CRANFIELD UNIVERSITY

ALICIA RODRIGUEZ SIXTOS HIGUERA

FUNGAL INTERACTIONS AND CONTROL OF AFLATOXINS IN MAIZE, PRE-AND POST-HARVEST UNDER DIFFERENT CLIMATE CHANGE SCENARIOS

APPLIED MYCOLOGY GROUP
SCHOOL OF WATER, ENERGY AND ENVIRONMENT

PhD THESIS
Academic Year: 2013 - 2017

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This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

ABSTRACT

Aspergillus flavus is a ubiquitous fungus that contaminates maize, the main risk from infection is the production of the carcinogenic mycotoxin aflatoxin B_1 (AFB_1). One strategy to control *A. flavus* contamination is the use of biocontrol agents (BCAs). The aim of this project was to examine the fungal diversity of Mexican maize cultivars and isolate potential BCAs which could control AFB_1 contamination of maize under existing and future climate change scenarios.

The four Mexican maize cultivars had low moisture content, below that which would cause any mould spoilage. *A. flavus* and other associated mycobiota were enumerated, isolated and identified. Eight candidate BCAs were screened for potential antagonism and dominance of toxigenic strains of *A. flavus* including a type strain. This showed that the Index of Dominance of the BCAs vs *A. flavus* was influenced by strain and water activity (a_w). On maize-based media, at 50:50 inoculum ratios four potential BCAs, an atoxigenic Afl^- MEX02, *T. atroviride* MEX03, *T. funiculosus* MEX05 and *C. rosea* 016 were effective in reducing AFB_1 production. The atoxigenic Afl^- MEX02 *A. flavus* strain decreased AFB_1 production by >95% by the toxigenic strain. These BCAs were then tested in more detail with different inoculum ratios including the atoxigenic *A. flavus* strain. The 50:50 ratios were used to analyse the expression of two key genes of the aflatoxin biosynthetic pathway, *aflR* (regulatory) and *aflD* (structural). For type strain of *A. flavus* (NRRL 3357) *aflD* relative gene expression was stimulated by the BCAs at 0.98 and 0.93 a_w. The toxigenic MEX01 strain had *aflD* expression down-regulated at 0.98 a_w in the presence of all the BCAs. The atoxigenic strain isolated from Mexican maize was the most effective at inhibiting AFB_1 production under all a_w x temperature conditions examined on maize-based media giving >90% control. Additionally, the potential reduction of inoculum potential of *A. flavus* by the best four BCA candidates on senescent maize leaves was examined under different a_w levels. None of the four BCAs were able to reduce the conidial production by the toxigenic strain of *A. flavus*. The best candidate BCAs, atoxigenic Afl^- MEX02 and *C. rosea* 016 were examined for efficacy in stored maize grain under

different antagonist: pathogen ratios of 25:75; 50:50 and 75:25 initial inoculum. The relative gene expression of the treatment 50:50 ratio was analysed. The expression of both genes was down-regulated in the presence of the BCAs. Also the atoxigenic *A. flavus* trains had a lower expression compared to the control. This resulted in >60% control of AFB_1 production by the atoxigenic strain under the a_w x temperatures tested. For the *C. rosea* 016 strain this was only affected with relatively freely available water. The two best BCA candidates were examined for efficacy and control of toxigenic *A. flavus* strain growth and AFB_1 production on maize cobs of different ripening ages which also represented different a_w and nutritional levels. Using 50:50 antagonist:pathogen ratios of inoculum this showed that the BCAs down-regulated the expression of the *aflD* and *aflR* genes in the aflatoxin biosynthetic pathway. However, there was no effect on growth or AFB_1 production. The resilience of the candidate BCAs was tested under climate change scenarios (a_w x temperature x CO_2). This showed that at the different ripening stages *A. flavus* was able to grow at similar rates to the control and that AFB_1 production was unaffected by the conditions and indeed by the presence of the BCAs examined. The results obtained are discussed in the context of the different minimisation strategies which can be employed to try and reduce exposure of consumers to this carcinogenic mycotoxin.

Keywords:

A. flavus, AFB_1, biocontrol agent, a_w, qPCR, maize

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
AFB_1	Aflatoxin B_1
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
a_w	Water activity
BLAST	Basic Local Alignment Search Tool
CAM	Coconut cream agar medium
cDNA	Complementary deoxy ribonucleic acid
cm^2	square centimetre
CO_2	Carbon dioxide
C_t	Threshold cycle
CTAB	Cetyl trimethylammonium bromide
DG18	Dichloran (18%) glycerol agar
DNA	Deoxy ribonucleic acid
dNTPs	2-Deoxynucleoside-5'-triphosphate
EDTA	Ethylenediaminetetraacetic acid
ERH	Equilibrium relative humidity
et al.	*et alii*
FAO	Food and Agricultural Organisation
FDA	Food and Drug Administration
g	gram
GMO	Genetically modified organism

h	Hour
HCl	hydrogen chloride
HPLC-FLD	High Pressure Liquid Chromatography-Fluorescence Detector
IARC	International Agency for Research on Cancer
ITS	Internal transcribed spacer
kbp	Kilo-base pair
k	kilo
L	Litre
ʎ	wavelength
log	logarithm
LOD	Limit of detection
LOQ	Limit of quantification
µ	Micro (10^{-6})
m	Milli (10^{-3})
M	Molar (mol/L)
MEA^{+}	Malt Extract Agar medium with Chloramphenicol
MeOH	Methanol
mRNA	Messenger Ribonucleic Acid
NA	Nutrient Agar
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
n	Nano (10^{-9})
NOI	Niche Overlap Index

PCR	Polymerase Chain Reaction
ppb	Parts per billion,
PPVP	polyvinylpyrrolidone
RH	Relative Humidity
RNA	Ribonucleic Acid
RNAse	Ribonuclease
qPCR	Real Time quantitative Polymerase Chain Reaction
TFA	Trifluoroacetic Acid
YES	Yeast Extract Sucrose medium

Chapter 1. General Introduction and Literature review

1.1 Maize

Maize (*Zea mays* L.) is one of the most important cereal grains, produced in 125 countries on nearly 100 million hectares. It is used for human and animal consumption, and production of starch, oil, sugar, alcoholic beverages and fuel. According to the International Grains Council in 2014/15 maize production was 1018.9 M tonnes and the consumption 994 M tonnes (International Grains Council (IGC), 2014). http://www.igc.int/en/markets/marketinfo-sd.aspx (13/02/2017)

Maize was domesticated in Mexico from its wild ancestor Teosinte (*Zea mays* ssp*. Parviglumis*) about 9000 years ago (Prasanna, 2012). Introduction to Europe, Asia and Africa occurred several hundred years ago. Because of this, maize has different derivatives adapted to diverse environmental conditions. Maize can grow at different altitudes, from sea level to 3000 meters, in temperate to tropic climates; its optimal growth occurs at temperature from 20-30°C depending on the cultivar (Dimsey, 2013; FAO, 2013).

Maize has different development stages: vegetative (V) and reproductive (R). the vegetative stage is determined by the collar method; the collar is where the leaf edge visually splits from the stalk (V Emergence – V Tasseling). The reproductive stage starts with the fertilization of the ear and the grain development (Figure 1.1,Table 1.1).

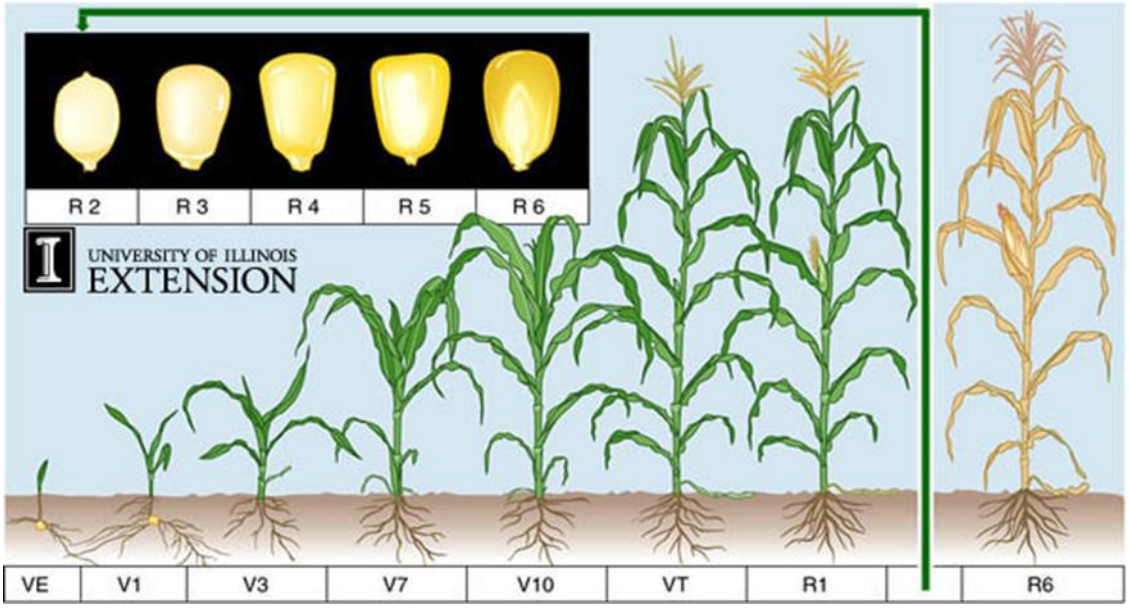


Figure 1.1 Stages of the maize plant development [online image](Nebraska-Lincoln, 2004)

Table 1.1 The different growth stages during silking of maize up to harvest and the associated moisture content on a wet weight basis (Ritchie *et al.*, 1992; Picot *et al.*, 2011)

		Reproductive stage	Kernel moisture content (%)
R1	Silking	Any silk is visible outside the husk; water stress cause pollen and silks desiccation	90
R2	Blister	Small white kernels with clear fluid	85
R3	Milk	Yellow kernels with milky fluid	80-70
R4	Dough	Accumulation of starch; stress will reduce kernel weight	70-60
R5	Dent	Kernel is drying and has a white layer of starch on the top; stress will reduce kernel weight	50-40
R6	Physiological maturity	Maximum dry matter accumulation	<25

1.2 Maize in Mexico and its economic importance

Mexico produces 25 M tonnes per annum of maize, 90% is white maize, meant for human consumption as nixtamalized dough, flour and other food preparations. The remaining is yellow corn used for livestock. In Mexico maize is grown in rain-fed land (81%); only 19% is grown in irrigated farms. Irrigated farms use commercial hybrids; rain-fed areas are mainly cultivated by small scale farmers using land native seed or *criollo* varieties (Turrent-Fernández *et al.*, 2012). A land native seed or 'landrace' is define as ' …dynamic population(s) of a cultivated plant that has historical origin, distinct identity, and lacks formal crop improvement, as well as often being genetically diverse, locally adapted, and associated with traditional farming systems' (Villa *et al.*, 2005). *Criollo* varieties are hybridization between landraces and improved maize. The use of commercial hybrids is expensive and difficult to maintain for the small farmers; they maintain 59 maize land races used as basic ingredients in regional cuisines (Prasanna, 2012; Turrent Fernández *et al.*, 2012). Many rural areas lack adequate infrastructure conditions for drying and storage of the grain, thus inadequate handling can lead to pest and fungal contamination, resulting in losses of up to 30% (Paterson and Lima, 2011; Plasencia, 2004).

1.3 Maize contamination with *Aspergillus flavus* and aflatoxins

Aspergillus flavus is the most common species that contaminates maize during pre-harvest due to its survival on crop debris. The spores are spread by wind or insects, which can result in kernel infection through the silks. The life cycle of *A. flavus* is shown in Figure 1.2. Maize is more susceptible to infection at the flowering stage when silks are browning. *A. flavus* can easily colonise the ears and kernels damaged by insects and if the conditions are conducive, germinate, grow and produce mycotoxins representing a serious health risk (Luo *et a*l., 2009; Reese *et al*., 2011). The fungus survives on crop debris, which is a major source of inoculum for infection of maize cobs later during silking. The kernels loose quality, *A. flavus* infection affects the endosperm but also the kernel changes its structure in response to the infection. Starch degradation and hexose mobilization, and an increase in free fatty acids are some of the

detrimental effects. It is unclear if the response is a plant defence mechanism or an increase in vulnerability to the invasive fungi (Figure 1.3,Dolezal *et al.*, 2014)

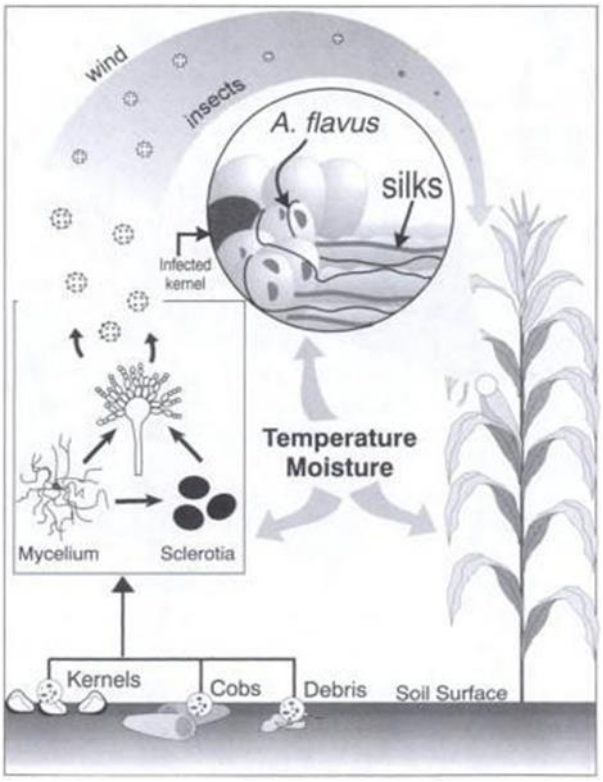


Figure 1.2 *A. flavus* life cycle on maize contamination (adapted from Payne, 1998)

a) b)

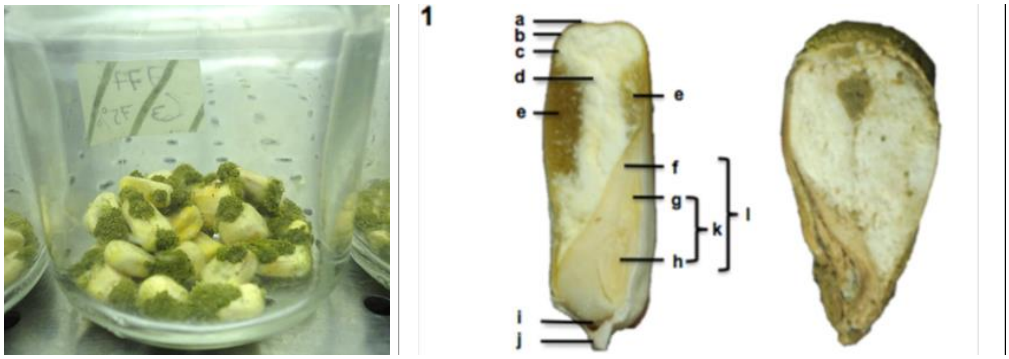


Figure 1.3 (a) Maize kernels inoculated with *A. flavus*. (b) Mature B73 kernels naturally infected with *A. flavus*. Frontal section, healthy kernel (left) compared to diseased kernel (right). Key: a-crown; b-pericarp; c-aleurone; d-starchy endosperm; e-hard endosperm; f-scutellar tissue; h-primary root; i-transfer cells; j-pedicel; k-embryo; and i-germ.(b; adapted from (Dolezal *et al*., 2014)

Aspergillus section *Flavi* species are ubiquitous saprophytes in soil, on crop debris and on senescing leaves. They have a high occurrence in tropical and subtropical regions. Section *Flavi* includes species such as *A. flavus, A. parasiticus* and *A. nomius*. Species such as *A. flavus* produces asexual conidial spores and sclerotia (Gugnani, 2003). The conidia are smooth and round and the colonies are yellow-green in appearance (Figure 1.4)

a) b)

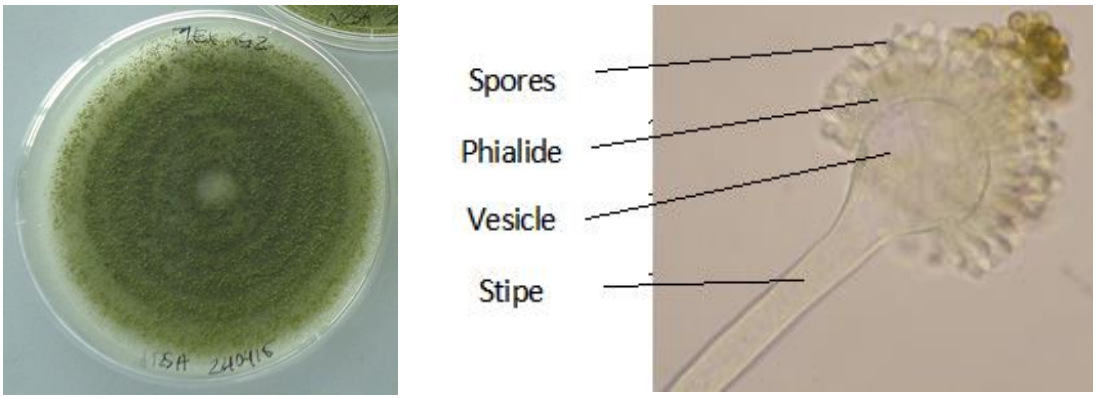


Figure 1.4 (a) Colony of *A. flavus* on Milled Maize Agar. (b) Morphological structure of the *Aspergillus* conidiophore.

A. flavus communities are diverse with both toxigenic and atoxigenic strains occurring in the maize ecosystem, especially in soil. The communities can be divided into S and L morphotypes, which are integrated by different vegetative compatibility groups (VCG). Morphotype S produces small sclerotia (<400 µm) and high aflatoxin production. Type L strains produce larger sclerotia (>400 µm) and low or no toxin production (Cotty and Mellon, 2006; Ehrlich, 2014). The toxigenic *A. flavus* strains produce aflatoxins B_1 and B_2; *A. parasiticus* produces B_1, B_2, G_1 and G_2; named due to the producer abbreviation A-fla-toxin (*A. flavus*) (Yu *et al*., 2011) and the fluorescence emitted under UV light (blue and green). Aflatoxin B_1 (AFB_1) is converted to aflatoxin M_1 when animals consume toxin contaminated feed. The International Agency for Research on Cancer (IARC) considers aflatoxins (AFs) as the most carcinogenic secondary metabolites naturally produced. AFB_1 is classified as a class 1A human and animal carcinogen, aflatoxin M_1 and B_2 are in group B2, as probable human

carcinogens. Aflatoxins are immunosuppressive, mutagenic, teratogenic and hepatocarcinogenic (IARC, 2012).

AFB_1 is a 6-methoxydifurocoumarone, insoluble in non-polar solvents and soluble in moderately polar solvents (chloroform, methanol). They are unstable in extreme pH levels (<3, >10) and oxidizing agents. The lactone ring is susceptible to alkaline hydrolysis; they are degraded when reacting with ammonia, sodium and calcium hypochlorite (IARC, 2012).

1.3.1 Aflatoxin biosynthesis gene cluster

The gene cluster involved in the biosynthesis of aflatoxins has now been elucidated (Figure 1.5; Yu *et al*., 2004). The genes involved in aflatoxin production are clustered together. There are two key regulatory genes in this pathway, *aflR* and *aflS*. *aflR* is a positive regulatory gene required for transcriptional activation which encodes a sequence-specific zinc binuclear DNA-binding protein; *aflS* encodes a transcriptor enhancer that interacts with *aflR* to activate the structural genes. If the *aflR* protein is missing there is no aflatoxin production. A key early structural gene in the pathway is *aflD* encoding for a reductase to change the first stable product of the pathway, norsoloronic acid (NA) to averantine (AVN) (Bhatnagar *et al*., 2006; Yu *et al*., 2011a). Nierman *et al*. (2015) recently reported the genome sequence of the *A. flavus* NRRL 3357 strain.

Schmidt-Heydt *et al*. (2010) suggested that in *A. parasiticus*, some of the genes in this cluster act together in different groups. They also showed that water activity (a_w) x temperature affected aflatoxin production. They suggested that the suppression of the ratio of the two regulatory genes (*aflR/aflS*) was important in relation to the relative amount of aflatoxin produced under different environmental conditions. Abdel-Hadi *et al*. (2012) utilized a microarray to examine the effect of a_w x temperature conditions on relative gene expression and related this to growth and AFB_1 production. They found that *A. flavus* can grow over a wide range of temperature x a_w, but AFB_1 production was over a narrower range. Yu *et al*. (2011) using RNA-Seq technology studied the *A. flavus* transcriptome at 30°C which was found to be the temperature conducive

to AFB_1 production, while 37°C was considered to be non-conducive. However, they did not examine the effect of interactions with water, temperature or CO_2 stress. At 30°C they found that cluster 54 (aflatoxin) was up regulated, including the expression of the *aflD*. The regulatory gene *aflR* was expressed at both temperatures; however they determined that 37°C downregulated the transcription of *aflR* and *aflS*, when compared to 30°C. Dolezal *et al.* (2013) analysed the transcriptome of *A. flavus* during colonization of maize grain from the inbred line B73, using the Affymetrix GeneChip® microarray. During the interaction 8 transcription factors were up-regulated, including the *aflR* gene. Furthermore, they examined the expression profile of the secondary metabolites gene cluster. For the aflatoxin cluster (54) there were indications that > 50% of the genes were over expressed, including the 'backbone' enzymes, which are necessary to start the biosynthetic process. Recently, Medina *et al.* (2015) analysed the effect of interactions between three climate change factors (a_w x temperature x CO_2) on AFB_1 and relative expression of the *aflD* and *aflR* genes. This study showed that *aflD* expression was increased under water stress conditions, elevated temperature and elevated CO_2 (0.99 a_w/30°C and 350 ppm CO_2 vs 0.92 a_w/ 37°C, CO_2 1000 ppm) and for the *aflR* regulatory gene at 37°C, with CO_2 650, and with 1000 ppm at 0.95 and 0.92 a_w. This suggests that interacting factors are an important consideration in understanding the functional importance of the genes involved in AFs production in developing control strategies.

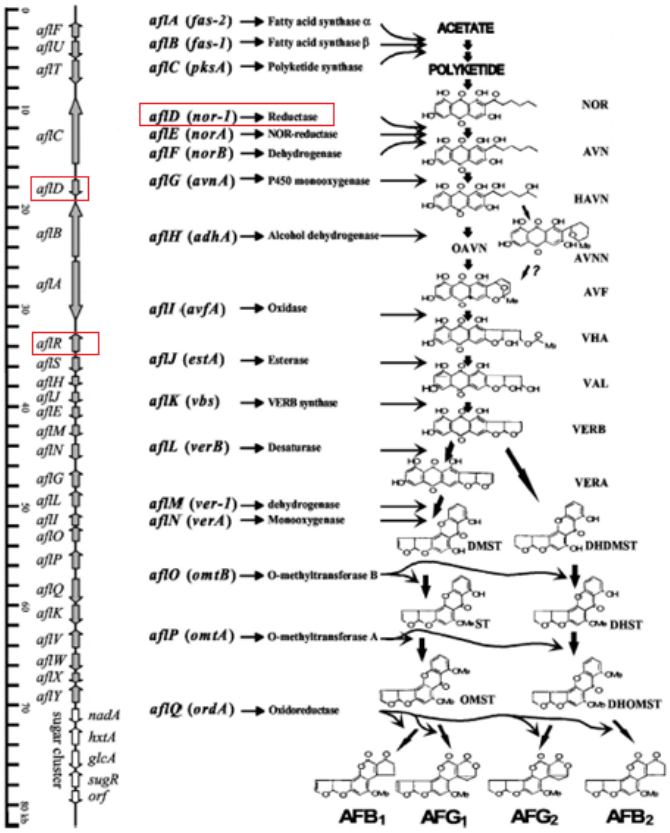


Figure 1.5 The gene cluster responsible for aflatoxin biosynthesis in *A. flavus* and *A. parasiticus*. The genes highlighted are *aflD,* an early structural gene and *aflR a* key regulatory gene in the biosynthetic pathway for aflatoxin production (adapted from Yu *et al.*, 2004).

