bulgar wheat is a semi-processed durum wheat, steamed to gelatinise the starch content, before being dried and cracked (Dick and Matsuo, 1988). The partial boiling may make the nutrients in the substrate more easily accessible compared to unprocessed whole grains and the gelatinisation of starch may influence water holding properties, characteristic of many gels. This solid substrate also does not compact so allowing effective O_2 supply in the substrate. However, the target yield of 9.9 log spores g^{-1} fresh weight substrate is a minimum requirement for economic

production and downstream processing. With sorghum grain at 0.96 a_w this was achievable in the present study.

In the present study significantly higher toxin was detected from wetter maize grain inoculated with mixed cultures of modified AFL^- and AFL^+ compared to drier maize regardless of the source of $AFL2^-$ spores. In both cases, spores which were from the 0.92-glycerol modified treatment gave the best control of toxin production. Perhaps 0.92-glycerol modification resulted in an accumulation of a mixture of compatible solutes which may enable the spores to tolerate and continue growing quickly during environmental fluctuations and hence better inhibition of toxin production. Previous studies have shown that physiologically manipulated BCAs are able to give the best control of pathogens. Mokiou and Magan (2008) demonstrated that *Pichia anomala* inhibited growth and ochratoxin A (OTA) production when grown in mixed cultures with *Penicillium verrucosum* on moist wheat. This suggests that ecophysiological manipulation of such BCAs can result in improved ecological competence of such formulations and effective biocontrol.

Interestingly, although the 0.92-proline modified spores germinated fastest under water stress, this treatment did not inhibit toxin production at all. Instead, it led to significantly higher toxin levels associated with the positive control ($AFL1^+$ alone). The reasons for these results are not clear, but we suspect possible leaking of accumulated Proline from modified spores into their immediate environment which is constituted by a solution of higher a_w into which the unmodified spores were suspended. This is despite the use of isotonic solutions of PEG 200 which according to Mokiou and Magan (2008)

can lead to an increase in the concentrations of compatible solutes synthesised during the process of modification. Since both the modified AFL2$^-$ and unmodified AFL1$^+$ were harvested in isotonic solutions. Perhaps the modifications are only transient, thus mixed cultures provide a new environment into which both modified AFL2$^-$ and unmodified AFL1$^+$ adapt. Depending on their osmotic potentials the two will equilibrate the total water potential of their cytoplasms to that of their immediate environment. Thus, during interaction a balance between the osmotic potential of the two interacting spores is reached. Possible implications for influx of proline and glucose into the spores have already been discussed. Since germination precedes aflatoxin biosynthesis, the additional glucose and proline that would accumulate into the unmodified AFL$^+$ is likely to lead to increased toxin production (Payne and Hagler Jr., 1983) regardless of the presence of the AFL$^-$.

For effective use in controlling the aflatoxin production by *A. flavus* it is essential that potential BCAs are able to tolerate a wide range of environmental factors. Although this approach has been tested on other BCAs before, this is the first time it has been used to develop ecologically competent atoxigenic *A. flavus* strains for control of aflatoxin contamination of maize. The approach provides opportunities for wide–spread implementation of this intervention in geographically diverse settings.

CHAPTER 8: Conclusions and Future Directions

8.1 Conclusions

1. This study examined the mycobiota and mycotoxins in maize from five climatic regions of Lesotho in two seasons for the first time
   a) based on molecular sequencing data *Fusarium verticillioides*, *F. proliferatum* and *F. subglutinans* were the main Fusaria isolated from the maize samples.
   b) other species included *Aspergillus flavus and A. parasiticus* which were more prevalent in the southern areas of Lesotho. *Aspergillus ochraceus* and *A. niger* were also isolated.
   c) targeted metabolomics analyses showed that the main mycotoxins contaminating the stored maize samples in the two seasons were fumonisins, moniliformin, beauvericin and fusaproliferin.
2. Examination of atoxigenic *A. flavus* strains from Lesotho and Egypt for *in vitro* and *in situ* competitiveness against two toxigenic strains of *A. flavus* and subsequent inhibition of aflatoxin B_1 (AFB_1) production under different environmental conditions revealed that:
   a) temperature, a_w and substrate influenced the types of interaction between atoxigenic and toxigenic strains.
   b) depending on temperature and a_w the two groups of fungi are antagonistic to each other; either mycelial contact or at a distance.

c) atoxigenic strains varied in their ability to inhibit AFB_1 accumulation and that their efficacy also depended on the toxigenic strain targeted.

d) by changing ratios of atoxigenic to toxigenic strains *in vitro* under different a_w x temperature regimes gave up to 90% inhibition of AFB_1 production.

e) control of AFB_1 was best in stored maize when more water was available (0.96 a_w) than under water stress (0.90 a_w). Overall, the best control level achieved was 77% reduction in AFB_1 relative to the controls.

3. The possible mechanisms of action were examined to better understand the similarities and differences between AFL^- and AFL^+ strains of *A. flavus*. This showed that:

    a) temperature and a_w and their interactions influenced mycelial growth and C-source utilization patterns found in maize of both AFL^- and AFL^+ strains.

    b) there was no real difference in growth of the two groups of strains

    c) based on carbon source utilization patterns, niche size of the two groups of strains increased with an increase in temperature and a_w.

    d) based on the computed values of niche overlap indices regardless of temperature, when water is freely available the two groups of strains co-existed (NOI value >0.90). However, under

moisture stress, the nutritional competitiveness of the strains was variable.

e) temporal carbon source utilisation sequences (TCUS) determined with Bioscreen C showed that when water is freely available (0.99 a_w) most carbon sources are utilised by the two groups of strains.
f) time to detection (TTD) of each strain was shortest on monosaccharides when water was freely available (0.99 a_w).
g) under moisture stress (a_w = 0.90 a_w) the least number of CS was utilised and the CS sources used include proline, glutamine and alanine in addition to monosaccharides.

4. Further experiments to elucidate the mechanistic basis of toxin inihition by AFL^- strains were carried out and these included comparative studies of spore germination rate, germ tube elongation, sporulation and hydrolytic enzymes production capacity of atoxigenic and toxigenic strains.
   a) temperature and a_w and their interactions had a significant influence on the germination, sporulation and extracellular enzyme production capacity.
   b) there were no differences between AFL^- and AFL^+ strains in terms of sporulation
   c) the rates of germination rate, germ tube extension were variable between the two groups of strains and therefore no clear distinctions could be drawn between AFL^- and AFL^+ strains in this respect.

d) Screening of the two groups of strains for hydrolytic enzyme production under different environmental conditions showed that of 19 enzymes, some including esterase, lipase acid phosphatase and N-acetyl-β-glucosaminidase were produced in significant amounts. These were subsequently quantified using *p*-nitrophenyl substrates:

- there was greater total enzyme activity at 0.96 a_w than under drier conditions (0.90 a_w).
- there were no differences between AFL^- and AFL^+ strains in lipase and esterase activity. However acid phosphatase and N-acetyl-β-glucosaminidase activity were variable between the two groups of strains and therefore no clear distinctions could be drawn between AFL^- and AFL^+ strains in this respect.

5. Physiological manipulation of conidia of strain $AFL2^-$ was done *in vitro* by modifying maize-based media with different solutes and on sorghum grain as a solid substrate under different a_w levels.The quality of the harvested conidia was tested and compared for germination rates and germ tube extension on tap water agar (TWA) modified with PEG to 0.96 and 0.92 a_w. Treatments that exhibited better germination rate and germ tube elongation rate were subsequently evaluated for ability to inhibit toxin production in mixed cultures 50:50 with unmodified conidia of AFL^+ This showed that:

   a) conidia produced on milled maize agar modified with proline to 0.92 a_w germinated fastest on water agar (TWA) at 0.92 and 0.96 a_w.

b) conidial inoculum produced on glucose modified maize medium at 0.92 a_w had the fastest germ tube extension rates on TWA modified to 0.96 a_w.

c) on sorghum solid substrate the spore production was maximum (log 9.2 spores/g dry weight sorghum) when the substrate was adjusted to 0.96 a_w and incubated at 25^oC for 10 days.

d) The conidial inoculum from the 0.92 a_w modified sorghum grain substrate completely inhibited AFB_1 production on 0.90 a_w maize grain.

e) conidia produced on 0.92-glycerol modified maize agar inhibited AFB_1 production better at 0.96 a_w on maize grain when applied as a 50:50 ($AFL2^-$:$AFL1^+$) mixed inoculum with initial spore concentration of each strain adjusted to 10^4 spores ml^{-1}.

8.2 Further work

1. Based on the findings on toxigenic mycobiota associated with stored maize from different regions of Lesotho, future research should concentrate on:

a) distribution of *Aspergillus* section *Flavi* in soils of maize fields in the Senqu River Valley. There is strong evidence to suggest this rain shadow area may be a reservoir of strains with aflatoxin production potential.

b) climate change may influence the dominant mycotoxins contaminating maize and this should be examined.

c) toxicological studies are imperative to correlate the prevalence of malnutrition of children <5 yrs with consumption of maize. Maize porridge is often used as weaning gruel by most people in the rural areas.

d) there is also need to understand the toxicology of the so called 'emerging mycotoxins' such as monilifornin, fusaproliferin and beauvericin.

e) ecophysiological factors affecting the growth and production of these emerging mycotoxins is also important for their control.

2. The potential for use of a mixture of atoxigenic strains should also be evaluated. AflaSafe in West and East Africa consists of a mixture of 4 local strains. This may be worthwhile testing in Lesotho provided that the effect of competition between the strains is understood.

3. Molecular studies that would elucidate the changes in expression of some of the genes that encode for aflatoxin production owing to co-inoculation of AFL^- and AFL^+ on maize need to be conducted.

4. A lot of factors that might explain competitive exclusion of AFL^+ by AFL^- were done in axenic cultures in the current study. It is therefore important to assess the dominance of one strain over the other during competition on natural substrates and this might require

a) use of labeled AFL^- and AFL^+ strains in such cultures in order to monitor differences in germination rate, germ tube length extension, mycelial growth rate and sporulation, during interactions. Pyrosequencing might also be used to differentiate between these two groups of strains in respect of these ecophysiological parameters.

5. Comparative studies on competitiveness of AFL^- and AFL^+ should be investigated under different regimes of CO_2 to better understand the possible effects of climate change on the ecophysiology of these two groups of fungi.

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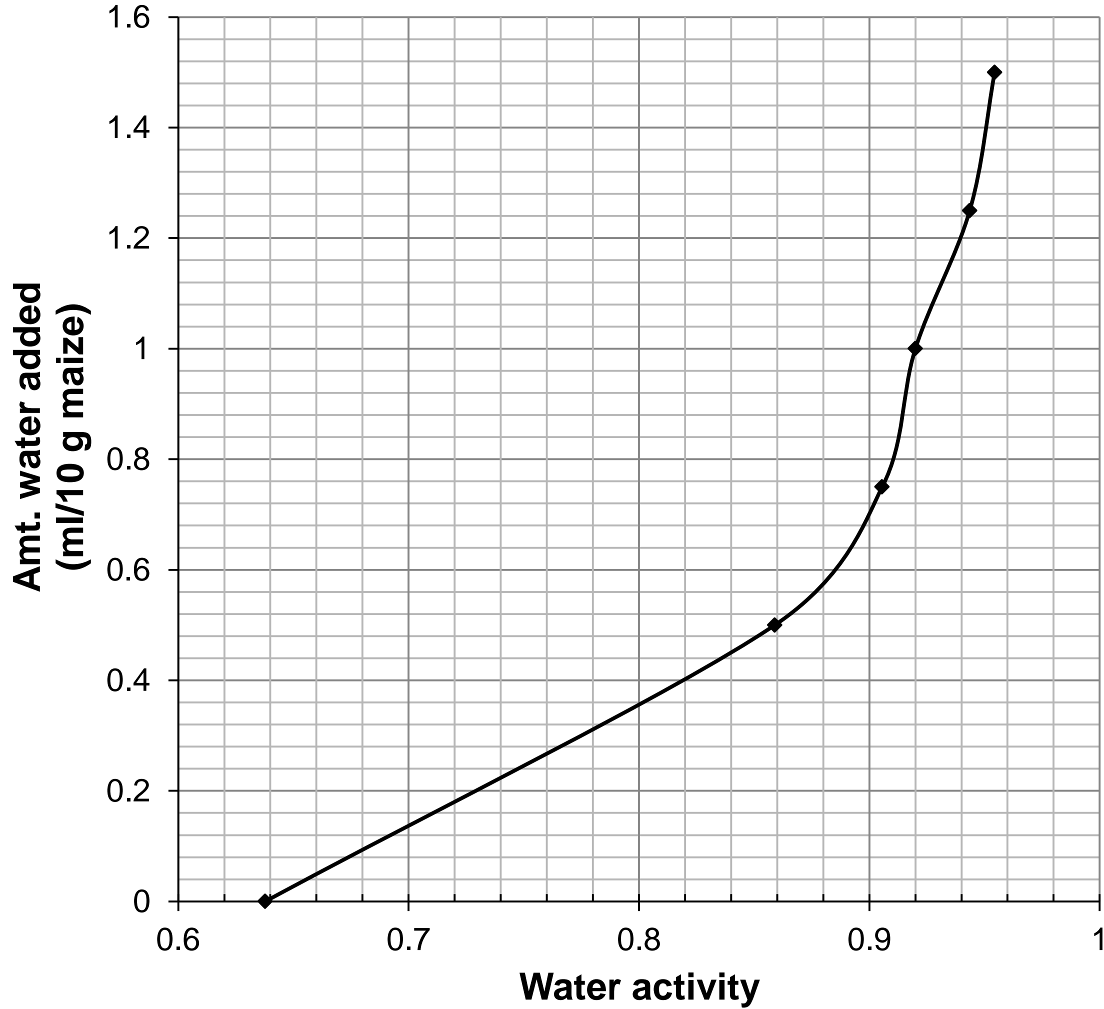
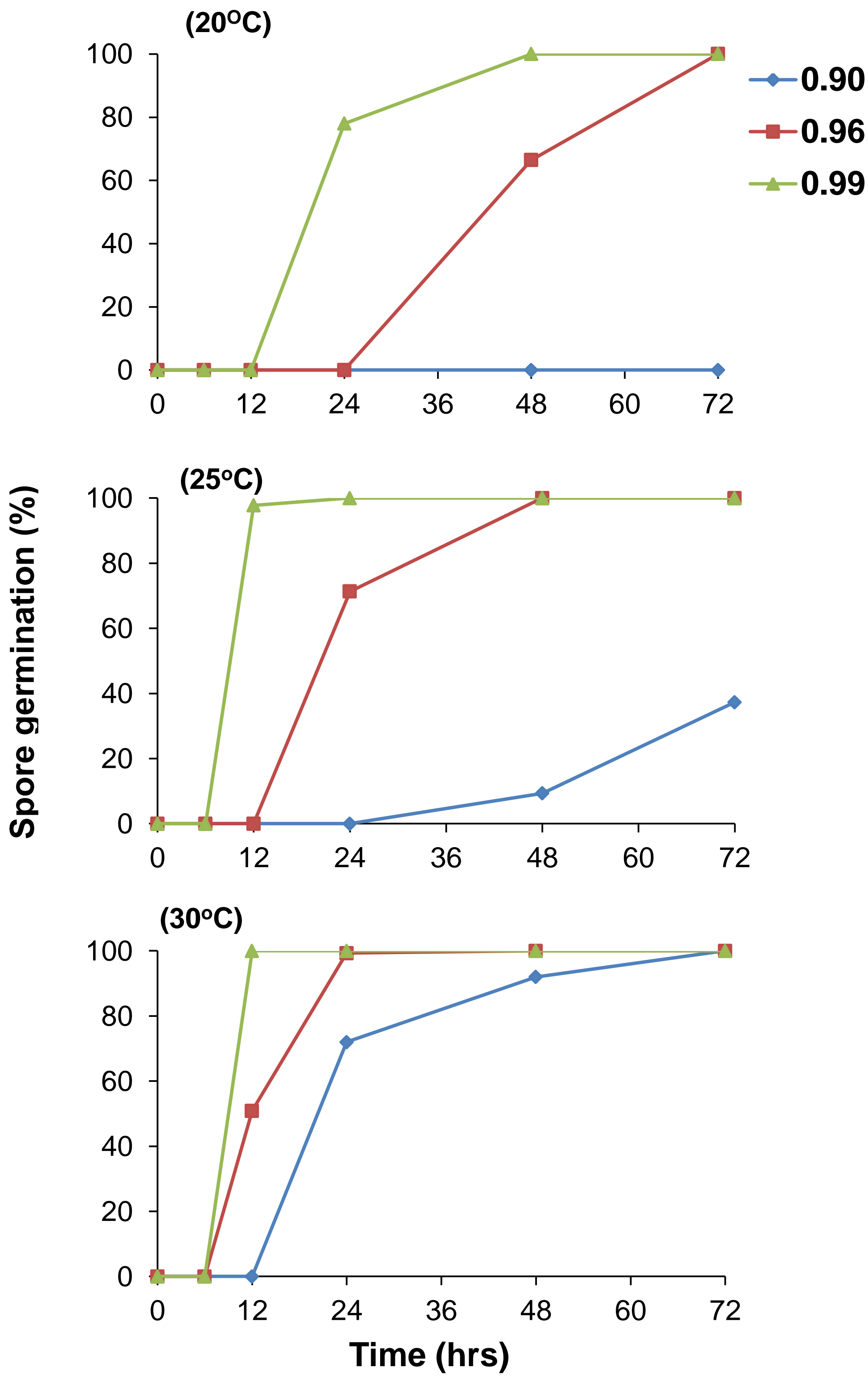
APPENDICES