1.4 Environmental conditions for fungal colonisation of food matrices

Maize colonization by fungi (e.g. *Aspergillus, Fusarium, Penicillium* species) is influenced by biotic and abiotic factors. The most important abiotic factors are a_w, temperature, pH and the intergranular gas composition. These may act individually or interact with each other (Magan and Aldred, 2007a). The optimum and marginal boundary conditions of temperature x a_w interactions for growth and mycotoxin production are important as these have been developed and modelled for many mycotoxigenic fungi, including *A. flavus* (Sanchis and Magan, 2004). This information is important because the window in which AFs can be produced has been identified and when developing control strategies to

minimise mycotoxin production in different food raw commodities this context needs to be borne in mind.

1.4.1 Water availability, growth and aflatoxin production

In raw food substrates (e.g. maize grains) some of the water is bound strongly to the protein, carbohydrates and lipid components, with a proportion weakly bound and available for microbial development. To determine the water available for microbial growth Scott (1957) developed the concept of a_w; this was defined as the ratio of the vapour pressure of water in the substrate (P) to the vapour pressure of pure water (Po), at the same pressure and temperature. The a_w value of pure water is 1.00. The a_w is related to the equilibrium of relative humidity (ERH). ERH is the ratio of the vapour pressure of the air to its saturation vapour pressure (Equation 1.1). When vapour and temperature are in equilibrium, a_w of the substrate is equal to the ERH of the air.

$$\mathrm{aw} = \frac{\mathrm{P}}{\mathrm{Po}} = \frac{\mathrm{ERH}(\%)}{100}$$

Equation 1.1 Formula to calculate the relation a_w with ERH

The water content (moisture content, m.c.) of a substrate and the relationship with a_w is given by a moisture sorption isotherm at a constant temperature and pressure. It is important to develop a moisture sorption curve for the substrate (e.g. maize grains) because the relationship between m.c. and a_w will be different for different grains. Figure 1.6 shows a diagrammatic representation of the adsorption and desorption curves and the slight difference that occur whether absorbing or loosing water. This difference is called the hysteresis effect. Reducing the amount of free water will reduce the growth of microorganisms. It should be noted that the sorption curve varies with the grain type, depending on the composition of carbohydrates or lipids and on the temperature.

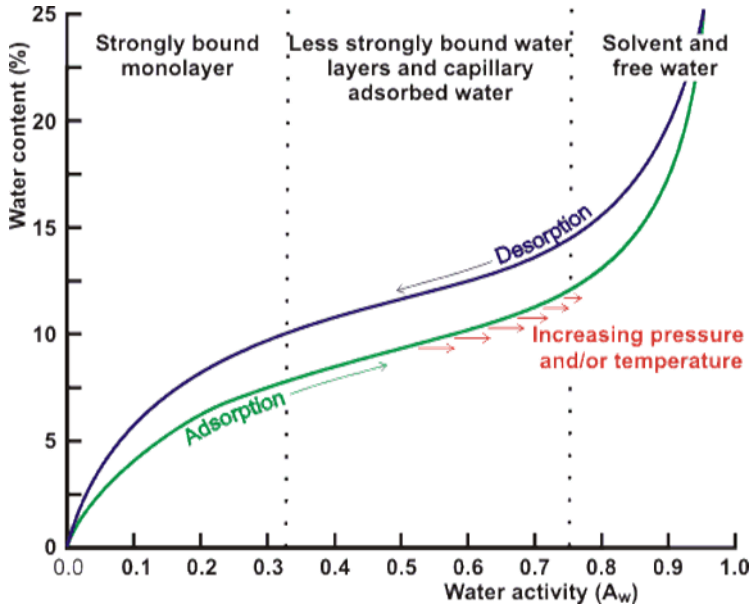


Figure 1.6 Example of a moisture sorption curve for maize [online image] (Accessed 26 November 2013) (adapted from http://www1.lsbu.ac.uk/water/activity.html).

The water content of the maize when ripe is usually 18-24% and is subsequently dried to about 15% (=0.70 a_w). If the maize grain is not dried correctly it can be contaminated with fungi that can decrease the grain quality and become a health risk due to mycotoxin contamination (Magan *et al.*, 2003; Magan and Aldred, 2007a; Magan *et al.,* 2010). Table 1.2 summarizes the relationship between a_w and moisture content for maize.

A. flavus can be classified as a xerophile, which is defined as a fungus which has at least one part of its life cycle (germination, growth or sporulation) at ≤0.85 a_w (Pitt, 1975). Because *A. flavus* is a xerophilic fungus it has some advantages over other species, especially in dry and hot conditions where many other fungi cannot effectively grow.

Table 1.2 Relationship between moisture content (wet weight basis, %) and water activity (a_w) for maize at 25°C. (adapted from Sanchis and Magan, 2004)

a_w	Moisture content (%, wet weight basis)
0.98	30-32
0.95	26-27
0.90	23-24
0.80	16-17
0.70	15-16

1.4.2 Temperature, growth and aflatoxin production

Temperature is one of the most important factors influencing fungal germination, growth and mycotoxin production. The typical temperature for the maize growing season until harvest is between 20 to 30°C, which is optimum for growth of *A. flavus*. The relationship between a_w, temperature and growth and AFs production by *A. flavus* has been examined previously on conducive YES media (Schmidt-Heydt *et al.*, 2010; Abdel-Hadi *et al.*, 2012). Its optimum growth conditions were 30-35°C and marginal conditions were 15 and 42°C at 0.99 a_w. The conditions for AFB_1 production were optimum at 25-30°C at 0.99 a_w and 30-35°C at 0.95 a_w. Sanchis and Magan (2004) reported 35°C/0.95 a_w for optimum growth and 33°C/0.99 a_w as optimum for mycotoxin production.

1.4.3 pH, growth and aflatoxin production

Fungi can grow over a range from pH 2–10, with the optimum around pH 5.0. Most spoilage and mycotoxigenic fungi prefer slightly acidic conditions for optimum growth (Magan, 2007). The pH of maize is approx. 5.0. pH becomes an important factor as feed maize is often treated with organic acids (propionic/sorbic/benzoic acids and their salts). These are most effective at around pH 4-5 because inhibitory effects are maximised when 50% of the acid is dissociated. However, often these are fungistats and the coverage of the treatment needs to be effective as untreated or under treated areas can result in growth of fungi, especially *A. flavus*, resulting in AFs contamination. Arroyo *et*

al. (2005) analysed the interaction between pH, a_w and preservatives (calcium propionate; potassium sorbate) on growth and ochratoxin A (OTA) production by *P. verrucosum*. The preservatives were effective at pH 4.5, but not at pH 6 even when used at the recommended concentrations. OTA production was also strain dependent. However, OTA production was often higher at pH 6. Thus under some circumstances pH will be important, especially for feed maize.

1.4.4 Gas composition

Fungal growth can be altered if the proportions of the atmospheric gases are changed. The intergranular composition of air becomes important, especially in stored grain. Grain is alive and respiring even when stored at a safe moisture content. Most mycotoxigenic fungi have been shown to be very tolerant of elevated CO_2 conditions (Magan and Aldred, 2007a; Magan *et al*., 2010).

Magan and Lacey (1984a) found that reduced O_2 (< 1%) and elevated CO_2 (5-10%) had an impact on fungal growth of a range of spoilage grain fungi over a range of a_w levels. Pateraki *et al*. (2007) studied the effect of controlled atmospheres on *Aspergillus carbonarius* germination, growth and OTA production. They exposed spores and mycelial colonies to different combinations of O_2, CO_2, N_2 for 24, 48 h and 2, 5, 10 days respectively. They showed that CO_2 at high concentration (50% CO_2) was ineffective as an inhibitor of spore germination, although it delayed the rate of germination. Overall, OTA could not be controlled by up to 50% CO_2, regardless of a_w level. Giorni *et al.* (2008) examined up to 75% CO_2 x a_w levels *in vitro* and *in situ* on *A. flavus* growth and AFB_1 production. Both mycelial extension and AFB_1 production were reduced to some extent by these CO_2 levels.

1.5 Fungal interactions

Maize and other cereals are colonised by a wide community of fungi. These fungi, under conducive environmental conditions, will interact with each other and influence the dominance of some components of the fungal communities (Magan and Aldred, 2007a). Fungi adapt using different strategies: combative species (C-selected) grow and occupy the niche rapidly; stress tolerant species

(S-selected) survive under environmental stress; ruderal species (R-selected) reproduce and use readily available sugars to colonise the substrate. Sometimes, fungal competitiveness is influenced by combinations of these strategies (C-R, S-R, C-S, C-S-R) especially under fluctuating environmental conditions. For those fungi using S and C strategies the production of secondary metabolites is important and probably influences the occupation and dominance of certain individual or groups of species (Magan and Aldred, 2007b, 2008). This is often a dynamic process with shifts in the dominance of species in the ecological niche due to changes in environmental conditions. This will impact on the ability of fungal species to colonise the substrate. There are different measures used to examine these interactions between the fungal communities of a specific niche, in this case, maize grain. Studies are conducted both *in vitro* and *in situ* to better understand the relationship between fungi, the relative dominance and the potential role of mycotoxins in dominance (Magan and Aldred, 2007b).

1.5.1 Interactions and the Index of Dominance

Magan and Lacey (1984b) developed a numerical scoring system to examine the interactions between fungal species at a colony level. Each interacting species was given a score. The scoring system is shown below for each interacting species:

- 1:1, mutual intermingling
- 2:2, mutual antagonism on contact
- 3:3, mutual antagonism at distance
- 0:4, dominance by the latter over the former species
- 0:5, dominance at a distance by the latter species.

Thus, the interactions between one species and a range of species/strains could then be added together to obtain an Index of Dominance (I_D). They were able to demonstrate that the I_D varies with a_w x temperature interactions and was not directly related to the growth rate of specific species *in vitro* or indeed *in situ* in wheat grain (Magan and Lacey, 1984a, 1985). Studies by Magan and Aldred (2008) showed that a_w x temperature interactions will influence the type

of interactions which might occur and the total I_D and that the type of interactions are in a state of flux and change with interacting environmental conditions. There is thus a need to examine the interactions between *A. flavus* and other fungi, which colonize maize grain. It is possible that the production of mycotoxins can give some fungi an ecological advantage over other species. In addition, this approach may help identify strains that are antagonistic or competitive against *A. flavus* and provide candidates for biocontrol of this aflatoxigenic species.

1.5.2 Niche Overlap Index (NOI)

Studies have demonstrated that the fungi consume the carbon source (CS) in different substrates at different rates. Wilson and Lindow (1994) examined the ability of different ice nucleation bacteria (*Pseudomonas syringae)* to colonise the phyllosphere surfaces of leaves by initially comparing the ability to utilise different Carbon compounds in *in vitro* assays. They suggested that this kind of data on the relative number of C-sources utilised commonly and those utilised by species individually, e.g. pathogen and a biocontrol agents, could be used to develop a Niche Overlap Index (NOI, Equation 1.2). This would help to better understand whether they occupied the same or different niches.

$$\text{NOI} = \frac{\text{no. of C-sources in common between two fungi}}{\text{Total no. of C-sources utilized by tested}}$$

Equation 1.2 Formula to calculate Niche Overlap Index

The value obtained ranged from 0 to 1. A score of >0.90 meant coexistence in the same ecological niche; a score of <0.90 defined occupation of different niches. This approach was extended by Marín *et al.* (1998) to examine the C-source utilisation patterns of different mycotoxigenic and non-mycotoxigenic maize fungi under different interacting a_w x temperature conditions. Later Arroyo *et al.* (2008) examined the influence of preservative concentrations on the NOI, and Giorni *et al.* (2009), the interaction between *F. verticillioides* and *A. flavus*.

Giorni *et al.* (2009) used the NOI of key carbons sources in maize only, modifying a_w and temperature, to determine the level of co-existence of *A.*

flavus and *F. verticillioides*. They demonstrated that *A. flavus* had nutritional dominance at 30°C, more under drier conditions and *F. verticillioides* dominated at 20°C, and 0.95 and 0.90 a_w (Figure 1.7). The consumption of different CS was linked to nutritional dominance only at extreme conditions, because most of the time they occupied separate niches. These results showed that a_w and temperature can change the CS consumption and the NOI of each fungus.

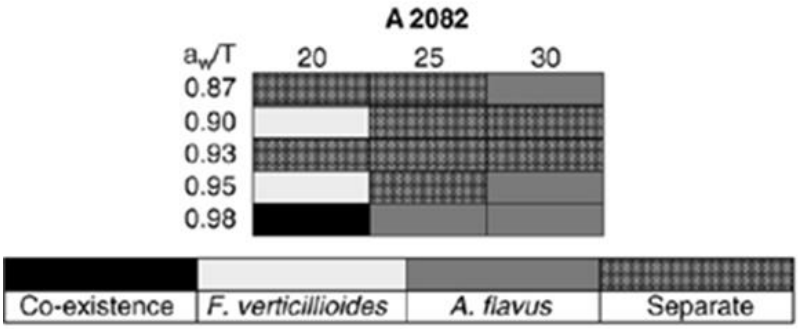


Figure 1.7 Schematic representation of NOI for the different conditions of temperature (20, 25 and 30°C) and a_w (0.87, 0.90, 0.93, 0.95 and 0.98) of *A. flavus* with *F. verticillioides* as the target pathogen (adapted from Giorni *et al.,* 2009).

More recently, Mohale *et al.* (2013a) studied the NOI of two toxigenic *A. flavus* strains and three atoxigenic ones at different temperatures (20-30°C) and a_w (0.90–0.99) levels. They tested the utilization of the carbon sources (CS) in maize as done previously by Giorni *et al.* (2009). The number of CS utilized was similar for both types of *A. flavus* strains. The number was decreased when the temperature and a_w were lowered. The NOI data at 0.96 and 0.99 a_w suggested that the strains co-existed, while under water stress (0.90 a_w), the toxigenic strains dominated or occupied different niches. Interestingly, by using rates of C-sources utilisation patterns using a rapid bioassay system it was possible to examine the relative rates of utilisation of the battery of C-sources in maize under different a_w x temperature conditions (Mohale *et al.,* 2013a). However, this showed little difference between the toxigenic and atoxigenic strains, suggesting that other mechanisms of competiveness may be important. Similar studies with *F. verticillioides* and biocontrol agents also suggested that this may not be the main mechanism of action (Samsudin *et al.*, 2016).

1.6 Impacts of climate change (CC) on *A. flavus* and aflatoxin production

According to the Intergovernmental Panel on Climate Change (IPCC) report, the projections of four CC scenarios are based on greenhouse gases (GHG) and air pollutant emissions. This is a mitigation scenario, two intermediate and one with very high GHG emissions. The projections show that in the next 50 years the temperature may increase by between 1.5 – 4.8°C and the CO_2 level between 400 to > 1000 pm, depending on the scenario (IPCC, 2014). The environmental changes caused by CC have received significant attention in terms of potential impacts on staple food production systems and the impact that might occur on crop agronomy and pests and diseases (Tirado *et al*., 2010; IPCC, 2014) . CC models predict that certain regions of the world will be hotspots, especially in parts of Africa, Mediterranean region, South America and the far east, where food security could be compromised (Fraser *et al.*, 2013). Hotspots, defined by de Sherbinin (2014) as 'regions particularly vulnerable to current or future climate impacts and where human security may be at risk', will suffer extreme changes. CC interacting factors will have an impact on the physiology of crop plants, including maize, and perhaps influence the susceptibility to *A. flavus* and *A. parasiticus* infection and AFs contamination during water and heat stress, representing a significant food security risk (Medina *et al.*, 2015; Fountain *et al.,* 2014).

As the crop production patterns change, the distribution of fungal pathogens may change, with drier conditions and perhaps more xerophilic fungi becoming important in food commodities displacing other less tolerant fungi (Magan *et al.*, 2011). Models of crop pest and disease movement suggest that they are migrating away from the Equator since 1960, at between 2.7-5 ± 0.8 km/yr. The authors take into account the bias of the ability of the northern countries to detect the pests earlier (Bebber *et al.*, 2013). In Mexico, this will have major effects on small-scale farmers working under rain-fed conditions. CC models for 2050 predict that the distribution pattern of maize will change due to a temperature increase of 3.5°C (Bellon *et al.*, 2011; Prasanna, 2012; Ureta *et al.*, 2012). Another possible effect of CC could be a diminishing of the host defence,

even in resistant cultivars impacting on fungal disease and perhaps mycotoxin contamination (Váry *et al.*, 2015).

Recent studies on other mycotoxigenic fungi of coffee suggest that the impact of CC factors may vary with the genus or species (Akbar *et al.*, 2016). Thus, while a stimulation of OTA production was observed for *A. westerdijkiae*, there was less effect on *A. carbonarius* strains, both *in vitro* and *in situ*. Recently, Borisade and Magan (2015) showed that for pest control using entomogenous fungi, CC factors reduced the efficacy and relative control achieved. Surprisingly, there is very little, if any knowledge on the impact of CC factors on biocontrol agents of fungal diseases, and none in relation to AFs control using atoxigenic *A. flavus* strains or other antagonist. If biocontrol is compromised, then alternative formulations or more resilient strains of biocontrol agents may be necessary. Recently Váry *et al.* (2015) acclimatized the host (susceptible and resistant wheat cultivars) and the pathogens (*Fusarium graminearum* and *Zymoseptoria tritici*) of wheat under normal and high CO_2 concentrations (390 and 730 ppm). For the trials they used the second generation of the wheat and 20^{th} subculture of the pathogen, acclimatized to both CO_2 concentrations. The pathogenicity of the acclimatized fungi was increased by 29%. When the susceptible wheat cultivar and the pathogen were acclimatized, the disease was increased by 14%, also reducing the grain number and weight. It would be interesting to obtain information on whether this resulted in an increase in deoxynivalenool (DON) production by *F. graminearum* (Váry *et al.*, 2015).

1.7 Control strategies for *Aspergillus flavus* contamination and aflatoxin consumption

1.7.1 Legislation

Worldwide regulations have been established since 1970 to protect the consumer from the known effects of mycotoxins since the discovery of AFs in the early 1960s. Many countries have very strict legislation with regard to the allowable levels of contamination of raw cereals/nut commodities world-wide. The FAO reported 100 countries with mycotoxins regulations in 2003 (Food Quality and Standards Service, 2004). The EU regulations include AFB_1, AFM_1,

total aflatoxins (B_1, B_2, G_1, and G_2)(European Commission No.1881/2006). The limit set for AFB_1 in cereals is 2.0 ppb; the sum of the 4 aflatoxins (B_1, B_2, G_1 and G_2) is 4.0 ppb (European Commission, 2010). Mexico has established AFs legislative limits via the Mexican Official Standards published by the Ministry of Public Health. However, there are no regulations for other mycotoxins. The Mexican Official Standard 187-SSA1-2002 (Secretaria de Salud, 2002) sets the maximum limit of 12 ppb of AFB_1 in maize dough and nixtamalized maize tortillas. The NOM-188-SSA1-2002 has set the contamination limit of 20 ppb total AFs in cereals for human consumption. If the cereal is contaminated with 21 – 300 ppb, the cereal can be only used for feed (Secretaría de Salud, 2002).

1.7.2 Biocontrol

A lot of effort has been focused on ways to reduce the risk of AFs contamination, especially in maize (Table 1.3) the use fungicides, improvement of the management with adequate irrigation and fertilization, resistant hybrids or biocontrol using non-toxigenic strains have all been strategies examined.

Table 1.3 Crop, insect and soil management practices to manage aflatoxin contamination (Abbas *et al*., 2009).

Strategy	Method	Rationale
Avoidance	Early planting, supplemental irrigation, short season hybrids	Reduce heat and moisture stress
Fertility management	Provide adequate nutrition	N- deficient corn more susceptible
Insecticide application	Appropriate timing of application to control insect damage to ears	Insect responsible for enhanced ingress into grain
Bt Hybrids	Hybrids engineered with resistance to ear-damaging insect	Insects responsible for penetration into grain
Natural resistance to insects	Breeding and selection hybrids for resistance	
Biological control	Use of nontoxigenic isolates of *A. flavus*	Competitive displacement of toxigenic isolates
Fungicides	Control phyllosphere fungi	Reduce inoculum density
Soil management	Incorporation of crop residues	

GMOs are not legally allowed to be grown in Mexico. There are concerns about the risk and impacts on diversity of maize cultivars and effects on gene flow, with potential for genetically modified maize entering native varieties. The potential effects of such interactions are unknown (Vargas-Parada, 2014). Thus, in Mexico, research has focused on the production of commercial hybrids with resistant germplasm using landraces resistant to mycotoxin contamination (Wisniewski *et al.*, 2002; Brown *et al.*, 2004; Ortega-Beltran *et al.*, 2014). Dolezal *et al*. (2014) found that the gene expression during *A. flavus* invasion is related to host resistance. However, the maize genes related to resistance need to be identified, for use as genetic markers.

Bacteria, yeasts and filamentous fungi including atoxigenic strains of *A. flavus* have been used as biocontrol agents to try and decrease AFs contamination of maize and groundnuts. Table 1.4 shows examples of atoxigenic *A. flavus*

strains being used to control *A. flavus* infection and AFB_1 production in different commodities. The strains are atoxigenic due to a deletion or insertion in the aflatoxin biosynthetic pathway and are from a different VCG than the toxigenic tested (Cotty and Mellon, 2006; Ehrlich, 2014; Bandyopadhyay *et al.*, 2016).

Table 1.4 Reduction of aflatoxin B_1 contamination by atoxigenic *A. flavus* strains (adapted from Abbas *et al*., 2009).

Crop	Results	Atoxigenic strain used	Citation
Cotton	90% AFB_1 inhibition	AF-36	(Cotty *et al.*, 2007)
Peanuts	98% aflatoxin inhibition	NRRL21882 active ingredient of Afla-Guard®	(Dorner and Lamb, 2006)
Maize	80% aflatoxin reduction	CT3 and K49	(Abbas *et al.*, 2006)
Peanuts	99% *Aspergillus* population reduction in soil	AF051	(Lyn *et al.*, 2009)
Maize	70-99% toxin reduction	Aflasafe™	(Atehnkeng *et al.*, 2008, 2014)
Maize	80% toxin inhibition	Three *A. flavus* strains isolated in Lesotho, Southern Africa	(Mohale *et al.*, 2013b)

Sultan and Magan (2011) isolated a *Streptomyces* strain (AS1) from peanuts which produced metabolites which were effective against germination and mycelial growth of *A. flavus* strains. Indeed, the extracts were more effective than the cells of the actinomycete itself. In studies with the extracts obtained from the AS1 isolate, AFB_1 production was inhibited on stored peanuts. Verheecke *et al*. (2015) studied six *Streptomyces* sp. and their effect on AFB_1 production by *A. flavus in vitro*. The six strains significantly decreased AFB_1 production by >90%. One strain was able to reduce *aflR* relative expression compared to the control; in contrast the *aflD* expression was unaffected.

Formenti *et al.* (2012) studied different chemical fungicides and a commercial biocontrol bacterium (Serenade: *Bacillus subtilis*) at different a_w levels (0.99, 0.98, 0.95 a_w) against *F. verticillioides* and *A. flavus*. All concentrations of Serenade affected the fungal growth; concentrations of 10^6 and 10^8 were the

most significant. The inhibitory effect diminished with less available water. It also reduced mycotoxin production by >99%. They conclude that the effect was due to competitive exclusion, but the bacteria were sensitive to lowered a_w.