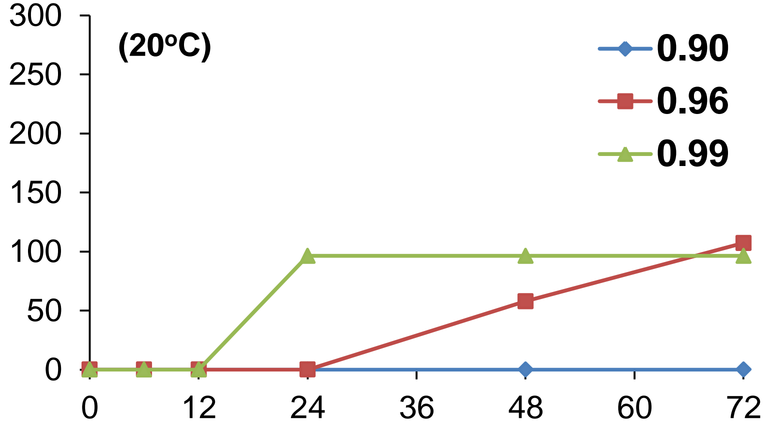
Appendix 1: Relationship between the amount of water added and a_w of maize.

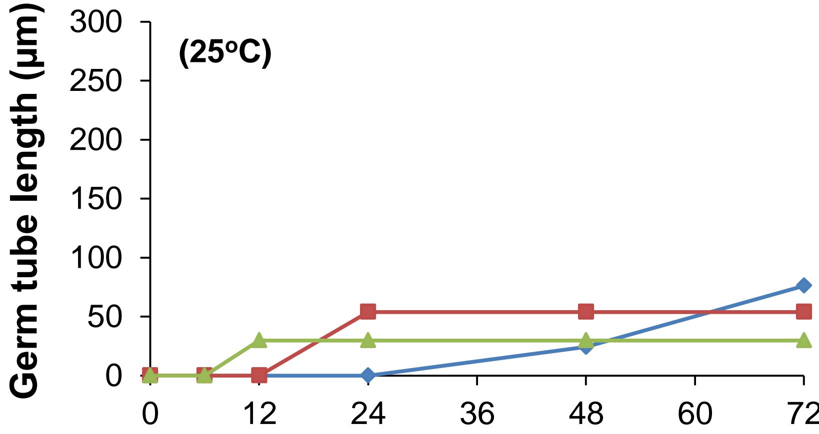


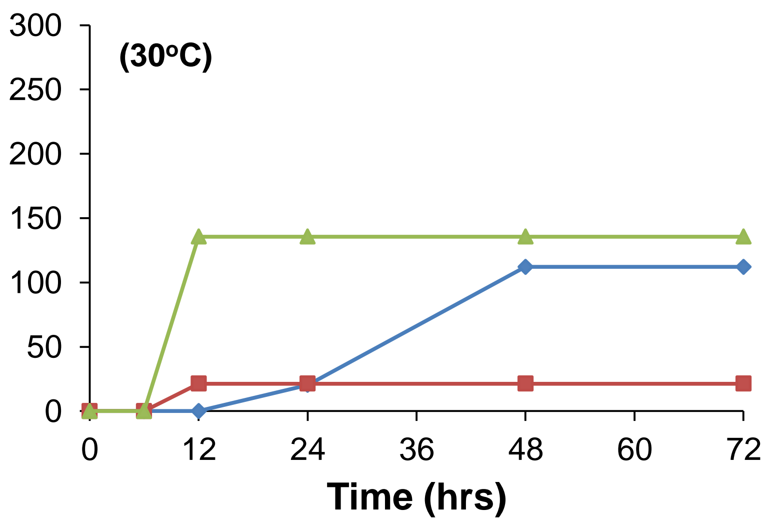
Appendix 2A: Effect of temperature and water activity on spore germination of atoxigenic *A. flavus* strains AFL3⁻ on milled maize agar over a 72 h period.