Mohale *et al.* (2013b) studied the effect of three atoxigenic *A. flavus* strains against two toxigenic strains under different temperature (20, 25 and 30°C), a_w (0.99, 0.96 and 0.90) and substrate conditions (*in vitro*: Malt Extract and Milled Maize Agar; *in situ*: stored maize grain). They used different pathogen:anatagonist inoculum ratios (100:0, 25:75, 50:50, 75:25, 0:100), to examine and the impact on AFB_1 production. They found that the strains were mutually antagonistic. The antagonism was on contact; however, the interaction changed to mutual antagonism at a distance depending on the temperature. At 30°C the overall Index of Dominance (I_D) was better for the toxigenic strain. AFB_1 production was reduced in all a_w treatments after 28 days with inhibition of up to 80%. These results demonstrate that these atoxigenic *A. flavus* strains were able to control AFB_1 contamination in stored maize.

Bandyopadhyay *et al.* (2016) developed a biocontrol product called Aflasafe™ for soil application, registered in 4 African countries and in developmental stage in 9. It is formulated from native atoxigenic *A. flavus* strains selected from different VCGs from the toxigenic strains. The product is applied on soil before the flowering period. The formulation is made with colonised sorghum grains (carrier and food source) as it gives an advantage to the atoxigenic strain. It reduces AF in >80% compared to untreated crops and the protection apparently continues during post-harvest storage.

Recently, Al-Saad *et al.* (2016) analysed the interaction between potential bacterial biocontrol agents and a toxigenic *A. flavus* strain on nutrient (0.98 and 0.94 a_w) and maize media (0.995 and 0.98 a_w) at 35°C. They examined the effects on gene expression (*aflD* and *aflR*) and on AFB_1 production using inoculum ratios of antagonist:pathogen cells/spores of 50%:50%. They found that gene expression was significantly decreased compared to the control. However, AFB_1 production was not correlated with gene expression for all the bacterial strains examined. In some cases, despite the effect on the biosynthetic

genes a stimulation of AFB_1 occurred. This suggests that other key genes may not be affected and this allowed the production of AFB_1. This study also showed that inoculum ratios and nutritional parameters need to be studied in more detail to understand the interaction between bacterial biocontrol agents and toxigenic strains of *A. flavus*.

Knowing the *A. flavus* life cycle on maize and its weak points helps to target application of BCAs to reduce infection and contamination with AFB_1. Initially, isolation of potential BCAs from four Mexican cultivars was done, to enable control to be examined in the weak points of the *A. flavus* life cycle. One such point is the soil surface, where the crop debris or the maize grains can be a source of inoculum. This is also linked to the spread of inoculum of *A. flavus* to the maize cobs during silking where it enters through the silks and via insect damage. Analysis of how simulated relevant environmental conditions will affect the interaction between the potential antagonists and pathogen are important.

1.8 Aim and Objectives

Aim

The aim of this project was to better understand the interactions between *A. flavus* and other mycobiota which contaminate Mexican maize to identify potential BCAs to inhibit aflatoxin production, under existing and under future CC scenarios. This approach could be useful to identify antagonistic strains which can control AFB_1 production by toxigenic strains of *A. flavus* by examination of effects on relative aflatoxin biosynthetic gene expression (*alfR* and *aflD*) and control of AFB_1 in Mexican maize. In addition, potential for control of inoculum production on crop residue under different environmental conditions was examined.

The research was carried out by addressing a series of linked objectives. These have been presented as Chapters with specific questions answered in each one to address the overall aim.

Chapter 2. Isolation of potential BCAs from four Mexican maize cultivars

a) Determine the mycobiota of four Mexican maize cultivars from different regions of Mexico, used for making tortillas.

b) Analyse the a_w and the moisture content of each cultivar and its mycobiota contamination levels.

c) Isolation of potential BCAs and fungal populations using selective media with different a_w levels.

d) Identify the mycotoxigenic fungal species present and confirm the ability of strains of the *Aspergillus* section *Flavi* to produce AFs, both qualitatively and quantitatively.

Chapter 3. Screening of potential biocontrol agents obtained in Chapter 2, *in vitro* in interactions with toxigenic *Aspergillus flavus* strains for antagonism and control of aflatoxin B_1 production:

a) Screen the effect of eight potential BCAs on *A. flavus* growth during co-inoculation on 3% milled maize agar (MMA) under different water activity (a_w) levels.

b) Quantify the relative antagonism and dominance of the BCA candidates against the two toxigenic *A. flavus* strains (NRRL 3357; MEX01) using an Index of Dominance (I_D) under different a_w and temperature conditions.

c) Examine the effect of 50:50 mixed inocula of the best candidate BCAs with toxigenic strains of *A. flavus* on AFB_1 production on 3% MMA at different a_w and temperature conditions.

d) Examine the effect of different inoculum ratios of the best candidate BCAs vs toxigenic *A. flavus* strain respectively (100:0; 75:25, 50:50, 25:75 and 0:100 spore ratios) on AFB_1 production on the MMA at different a_w levels and 30°C.

Chapter 4. Impact of the biocontrol agents on *Aspergillus flavus* conidial production on senescent maize leaves under different water activity regimes

a) Construct a moisture adsorption curve with senescent maize leaves

b) Compare the effect of 50:50 mixed spore inoculum of the BCAs with the toxigenic *A. flavus* strain (MEX01) on relative sporulation of the toxigenic *A. flavus* at 0.98 a_w (=-2.8 MPa water potential) and 0.93 a_w (= -9.8 MPa) at 30ºC on senescent maize leaves.

c) Examine the effect of the atoxigenic *A. flavus* strain on sporulation of the toxigenic strain (MEX01) on a selective coconut cream agar under the treatment conditions detailed in (b).

Chapter 5. Efficacy of the best biocontrol agents on aflatoxin B_1 production in stored maize grain under different a_w x temperature conditions

a) Develop a moisture adsorption curve for gamma irradiated maize grains

b) Compare the effect of different inoculum ratios of the best BCAs + toxigenic *A. flavus* strains (NRRL 3357; MEX01) on relative gene expression of *aflD* (structural) and *aflR* (regulatory) genes by *A. flavus* at 0.98 and 0.93 a_w at 30°C on maize stored for 10 days.

c) Evaluate the effect of the BCAs on AFB_1 production by the two toxigenic *A. flavus* strains (NRRL 3357 and MEX01).

Chapter 6. Impact of the best two biocontrol agents on control of aflatoxin B_1 on maize cobs of different ripening stages

a) Measure the a_w of maize cobs at different ripening stages (Milk R3, Dough R4 and Dent R5)

b) Examine the efficacy of the atoxigenic *A. flavus* strain (Afl^- MEX02) and *C. rosea* 016 on control of AFB_1 production by toxigenic *A. flavus* strain

(MEX01) when co-inoculated in 50:50 conidial inoculum ratio in maize cobs of different ripening stages (R3, R4 and R5) at 30°C.

c) Evaluate the effect of the BCAs on the relative toxigenic *A. flavus* strain gene expression of *aflD* and *aflR* in the different ripening stages of the maize cobs in (b)

d) Quantify the effect of the two BCAs on AFB_1 production by the toxigenic *A. flavus* strain

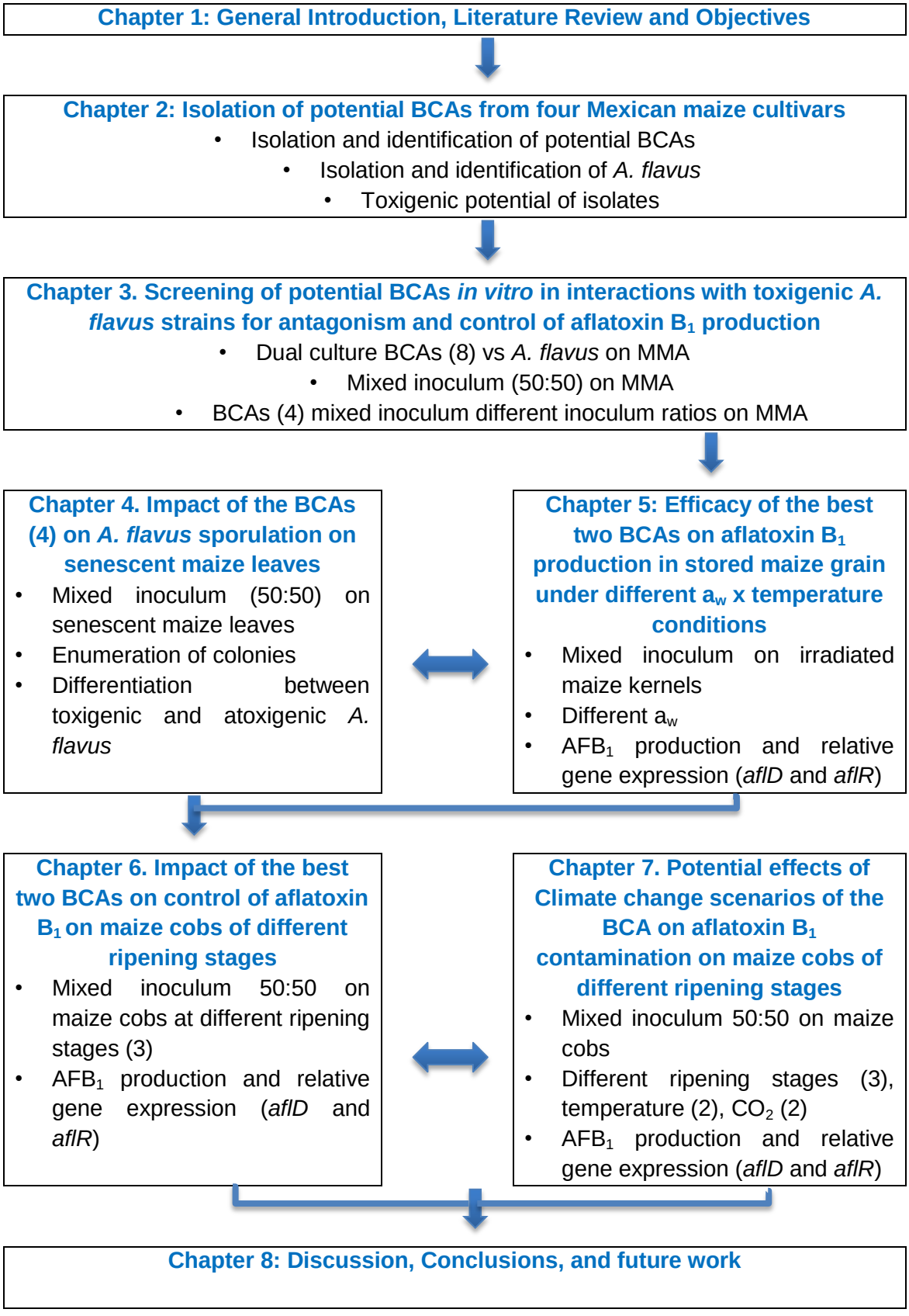
Chapter 7. Potential effects of climate change scenarios on the biocontrol agents and on control of *A. flavus* growth and aflatoxin B_1 contamination of maize cobs of different ripening stages

a) Evaluate the impact of interactions between a_w, temperature (30 and 37˚C) and CO_2 (400 and 5000 ppm) on the atoxigenic *A. flavus* (Afl^- MEX02) strain and *C. rosea* 016 BCAs and efficacy against the growth of toxigenic *A. flavus* MEX01 strain when inoculated in 50:50 inoculum ratio during co-inoculation on maize cobs at different ripening stages (R3, R4 and R5) under different environmental conditions.

b) Examine the influence of CC factors in (a) and BCAs on relative expression of two key genes of aflatoxin biosynthetic pathway (*aflD* and *aflR*) in the toxigenic *A. flavus* strain.

c) Quantify the effect of the two BCAs on AFB_1 production by the toxigenic *A. flavus* strain under normal and extreme CC conditions detailed in (a).

Chapter 8. Overall Discussion, Conclusions and Future Work.


Chapter 1: General Introduction, Literature Review and Objectives
Chapter 2: Isolation of potential BCAs from four Mexican maize cultivars
• Isolation and identification of potential BCAs
• Isolation and identification of A. flavus
• Toxigenic potential of isolates
Chapter 3. Screening of potential BCAs in vitro in interactions with toxigenic A. flavus strains for antagonism and control of aflatoxin B1 production
• Dual culture BCAs (8) vs A. flavus on MMA
• Mixed inoculum (50:50) on MMA
• BCAs (4) mixed inoculum different inoculum ratios on MMA
Chapter 4. Impact of the BCAs (4) on A. flavus sporulation on senescent maize leaves
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• Enumeration of colonies
• Differentiation between toxigenic and atoxigenic A. flavus
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• Mixed inoculum on irradiated maize kernels
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• Mixed inoculum 50:50 on maize cobs at different ripening stages (3)
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Chapter 7. Potential effects of Climate change scenarios of the BCA on aflatoxin B1 contamination on maize cobs of different ripening stages
• Mixed inoculum 50:50 on maize cobs
• Different ripening stages (3), temperature (2), CO2 (2)
• AFB1 production and relative gene expression (aflD and aflR)
Chapter 8: Discussion, Conclusions, and future work

Chapter 2. Isolation of potential BCAs from four Mexican maize cultivars

2.1 Introduction

Maize is one of the most important grains produced in 125 countries. It can grow at different altitudes, from sea level to above 3000 meters, from temperate to tropical regions, and is thus adapted to different environmental conditions (FAO, 2013). Maize can be contaminated by mycotoxigenic fungi during different stages, pre-harvest, during silking, harvest, drying and storage; maize is also more prone to contamination by mycotoxigenic fungi during drought stress episodes.

In Mexico maize is grown in rain-fed land and irrigated farms. Irrigated farms use commercial hybrids. In rain-fed areas, mainly cultivated by small scale farmers, native land seed or *criollo* varieties are used (Turrent Fernández *et al*., 2012). Many rural areas lack adequate infrastructure conditions for drying and storage of the grain, thus inadequate handling can lead to pest and fungal contamination, resulting in losses of up to 30% (Plasencia, 2004; Paterson and Lima, 2011).

Fungal colonization will affect grain quality, causing economic losses and representing a health risk due to mycotoxin contamination. Maize fungal contamination by, e.g., *Aspergillus, Fusarium,* and *Penicillium* species, is influenced by biotic and abiotic factors. The most important abiotic factors are water availability (a_w), temperature, pH and intergranular gas balance. These may act individually or interact with each other (Magan and Aldred, 2007a). Thus it is important to identify the predominant colonizing fungi of different maize cultivars and identify those which can contaminate the grain with mycotoxins (Samson *et al.*, 2010).

The objectives of this chapter were to:

a) Determine the mycobiota of four Mexican maize cultivars from different regions of Mexico, used for making tortillas.
b) Analyse the a_w to support growth and the moisture content of each cultivar and its mycobiota contamination levels.
c) Isolation of potential BCAs and fungal populations using selective media with different a_w levels.
d) Identify the mycotoxigenic fungal species present and confirm the ability of strains of the *Aspergillus* section *Flavi* to produce aflatoxins.

2.2 Materials and Methods

2.2.1 Mexican maize cultivars

The maize grain types examined in this study were Purple Mexican Maize and White Mexican Maize; these cultivars are used for white tortilla production. The Yellow Maize, San Dionisio Oaxaca was from San Dionisio Ocotlan, Oaxaca in south-western Mexico kindly supplied by Dr. Doralinda Guzman from the Research Centre CINVESTAV at Irapuato, Guanajuato, Mexico. The White Maize Asgrow 773 was from Los Mochis, Sinaloa in north-western Mexico. This was kindly supplied by Dr Roberto Parra, TEC de Monterrey at Monterrey, Nuevo Leon, Mexico. The maize samples were all stored at 4°C until they were examined.

2.2.2 Determination of water activity and moisture content

A sub-samples of each maize grain cultivar was placed in a water activity meter container and placed in the AQUALAB® Series 3TE (Decagon Devices Inc., Pullman, Washington, USA) to measure the a_w of the sample at 25°C.

A known weight of maize grain (10 g) was weighed in a glass beaker and recorded. The samples were dried in an oven at 105°C overnight. Afterwards the maize was cooled and stored in a desiccator with silica gel until a constant weight was obtained and weighed; the “dry weight” was recorded. The moisture content was then calculated.

$$\text{Moisture content (dry basis)} = \left(\frac{\text{wet weight-dry weight}}{\text{wet weight}}\right) \times 100$$

Equation 2.1 Formula to calculate the moisture content based on a wet weight basis

2.2.3 Fungal isolation and identification

a) Direct plating

To determine the mycobiota present on the maize grain samples, 5 grains were directly plated on Malt Extract Agar containing Chloramphenicol (MEA: 50 g/L CM0059, OXOID Ltd, Basingstoke, UK; chloramphenicol, BP904100, Fisher Scientific) and Dichloran 18% Glycerol Agar (DG18: 15.75 g CM0729, OXOID Ltd, Basingstoke, UK; glycerol 180 g, water 1000 mL), in 9 cm Petri plates and incubated at 25°C for 7 days. Three replicates (Petri plates; 9 cm diameter) per maize type were used. The fungal colonies on and around the maize grains were macroscopically examined using a dissecting stereomicroscope. The different genera were identified (Samson et al., 2010) into *Aspergillus* section *Flavi*, *Nigri, Circumdati, Fusarium* spp., *Eurotium* spp., *Penicillium* spp. and Others that included *Trichoderma* spp., *Rhizopus* spp., *Mucor* spp., *Alternaria* spp.. Afterwards, all fungi were sub-cultured on Malt Extract Agar with Chloramphenicol (MEA^+) and incubated at 25°C for 7 days. Subsequently, they were kept at 4°C until molecular identification and aflatoxin production analysis, from strains of the Section *Flavi* group.

b) Serial Dilution

For serial dilution, 10 g sub-samples of maize were ground with a pestle and a mortar and placed in a 25 mL Universal glass bottle. A 1 g sub-sample was mixed with 9 mL sterile water containing Tween®80 (278632500, ACROS Organics™) and the mixture soaked at 25°C for 2 hrs. The Universal bottle containing the mixture was shaken vigorously for 3 mins with a vortex mixer. Serial dilution was done with 1 mL being transferred to 9 mL sterile water, to obtain dilutions between 10^{-1} and 10^{-3}. From each dilution, an aliquot (0.2 mL) was removed with a sterile tip, decanted and spread-plated with a sterile glass spreader on each one of the three plates from each media, MEA^+, DG18 and ½ Nutrient Agar Media (½ NA: Nutrient broth 7.5 g CM0003, OXOID Ltd, Basingstoke, UK; Agar Technical No.3, 20 g LP0013 OXOID Ltd, Basingstoke, UK; cycloheximide 25 µg/mL, water 1000 mL). The Petri plates were incubated

for 5-7 days at 25°C and the populations counted in the range 5-50 colonies per plate, the fungal genera and fungal populations present enumerated. In all cases three replicates per treatment (dilution) were used.

2.2.4 Molecular analysis

a) *DNA extraction*

DNA extraction of the different isolates including *Aspergillus, Fusarium, Penicillium* and *Trichoderma* was carried out. For this, the fungal spores were taken with a loop from colonies cultured on MEA^{+} and inoculated on Yeast Extract Sucrose Medium (YES: yeast extract 20 g, sucrose 150 g, $MgSO_4.7H_2O$ 1 g, agar 20 g, water 1000 mL), and incubated at 25 or 30°C for 5 days. The mycelium was removed from the agar medium with a pre-frozen spatula and ground to fine powder in a pre-frozen mortar with a pestle. A 100 mg of sub-sample of the powder was transferred into a 2 mL Eppendorf tube and mixed with 500 µL of CTAB buffer lysis (5 g D-sorbitol, 2 g N-lauroylsarcosine, 1.6 g L^{-1} CTAB, 1.4M NaCl, 20 mM Na_2 EDTA, 2 g PVPP, 0.1 M Tris-HCl, pH 8.0; 200 mL) containing 5 µL of β-mercaptoethanol. The tube was shaken by hand for 15 s. Ten µL of proteinase K solution (10 mg/mL) were added to the tube and the contents vortexed for 30 s. The sample was then incubated at 65°C for 1 h. The sample was centrifuged at 13,000 rpm for 5 min at 4°C. The supernatant was transferred by pipetting into a new 2 mL Eppendorf tube to which 500 µL of chloroform (HPLC grade) were added mixed briefly by vortexing for 30 s. After centrifugation at 13,000 rpm for 20 min at 4°C, the upper layer was transferred to a new 2 mL Eppendorf tube. Next, 10 µL of RNase solution (10 mg/mL) were added and the sample was incubated at 37°C for 1 h. After adding 500 µL of chloroform, the tube was vortexed for 30 s and centrifuged at 13,000 rpm for 5 min at 4°C. The upper layer was transferred to a new Eppendorf and mixed with 500 µL of cold isopropanol. Later, the sample was centrifuged at 10,000 rpm for 2 min at 4°C and the supernatant removed. After adding 1 mL of 70% ethanol (HPLC grade), the sample was vortexed briefly and centrifuged at 13,000 rpm for 2 min at 4°C. The supernatant was discarded and the DNA resuspended in

100 µL Tris-EDTA (TE) buffer pH 8.0 and stored at -20°C until used as template for PCR amplification.

b) *PCR amplification*

PCR reactions for the ITS1-5.8S-ITS2 region were performed with two primer pairs ITS1-ITS2 and ITS3-ITS4 (Table 1.2; Bellemain *et al*., 2010). The PCR reaction mixture consisted of Buffer 10x (5 µL), Cl_2Mg (3 µL), dNTPS (1 µL), Taq polymerase (0.5 µL) and 2 µL of each primer (10 µM) in a total reaction volume of 50 µL. The amplification program was: 1 cycle at 94°C for 5 min, 40 cycles of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C, and finally 1 cycle of 5 min at 72°C. The PCR products were separated on a 2% agarose 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 v.7.09.02 image acquisition software (Syngene, Cambridge, UK). Amplification products were purified and sequenced at Macrogen (Netherlands). The sequences were analysed with Sequence Scanner (v. 1.0) and CLC Sequence Viewer (v. 6.6.1) software. Sequences were aligned and compared with other sequences previously deposited in the National Center for Biotechnology Information database (NCBI, http://www.ncbi.nlm.nih.gov). The identification of fungal species was based on the BLAST tool of the NCBI database. When assigning a species name to the isolate, only BLAST search results showing >99% similarity with the isolate's ITS sequence, was considered.

Table 2.1 Primers with their nucleotide sequences

Primers forward	**Primer sequence**	**Position**
ITS1	TCCGTAGGTGAACCTGCGG	1751-1779
ITS3	GCATCGATGAAGAACGCAGC	2024-2045
Primers reverse	**Primer sequence**	**Position**
ITS2	GCTGCGTTCTTCATCGATGC	2024-2043
ITS4	TCCTCCGCTTATTGATATGC	2390-2409

2.2.5 Screening for mycotoxin production

a) *Qualitative screening for fluorescent mycotoxin production on a Coconut Cream Agar Medium*

Coconut cream Agar medium (CAM; Blue Dragon Coconut Cream: Coconut Extract (78%), Water, Emulsifier: E471, Stabiliser: E466, 100 g, hot distilled water 100 mL, Agar Technical No.3, 20 g LP0013 OXOID Ltd, Basingstoke, UK; water, 900 mL) was used to determine mycotoxin production in a qualitative way based on fluorescence under UV light. Isolates belonging to the *Aspergillus* section *Flavi, Nigri* and *Circumdati* were inoculated on MEA^{+} media for 7 days. Spores from these cultures were taken with a needle and point inoculated in three equidistant points on the CAM. An *A. flavus* type strain (NRRL 3357) kindly provided by Prof. Deepak Bhatnagar from Agricultural Research Service (ARS), United States Department of Agriculture (USDA) was used as the positive control for aflatoxin production. The plates were incubated at 25°C and observed under UV light on day 2-5. A blue or greenish fluorescent ring around the colony was considered an indicator of aflatoxin or ochratoxin production.

b) *Quantitative analyses of* A. flavus *isolates for aflatoxin* B_1 *production*

Cultures of seven *A. flavus* isolates were also grown on the YES conducive medium for 10 days at 25°C. A sterile cork-borer (4 mm diameter) was used to

obtain colony samples across each colony (6-8 plugs). The plugs were placed in pre-weighed 2 mL Eppendorfs and weighed. The AOAC method (AOAC, 2000) was used to quantify aflatoxin production.