Appendix 2B: Effect of temperature and water activity on germ tube extension of atoxigenic *A. flavus* strains AFL3$^-$ on milled maize agar over a 72 h period.



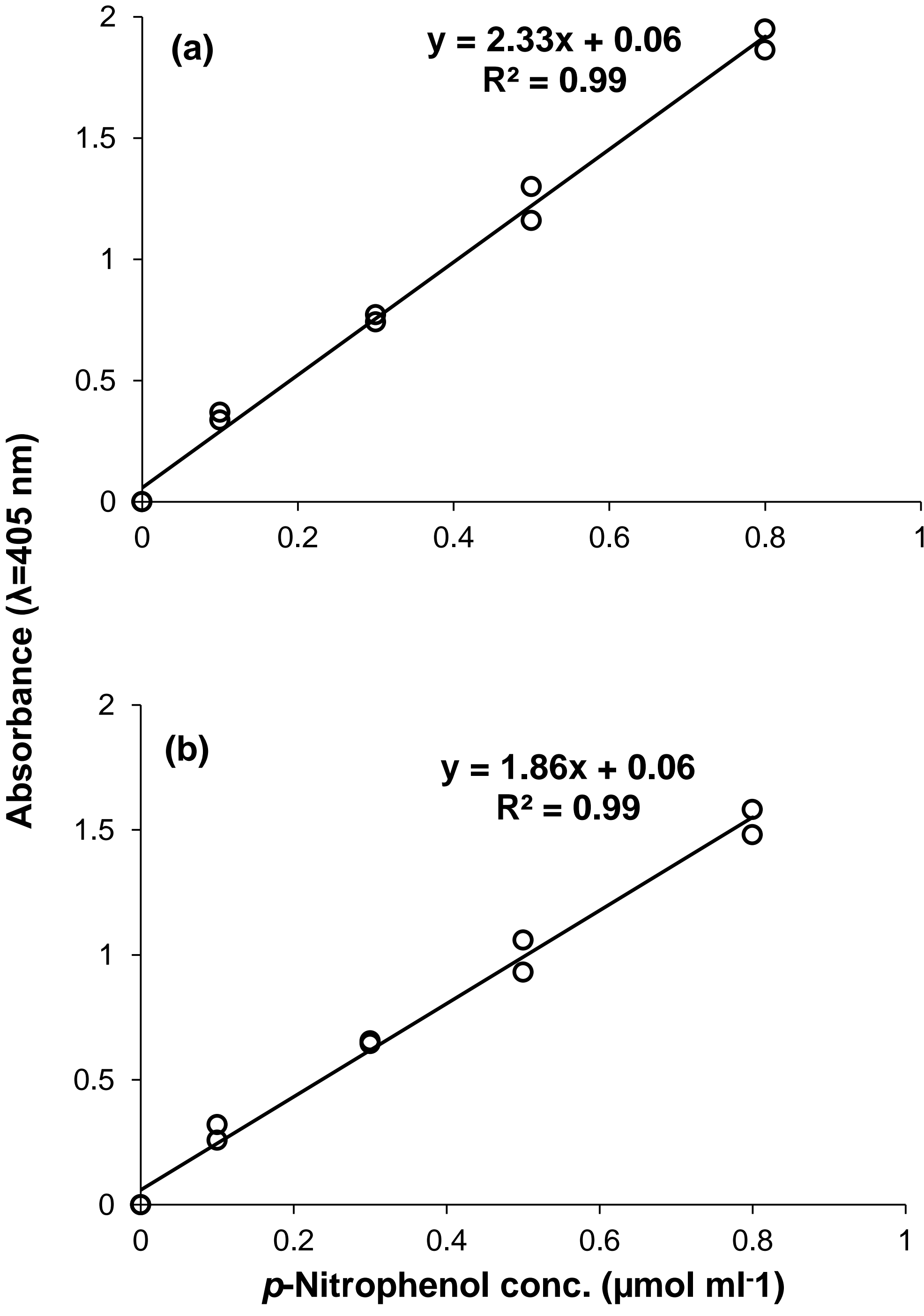




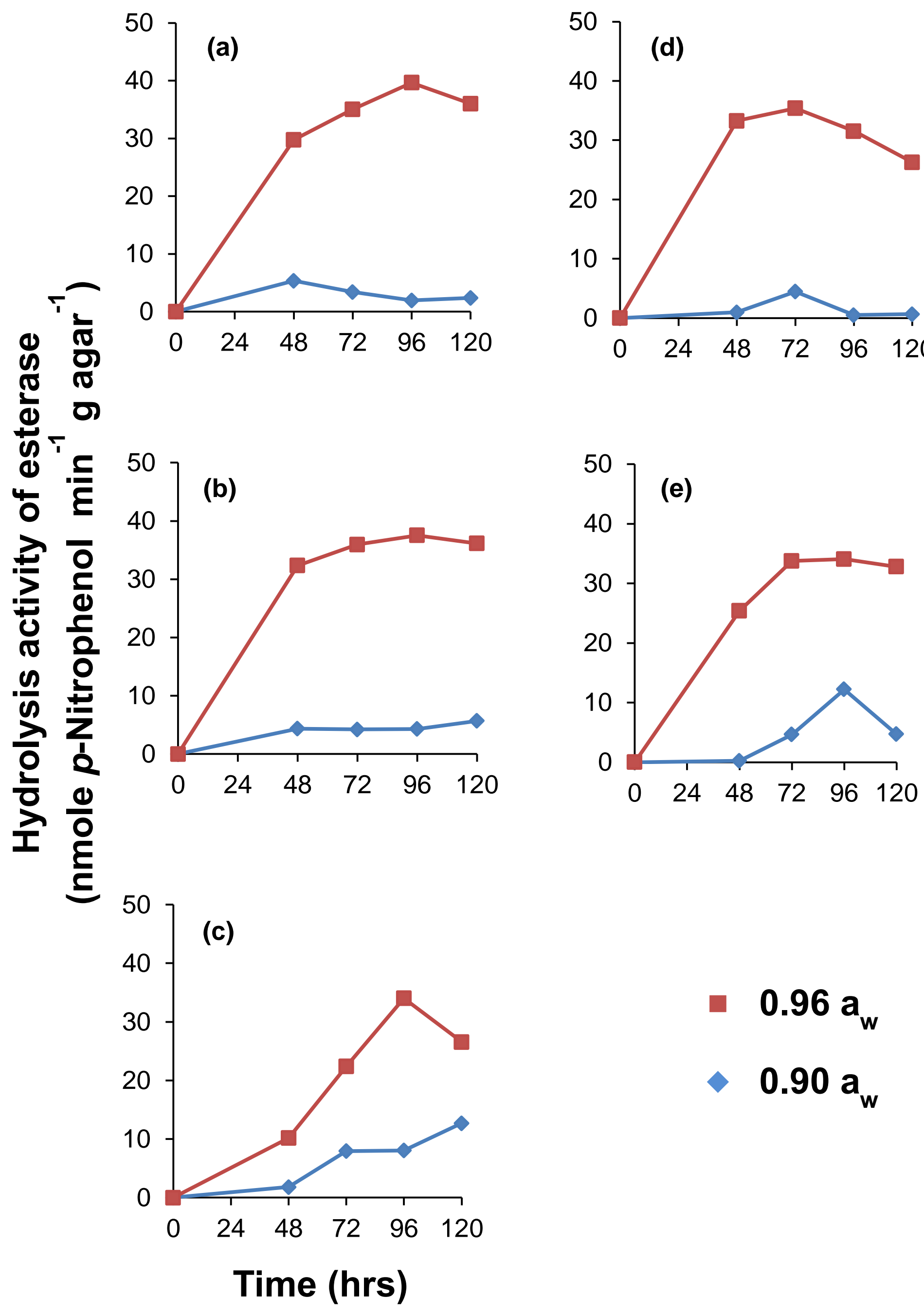
Appendix 3A: API ZYM colour chart

Quantity of hydrolysed substrate		0 nanomoles	5 nanomoles	10 nanomoles	20 nanomoles	30 nanomoles	40 nanomoles
Activity mark		0	1	2	3	4	5
Control	1						
2-naphthyl phosphate	2						
2-naphthyl butyrate	3						
2-naphthyl caprylate	4						
2-naphthyl myristate	5						
L-leucyl-2-naphthylamide	6						
L-valyl-2-naphthylamide	7						
L-cystyl-2-naphthylamide	8						