2.2.5.1 **Aflatoxin quantification using HPLC-FLD**

a) *Aflatoxin extraction*

A mL of chloroform (HPLC grade) was added to the plugs in the Eppendorf tubes which were shaken for 1 h at 180 rpm and then centrifuged at 10,000 rpm for 10 min. 800 µL of the chloroform extract was carefully placed in a new 2 mL Eppendorf tube with a pipette and dried overnight. The biomass was discarded.

b) *Aflatoxins standard preparation*

Aflatoxin Mix 1 (002021, Romer Labs UK Ltd, Cheshire, UK) dissolved in acetonitrile contains 2.0 µg/mL of AFB_1 & AFG_1 and 0.5 µg/mL of AFB_2 & AFG_2, respectively. An aliquot of 0.2 mL of Aflatoxin Mix containing 0.4 µg of AFB_1 was transferred to a 2 mL Eppendorf tube and evaporate to dryness overnight in the fume cupboard. Afterwards the aliquot was derivatised and diluted to obtain working standards at different concentrations (400, 200, 100, 50, 10, 1 and 0.5 ng AFB_1/mL) for a seven-point standard curve.

c) Derivatisation

200 µL of hexane (HPLC grade) was added to the Eppendorf tube, which was shaken by hand. 50 µL of Trifluoroacetic Acid (TFA) was added and the mix was vortexed for 30 s. The reaction was then left for 5 min. After, 950 µL of a water:acetonitrile mix (9:1, v/v) was added, vortexed for 30 s then left for 10 min for separation of the layers. The upper layer was discarded (300 µL) and the rest filtered through syringe nylon filter Nylon 13 mm, 0.22 µm (Jaytee Bioscience Ltd, UK) into an amber silanized 2 mL HPLC vials (Agilent Technologies, Inc, Santa Clara, CA, USA). The samples were kept at -20°C until injection.

d) *HPLC-FLD analysis*

The samples were injected in an Agilent 1200 series HPLC (Agilent, Berkshire, UK) (Agilent 2), for reverse-phase separation with Fluorescence Detector (FLD) (λ excitation 360 nm; λ emission 440 nm). The analytical column was a Phenomenex Luna (Phenomenex Luna C_{18} column ODS2 150 x 4.6 mm, 5 µm). The mobile phase was methanol:water:acetonitrile (30:60:10 v/v/v), using an isocratic pump with a flow of 1 mL/min. The injection volume was 20 µL and the run time 25 min. At the beginning of each run the standards were injected, then between each 6-10 samples and at the end of the run. The solvents used were HPLC grade. The standard curves were made by plotting in Excel (Microsoft® Excel®) the area obtained with the HPLC software (ChemStation for LC Systems Rev.B.04.02 SP1 (208), Agilent Technologies 2001-2010) against the concentration of AFB_1 standard injected. The standard curve was made to find the correlation using linear regression. Aflatoxin produced by each isolate was quantified in ng/g of agar. LOD and LOQ were calculated as LOD = 3.3σ/slope and LOQ=10σ/slope. σ=standard deviation of the sample and slope of the calibration curve (Medina and Magan, 2012).

2.2.6 Statistical Analysis

All treatments were randomized and replicated at least three times. The data was assessed for normality using Shapiro-Wilk test and Levene test for variance homogeneity. When the assumptions were achieved, the data was analysed with ANOVA and Tukey test for post–hoc analysis. The data was transformed with log and if still the assumptions were not met, non-parametric analysis was used, Kruskal-Wallis and then Mann-Whitney to check the difference between treatments. R version 3.1.2 (2014-10-31) -- "Pumpkin Helmet". Copyright (C) 2014 The R Foundation for Statistical Computing.

2.3 Results

2.3.1 Water activity and moisture content of Mexican maize cultivars

Table 2.2 shows the a_w and the moisture content of the four Mexican maize cultivars was analysed. White Mexican Maize had the lowest MC (7.7%) and Yellow San Dionisio Oaxaca Maize had the lowest a_w (0.434 a_w). All the cultivars represented very dry maize samples, which would not support fungal growth.

Table 2.2 Water activity and moisture content of the Mexican maize cultivars

Mexican maize cultivar	a_w	MC %
Purple Mexican Maize	0.569	8.3
White Mexican Maize	0.558	7.7
Yellow San Dionisio Oaxaca Maize	0.434	8.7
White Sinaloa Maize	0.561	11.0

2.3.2 Frequency of isolation and fungal populations isolated from Mexican maize cultivars

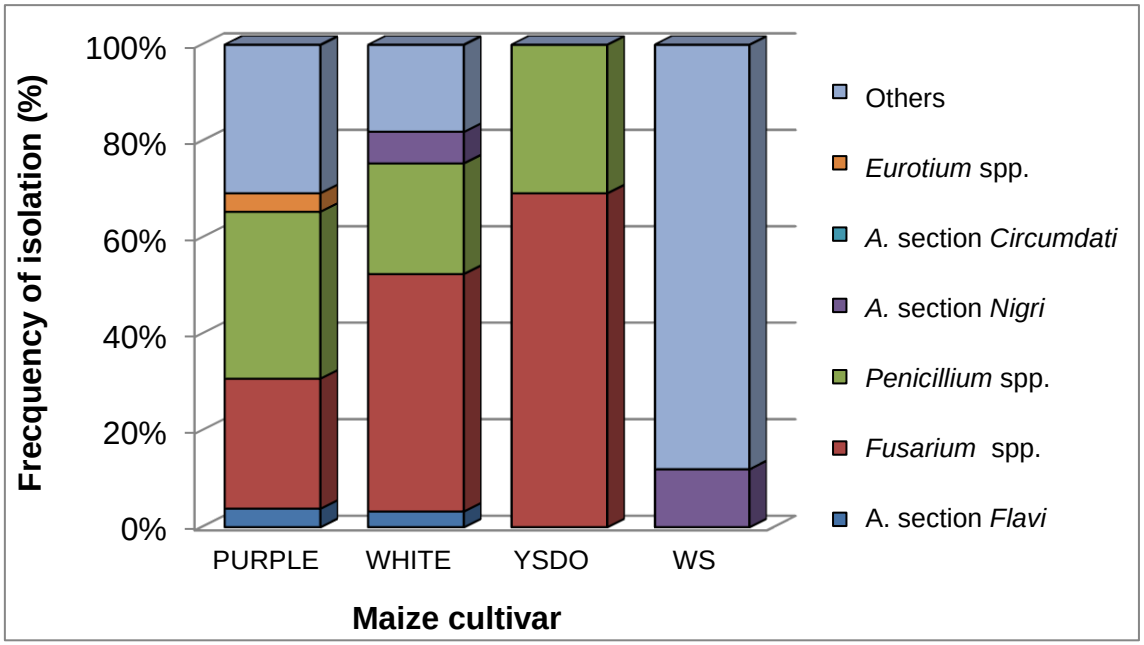
a) *Frequency of fungal isolation*

The mycobiota of the Mexican cultivars was analysed by direct plating on MEA^+ which has freely available water (0.995 a_w, Figure 2.1a) and DG18 with a lower available water (0.95 a_w, Figure 2.1b). On MEA^+ the predominant genera were *Fusarium* spp. *and Penicillium* spp*.* and other genera grouped in the "Others" category (*Trichoderma* spp.*, Epicoccum* spp. and *Rhizopus* spp.). *Penicillium* spp. represented 35% of the isolates from Purple maize from 26 total colonies. *Fusarium* spp. represented >50% on White (36 colonies) and Yellow SDO maize (13 colonies). Purple, White maize and White Sinaloa samples had a higher diversity than Yellow SDO with 5, 6 and 4 different genera, respectively. The Purple and White maize samples were contaminated with *Aspergillus*

section *Flavi*. The Yellow SDO cultivar was the least contaminated, with two genera, *Fusarium* (69%) and *Penicillium* spp. (31%).

On DG18, 12 different genera were isolated, the predominant colonies present were from *Aspergillus* section *Flavi,* other *Aspergillus* spp. *and Penicillium* spp.. Others were occasionally isolated like *Rhizopus* spp., *Mucor* spp., *Aspergillus clavatus, Alternaria* spp.. *Eurotium* spp. was isolated in 80% of Purple maize. On White maize, the genera predominantly isolated was *Penicillium* spp., with 34% frequency of isolation. On DG18 the frequency of isolation of fungi from Yellow SDO was higher than on MEA^{+} and *Eurotium* spp. represented 40% of the isolates. White maize and White Sinaloa cultivars had higher fungal diversity compared to the other cultivars. *Aspergillus* section *Flavi* was isolated from White maize in 14% and from White Sinaloa in 5%. *A.* section *Circumdati* was isolated from Yellow SDO and White Sinaloa maize. Figure 2.2 shows examples of the isolates growing from direct plated maize grains.

a) MEA^{+}



b) DG18

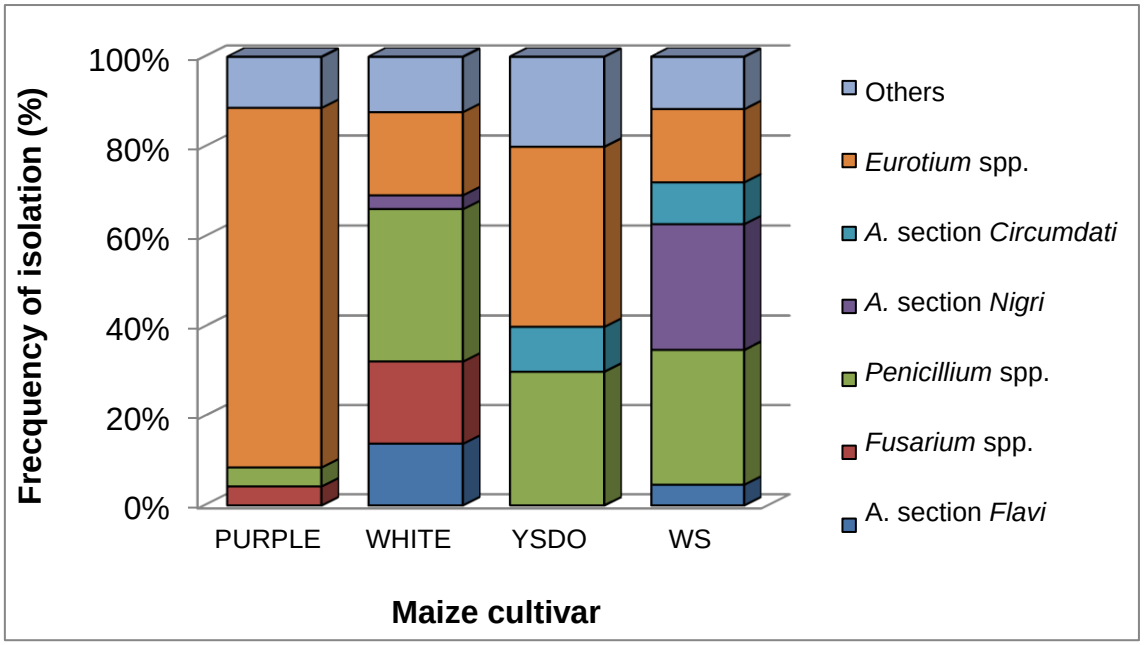


Figure 2.1 Frequency of total fungal isolation from four Mexican cultivars on MEA^{+} (a) and DG18 (b) after 7-day incubation at 25˚C. A.=*Aspergillus*, YSDO= Yellow San Dionisio Oaxaca, WS= White Sinaloa.

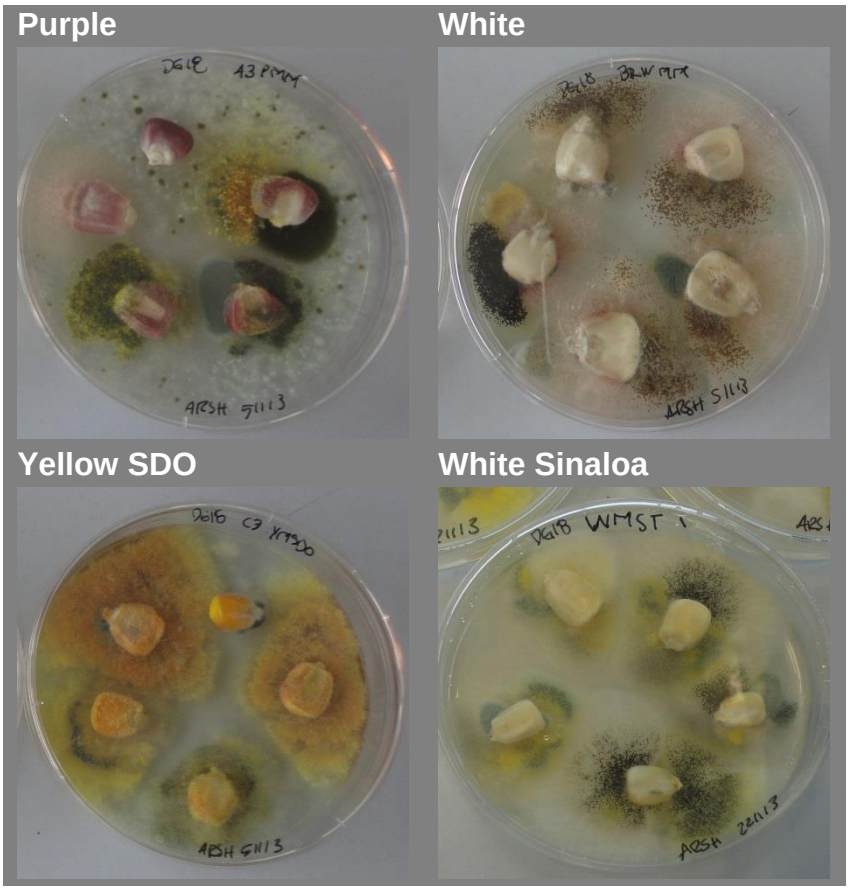


Figure 2.2 Mycobiota isolated from the four maize cultivars, on DG18 after seven days incubation at 25˚C.

b) <u>Fungal populations on maize cultivars</u>

The fungal and bacterial populations were isolated from the different maize cultivars using three different media (MEA^+, DG18 and ½ NA. Table 2.3 shows the mean total log CFU/g dry weight of maize per cultivar on these media. On both MEA^+ and DG18 the log populations on the White and White Sinaloa maize were significantly higher ($p<0.05$) than those isolated from the others (9-10 and 6-7 log CFU/g vs <1 log CFU/g respectively). On ½ NA, White Sinaloa and Purple cultivars appeared to be highly contaminated with bacteria. The other two cultivars (White and Yellow SDO) had none.

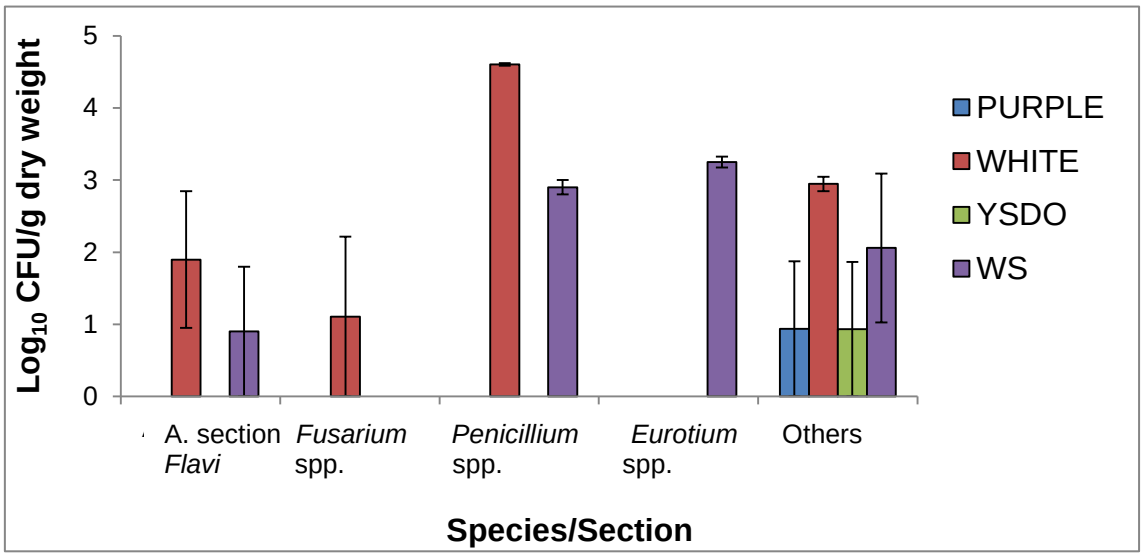
Table 2.3 Mean of total Log_{10} CFU/g of maize grain samples, on three different media, incubated at 25ºC.

Mexican maize cultivar	MEA$^+$	DG18	½ NA
Purple Mexican Maize	0.94	0.94	2.91*
White Mexican Maize	10.55*	7.13*	0.00*
Yellow San Dionisio Oaxaca Maize	0.93	0.93	0.00*
White Sinaloa Maize	9.11*	6.35*	4.70

The data are means of three replicates. Means followed by asterisks indicate significant difference between treatments ($p<0.05$).

Figure 2.3 shows the different fungal population diversity isolated from the four maize cultivars on (a) MEA$^+$ and (b) DG18 media at 25°C. On MEA$^+$ the main genera isolated were *Penicillium* spp. and a range of other species (*Mucor* spp., *Epiccocum* spp. and *Acremonium* spp.). White Sinaloa had *Eurotium* spp. *Penicillium* spp. in higher populations. Both White cultivars were contaminated with *Aspergillus* section *Flavi* and had significantly higher CFU/g maize than the Purple and Yellow SDO cultivars. On DG18, the isolation of fungal populations was lower than that on MEA$^+$ for all four cultivars. Almost all the whole populations consisted of *Penicillium* species and a range of other fungi imperfecti.

a) MEA$^+$



b) DG18

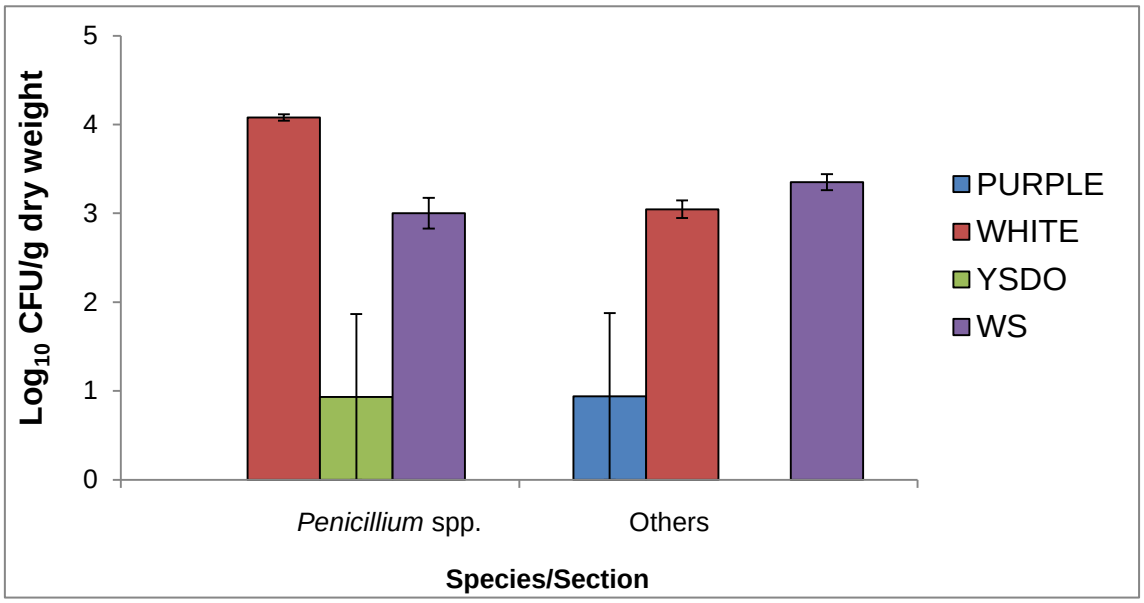


Figure 2.3 Mean fungal populations (log_{10} CFU/g of maize) isolated from the four maize cultivars on (a) MEA$^+$ and (b) DG18 at 25°C. YSDO= Yellow San Dionisio Oaxaca, WS= White Sinaloa.

2.3.3 Molecular Identification of the mycobiota isolates and relative aflatoxin production by *Aspergillus* section *Flavi* isolates from the Mexican maize samples.

After the isolation and morphological characterization, it was necessary to confirm the identity of the fungi isolated from the different maize cultivars with molecular techniques with the ITS region as detailed previously.

Table 2.4 shows the strains, which were characterized morphologically and molecularly identified as being in genus *Aspergillus.* Seven strains were confirmed as *A. flavus*, four as *A. niger*, and others as *A. westerdijkiae, A. terreus, A. candidus* and *A. wentii*. The latter was identified only morphologically.

The ability of the isolates to produce mycotoxin was screened qualitatively by fluorescence on CAM under UV light (Figure 2.4). *A. flavus* was checked for aflatoxin and *A. niger* for ochratoxin A production. This showed that six strains produced a blue fluorescence around the colony indicating potential positive result for aflatoxins production, but none of the *A. niger* isolates were positive for toxin production.

HPLC-FLD was subsequently used to quantify the amount of AFB_1 produced by each isolate of *A. flavus*. The seven strains were grown on conducive media (YES) for 10 days at 25°C, and the production of AFB_1 quantified. The production ranged from 0.45 to 1,573 ng/g. *A. flavus* strain named “O”, produced high amount of AFB_1, in subsequent experiments it was coded “MEX01”. One of the isolates, *Aspergillus* “X”, was negative on CAM but positive when grown on YES and tested with HPLC-FLD, although the amount of toxin produced was very low. This strain was classified as atoxigenic “Afl^- MEX02” in subsequent experiments.

Other strains isolated from the maize grain were also identified as a *Fusarium species, Talaromyces funiculosus* and *Trichoderma atroviride* (Table 2.5). The isolates chosen for the screening of potential biocontrol agents against toxigenic *A. flavus* strains (type strain NRRL 3357 and isolated “MEX01”) and AFB_1

inhibition are highlighted in bold and underlined letters (Table 2.4 and Table 2.5).

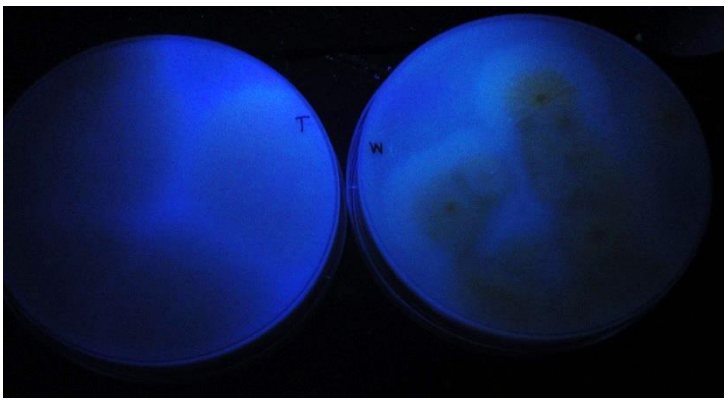


Figure 2.4 *Aspergillus* spp. strains screened qualitatively by fluorescence on CAM under UV light after 5-day incubation at 25°C. At the left strain "T" with blue fluorescence and to the right, strain "W" did not present fluorescence.