N-benzoyl-DL-arginine-2-naphthylamide	9						
N-glutaryl-phenylalanine-2-naphthylamide	10						
2-naphthyl phosphate	11						
Naphthol-AS-BI-phosphate	12						
6-Br-2-naphthyl-αD-galactopyranoside	13						
2-naphthyl-βD-galactopyranoside	14						
Naphthol-AS-BI-βD-glucuronide	15						
2-naphthyl-αD-glucopyranoside	16						
6-Br-2-naphthy-βD-glucopyranoside	17						
1-naphthyl-N-acetyl-βD-glucosaminide	18						
6-Br-2-naphthyl-αD-mannopyranoside	19						
2-naphthyl-αL-fucopyranoside	20						

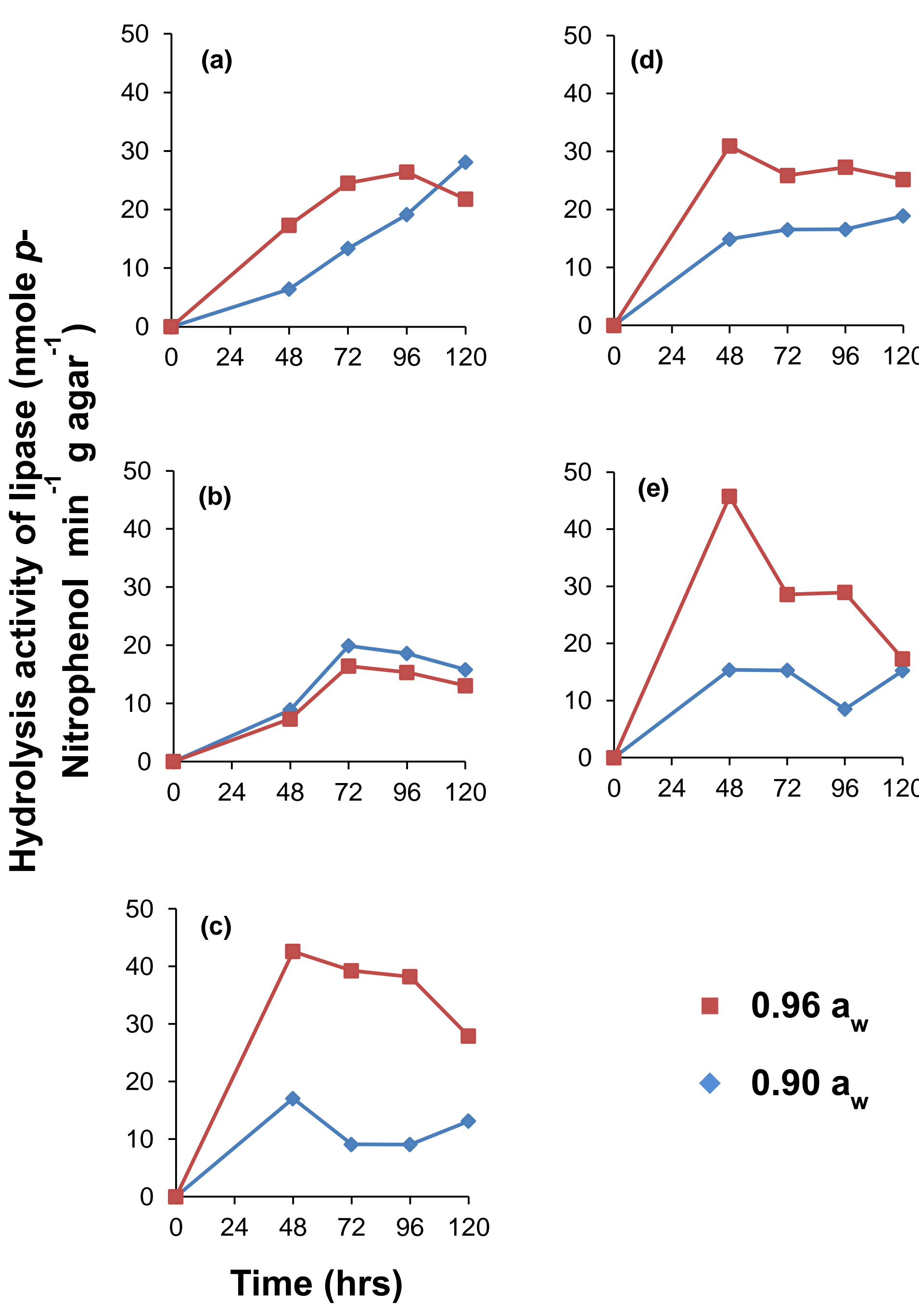
Appendix 3B: Absorbance coefficients at λ=405 nm of *p*-Nitrophenol at pH (a) 7.5 and (b) 4.8.