Table 2.4 Molecular and morphological identification of *Aspergillus* spp. and aflatoxin B_1 production on CAM and HPLC-FLD

Isolate name	Food matrix Maize	Molecular identification	Morphological characterization	Toxin production	
				CAM	HPLC (ng/g)[a]
Aspergillus L	White	*Aspergillus* sp.	*A. wentii*	-	-
Aspergillus M	Purple	*A. flavus*	*A. flavus*	+	197.48 ±42.85
Aspergillus N	Purple	*A. flavus*	*A. flavus*	+	0.55 ±0.33
Aspergillus O	**White**	***A. flavus***	***A. flavus***	**+**	**1573.00 ± 614.91**
Aspergillus X	**White Sinaloa**	***A. flavus***	***A. flavus***	**-**	**2.30 ±1.06**
Aspergillus Y	White Sinaloa	*A. flavus*	*A. flavus*	+	0.45 ±0.15
Aspergillus T	White	*A. flavus*	*A. flavus*	+	N/A[b]
Aspergillus U	White	*A. flavus*	*A. flavus*	+	N/A[b]
Aspergillus H	Purple	*A. niger*	*A. niger*	-	N/A
Aspergillus I	White	*A. niger*	*A. niger*	-	N/A
Aspergillus J	Purple	*A. niger*	*A. niger*	-	N/A
Aspergillus V	White	*A.niger*	*A. niger*	-	N/A
Aspergillus W	Yellow SDO	*A. ochraceus*	*A. westerdijkiae*	-	N/A
Aspergillus #30	White Sinaloa	*A. terreus*	*A. terreus*	N/A	N/A
Aspergillus #32	White Sinaloa	*A. candidus*	*A. candidus*	N/A	N/A

[a] Mean of three replicates ± standard deviation
[b] AFB_1 production was not analysed

Table 2.5 Molecular and morphological identification of isolates

Isolate name	Food matrix Maize	Molecular identification	Morphological characterization
Fusarium K	White	*Fusarium* sp.	*Fusarium* sp.
***Penicillium* #56**	**White Sinaloa**	***T. funiculosus*[a]**	***Penicillium* sp.**
***Penicillium* #57**	**White Sinaloa**	***T. funiculosus*[a]**	***Penicillium* sp.**
***Trichoderma* GW#43**	**White**	***T. atroviride***	***Trichoderma* sp.**

[a] Kindly identified by Prof. Jens Frisvad.

2.4 Discussion

This study showed that all four Mexican cultivars had a_w levels and moisture contents lower than the 0.70 a_w and 14% MC, which are recommended for safe storage. Even though the grains were contaminated, no fungal development would have taken place during storage. The diverse contamination was probably due to pre-harvest colonization during silking and through post-harvest handling and drying (Magan and Aldred, 2007a). In Mexico the maize cobs are usually harvested with 25 to 30% moisture content, and then sun-dried until reaching a moisture content of 12-14% (Plasencia, 2004). The storage facility depends on whether the maize is grown by rural farmers or by larger agronomic producers. The small-scale farmer sometimes uses *ad hoc* storage facilities of poor quality while larger industries use silos with the modern controls for ventilation and temperature control. For rural farmers the risks from aflatoxin contamination are significantly higher because of slower and uneven drying of the maize cobs/grain (Mohale *et al*., 2013c; Ordoñez Morales, 2015; Pitt *et al*., 2013).

Based on the frequency of isolation it was determined that the predominant fungi isolated on MEA^+ were *Fusarium* spp. in a range of 27 to 69%, *Penicillium* spp. from 23 to 35% and diverse genera grouped in the "Others" category. *Aspergillus* section *Flavi* was present in Purple and White maize samples but in a low frequency of isolation. The predominant fungi isolated on DG18 were *Penicillium* spp. with 4 to 34% and *Eurotium* spp. from 16 to 80% on the four cultivars; *Aspergillus* section *Flavi* was present in White and White Sinaloa in 14 and 5% respectively.

There seemed to be a contrast between the White and Purple/Yellow maize cultivars examined. Both White maize cultivars had very high populations of fungi (log 9-10 CFU/g, $p<0.05$) on MEA^+ while the others had < log 1 CFU/g. The same pattern was observed on DG18. *Penicillia* appeared to predominate. On the White maize there were log 2 CFU/g of *Aspergillus* section *Flavi* isolates.

Both white maize cultivars, White and White Sinaloa, were the most contaminated, the latter was obtained from north-western Mexico. The Yellow SDO maize used in this experiment was a native land-race or criollo from south-western part of Mexico; although the importation of yellow maize has increased in Mexico with a higher risk of aflatoxin contamination (UNCTAD, 2012).

Montes *et al*. (2009) screened the mycobiota of 12 yellow and 10 white maize hybrids in Tamaulipas, northeast Mexico using PDA. They found out that the yellow hybrids were more contaminated than the white ones. The grains were collected after harvested and used for morphological identification by surface sterilization and direct plating on potato dextrose agar. They found that the predominant fungal genera were *Fusarium* spp. 32%, *Aspergillus* spp. 20% and *Penicillium* spp. 14%; *A. flavus* was present in all the hybrids except two white genotypes.

Arrúa Alvarenga *et al*. (2012) analyzed different types of maize (white, yellow and coloured) from 14 states across Mexico; the samples were collected from silos, fields and crop residues and the fungi isolated on malt salt agar and potato dextrose agar. The results were reported by State, not by maize type. They only reported mycotoxigenic genera (*Fusarium, Penicillium* and *Aspergillus*). The predominant fungi were *Fusarium* spp. present in 99% of the maize grain samples from Quintana Roo; *A. flavus* in 62.5% of the grain from Hidalgo and *Penicillium* spp. in 61% isolated from Chiapas. Unfortunately the total fungal populations were not assessed.

Ordoñez Morales (2015) studied mycotoxin contamination of maize grain during postharvest by freezer blotter test. Thirty-one samples were collected from different types of silos and experimental plots in Mexico and Puebla (southcentral states). In the State of Mexico, the predominant genera were *Aspergillus* spp., *F. verticillioides* and *F. graminearum*; in Puebla, *F. verticillioides*. Toxin production was tested using ELISA, with fumonisin and deoxynivalenol levels below the 2-4 ppm recommended by the FDA; although *Aspergillus* spp. were isolated, AFB_1 was not detected.

In the present study 7 out of 8 isolates from *Aspergillus* spp. were identified as *A. flavus* with morphological and molecular techniques. *A. parasiticus* was absent from these maize grains. Aflatoxin production was tested on CAM and HPLC-FLD. One strain was negative on CAM but positive when analyzed by HPLC-FLD, producing small quantities of aflatoxin; this strain was examined for potential antagonism against the toxigenic strains. The remaining *A. flavus* isolates were all positive for aflatoxin production with both methods.

Other researchers have worked in the north of Mexico looking for specific isolates of *Aspergillus* section *Flavi* from soil in Sonora (Ortega-Beltran *et al.*, 2015) or analyzing the diversity of *Fusarium* species isolated from maize grains in Sinaloa (Leyva-Madrigal *et al*., 2015). Recently, Samsudin (2015) compared maize samples from 3 different countries, France, Malaysia and Mexico. The French and Mexican maize had < 0.6 a_w and the Malaysian maize was very wet with an a_w of 0.97. On unsterilized grains, the predominant genus in the French maize was *A. terreus*, on the Malaysian and Mexican maize this was *F. verticillioides*. *A. flavus* was isolated from both the French and the Mexican maize.

Ortega-Beltran *et al.* (2015) analyzed soil from four agro-ecological zones with different elevations (30–1700 metres). They isolated *A. flavus* S and L strains, *A. parasiticus* and *A. tamarii*. 95% of the isolates tested for aflatoxin production were positive. Mohale *et al*. (2013c) isolated mycotoxigenic fungi from stored maize of five agro-ecological regions in Lesotho, with elevations ranging between 1,400 to 3,500 meters. They analysed the presence of toxigenic fungi. *F. verticillioides, F. proliferatum* and *F. subglutinans* were the predominant fungi, contaminating 50% of 42 the samples. They found that 7 isolates out of 14 of *Aspergillus* section *Flavi* were *A. parasiticus*. All the isolates (7) were positive for aflatoxin production on CAM and HPLC-FLD.

2.5 Conclusions

The four Mexican grain samples had very low moisture contents for safe storage and avoidance of any post-harvest fungal spoilage, although they had a high diversity. The fungal populations were high on the White maize cultivars when compared to the Purple and Yellow SDO maize examined. The maize had similar genera to the data obtained previously on Mexican maize. *Fusarium*, *Aspergillus*, and *Penicillium* spp. colonized the four Mexican maize cultivars. These mycotoxigenic fungi represent a risk for food security due to potential mycotoxin production, under conducive environmental conditions. Seven *A. flavus* isolates were positive for aflatoxin production. Two isolates were subsequently chosen for more detailed biocontrol screening assays. The strain with higher AFB_1 production, was coded “MEX01” and the strain that was negative on CAM and a very low AFB_1 producer, was coded “Afl^- MEX02 “. From the isolates obtained from the maize grains, three other strains were select to be tested as potential biocontrol agents against toxigenic *A. flavus* “MEX01”, *T. atroviride* “MEX03” and *T. funiculosus* (MEX04 and MEX05).

Chapter 3. Screening of potential biocontrol agents *in vitro* in interactions with toxigenic *Aspergillus flavus* strains for antagonism and control of aflatoxin B_1 production

3.1 Introduction

Aspergillus flavus causes economic losses and health risks to consumers and in the animal feed chain due to aflatoxin B_1 (AFB_1) production, considered by the International Agency of Research on Cancer as the most potent carcinogenic natural compound. Because of this risk, there are different strategies to prevent and control *A. flavus* invasion and aflatoxin contamination pre- and post-harvest. One strategy to control *A. flavus* contamination is the use of natural antagonistic biocontrol agents (BCAs). A number of studies have demonstrated the potential of atoxigenic strains of *A. flavus*, other filamentous fungi, yeasts or bacteria to control toxigenic *A. flavus* strains (Mohale *et al*., 2013c; Verheecke *et al.*, 2014; Al-Saad *et al*., 2016) The interactions among the fungi that colonise the ripening cobs of maize are dynamic and influenced by environmental conditions. The temperature will rise, causing drought and changes on fungal diseases and toxin production. It is necessary to understand the ecophysiology of *A. flavus* and the interaction with potential biocontrol candidate under different environmental conditions (Medina *et al*., 2014, 2015).

Thus, a total of eight potential BCAs from Mexican maize and other sources were screened for the ability to effectively antagonise two toxigenic strains, *A. flavus* wild-type (NRRL 3357) and a toxigenic Mexican isolate (MEX01). This was combined with efficacy assays to examine relative inhibition of AFB_1 production by the toxigenic strains under different environmental factors on a maize-based medium.

The objectives of this Chapter were to:

a) Analyse the effect of the potential BCAs (n=8) on *A. flavus* (n=2) growth during co-inoculation on a 3% milled maize agar (MMA) under different water activity (a_w) levels.
b) Quantify the relative antagonism and dominance of the BCA candidates against the two toxigenic *A. flavus* strains (NRRL 3357; MEX01) using the Index of Dominance (I_D) under different a_w and temperature conditions.
c) Test the effect of 50:50 mixed inoculum of the best candidate BCAs (n=4) with toxigenic strains of *A. flavus* on AFB_1 production on 3% MMA at different a_w and temperature conditions.
d) Examine the effect of different inoculum ratios of the BCA vs *A. flavus* strain respectively (100:0; 75:25, 50:50, 25:75 and 0:100 spore ratios) on temporal AFB_1 production on the MMA at different a_w levels and 30°C.

3.2 Materials and Methods

3.2.1 Fungal and bacterial strains

Two *A. flavus* toxigenic strains were used in this experiment, a type strain from the USDA (NRRL 3357; Mohale *et al.*, 2013b; Medina *et al.*, 2015) and an isolate from white Mexican maize grain designated with the code "MEX01". Eight candidate BCAs were used, four fungi and three bacteria. Three potential fungal BCAs were isolated from Mexican maize; an *A. flavus* strain (Afl^- MEX02), a low AFB_1 producer; *Talaromyces funiculosus* (MEX04 and MEX05; from White maize Sinaloa Asgrow 773) and *Trichoderma atroviride* (MEX03*;* from White maize). *Clonostachys rosea* 016 strain was the other fungal BCA, previously used for biocontrol of *Fusarium* (Palazzini *et al.*, 2013; Samsudin and Magan 2016). It was kindly supplied by Dr Jurgen Kohl, PRI, Wageningen, NL. The bacterial BCAs were a *Streptomyces* sp. AS1, used previously as a biocontrol agent for toxigenic *A. flavus* (Sultan and Magan, 2011) and *Enterobacter hormaechei* used against *F. verticillioides* (Pereira *et al.*, 2010; 2011). These strains were kindly supplied by Dr. Yousef Sultan from Egypt and by Prof. Miriam Etcheverry, Univesity of Río Cuarto, Argentina, respectively. The third bacterial BCA was isolated from Mexican maize, designated as BY #84.

3.2.2 Media and inoculation preparation

All strains were stored at 4°C, sub-cultured on MEA^+ when needed and incubated for 7 days at 25°C. For fungi, the spores were harvested from colonies by pouring 9 mL of sterile water containing 0.025% Tween® 80. The surface of the colony was agitated with a sterile glass rod and decanted into a 25 mL Universal bottle. The spore concentration was checked with a haemocytometer (Helber chamber depth 0.02 mm; © Marienfeld-Superior, Germany) and a compound microscope (Olympus BH-2). This was diluted as required to obtain the target concentration of $1x10^6$ spores/mL. The spore suspensions were kept at 4°C until their use. Bacteria were grown on ½ NA

media for 4 days, the cells removed as described previously, counted and stored at 4°C until use.

The MMA was prepared using Mexican nixtamalized white maize flour to have similar levels of nutrients as the white maize consumed in Mexico. The MMA was prepared using 30 g of MASECA® flour and 20 g of Agar Technical No.3 (LP0013 OXOID Ltd, Basingstoke, UK), in 1000 mL of water. The modified a_w media were modified with glycerol/water solutions to achieve the required a_w levels of 0.98, 0.95 and 0.93 (Dallyn and Fox, 1980).

3.2.3 Dual-culture assays of *A. flavus* and potential biocontrol candidates on milled maize agar

The fungal spore suspension for pathogen and antagonist, was made as described before, from a seven-day old culture on MEA$^+$ incubated at 25°C, counted with a haemocytometer (Helber chamber) and a compound microscope (Olympus BH-2). The spores were taken with a sterile loop and point inoculated on the media at 5, 2.5 and 2 cm for 0.98, 0.95 and 0.93 a_w, respectively. For bacterial candidates streak inoculation was used (Figure 3.1). Three replicates per pathogen:antagonist of the same a_w were inoculated, kept in closed polyethylene bags and incubated at 25°C for 9-15 days. Controls consisted of the individual toxigenic strains (*A. flavus*) grown singly on the same media treatments. Growth rates were obtained by measuring the colonies in two directions at right angles to each other (diameter) after 1, 3, 5, 7 and 9 days for 0.98 a_w and until 15 days for 0.95 and 0.93 a_w. The measurements were averaged and expressed as colony diameter (mm). The macroscopic interactions between colonies of the BCAs and toxigenic *A. flavus* strains were done by scoring the fungi individually depending on the outcome of the competition on the agar surface. Thus mutually intermingling strains were given scores of 1:1, mutual antagonism on contact 2:2, mutual antagonism at a distance scores of 3:3, dominance of one strain over another was given the scores of 4:0, and dominance at a distance resulted in scores of 5:0, respectively (Magan and Lacey, 1984; Magan *et al.*, 2010). The relative scores of the three replicates under each different condition were added together to

obtain an overall Index of Dominance (I_D) for each species on MMA at 25°C and three a_w levels (0.98, 0.95 and 0.93).

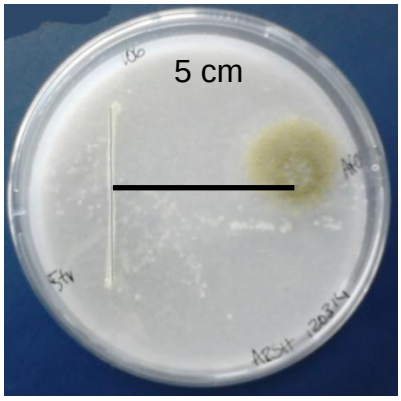


Figure 3.1 Illustration of point inoculation of *A. flavus* toxigenic strain and streak inoculation of *Streptomyces* sp. AS1 at 0.98 a_w.

3.2.4 Co-cultivation of *A. flavus* and potential biocontrol agents on milled maize agar

The interaction between the toxigenic *A. flavus* strains and the biocontrol candidates on aflatoxin production was analysed using a mixed inoculum under different environmental conditions.

a) Mixed inoculum 50:50 ratio

The conditions for the mixed inoculum 50:50 ratio ($1x10^6$ spores/mL total mix) were carried out at two a_w levels (0.98 and 0.95 a_w) and two temperatures (25 and 30°C) on MMA. The spore suspension was made up as described before in Section 3.2.2. The spore/cell inocula were mixed in a 25 ml Universal bottle (50%:50%), stored overnight at 4°C. After equilibration at the target experimental temperature 0.2 mL of the mixed inoculum were taken and spread plated with a sterile glass spreader over the agar surface. Treatments and three replicates of the same a_w were kept in closed polyethylene bags and incubated at 25 or 30°C for 10 days. Controls consisted of the individual strains grown singly on the same media.

b) Mixed inocula of antagonist:pathogen at different inoculum ratios

The fungal candidates that were antagonistic to *A. flavus* and affected AFB_1 production at 50:50 ($5x10^5$:$5x10^5$ spores/mL) mixed inoculum were selected to analyse the effect of different inoculum ratios in more detail. Toxigenic *A. flavus* (NRRL 3357; MEX01) were co-inoculated with the biocontrol candidates at different inoculum ratios on MMA (100:0; 75:25; 50:50; 25:75; 0:100; Samsudin and Magan, 2016). The media was modified with glycerol to achieve the required a_w levels of 0.98 and 0.93 as detailed in Section 3.2.2. The spores/cell suspensions were prepared and counted to obtain concentrations of $1x10^6$ spores/mL. Afterwards, the spore suspensions were vortexed and mixed in 25 mL Universal bottles in different proportions (Table 3.1). The mixed inoculum (0.2 mL) was spread with a sterile glass spreader over the media surface. The Petri plates were stored in plastic environmental chambers containing 500 mL beakers of glycerol/water mixture to maintain the ERH of the atmosphere at the target a_w level of the media treatments. Four replicates per treatment were used in these assays. The experiment was incubated at 30°C for 10 and 20 days. After these periods the AFB_1 was quantified and biomass analysed with real-time quantitative PCR (qPCR) for specific genes of the aflatoxin biosynthetic cluster. The fungal biomass was removed from the media with a pre-frozen sterile spatula, put in aluminium foil, frozen in liquid N_2 and stored at -80°C until RNA extraction was performed. The qPCR method for the analysis of *aflD* and *aflR* relative gene expression is described later.

Table 3.1 Summary of the different treatments and ratios of pathogen vs antagonist used in these experiments

Treatment	*A. flavus* toxigenic (NRRL or MEX01)	BCAs
Positive Control	100	0
1	75	25
2	50	50
3	25	75
Negative control	0	100

3.2.5 Relative gene expression using qPCR

RNA extraction: the 10 day treatments were used in duplicate, and the positive controls and the 50:50 mixtures included. The negative control of Afl^- MEX02 was also extracted. Total RNA was extracted according to the Spectrum™ Plant Total RNA Kit protocol (Sigma-Aldrich, MO, USA) as described by the manufacturer and it was subsequently used for cDNA synthesis.

A mycelial sample of 100 mg was placed in a sterile microcentrifuge tube with glass beads. It was re-suspended in 750 µL of lysis solution previously supplemented with β-mercaptoethanol 10 µL/mL.

After vortexing the sample (30s), the tubes were frozen in liquid N_2. They were then incubated at 56°C for 3 mins, and centrifuged at maximum speed for a further 3 mins. The supernatant was transferred, avoiding the pellet, into the Filtration Column (blue) inside a 2 mL Collection Tube. Subsequently it was centrifuged at maximum speed for 1 min and the Binding solution (750 µL) added to the lysate, and mixed by pipetting 5 times. The mixture was pipetted (700 µL) into the Binding column (red) and centrifuge at maximum speed for 1 min; the flow-through liquid was discarded. The rest of the mix was added to the Binding column, centrifuged and the flow-through discarded. To wash the column after the RNA was bound to it, 500 µL Wash Solution 1 was added to the column and it was centrifuge at max speed for 1 min. The flow-through liquid was discarded between each wash. 500 µL of Wash Solution 2 was added, the column centrifuged at maximum speed for 30 s, with the flow-through liquid discarded and repeated. The column was dried by centrifugation at a maximum speed for 1 min, and then the collection tube discarded. The column was transferred to a new-labelled collection tube. The elution solution was pipetted (50 µL) into the centre of the column and centrifuged at maximum speed for 1 minute. The RNA was stored at -80°C until cDNA synthesis. The RNA concentration was checked with Picodrop® µL spectrophotometer (PICOPET01, Picodrop Ltd., Hinxton, UK) ratio A260/A280, and the integrity with an agarose gel (1%).

cDNA synthesis: the reverse transcription protocol was done with Oligo-dT primers of Omniscript® RT kit (Qiagen, Hilden, Germany). The RNA template and the reagents were thawed on ice. The RNA was diluted 1:10 with RNase-free water. Each component was vortex and centrifuge (4°C). The PCR reaction mixture consisted of 10x buffer RT (2 µL), dNTP Mix (5 mM each dNTP, 2 µL), Oligo-dT primers (2 µL), Omniscript Reverse Transcriptase (1 µL), RNAse free water (8 µL) and RNA template (5 µL) in a total reaction volume of 20 µL in Individual PCR tubes with flat caps (Bio-Rad Laboratories Ltd., Hertfordshire, UK) The amplification program used was: 1 cycle at 94°C for 5 min, 40 cycles of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C, and finally 1 cycle of 5 min at 72°C using the thermal cycler Techne™ TC-512 (Techne Inc., NJ, USA).

qPCR: the expression of two genes of the aflatoxin biosynthetic pathway was analysed, the structural gene *aflD* and the regulatory gene *aflR.* The *β-tubulin* gene was used as the reference gene. The primers and the probes were the same as those used previously in other studies and are shown in Table 3.2 (Abdel-Hadi *et al*., 2010; Abdel-Hadi *et al.*, 2012; Medina *et al.*, 2015). The primer pairs norTaq1, norTaq2 and the probe norProbe were designed from the *aflD* gene. AflRTaq1, aflRTaq2 and the probe aflRProbe were designed from the *aflR* gene. The probes of the aflatoxin genes were labelled with the reporter molecule 6-carboxyfluorescein (FAM) at the 5' end and the BlackHole Quencher 2 (BHQ2) at the 3' end. The pair benTaq1, benTaq2 and benProbe were based on the *β-tubulin* gene. The bepProbe was labelled with the reporter cyanine-5 (CY5) at 5' end and the BlackHole Quencher 2 (BHQ2) at the 3' end.

Table 3.2 Nucleotide sequences of primers and probes for qPCR design from *aflD, aflR* and *β-tubulin* genes

Primer pairs	Gene	Nucleotide sequences (5´-3´)	Position
norTaq1	*aflD*	GTCCAAGCAACAGGCCAAGT	516[a]
norTaq2		TCGTGCATGTTGGTGATGGT	562[a]
norProbe		[FAM]TGTCTTGATCGCGCCCG[BHQ2]	537[a]
aflRTaq1	*aflR*	TCGTCCTTATCGTTCTCAAGG	1,646[b]
aflRTaq2		ACTGTTGCTACAGCTGCCACT	1,735[b]
aflRProbe		[FAM]AGCAGGCACCCAGTGTACCTCAAC[BHQ2]	1,689[b]
benTaq1	*β-*	CTTGTTGACCAGGTTGTCGAT	65[c]
benTaq2	*tubulin*	GTCGCAGCCCTCAGCCT	99[c]
benProbe		[CY5]CGATGTTGTCCGTCGCGAGGCT[BHQ2]	82[c]

[a] Positions are in accordance with the published sequence of the *aflD* gene of *A. flavus* (GeneBank accession no. XM_002379908.1).