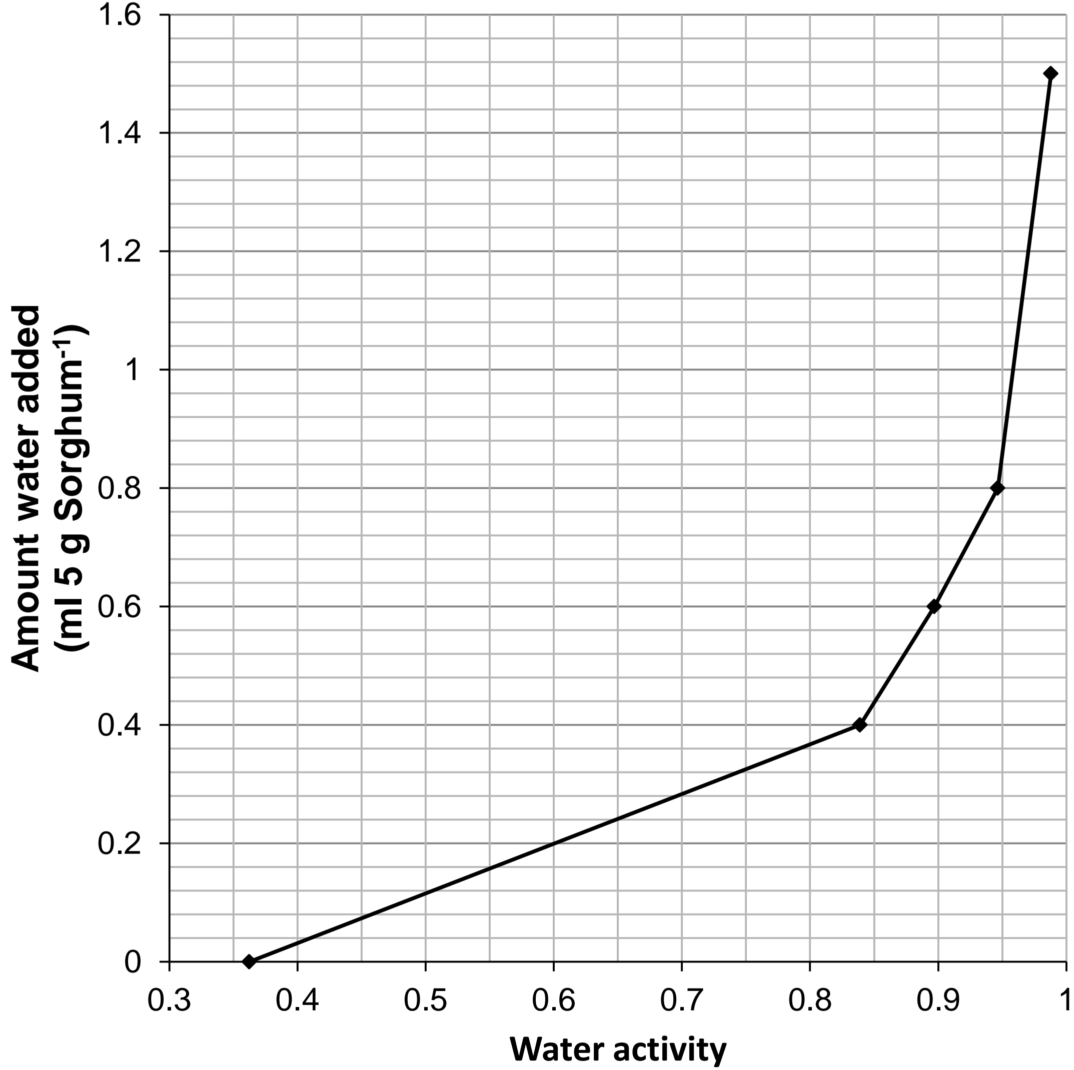
Appendix 3C: Comparison of esterase activity between atoxigenic strains (a) AFL1$^-$, (b) AFL2$^-$ & (c) AFL3$^-$ and toxigenic strains (d) AFL1$^+$ & (e) AFL2$^+$ of *A. flavus* grown on CYEA amended to (0.90, 0.96 a_w) over a period of 120 h.



Appendix 3D: Comparison of lipase activity between atoxigenic strains (a) AFL1^{-}, (b) AFL2^{-} & (c) AFL3^{-} and toxigenic strains (d) AFL1^{+} & (e) AFL2^{+} of *A. flavus* grown on CYEA amended to (0.90, 0.96 a_w) over a period of 120 h.



Appendix 4: Relationship between the amount of water added and a_w of sorghum.



Appendix 5A: Publications

Publication 1:

Biocontrol Science and Technology

Volume 23, Issue 7, 2013

Effect of environmental factors on *in vitro* and *in situ* interactions between atoxigenic and toxigenic *A. flavus* strains and control of aflatoxin contamination of maize

DOI: 10.1080/09583157.2013.794895

Sejakhosi Mohale[a], Angel Medina[a] & Naresh Magan[a*]

pages 776-793

Publishing models and article dates explained

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Published online: 17 Jul 2013

Publication 2: (In press)

ARTICLE IN PRESS

FUNGAL BIOLOGY XXX (2013) 1–10

journal homepage: www.elsevier.com/locate/funbio

Comparison of growth, nutritional utilisation patterns, and niche overlap indices of toxigenic and atoxigenic *Aspergillus flavus* strains

*Sejakhosi MOHALE, Naresh MAGAN, Angel MEDINA**

Applied Mycology Group, Cranfield Health, Cranfield University, Cranfield, Bedford MK43 0AL, UK

Publication 3: (In press)

Mycotoxin Res
DOI 10.1007/s12550-013-0176-9

ORIGINAL PAPER

Mycotoxigenic fungi and mycotoxins associated with stored maize from different regions of Lesotho

Sejakhosi Mohale · Angel Medina · Alicia Rodríguez ·
Michael Sulyok · Naresh Magan

Received: 27 March 2013 / Revised: 16 July 2013 / Accepted: 31 July 2013
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Appendix 5B: Conferences, meetings and seminars attended

Conference 1.

Fungal Interactions [British Mycological Society Annual Meeting]
3–6 September 2012
Universidad de Alicante, Alicante University, Spain

Poster:

“Effect of environmental factors on *in vitro* and *in situ* interactions between atoxigenic and toxigenic *A. flavus* strains and control of aflatoxin contamination of maize”

(**MOHALE Sejakhosi**, Medina Angel and Naresh Magan)

Oral presentation:

“Temporal carbon utilisation sequence (TCUS): New insights on how fluctuating environment influences nutritional utilisation by atoxigenic and toxigenic *Aspergillus flavus* strains”

(**MOHALE Sejakhosi**, Medina Angel and Naresh Magan)

Conference 2.

BMS joint meeting with Society for Applied Microbiology

Royal Society

A BMS joint meeting with Society for Applied Microbiology on 9th January 2013.

The Winter Meeting 2013 programme includes sessions on:

- Food mycology
- Emerging technologies in applied microbiology

Conference 3.

The final MycoRed International Conference will take place in **Martina Franca, Italy, from 27 to 30 May 2013**, closing the successful series of MycoRed International Conferences held in Vienna, Austria - 2009, Penang, Malaysia 2010, Cape Town, South Africa, 2011, Mendoza, Argentina, 2011, Ottawa, Canada 2012.

Short oral presentation:

"Development of atoxigenic *A. flavus* strains for biocontrol of toxigenic strains from Lesotho: Environmental and competitive exclusion considerations"

(**MOHALE Sejakhosi**, Medina Angel and Naresh Magan)

Conference 4

Mycotoxins & Phycotoxins
Gordon Research Seminar

Emerging Technologies for the Discovery, Detection, and Risk Assessment of Harmful Biotoxins

June 15-16, 2013
Stonehill College
Easton, MA

Poster:

"Development of atoxigenic *A. flavus* strains for biocontrol of toxigenic strains from Lesotho: Environmental and competitive exclusion considerations"

(**MOHALE Sejakhosi**, Medina Angel and Naresh Magan)

Conference 5.

Mycotoxins & Phycotoxins

June 16-21, 2013
Stonehill College
Easton, MA

Poster:

"Development of atoxigenic *A. flavus* strains for biocontrol of toxigenic strains from Lesotho: Environmental and competitive exclusion considerations"

(**MOHALE Sejakhosi**, Medina Angel and Naresh Magan)

Appendix 5C: Selected posters presented

Poster 1: Presented in Alicante, Spain

Cranfield Health

Effect of environmental factors on *in vitro* and *in situ* interactions between atoxigenic and toxigenic *A. flavus* strains and control of aflatoxin contamination of maize

Sejakhosi Mohale, Angel Medina and Naresh Magan

Applied Mycology Group, Cranfield Health, Cranfield University, Cranfield Bedford MK43 0AL, U.K.