[b] Positions are in accordance with the published sequences of *aflR* gene of *A. flavus* (GeneBank accession no. AF441435.2).

[c] Positions are in accordance with the published sequences of *β-tubulin* (benA56) gene of *A. flavus* (GeneBank accession no. AF036803.1). (Medina *et al.*, 2015).

The qPCR was done with the Thermal Cycler Bio-Rad CFX96 Real Time System C1000 (Bio-Rad Laboratories Ltd., Hertfordshire, UK). The reaction mixture was prepared in an Eppendorf and afterwards distributed in individual PCR tubes (Bio-Rad Laboratories Ltd., Hertfordshire, UK). The mix contained 6.25 µL Premix Ex Taq™ (Takara Bio, Inc., Otsu, Japan), 0.25 µL of each primer (µL at 41.6 µM), 0.25 µL of each probe (µL at 16.6 µM), ROX 1%, 1.5 µL of cDNA template (diluted 1:10) for a final volume of 12.5 µL. They were prepared in duplicate for each sample per gene and two non-template controls (NTC) were added in each run. The qPCR conditions were: 40 cycles at 95°C for 10 min, 95°C for 15 s, 55°C for 20 s and 72°C for 30 s.

The data analysis was made with the software Bio-Rad CFX Manager, Version 3.1 (Bio-Rad Laboratories Ltd., Hertfordshire, UK). Relative quantification of the

expression of *aflD* and *aflR* genes was performed with the reference gene *β-tubulin* as an endogenous control to normalise the quantification of the mRNA target for differences in the amount of total cDNA added to each reaction in the relative quantification assays and used for all treatments. The expression ratio was calculated as previously described by Livak and Schmittgen (2001). Before using the above method, it was tested to show that experimental treatments did not influence the expression of the internal control gene, and the amplification efficiencies of the target and reference genes were practically equal (87.5% for *aflD* and 92.1% for *β-tubulin* and 93.1% for *aflR* and 95.2% for *β-tubulin* genes, respectively). This method allows calculation of the expression ratio of a target gene between a tested sample and its relative calibrator ("control" sample). In this study, to analyse the effect of the BCAs on target gene expression the calibrator corresponded to *A. flavus* NRRL 3357 and *A. flavus* MEX01 grown on MMA at different a_w levels.

3.2.6 Aflatoxin extraction, analysis with HPLC-FLD

The aflatoxin extraction from culture media was made according to AOAC method (AOAC, 2000), with a sterile cork borer 8 plugs of 4 mm diameter were taken across the ten-day old colony, placed in pre-weighed 2 mL Eppendorf safe-lock tubes and weighed. The samples were extracted, derivatised and analysed with HPLC-FLD as described before in Chapter 2, Subsection 2.2.5.1.

3.2.7 Statistical Analysis

All treatments were randomized and replicated at least three times. *A. flavus* toxigenic strains (MEX01 or NRRL 3357) growth and AFB_1 production were analysed for all the BCAs, 3 plates per co-inoculation at one a_w (0.93, 0.95 or 0.98) and one temperature (25 or 30˚C). AFB_1 production data was transformed using ($\log_{10}$+1) before statistical analysis. Shapiro-Wilk test and Levene's test were used to assess normality and variance homogeneity, respectively. When the assumptions were met, the data was analysed with one-way ANOVA. To compare the significant differences between treatments Tukey's Honest Significant Difference (HSD) was used. Otherwise non-parametric tests were

applied, Kruskal-Wallis to analyse if there was difference between treatments and Mann Whitney to compare each treatment with the control. R version 3.1.2 (2014-10-31) -- "Pumpkin Helmet". Copyright (C) 2014 The R Foundation for Statistical Computing.

3.3 Results

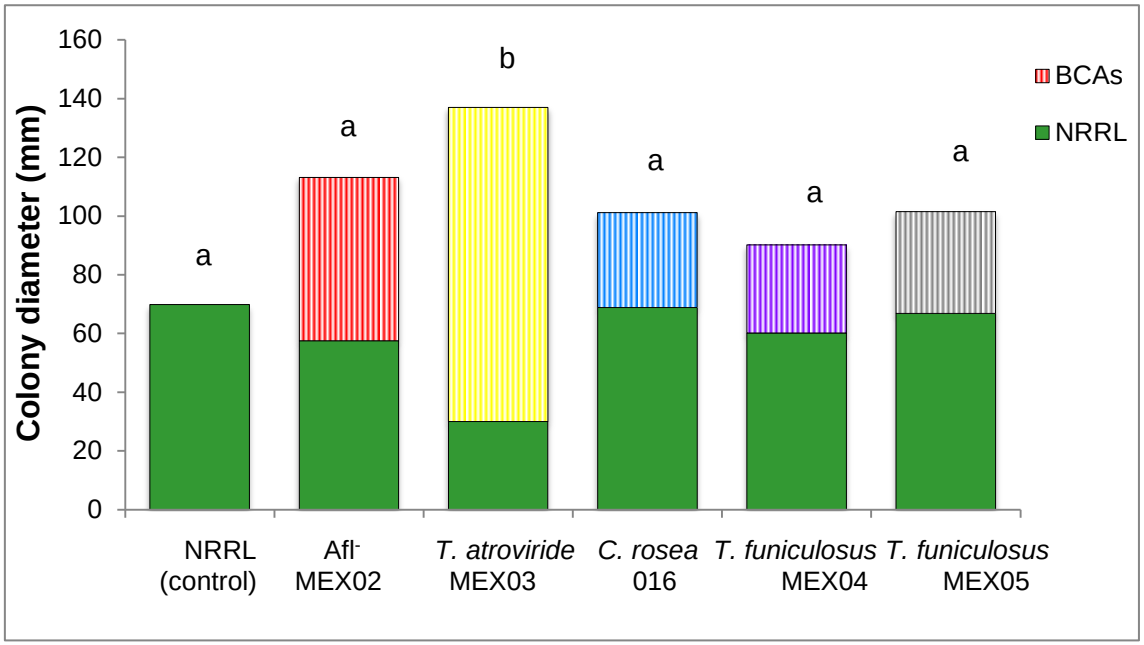
3.3.1 Interaction between *A. flavus* and potential BCAs on dual-culture

The effect of the BCAs on *A. flavus* growth during co-culture was by measuring the relative growth for nine days. Figure 3.2a compares the colony diameter of the toxigenic *A. flavus* (NRRL 3357; MEX01 strains; Figure 3.2b) at 0.98 a_w. The growth of the *A. flavus* NRRL 3357 strain was significantly affected during interaction with *T. atroviride* MEX03, compared to the other BCAs. Notably, the results obtained with toxigenic MEX01 isolate were similar; the growth was significantly reduced by *T. atroviride* MEX03 only.

At increased water stress levels (0.95 and 0.93 a_w) the effects on interacting co-cultures were measured after 15 days. Figure 3.3 shows the effect of such interactions on the growth of both toxigenic strains. At 0.95 a_w, NRRL 3357 growth was unaffected by interaction with the candidate BCAs. Meanwhile within treatments (BCAs, same a_w and temperature), *T. atroviride* MEX03 and *T. funiculosus* MEX05 significantly ($p<0.05$) increased the growth of the toxigenic NRRL 3357 strain compared to the atoxigenic Afl^- MEX02 strain. At the highest water stress level tested (0.93 a_w) there was little effect on the growth of the toxigenic strain by any of the candidate BCAs.

The growth of the toxigenic MEX01 isolate was affected by the interaction with *C. rosea* 016 at 0.95 a_w (Figure 3.2b). However, the other BCAs did not have a significant effect on the growth of the toxigenic strain when compared to the control. Again at 0.93 a_w the growth of the toxigenic strain was unaffected by the potential BCAs.

a) Toxigenic strain NRRL 3357



b) Toxigenic strain MEX01

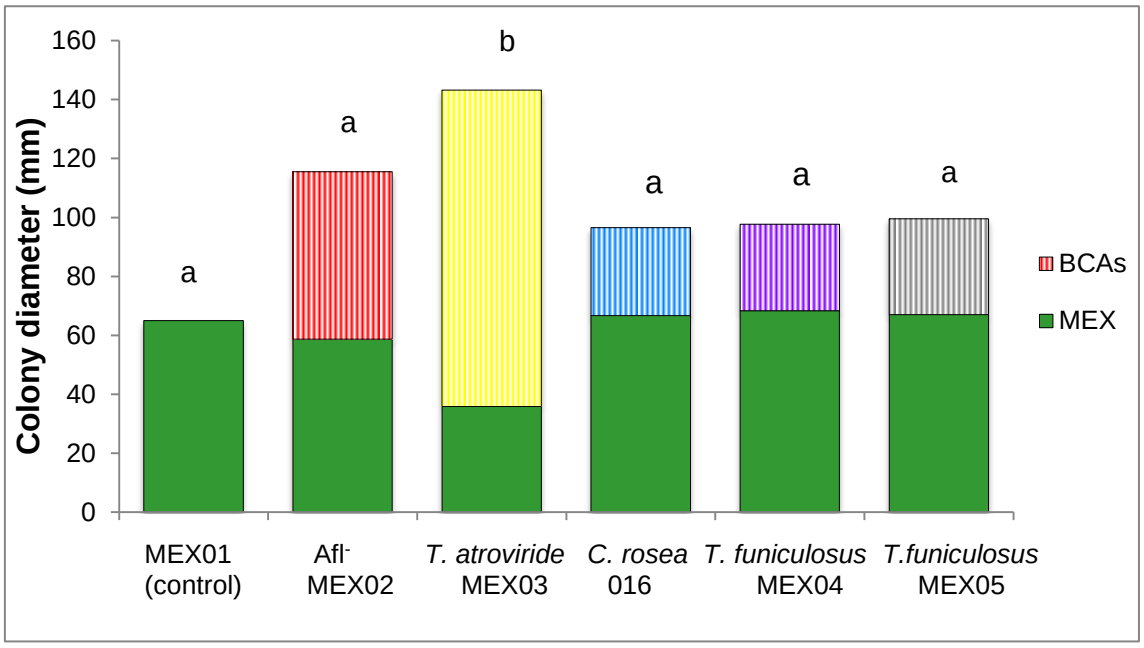
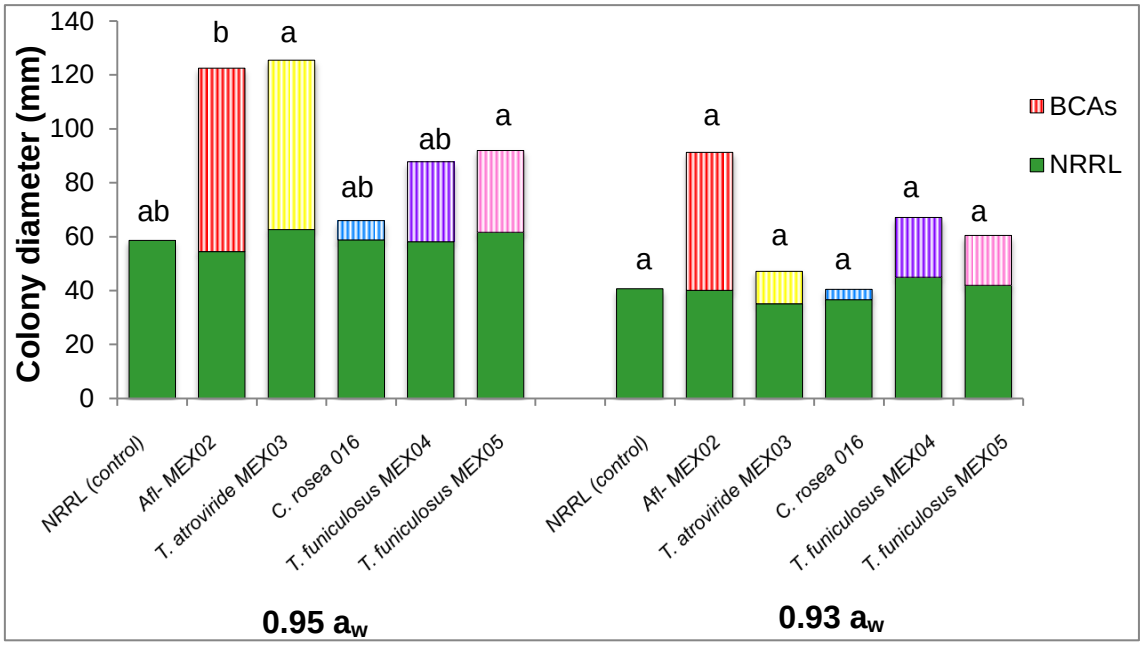


Figure 3.2 Fungal colony diameter of *A. flavus* interacting with BCAs, after 9-day incubation at 25°C on MMA at 0.98 a_w (a) NRRL 3357 and (b) toxigenic isolate MEX01. Different letters indicate significant difference ($p<0.05$) between *A. flavus* by Tukey's Honest Significant Difference (HSD).

a) Toxigenic strain NRRL 3357



b) Toxigenic strain MEX01

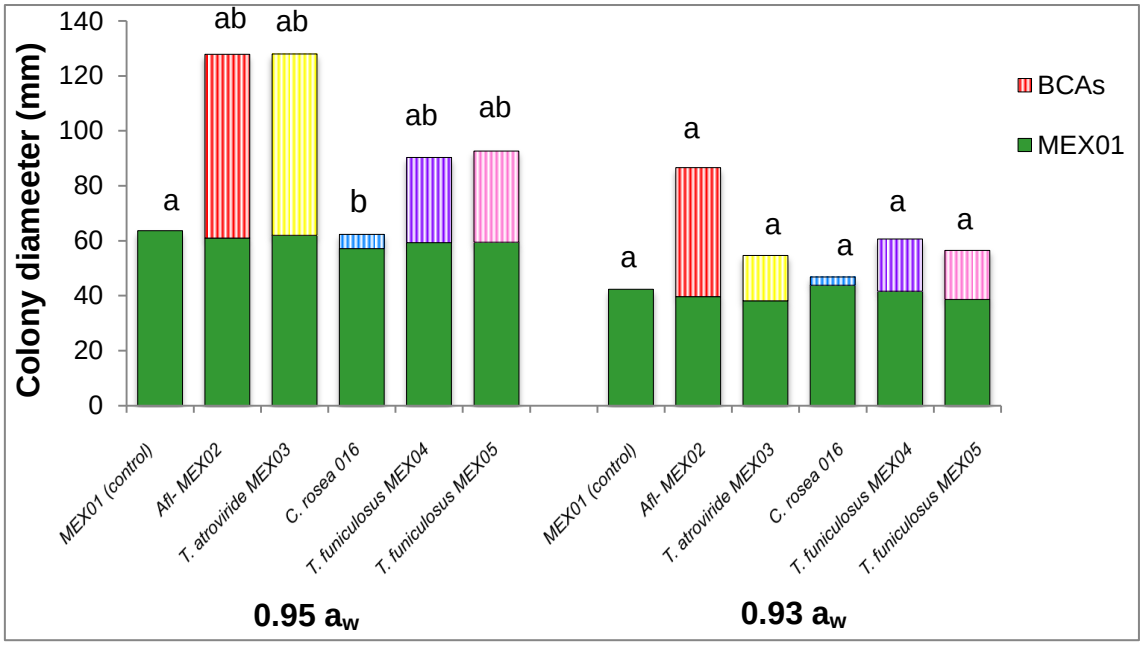


Figure 3.3 Fungal colony diameter of *A. flavus* interacting with potential biocontrol after 15-day incubation at 25°C on MMA at 0.95 and 0.93 a_w (a) NRRL 3357 and (b) toxigenic MEX01 isolate. Different letters indicate significant difference ($p<0.05$) between *A. flavus* by Tukey's Honest Significant Difference (HSD).

a) Interactions and Index of Dominance

The macroscopic interactions between the colonies were numerically classified and the Index of Dominance (I_D) obtained at different a_w levels. Table 3.3 shows the type of interactions and the total I_D, the first score is for the BCA candidate and the second is for toxigenic NRRL 3357 *A. flavus* strain. At 0.98 a_w the interactions were mutual antagonism or the BCA dominated. However, under more water stress, 0.95 a_w, the interactions were mutual antagonism or the toxigenic NRRL 3357 strain dominated. With less available water (0.93 a_w) *A. flavus* was dominated by *T. atroviride* MEX03 and *T. funiculosus* MEX04. The *T. atroviride* MEX03 had the highest I_D score (10) at the three a_w levels tested. The *T. funiculosus* MEX05 had a total score of 8 and the Afl$^-$ MEX02 6. The latter was mutually antagonistic on contact under all the conditions tested. *C. rosea 016* was competitive and dominated the toxigenic *A. flavus* strain but only at 0.98 a_w (score of 4:0). The colour of the *A. flavus* also was noticeably a pale green. The bacterial isolate BY#84 was dominated by toxigenic *A. flavus* under all the conditions tested. The *Streptomyces* sp. AS1 and *E. hormaechei* were antagonistic on contact and at distance, respectively, but only with freely available water. Under water stress both were dominated by the toxigenic *A. flavus* strain.

Table 3.3 Interaction scores and Index of Dominance of *A. flavus* NRRL 3357 with potential biocontrol agents on MMA at 25°C and different water activities

Species	0.98	0.95	0.93	I_D
Afl$^-$ MEX02	2/2	2/2	2/2	**6/6**
T. atroviride MEX03	4/0	2/2	4/0	**10/2**
C. rosea 016	4/0	0/4	0/4	**4/8**
T. funiculosus MEX04	4/0	2/2	4/0	**10/2**
T. funiculosus MEX05	4/0	2/2	2/2	**8/4**
E. hormaechei	2/2	0/4	0/4	**2/10**
Streptomyces sp. AS1	3/3	0/4	0/4	**3/11**
Bacteria BY#84	0/4	0/4	0/4	**0/12**

Table 3.4 shows the interaction between the potential BCAs and the toxigenic MEX01 strain. At 0.98 a_w the BCAs were able to dominate MEX01 toxigenic strain or antagonise it on contact. *T. funiculosus* MEX04 and MEX05 had the highest total I_D score (12) under the three different conditions by dominating the toxigenic strain on contact; *T. atroviride* MEX03 had a score of 8 and the Afl$^-$ MEX02 6, mainly due to mutual antagonism. The *E. hormaechei* bacterial strain was dominated by the toxigenic MEX01 under all conditions. The *Streptomyces* AS1 and the bacterial isolate were mutually antagonistic on contact under freely available water conditions. The three bacteria were dominated by MEX01 strain under water stress.

Table 3.4 Interaction scores and Index of Dominance of *A. flavus* MEX01 with potential BCAs on MMA at 25°C and different a_w

Species	0.98	0.95	0.93	I_D
Afl$^-$ MEX02	2/2	2/2	2/2	**6/6**
T. atroviride MEX03	4/0	2/2	2/2	**8/4**
C. rosea 016	4/0	0/4	0/4	**4/8**
T. funiculosus MEX04	4/0	4/0	4/0	**12/0**
T. funiculosus MEX05	4/0	4/0	4/0	**12/0**
E. hormaechei	0/4	0/4	0/4	**0/12**
Streptomyces sp. AS1	2/2	0/4	0/4	**2/10**
Bacteria BY#84	2/2	0/4	0/4	**2/10**

Figure 3.4 exemplifies the interaction of the toxigenic *A. flavus* strains (NRRL 3357 or MEX01) with the Afl$^-$ MEX02, *T. atroviride* MEX03 and *T. funiculosus* MEX05 at 25°C and the three different a_w levels examined. The Afl$^-$ MEX02 strain showed mutual antagonism on contact with the toxigenic strains under all conditions. *T. atroviride* MEX03 and *T. funiculosus* (MEX04 and MEX05) showed dominance on contact at 0.98 a_w and mutual antagonism on contact at 0.95 and 0.93 a_w. The latter strains changed their ability to interact with the toxigenic strains as water stress was imposed.

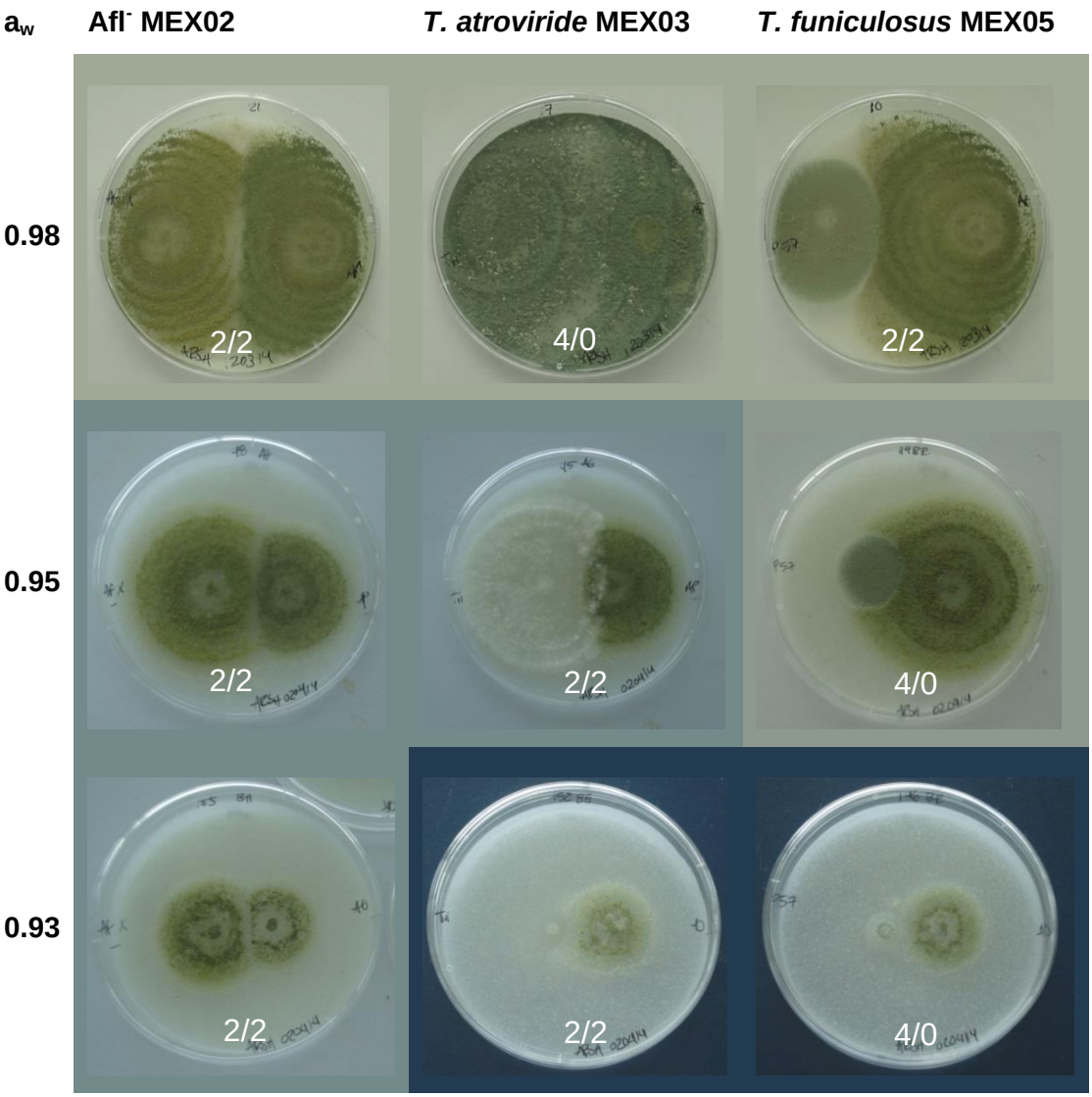


Figure 3.4 Examples of interactions between potential biocontrol agents and *A. flavus* at three water activities (0.98, 0.95 and 0.93 a_w) at 25°C on a MMA medium.

3.3.2 Mixed inoculum of toxigenic *A. flavus* strains and BCAs on MMA

a) 50:50 ratios

Table 3.5 shows the effect of BCAs on AFB_1 production by the toxigenic NRRL 3357 strain with the different candidates at 25 and 30°C and the 3 a_w levels examined. At 25°C and with relatively freely available water (0.98 a_w) the AFB_1 production by the NRRL 3357 strain was significantly decreased by Afl- MEX02,

T. atroviride MEX03 and *T. funiculosus* MEX05. Similar results were obtained at the same temperature with lower available water (0.95 a_w) with the same three BCAs reducing AFB_1 production. At the higher temperature (30°C) and 0.98 a_w there was no significant effect compared to the control and between treatments. At 0.95 a_w and 30°C AFB_1 production was stimulated by the *Streptomyces* sp. AS1 and the bacterial isolate. AFB_1 production was generally higher at 0.98 a_w than other a_w levels tested at both temperatures

Table 3.4 shows the production of AFB_1 by the toxigenic MEX01 strain at different temperatures and two a_w levels. At 25°C and 0.98 a_w, AFB_1 production was reduced by Afl^- MEX02 and *T. atroviride* MEX03. In contrast, the presence of the bacteria *E. hormaechei* stimulated AFB_1 production. At 0.95 a_w the Afl^- MEX02 strain inhibited AFB_1 production. *C. rosea* 016 was also effective under these conditions. At 30°C and 0.98 a_w, five of the BCA candidates affected AFB_1 production. Of those screened, the Afl^- MEX02, *T. atroviride* MEX03 and *T. funiculosus* MEX05 were the best potential strains for control of AFB_1 production. At 0.95 a_w and 30°C only the Afl^- MEX02 strain inhibited AFB_1 production.

Table 3.5 Effect of mixed inocula ($1x10^6$/mL) of BCAs on AFB_1 production by type strain *A. flavus* NRRL 3357 on MMA, at 25 and 30°C.

Species	AFB_1 ng/g of agar			
	25°C		30°C	
	0.98 a_w	0.95 a_w	0.98 a_w	0.95 a_w
NRRL 3357 (control)	2405.0±1171.9^{ab}	162.2±34.4^{abc}	1045.2±273.0^{ab}	31.2±12.7^{bc}
Afl$^-$ MEX02	**3.1±0.7^{d}**	**2.8±0.1^{e}**	238.7±223.8^{b}	6.9±1.9^{c}
T. atroviride MEX03	**2.5±0.1^{d}**	20.8±8.9^{cde}	84.0±35.3^{b}	202.9±71.7^{b}
C. rosea 016	524.1±121.9^{bc}	159.7±134.9^{bcd}	1167.0±254.6^{ab}	96.5±47.2^{b}
T. funiculosus MEX04	3588.6±155.5^{ab}	54.7±4.8^{bcd}	2152.0±1914.1^{ab}	10.3±3.5^{c}
T. funiculosus MEX05	**253.4±183.79^{c}**	**4.5±1.4^{de}**	242.3±77.4^{ab}	5.4±1.8^{c}
E. hormaechei	1888.6±191.0^{ab}	262.1±91.3^{ab}	56.5±40.3^{b}	152.5±79.4^{b}
Streptomyces sp. AS1	13903.2±10291.5^{a}	1619.1±287.6^{a}	11467.0±5838.6^{a}	**8454.0±1828.3^{a}**
Bacteria BY#84	5062.3±9.5^{a}	291.2±225.5^{abc}	6944.4±2232.1^{a}	**8823.5±1299.2^{a}**

Data are means of triplicates ± standard error. Means of the same treatment followed by different letters are significantly different ($p<0.05$). Underlined and bolded numbers mean reduction and bold numbers mean stimulation of AFB_1 production.

Table 3.6. Effect of mixed inocula (1x10^6/mL) of BCAs on AFB_1 production by the toxigenic *A. flavus* MEX01 isolate on MMA, at 25 and 30°C.

Species	AFB_1 ng/g of agar			
	25°C		30°C	
	0.98 a_w	0.95 a_w	0.98 a_w	0.95 a_w
MEX01 (control)	3131.3± 3024.2^{b}	15195.6±1663.6^{a}	56174.3±10918.6^{a}	57939.5±4086.1^{ab}
Afl$^-$ MEX02	**7.3±3.1^{d}**	**22.6±4.2^{c}**	**40.8±18.1^{d}**	**144.4±76.8^{c}**
T. atroviride MEX03	**15.4±2.1^{cd}**	9375.0±2196.4^{ab}	**19.4±3.7^{d}**	77833.1±7972.0^{ab}
C. rosea 016	572.8±223.2 ^{b}	**1203.7±372.0^{b}**	**152.5±70.2^{cd}**	45718.2±4747.7^{b}
T. funiculosus MEX04	247.4±57.7^{bc}	22470.6±18252.5^{ab}	**3613.3±1970.6^{bc}**	121285.6±5882.8^{a}
T. funiculosus MEX05	91.5±53.2^{bcd}	22424.8±10447.3^{ab}	**37.4±14.2^{d}**	147300.9±11947.2^{a}
E. hormaechei	**85641.4±12730.1^{a}**	2973.8±164.7^{ab}	5440.1±1571.1^{ab}	37368.4±4944.3^{b}
Streptomyces sp. AS1	229.7±135.3^{bcd}	1594.0±209.9^{ab}	15994.9±3085.1^{ab}	78518.3±5149.2^{ab}
Bacteria BY#84	2970.1±2061.0^{b}	3169.9±1320.0^{ab}	7079.0±4863.0^{ab}	63890.6±4002.7^{ab}

Data are means of triplicates ± standard error. Means of the same treatment followed by different letters are significantly different ($p<0.05$). Underlined and bolded numbers mean reduction and bold numbers mean stimulation of AFB_1 production.

b) Effect of different inoculum ratios of toxigenic A. flavus and BCAs on relative gene expression of aflatoxin biosynthetic genes

The effect of the best BCAs were chosen from the 50:50 ratio study, and tested at different inoculum ratios. The biocontrol candidates were Afl$^-$ MEX02, *T. atroviride* MEX03, *C. rosea* 016 and *T. funiculosus* MEX05. Different inoculum ratios were inoculated on MMA, at 0.98 and 0.93 a_w, incubated at 30°C for 10 and 20 days. To analyse the relative gene expression of *aflD* (structural gene) and *aflR* (regulatory gene) the 50:50 ratio treatments were used. The relative gene expression of the toxigenic strains (100:0 ratio) was used as a positive control and calibrator. Figure 3.5a shows that at 0.98 a_w the expression of *aflD* was up-regulated 8 fold during the interaction with the Afl$^-$ MEX02 and 4.7 fold with *T. atroviride* MEX03 compared to the calibrator (NRRL 3357). Under the same conditions, all the BCAs down-regulated the expression of the structural gene by approx. 50-90% during interaction with the toxigenic MEX01 strain, (Figure 3.5b). Also, the negative control Afl$^-$ MEX02 (0:100 ratio) had a lower expression compared to toxigenic MEX01. Under water stress, the candidate BCAs stimulated *aflD* expression of the toxigenic NRRL strain (Figure 3.5c). Contrary to the results obtained with toxigenic MEX01, the Afl$^-$ MEX02 strain down-regulated the expression and *C. rosea* 016 increased it by 2.9 fold (Figure 3.5d). Unfortunately, in some treatments and replicates the variability in the *aflD* and *aflR* expression was higher than 0.5 Ct within biological (n=3) replicates and the data was not used.

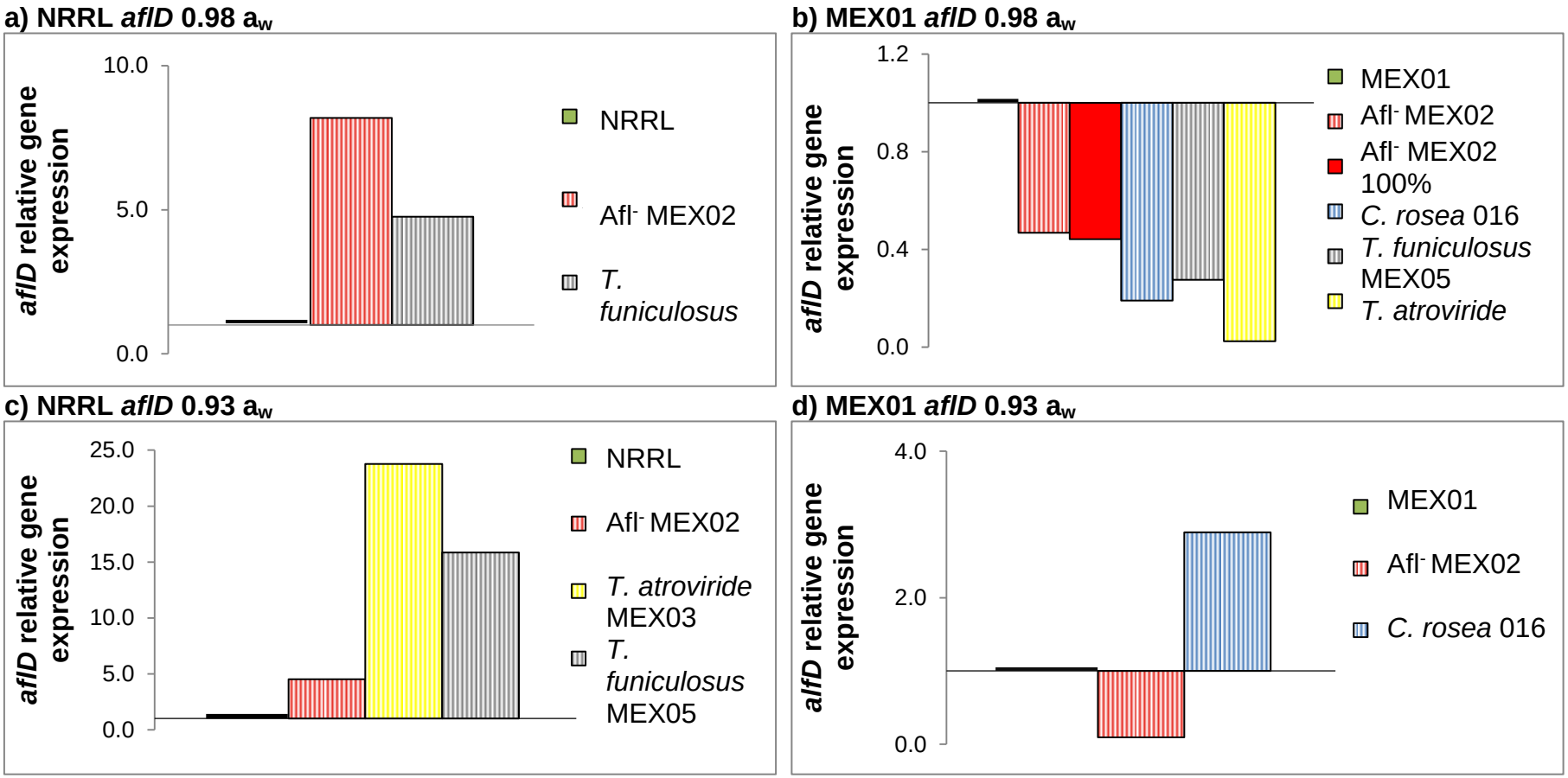


Figure 3.5 Relative gene expression values of *aflD* in *A. flavus* toxigenic strains (NRRL 3357 and MEX01) inoculated with the BCAs (50:50 ratio) on MMA incubated at 30°C for 10 days at 0.98 and 0.93 a_w. Toxigenic strain (100%) was used as the calibrator. Means of n=4-6 replicates.

c) *Effect of different inoculum ratios of BCAs and toxigenic strains on AFB_1 production*

Table 3.7 shows the effect of the BCA candidates on AFB_1 production by the toxigenic NRRL 3357 strain during co-inoculation at different inoculum ratios. The treatment conditions were with relatively freely available water (0.98 a_w) and water stress (0.93 a_w) at 30°C, when incubated for 10 and 20 days. At 0.98 a_w NRRL 3357 type strain produced more AFB_1 than at 0.93 a_w. At 0.98 a_w and both time periods all the BCAs affected AFB_1 production regardless of the ratio. At 0.93 a_w and 10 days, *T. funiculosus* MEX05 could inhibit the production at a high inoculum concentration $7.5x10^5$ spores/mL (25:75% ratio). After 20 days at 0.98 a_w all the BCAs were effective in reducing or inhibiting toxin production. Under water stress, Afl^- MEX02 inhibited AFB_1 production at all ratios. *C. rosea* 016 was effective at (75:25% ratio) inoculum level and *T. funiculosus* MEX05 at 25% (2.5×10^5) and 75% ($7.5x10^5$).

The results obtained from co-inoculation of the BCAs with the toxigenic MEX01 strain are shown in Table 3.8. The BCA candidates inhibited AFB_1 production at 0.98 a_w after 10 and 20 days at all ratios. At 0.93 a_w and 10 days, although AFB_1 was low, even in the controls, *C. rosea* 016 inhibited the production at a high inoculum concentration $7.5x10^5$ spores/mL (25:75% ratio). Under the same conditions, *T. atroviride* MEX03 at a high inoculum concentration, actually stimulated AFB_1 production. After 20 days, the Afl^- MEX02 strain was effective regardless of the ratio. *C. rosea* 016 was effective at 25% and *T. funiculosus* MEX05 at the same inoculum level but also at 75%.

Table 3.7 AFB_1 production by type strain NRRL 3357 in mixed inoculum with BCAs at different ratios incubated for 10 and 20 days at 30°C on MMA modified to 0.98 and 0.93 a_w.

Days	a_w	BCAs	NRRL strain:BCA strain, AFB_1 (ng/g) ±SE				
			100%	75%:25%	50%:50%	25%:75%	100%
10	0.98	NRRL 3357 (control)	1234.0±289.0	-	-	-	-
		Afl$^-$ MEX02		**6.7±4.0***	**1.0±0.0***	**1.0±0.0***	**0.5±0.3***
		T. atroviride MEX03		**0.5±0.0***	**0.5±0.0***	**0.1±0.1***	-
		C. rosea 016		248.9±148.1	**390.8±64.7***	**43.3±19.2***	-
		T. funiculosus MEX05		**41.9±35.6***	**0.5±0.0***	**0.7±0.4***	-
	0.93	NRRL 3357 (control)	231.0±227.8	-	-	-	-
		Afl$^-$ MEX02		0.5±0.0	0.5±0.0	0.2±0.1	0.4±0.1
		T. atroviride MEX03		2.3±1.8	5.0±2.5	6.1±3.9	-
		C. rosea 016		292.5±291.3	0.5±0.0	12.7±12.2	-
		T. funiculosus MEX05		3.1±2.6	0.7±0.2	**0.1±0.1***	-
20	0.98	NRRL 3357 (control)	1217.6±305.6	-	-	-	-
		Afl$^-$ MEX02		**23.7±12.2***	**0.5±0.0***	**0.5±0.0***	nd
		T. atroviride MEX03		**0.1±0.1***	**nd**	**nd**	-
		C. rosea 016		408.4±184.4	**35.7±13.0***	**41.4±27.3***	-
		T. funiculosus MEX05		**8.6±1.4***	**1.1±0.5***	**nd**	-
	0.93	NRRL 3357 (control)	239.5±235.8	-	-	-	-
		Afl$^-$ MEX02		**nd**	**nd**	**nd**	nd
		T. atroviride MEX03		190.7±188.31	13.6±6.53	3.7±1.7	-
		C. rosea 016		**0.4±0.1***	1.4±0.5	7.7±7.2	-
		T. funiculosus MEX05		**nd**	1.4±0.8	**nd**	-

Data are mean of triplicates ± standard error. Means of the same treatment followed by asterisk are significantly different compared to the control ($p<0.05$). Bold and underlined means reduction of AFB_1 production. nd= not detected.

Table 3.8 AFB_1 production by *A. flavus* MEX01 in mixed inoculum with the four BCAs at different ratios incubated for 10 and 20 days at 30°C on a MMA modified at 0.98 and 0.93 a_w.

			MEX strain:BCA strain, AFB_1 (ng/g) ±SE				
Days	**a_w**	**BCAs**	**100%**	**75%:25%**	**50%:50%**	**25%:75%**	**100%**
10	0.98	MEX01 (control)	1216.1±458.4	-	-	-	-
		Afl$^-$ MEX02		**3.1±0.9***	**2.2±0.7***	**1.2±0.2***	**1.1±0.0***
		T. atroviride MEX03		**1.0±0.0***	**2.7±1.0***	**0.5±0.3***	-
		C. rosea 016		**0.4±0.4***	**0.3±0.3***	**1.6±0.5***	-
		T. funiculosus MEX05		**7.4±4.1***	**1.0±0.4***	221.8±209.8	-
	0.93	MEX01 (control)	3.3±0.1	-	-	-	-
		Afl$^-$ MEX02		**1.1±0.0***	**0.8±0.3***	**0.7±0.7***	nd
		T. atroviride MEX03		65.3±61.3	1393.5±1351.3	**582.7±252.14***	-
		C. rosea 016		19.3±15.7	0.8±0.8	**nd**	-
		T. funiculosus MEX05		1663.9±910.0	1091.6±896.4	2.7±0.5	-
20	0.98	MEX01 (control)	566.7±334.4	-	-	-	-
		Afl$^-$ MEX02		**1.4±0.5***	**0.9±0.0***	**nd**	nd
		T. atroviride MEX03		**1.3±1.1***	**1.9±1.7***	**nd**	-
		C. rosea 016		**0.2±0.2***	**nd**	**0.3±0.3***	-
		T. funiculosus MEX05		**0.8 ±0.3***	**0.5±0.3***	**0.3±0.3***	-
	0.93	MEX01 (control)	337.70±219.7	-	-	-	-
		Afl$^-$ MEX02		**1.0±0.0***	**nd**	**0.3±0.3***	**0.5±0.3***
		T. atroviride MEX03		972.5±792.4	2106.8±774.9	5031.9±4551.9	-
		C. rosea 016		92.1±51.1	**1.5±0.5***	7.3±5.7	-
		T. funiculosus MEX05		3059.3±2922.8	1360.8±1232.5	2307.6±1098.9	-

Data are mean of triplicates ± standard error. Means of the same treatment followed by asterisk are significantly different compared to the control ($p<0.05$). Bold and underlined means reduction of AFB_1 production. nd= not detected.

3.4 Discussion

This study examined the interactions between toxigenic *A. flavus* strains and potential BCAs under different environmental conditions. Specific water availability and temperature affected growth of pathogen and antagonists, the type of interactions and the overall I_D of the potential BCAs. Although the primary aim was for AFB_1 control, effects on relative growth of *A. flavus* under different interacting environmental conditions were also of interest.

At 0.98 a_w *T. atroviride* MEX03 grew faster and over the toxigenic *A. flavus* strains. Although growth of *T. atroviride* MEX03 was more sensitive to water stress, it was still dominant or antagonistic to the pathogen. Marín *et al*. (1998) tested *T. viride* against *A. parasiticus*, obtaining a score of 4 against 0, with dominance on contact. Longa *et al*. (2008) analysed a strain of *T. atroviride* as a potential biocontrol agent of soil-borne plant pathogens and found optimal activity at 25°C and 0.998 a_w. Daryaei *et al*. (2016) analysed another *T. atroviride* strain LU132 against *Rhizoctonia solani* on ½ PDA. They examined spores of the BCA produced under different temperatures and time periods and found that those harvested after 25 days at 30°C had best efficacy on contact.

T. funiculosus (MEX04 and MEX05) candidates had slower growth than toxigenic *A. flavus* strains examined (NRRL 3357 and MEX01) but were dominant or mutually antagonistic on contact under all conditions tested. Both antagonists had the highest I_D score of 12 with the toxigenic MEX01 and 10 with the NRRL 3357 strain. The *T. funiculosus* strains were isolated from stored Mexican maize. Previously, Pitt (2014) suggested that *Talaromyces* spp. are ubiquitous and commonly isolated from cereals, sometimes causing spoilage even though some *Talaromyces spp*. are used as BCAs (Zhai *et al*., 2016).

The Afl^- MEX02 strain of *A. flavus* was mutually antagonistic on contact with both toxigenic strains. The overall I_D scores were similar over all the environmental conditions tested indicating good tolerance of water stress. The

ability of the atoxigenic *A. flavus* strain to control AFB_1 production has been evaluated both in field assays and on grains, on a variety of different crops including cotton, peanuts and maize (Cotty and Bhatnagar, 1994; Dorner and Lamb, 2006; Abbas *et al.*, 2011). In the present study, the Afl^- MEX02 and toxigenic *A. flavus* strains were mutually antagonistic. This was similar to the results obtain by Mohale *et al.* (2013b) and Abdel-Hadi *et al.* (2011) with atoxigenic strains isolated from Lesotho (maize) and Egypt (peanuts).

C. rosea 016 strains have been commonly used for biocontrol of fungal pathogens, especially soil-borne ones (Luongo *et al.*, 2005; Palazzini *et al.*, 2013). There is only one example of efficacy against mycotoxigenic fungi (Samsudin and Magan, 2016). This recent study showed that a good control of fumonisin B_1 production was obtained. In the present study, the *C. rosea* 016 strain grew more slowly than *A. flavus* but was able to dominate on contact at 0.98 a_w. Morphological changes, especially in terms of sporulation behaviour and spore colour were also observed. This may be due to interactions between the mycelia as shown by Zhang *et al.* (2008), where penetration of nematodes was demonstrated by the action of extracellular hydrolytic enzymes. Previously, another strain of *C. rosea* 016 was examined for control of *Fusarium* species on wheat, maize and crop residue Luongo *et al.* (2005). Palazzini *et al*. (2013) used *Clonostachys* as a biocontrol agent against 7 species of *Fusarium* on wheat stalks. The study was made for 180 days under field conditions. They suggested that the success of *Clonostachys* was due to its ability to colonize decaying tissue faster than *Fusarium.* This has not been previously examined in relation to *A. flavus* survival on crop residue, especially under different interacting environmental conditions.

The toxigenic *A. flavus* strains dominated the *Streptomyces* AS1 under water stress (0.93 a_w). However, at 0.98 a_w the interaction was mutually antagonistic. The maize-based medium may not have been ideal as this strain was originally obtained from peanuts (Sultan and Magan, 2011). Generally, the bacteria were unable to outcompete the toxigenic *A. flavus* strains perhaps due to their relative sensitivity to water stress. For example, it is possible that anti-fungal

secondary metabolites are not produced on the MMA. Previous studies with the *Streptomyces* sp. AS1 strain (Sultan and Magan, 2011) showed that the metabolites produced by this strain on ½ NA were very effective in controlling growth and AFB_1 production *in vitro* and *in situ* in stored peanuts. The interactions on peanut-based medium were mutually antagonistic at a distance (3/3). Indeed, the *Streptomyces* AS1 was antagonistic to *A. flavus* in different media including MEA, ½ NA, YES and MS. However, the crude extracts of the *Streptomyces* were found to be more effective than the colony itself (Sultan and Magan, 2011). Also Verheecke *et al.* (2014) studied the interaction of *Streptomyces* isolates and *A. flavus* on yeast extract/malt extract agar (ISP-2) medium for 10 days at 28°C. Some strains showed antagonism on contact, dominance on contact or at distance. In the present study, four strains were selected for further studies to reduce AFB_1 directly on MMA. These were effective in reducing toxin production by >80%.

To score the potential BCAs a 50:50 spore mixture was used on MMA to analyse the effects on AFB_1 production. Of all the potential candidates**,** the atoxigenic Afl^- MEX02 strain was mutually antagonistic with both toxigenic *A. flavus* strains examined and this resulted in reduction of AFB_1 production. Against the toxigenic NRRL 3357 strain it was significantly effective in reducing the AFB_1 production >95% at 25°C at 0.98 a_w. With the toxigenic MEX01 strain it was effective in significantly reducing AFB_1 production >95% under all conditions tested. Mohale *et al.* (2013b) analysed the effect of atoxigenic strains on AFB_1 production, using different spore inoculum ratios on MEA^+, MMA and maize grain. *In vitro* the reduction was 80% at 0.99 and 0.96 a_w. The effect *in situ* was modified by the strain tested, the inoculum ratio and a_w.