Introduction

- There is interest in using atoxigenic strains of *Aspergillus flavus* to control production of aflatoxins (AFAs) in staple commodities by outcompeting the producing strains.
- It has been suggested that the use of geographically local atoxigenic strains is more effective for biocontrol of toxigenic *A. flavus* strains on maize, groundnuts and cotton.
- There is interest in a better understanding of the relationship between atoxigenic and toxigenic strains and effects on AFA production. Thus the role of competitive exclusion and the effect of environmental factors needs to be understood.
- **The objectives of this study were to: (a) examine the macroscopic interactions between toxigenic and atoxigenic strains using the Index of Dominance (I_D) approach, (b) relative ratios of atoxigenic and toxigenic strains on production of AFAs under different temperature and water availability conditions on a maize meal extract agar (MMEA) and maize.**

Methods/Materials

Strains: Atoxigenic (**AFl1⁻** (strain Af19LS), **AFl2⁻** (strain Af24LS) & **AFl3⁻** (strain EGP19); toxigenic **AFl1⁺** (strain Af18LS) and **AFl2⁺** (type strain NRRL3357) were isolated from maize or groundnuts.

Media: Maize meal extract agar (MMEA) was adjusted to 0.90, 0.96 & 0.99 water activity (a_w). A maize moisture adsorption curve was used to determine the amounts of water required to modify the a_w of maize kernels to 90 and 0.96 a_w.

Interactions studies: Plates were incubated at 20, 25 & 30°C. Types of interaction were identified and scored according to Magan & Lacey (1984). The I_D values were calculated by addition of numerical score of each interaction. Radii of growing colonies were measured and area calculated over a period of 10 days.

AFB_1 inhibition studies: Ratios of spore concentrations of 100, 75:25, 50:50, 25:75 and 100% of each atoxigenic and toxigenic strain was used in mixed spore inoculum and spread plate on the MMA plates under different environmental conditions. These plates/maize kernels were incubated for 14 days at 25°C.

AFB1 analyses: Extraction, immunoaffinity columns (maize studies), a derivatisation and HPLC analyses were used to quantify the amounts of aflatoxin produced.

Results

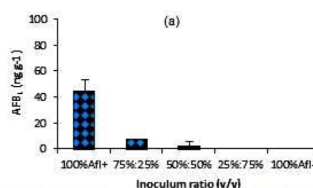
Table 1. Effect of a_w and temperature on numerical interaction scores and I_D between strain **AFl2⁻** (strain *Af*24LS) & **AFl1⁺** (strain Af18LS and NRRL3357) on (a) MMEA and (b) MEA. *In vitro*, the atoxigenic and toxigenic strains were mutually antagonistic to each other, either on contact or at a distance. On MEA at 30°C toxigenic strains dominated under wetter conditions while atoxigenic strains were dominant under moisture stress.

(a)

Strain	Temperature (°C)											
	20				25				30			
	0.99	0.96	0.90	I_D	0.99	0.96	0.90	I_D	0.99	0.96	0.90	I_D
NRRL3357	3/3[¶‡]	3/3	3/3	9/9	2/2	3/3	3/3	9/9	2/2	3/3	3/3	9/9
*Af*18LS	3/3	3/3	3/3	9/9	2/2	3/3	3/3	9/9	2/2	3/3	3/3	9/9
TOTAL I_D	6/6	6/6	6/6	18/18	6/6	6/6	6/6	18/18	6/6	6/6	6/6	18/18
(b)												
NRRL3357	2/2	2/2	3/3	7/7	2/2	2/2	2/2	6/6	0/4	0/4	4/0	4/8
*Af*18LS	2/2	2/2	3/3	7/7	2/2	2/2	2/2	6/6	0/4	0/4	4/0	4/8
TOTAL I_D	4/4	4/4	6/6	14/14	4/4	4/4	4/4	12/12	0/8	0/8	8/0	8/16

[¶] AFl1⁻ score/AFl1⁺ strain score

[‡] mutual intermingling (1), mutual antagonism on contact (2/2), mutual antagonism at a distance (3/3), dominance of one species on contact (4), dominant strain; 0 inhibited strain, dominance of one species at a distance (5); 0 inhibited strain.



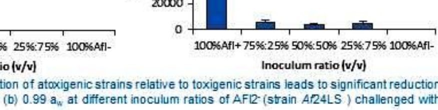


Figure 3. Increased inoculum concentration of atoxigenic strains relative to toxigenic strains leads to significant reduction in toxin production in vitro. (a) 0.96 and (b) 0.99 a_w at different inoculum ratios of AFl2⁻ (strain *Af*24LS) challenged with AFl1⁺ (strain *Af*18LS).

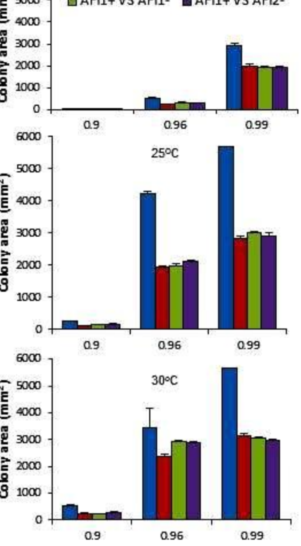


Figure 2. Colony areas of toxigenic strains were significantly greater when grown alone than when challenged with atoxigenic strains. The areas were significantly influenced by both temperature and a_w.

Conclusions

- Dominance which is a measure of competitiveness is not a determinant for AFB_1 inhibitory capability of atoxigenic strains in vitro.
- Efficacy of atoxigenic strains for control of AFB_1 is influenced by a_w.

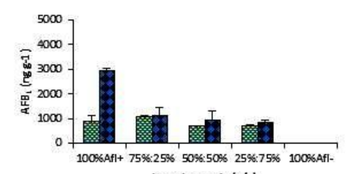


Figure 4. On maize kernels, atoxigenic strains controlled toxin production more efficiently at higher a_w levels than under moisture stress *and* the level of control was not significantly influenced by concentration of atoxigenic strain.

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References

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www.cranfield.ac.uk/health

Poster 2: Presented in Boston, Massachusetts, USA

Cranfield Health

DEVELOPMENT OF ATOXIGENIC *A. FLAVUS* STRAINS FOR BIOCONTROL OF TOXIGENIC STRAINS FROM LESOTHO: ENVIRONMENTAL AND COMPETITIVE EXCLUSION CONSIDERATIONS

Sejakhosi Mohale, Angel Medina and Naresh Magan

Applied Mycology Group, Cranfield Health, Cranfield University, Cranfield Bedford MK43 0AL, U.K.

Introduction

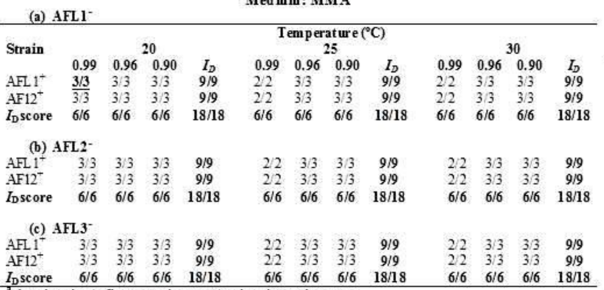
- There is interest in using atoxigenic strains of *Aspergillus flavus* to control production of aflatoxins (AFAs) in staple commodities by outcompeting the producing strains.
- It has been suggested that the use of geographically local atoxigenic strains is more effective for biocontrol of toxigenic *A. flavus* strains on maize, groundnuts and cotton.
- A better understanding of the relationship between atoxigenic and toxigenic strains and effects on AFA production is required. Thus the role of competitive exclusion and the effect of environmental factors needs to be better understood.
- **The objectives of this study were to: (a) examine the interactions between toxigenic and atoxigenic strains using the Index of Dominance (I_D) approach, (b) relative ratios of atoxigenic and toxigenic strain interactions on production of AFAs under different temperature and water availability conditions on maize-based matrices and in maize.**

Methods/Materials

- **Strains:** Atoxigenic **AFl1⁻** (strain *Af*19LS), **AFl2⁻** (strain *Af*24LS) & **AFl3⁻** (strain EGP19); toxigenic **AFl1⁺** (strain *Af*18LS) and **AFl2⁺** (type strain NRRL3357) were isolated from maize or groundnuts.
- **Media:** Maize meal extract agar (MMEA) was adjusted to 0.90, 0.96 & 0.99 water activity (a_w). A maize moisture adsorption curve was used to determine the amounts of water required to modify the a_w of maize kernels to 0.90 and 0.96 a_w.
- **Interactions studies**: Plates were incubated at 20, 25 & 30°C. Types of interaction were scored according to Magan & Lacey (1984). The I_D values were calculated by addition of numerical score of each interaction. Radii of growing colonies were measured and area calculated over a period of 10 days. The ability to utilise maize nutritional sources (21 different ones) were also compared under different a_w x temperature conditions (Giorni et al., 2009).
- **AFB_1 inhibition studies:** Ratios of spore concentrations of 100, 75:25, 50:50, 25:75 and 100% of each atoxigenic and toxigenic strain was used in mixed spore inoculum and spread plated on different a_w MMA treatment plates. These plates/maize kernels were incubated for 14 days at 25°C.
- **AFB_1 analyses:** Extraction, immunoaffinity columns (maize studies), derivatisation and HPLC analyses were used to quantify the amounts of aflatoxin B_1 produced.

Results

Table 1. Effect of a_w and temperature on numerical interaction scores and I_D between atoxigenic strains **AFl1⁻ AFl2⁻ & AFl3⁻** versus toxigenic strains **Afl1⁺ & Afl2⁺** on MMA. The atoxigenic and toxigenic strains were mutually antagonistic to each other, either on contact or at a distance



Medium: MMA

Strain	20				25				30			
	0.99	0.96	0.90	I_D	0.99	0.96	0.90	I_D	0.99	0.96	0.90	I_D
(a) AFL1⁻												
AFL1⁺	3/3	3/3	3/3	9/9	2/2	3/3	3/3	9/9	2/2	3/3	3/3	9/9
AF12⁺	3/3	3/3	3/3	9/9	2/2	3/3	3/3	9/9	2/2	3/3	3/3	9/9
I_D score	6/6	6/6	6/6	18/18	6/6	6/6	6/6	18/18	6/6	6/6	6/6	18/18
(b) AFL2⁻												
AFL1⁺	3/3	3/3	3/3	9/9	2/2	3/3	3/3	9/9	2/2	3/3	3/3	9/9
AF12⁺	3/3	3/3	3/3	9/9	2/2	3/3	3/3	9/9	2/2	3/3	3/3	9/9
I_D score	6/6	6/6	6/6	18/18	6/6	6/6	6/6	18/18	6/6	6/6	6/6	18/18
(c) AFL3⁻												
AFL1⁺	3/3	3/3	3/3	9/9	2/2	3/3	3/3	9/9	2/2	3/3	3/3	9/9
AF12⁺	3/3	3/3	3/3	9/9	2/2	3/3	3/3	9/9	2/2	3/3	3/3	9/9
I_D score	6/6	6/6	6/6	18/18	6/6	6/6	6/6	18/18	6/6	6/6	6/6	18/18

Temperature (°C)

[a] Atoxigenic *A. flavus* strain score/toxigenic strain score.

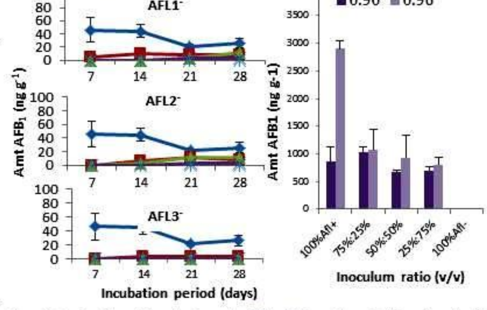


Figure 1. *In vitro* effect of 3 atoxigenic strains (AFL1⁻, AFL2⁻, AFL3⁻) on temporal production of AFB_1 grown in different ratios with a toxigenic strain at 0.96 a_w at 25°C. Bars indicate SEs. (Mohale et al., 2013).

Figure 2. Effect of strain AFL2⁻ on aflatoxin at different a_w levels when challenged with AFL1⁺. Bars indicate SEs.

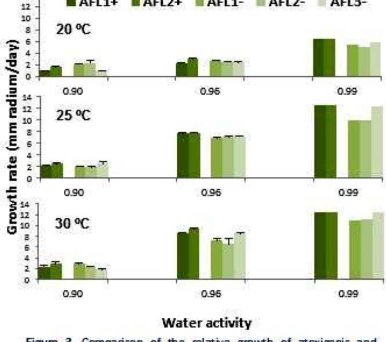


Figure 3. Comparison of the relative growth of atoxigenic and toxigenic strains of *A. flavus* at different temperatures and a_w levels.

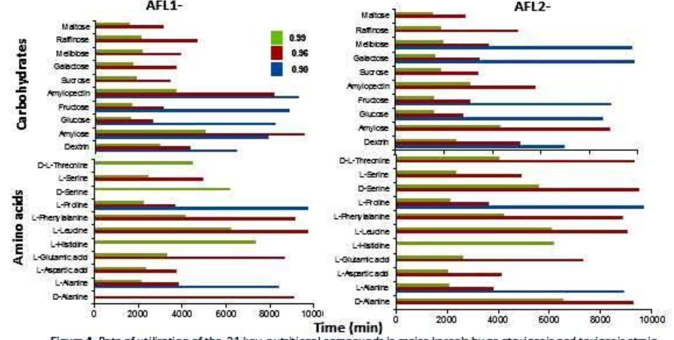


Figure 4. Rate of utilisation of the 21 key nutritional compounds in maize kernels by an atoxigenic and toxigenic strain of *A. flavus* using the BioScreen system (Medina et al., 2012). This shows that they were able to utilise a similar range of compounds regardless of temperature or aw level.

Conclusions

- It is important to consider the environmental conditions under which biocontrol agent is required when screening for efficacy of potential candidates for mycotoxin control
- Growth rate may not always be a measure of competitiveness in terms of controlling mycotoxin production
- The efficacy of atoxigenic strains to control AFB_1 production was influenced by temperature x a_w interactions and was better in vitro than on maize grain.
- The relative growth rate and ability to utilise maize nutritional components did not explain the ability to inhibit AFB_1 production in mixed inoculum on maize.
- Studies are investigating the relative ability for hydrolytic enzyme production by atoxigenic and toxigenic strains.

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