T. atroviride MEX03 had effects on AFB_1 production and also had high interaction scores. It decreased the toxin production by the toxigenic NRRL 3357 strain by >90% at 25°C and >95% of the MEX01 strain at 0.98 a_w at both temperatures. At 0.98 a_w the effect may partially be due to more rapid colonisation than *A. flavus*. Previously, Barberis *et al.* (2014) analysed *Trichoderma* spp. against *A. carbonarius* and found that toxin production was

decreased by >80% on a YES medium at 0.98 and 0.93 a_w. Reddy *et al*. (2009) studied a range of *Trichoderma* species as BCAs against *A. flavus* and AFB_1 production on stored rice. However, a_w and temperature were not modified. They found *T. viride* reduced AFB_1 production by 72%.

T. funiculosus (MEX04 and MEX05) had a high total I_D score. Only *T. funiculosus* MEX05 decreased the production of AFB_1 by the NRRL 3357 toxigenic strain production at 25°C by >85%. With the toxigenic MEX01 strain AFB_1 production was only decreased at 0.98 a_w and 30°C. Similarly, *C. rosea* 016 impacted on AFB_1 production of AFB_1 by >90% at 98 a_w/30°C and at 0.95 a_w/25°C. In contrast, *Enterobacter*, *Streptomyces* and a bacterial isolate stimulated toxin production. This contrasts with the results obtained by Samsudin and Magan (2016) in which *C. rosea* 016 inhibited >70% fumonisin B_1 production at 0.98 and 0.95 a_w in a 75:25 ratio (pathogen:antagonist). Also *Enterobacter* and *Streptomyces* were also used in the same study, decreasing fumonisin B_1 production. Pereira *et al.* (2010) tested *E. hormaechei* against *F. verticillioides,* in field trails for two years. They use *E. hormaechei* on maize ears and as a maize seed coating treatment. They found that it was effective in diminishing fungal infection and fumonisin B_1 production as a seed coating treatment.

After the 50:50 mixed inoculum study it was important to determine the effect of the biocontrol candidates using different inoculum ratios. Therefore the most effective strains were chosen for analysis on MMA at different inoculum ratios and different environmental conditions, with relatively freely available water and under water stress. The relative gene expression (*aflD* and *aflR*) and effects on AFB_1 production were analysed. The relative gene expression is influenced by abiotic conditions and by biotic factors such as interacting BCAs. The *aflD* relative gene expression of the toxigenic NRRL 3357 strain was up-regulated by the BCAs at both a_w levels tested. Meanwhile for the MEX01 strain the *aflD* gene expression was down-regulated at 0.98 a_w by all the BCAs and increased at 0.93 a_w by *C. rosea* 016. *The aflD* gene expression has been analysed during co-inoculation with different biocontrol agents previously. Recently, Verheecke

et al. (2015) found out that actinomycetes did not affect *aflD* expression. However, Al-Saad *et al.* (2016) co-inoculated *A. flavus* with four bacterial antagonist, and were able to decrease *aflD* expression. When *A. flavus* was grown without interaction with antagonists under stress conditions, *aflD* expression was variable, lower or higher compared to the reference gene (β-Tubulin) (Schmidt-Heydt *et al*., 2008; Abdel-Hadi *et al*., 2010, 2012; Medina *et al.*, 2015).

During co-inoculation, the four BCA strains atoxigenic Afl^- (MEX02), *T. funiculosus (*MEX05), *T. atroviride* (MEX03) and *C. rosea* 016 were successful in reducing the AFB_1 production by the toxigenic NRL 3357 strain by >90% at 0.98 a_w on both days and 0.93 a_w after 20 days. Toxin production by the toxigenic MEX01 strain was high at 10 days and 0.98 a_w and after 20 days under water stress. Of all the four candidates interacting with MEX01, the atoxigenic Afl^- MEX02 strain significantly inhibited AFB_1 production at all ratios regardless the environmental conditions. Previously, Mohale *et al.* (2013b) found that AFB_1 was significantly inhibited by atoxigenic strains when the inoculum was 50:50 or 75:50 with the pathogen.

The three other BCA candidates *T. funiculosus (*MEX05), *T. atroviride* (MEX03) and *C. rosea* 016, significantly reduced AFB_1 production by the toxigenic MEX01 strain at 0.98 a_w. However, this changed when water stress was imposed. For example, *T. atroviride* MEX03 caused an increase in AFB_1 production at a high inoculum ratio. Previously, Reddy *et al.* (2009) found that some *Trichoderma* spp. reduced AFB_1 production by *A. flavus* on rice. However, effects of environmental factors or pathogen:antagonist ratios were not examined. Schubert *et al.* (2008) analysed the influences or temperature, a_w and media on *T. atroviride* isolate. The strain was mesophilic growing between 25 and 30°C. The growth was faster at high a_w, decreasing under water stress conditions. Longa *et al.* (2008) found similar results; *T. atroviride* was affected by low a_w. *T. funiculosus* MEX05 was not effective at low a_w, although it has been reported to grow at 30°C and tolerate 0.90 a_w (Pitt and Hocking, 2009).

3.5 Conclusions

The initial screening for potential BCAs agents was successful. Four biocontrol agents isolated from the maize are the most effective against the two toxigenic *A. flavus* strains examined. The interactions and I_D scores between the BCAs and the toxigenic strains were modified by temperature, water availability and inoculum ratios. The Afl^- MEX02 isolate was mutually antagonistic with the toxigenic *A. flavus* strains under different environmental conditions. Based on 50:50 inoculations of the biocontrol candidates and the toxigenic strains, the atoxigenic Afl^- MEX02, *T. atroviride* MEX03*, T. funiculosus* MEX05 and *C. rosea* 016 reduced AFB_1 production >85%. Analysing different inoculum ratios, four potential biocontrol agents stimulated or decreased *aflD* expression and AFB_1 production. Under water stress, the most effective was the atoxigenic Afl^- MEX02 strain against both toxigenic *A. flavus* strains reducing AFB_1 production >65%. Additionally, *T. atroviride* MEX03*, T. funiculosus* MEX05 and *C. rosea* 016 were successful against the toxigenic MEX01 strain at 0.98 a_w, inhibiting the toxin > 95%. Therefore, these biocontrol candidates had the best potential for further studies. Also *C. rosea* 016 was included because it could colonise crop debris and perhaps reduce inoculum potential of *A. flavus*.

Chapter 4. Impact of the biocontrol agents on *Aspergillus flavus* conidial production on senescent maize leaves under different water activity regimes

4.1 Introduction

An important part of the *A. flavus* life cycle is the survival of inoculum in soil and on maize crop debris as sclerotia, mycelia and conidia, especially in “no till” production systems. Conducive environmental conditions allow sclerotial germination (Giorni *et al.*, 2012) or mycelial growth and conidial production. This can then allow transfer to ripening maize plants, either by insects or wind/rain. Thus, there is potential for using BCAs to minimise inoculum production in this phase of the *A. flavus* life cycle to reduce subsequent infection during silking. Atoxigenic *A. flavus* strains have been used as biocontrol agents (BCAs) to reduce AFB_1 contamination by competitive exclusion when applied to the soil (Bandyopadhyay *et al.*, 2016). It has been suggested that these atoxigenic strains displace the toxigenic ones thus reducing potential for subsequent toxin production. However, evidence of control of inoculum potential in soil/crop residue is lacking (Cotty and Bhatnagar, 1994; Dorner and Lamb, 2006; Abbas et *al.*, 2011). The key to reducing inoculum potential of toxigenic *A. flavus* is to interfere with the asexual sporulation or inhibition of sclerotial germination. This would potentially reduce inoculum size and perhaps the potential for AFB_1 production. In the present study *T. atroviride* MEX03, *T. funiculosus* MEX05 and *C. rosea* 016 were effective at decreasing AFB_1 production on maize-based media. The question arises as to whether under different environmental conditions these potential BCAs could inhibit asexual sporulation of toxigenic *A. flavus* strains.

The objectives of this chapter were:

a) Construct a moisture adsorption curve with senescent maize leaves to accurately modify to target a_w treatments.
b) Compare the effect of 50:50 mixed spore inoculum of the BCAs with the toxigenic *A. flavus* strain (MEX01) on relative sporulation of this pathogen at 0.98 (=-2.8 MPa water potential) and 0.93 a_w (=-9.8 MPa) at 30°C on senescent maize leaves.
c) Examine the effect of the atoxigenic *A. flavus* strain on asexual sporulation of the toxigenic strain (MEX01) on a selective coconut cream agar (CAM) under the treatment conditions detailed in (b).

4.2 Materials and Methods

4.2.1 Fungal strains

The fungal isolates used for this experiment were the toxigenic *A. flavus* MEX01 strain, as the positive control. The four BCAs were the atoxigenic Afl$^-$ (MEX02), *T. funiculosus (*MEX05), *T. atroviride* (MEX03) and *C. rosea* 016. These were chosen based on the previous studies (see Chapter 3).

4.2.2 Media and inoculum preparation

The fungi were point inoculated on 3% Milled Maize Agar (MMA) prepared as described before in Section 3.2.2. The spores were removed from seven-day-old MMA cultures using 9 mL of sterile water with Tween® 80 and agitating the colony surface with a sterile glass spreader to dislodge the conidia. The conidia were carefully removed with a sterile 1 mL tip and placed in a 25 mL sterile Universal bottle. The spore concentration was calculated using a haemocytometer and a compound microscope (Olympus BH-2). The spore suspensions were diluted with sterile water to obtain the target concentrations of $1x10^4$ spores/mL for the different strains.

a) Senescent maize leaves moisture content

The senescent maize leaves were cut, autoclaved in beakers and afterwards kept at room temperature until complete dryness. Subsequently, some samples were used to measure the a_w and the rest were kept for the experiment. The leaves were sub-sampled and cut (approximate 2.5 x 5 cm), weighed (0.5 g) in Petri plates and different amounts of water were added in the range of 0.025 - 0.500 mL. The Petri plates were closed with a flexible plastic film (Parafilm M®), shaken and left to equilibration overnight at 4°C. After this the sub-samples were allowed to equilibrate at 25°C and the a_w measured using an AQUALAB® Series 3TE (Decagon Devices Inc., Pullman, Washington, USA). Afterwards, the samples were dried overnight at 80°C to obtain the moisture content (MC). The adsorption curve was obtained by plotting the amount of water added against the a_w (Figure 4.1). An additional curve was plotted of the a_w relationship with the MC. The adsorption curve was used to determine the

volume of water needed to modify the leaves to obtain the target a_w treatments (0.93=-9.8 MPa water potential; and 0.98 a_w=-2.8 MPa water potential). When equilibrating the senescent maize leaves the addition of 0.025 mL of the spore suspensions were taken into account. Under sterile conditions the autoclaved maize leaves were placed in plastic chambers, the water added, shaken and left to equilibrate overnight at 4°C.

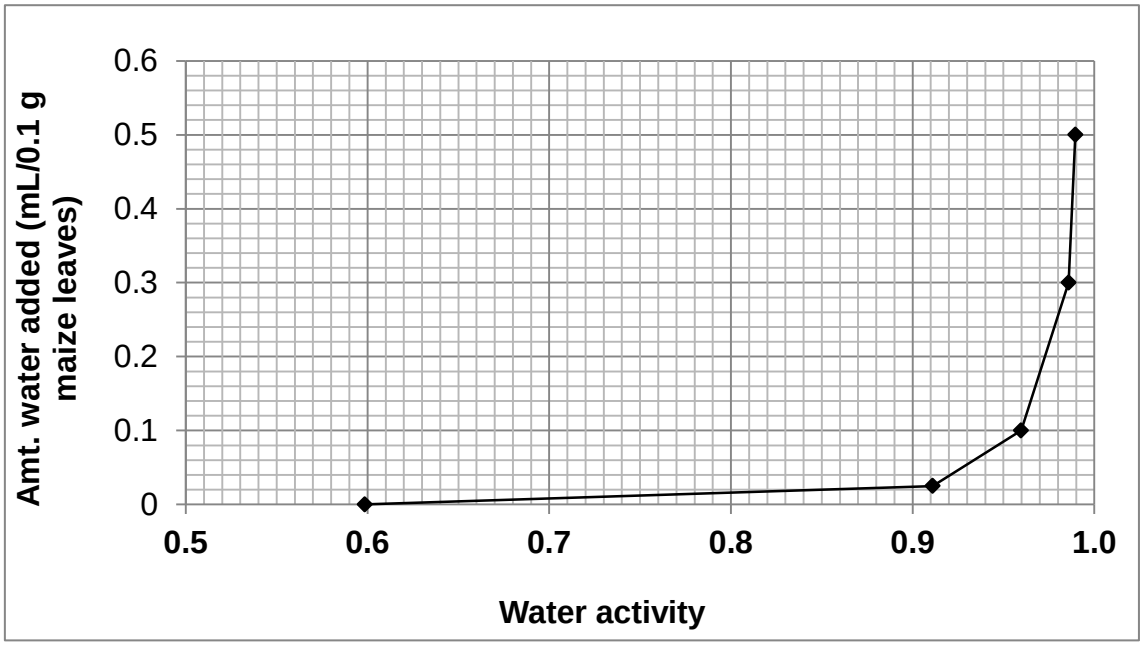


Figure 4.1 Adsorption curve to determine the amount of water needed to modify the senescent maize leaf water activity.

4.2.3 Co-cultivation of the potential biocontrol agents and *A. flavus* on senescent maize leaves

The interaction between the four candidate BCAs and the toxigenic *A. flavus* strain on conidial sporulation of the pathogen was examined with a 50:50 inoculum ratio at both water stress conditions (0.98 and 0.93 a_w) at 30°C. The spore suspension was made up as described before in Section 4.2.2. The mixed 50:50 inoculum ratio was made up in 25 mL Universal bottles. The leaves were sprayed with the different treatments using a painting airbrush (Spray Gun Set, Model 250-3, Badger Products, SHESTO Ltd., Watford, UK). The leaves were cut in similar pieces (approx. 2.5 x 5 cm), and four pieces were placed on aluminium foil under a sieve (Retsch, DIN-ISO 3310/1, 710 Mic,

Ser.Nr.56200426, Germany, Figure 4.2). The spore suspension of the different treatments was sprayed on the leaves (0.1 mL per four pieces). The amount of liquid sprayed and the air stream were determined beforehand. With sterile tweezers, two inoculated pieces were placed in a Petri plate prepared beforehand with a thin layer of water agar (Agar Technical No.3, 20 g LP0013 OXOID Ltd, Basingstoke, UK; water 1000 mL) modified with glycerol to achieve the required a_w levels (0.98 and 93 a_w) as described previously in Section 3.2.2. The leaf pieces were placed on the inverted lid, so that there was no contact between the water agar (above) and the leaf segments. The experiment had four replicates per treatment and a randomized design. Two leave pieces per petri plates, the treatments were positive control, negative control and 50%:50% pathogen:antagonist ($5x10^3$:$5x10^3$, 4 BCAs) were kept in a polyethylene bag and incubated at 30°C for 3 and 8 days. Between each treatment inoculation the airbrush, glass container and sieve were cleaned with sterile distilled water, IPA 70% and sterile distilled water again and then dried.

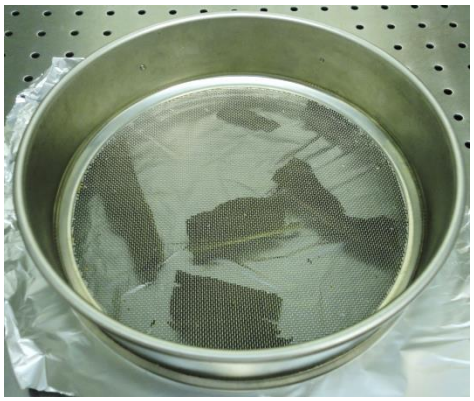


Figure 4.2 Senescent maize leaves pieces to be sprayed with the spore suspension.

a) <u>Sporulation assessment</u>

The spores were counted on day 3 and 8 for 0.98 a_w treatment and on day 8 for the 0.93 a_w. For sporulation assessment one leaf segment was removed from the Petri plate with sterile tweezers. Three leaf disc samples were taken randomly using a sterile cork-borer (diameter 0.7 cm, area of 0.38 cm^2 per piece) from the leaf segments. The three pieces were placed in a sterile

Eppendorf tube (safe cap) with 2 mL mix of sterile water with 0.05% Tween® 80. The Eppendorf was vortex to detach the conidia from the leaves for 30 s. The spores were counted to obtain the total number, as described before in Section 3.2.2 and expressed as spores per cm^2. An initial trial was made to determine the sporulation time of the *A. flavus* on maize leaf. Also, with the information that sclerotia on maize stalk can sporulate since day 1 (Giorni *et al.*, 2012)

b) Assessment of the production of viable conidia by the toxigenic A. flavus *using serial dilution*

The numbers of viable colonies arising from conidia of the mixed populations of the toxigenic *A. flavus* strain and the BCA populations was assessed after 3 and 8-day incubation. Following counting of spores, the suspension was diluted to reach the concentration of $1x10^3$ spores/mL. Twenty-five µL of the control and 50 µL of the treatment (50:50) were inoculated on MEA^+ and spread with a sterile glass spreader. The plates were stored in polyethylene bags and incubated at 25°C. The CFUs were counted on day 3 and the strain was confirmed on day 4. This was done for *T. atroviride* MEX05 which had a very fast growth rate. For populations of the toxigenic and atoxigenic strains the CFUs were assessed with CAM medium to differentiate between them. After 3 days the growth of colonies was examined under UV light, with a blue fluorescent ring around the colonies as an indicator of aflatoxin production. An initial trial was made to distinguish them on CAM, indicating that the atoxigenic Afl^- (MEX02) strain was negative for aflatoxin production on this medium.

4.2.4 Statistical Analysis

All treatments were randomized and replicated four times. The spore data and the CFUs were transformed using ($\log_{10}$ + 1) before statistical analysis. Shapiro-Wilk test and Levene's test were used to assess normality and variance homogeneity, respectively. When the assumptions were met the data was analysed with one-way ANOVA. Tukey's Honest Significant Difference (HSD) was used to compare the significant differences between treatments. When the assumptions were not achieved, non-parametric analysis was applied, Kruskal-Wallis and then Mann-Whitney to compare difference between treatment and

control. R version 3.1.2 (2014-10-31) -- "Pumpkin Helmet". Copyright (C) 2014 The R Foundation for Statistical Computing.

4.3 Results

4.3.1 Effect of the BCAs on *A. flavus* MEX01 sporulation on senescent maize leaves at different a_w levels.

Table 4.1 shows the effect of the mixed inoculum of each BCA on conidial production by the toxigenic *A. flavus* strain on senescent maize leaves. Under relatively wet conditions, 0.98 a_w, on day 3, there was no effect of the BCAs on the ability of the toxigenic *A. flavus* strain to produce asexual conidia. However, after 8 days at 0.98 a_w, the presence of *T. atroviride* MEX03, *C. rosea* 016 and *T. funiculosus* MEX05 resulted in an increase in conidial production by the toxigenic *A. flavus* strain. Additionally, the atoxigenic strain (Afl^- MEX02; negative control) produced more conidia than the toxigenic strain on senescent maize leaves without any competition. At 0.93 a_w there was very little if any sporulation after day 3. Thus, the data at this water availability level was assessed later after day 8 when both a_w treatments could be assessed for sporulation. At 0.93 a_w the asexual sporulation of the toxigenic *A. flavus* strain was unaffected by the presence of any of the BCAs tested. Indeed, with more available water (0.98 a_w) the presence of the three BCAs actually resulted in an increase in sporulation of the toxigenic *A. flavus* strain. Figure 4.3 shows the sporulation of the toxigenic *A. flavus* MEX01 strain on the senescent leaf segments.

Table 4.1 Sporulation of the toxigenic *A. flavus* strain (MEX01) (Log_{10} conidia/cm^2) during co-inoculation with different potential BCAs on senescent maize leaves at 0.98 (-2.8 MPa water potential) and 0.93 a_w (-9.8 MPa water potential) at 30˚C, harvested after 3 and 8 days.

Strains	Spores (Log_{10})/cm^2		
	Day 3	Day 8	
	0.98 a_w	0.98 a_w	0.93 a_w
Toxigenic MEX01 (positive control)	5.55±0.09[a]	4.61±0.17[b]	5.37±0.14[a]
Atoxigenic Afl^- MEX02 (negative control)	5.67±0.23[a]	6.10±0.21[a]	3.85±1.29[a]
BCAs			
+ Afl^- MEX02	5.33±0.07[a]	4.56±0.05[b]	5.72±0.19[a]
+ *T. atroviride* MEX03	5.20±0.18[a]	**5.60±0.15[a]**	5.38±0.18[a]
+ *C. rosea* 016	5.11±0.10[a]	**5.74±0.38[a]**	5.60±0.18[a]
+ *T. funiculosus* MEX05	5.55±0.09[a]	**5.92±0.18[a]**	5.78±0.14[a]

Different letters indicate significant difference ($p<0.05$) within treatments by Tukey's Honest Significant Difference (HSD). Bold numbers mean stimulation of conidial production.

a) b)

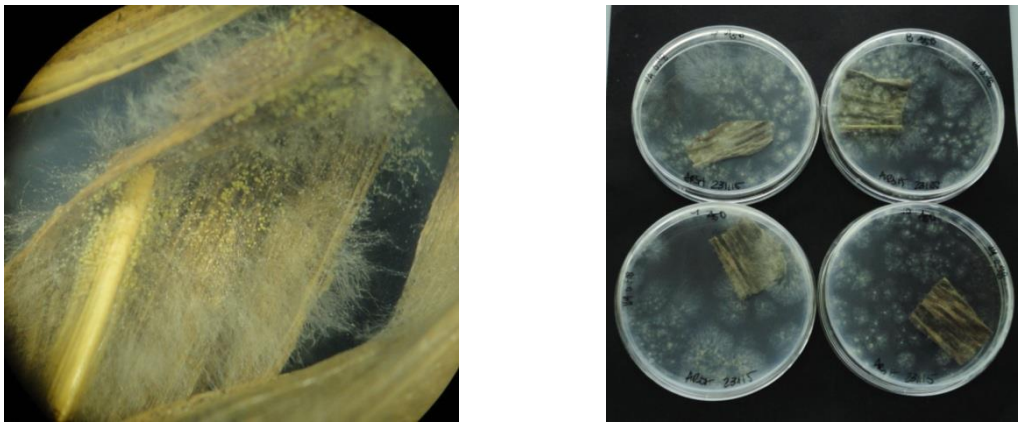


Figure 4.3 (a) *A. flavus* MEX01 colony growth on senescent maize leaves; (b) *A. flavus* MEX01 and Afl^- MEX02 co-inoculated on senescent maize leaves in 50:50 ratio at 0.98 a_w, 30°C after 8 days.

4.3.2 Effect of BCAs on viable spore production by *A. flavus* at different a_w levels based on serial dilution

After the quantification of conidial production by the toxigenic *A. flavus* strain the conidia were assessed for viability and enumerated using the serial dilution method. The toxigenic *A. flavus* MEX01 strain was the positive control. Figure 4.4 compares the viable conidial production by the toxigenic *A. flavus* MEX01 strain at both a_w levels after 3 and 8 days alone or with the BCAs. Overall there was no significant difference between the control and BCA treatments at both 0.98 and 0.93 a_w. At 0.98 a_w after 3 days there was no recovery of *C. rosea* 016 and *T. atroviride* MEX03 conidia. *T. funiculosus* MEX05 was recovered as Log 5.01 CFU/cm^2. On day 8 *T. atroviride* MEX03 was recovered from the 50:50 treatment (log 4.67 CFU/cm^2) in the 0.98 a_w treatment; and log 6.83 CFU/cm^2 for the *T. funiculosus* MEX 05. At 0.93 a_w, after 8 days, none of the BCAs could be recovered from the senescent maize leaves.

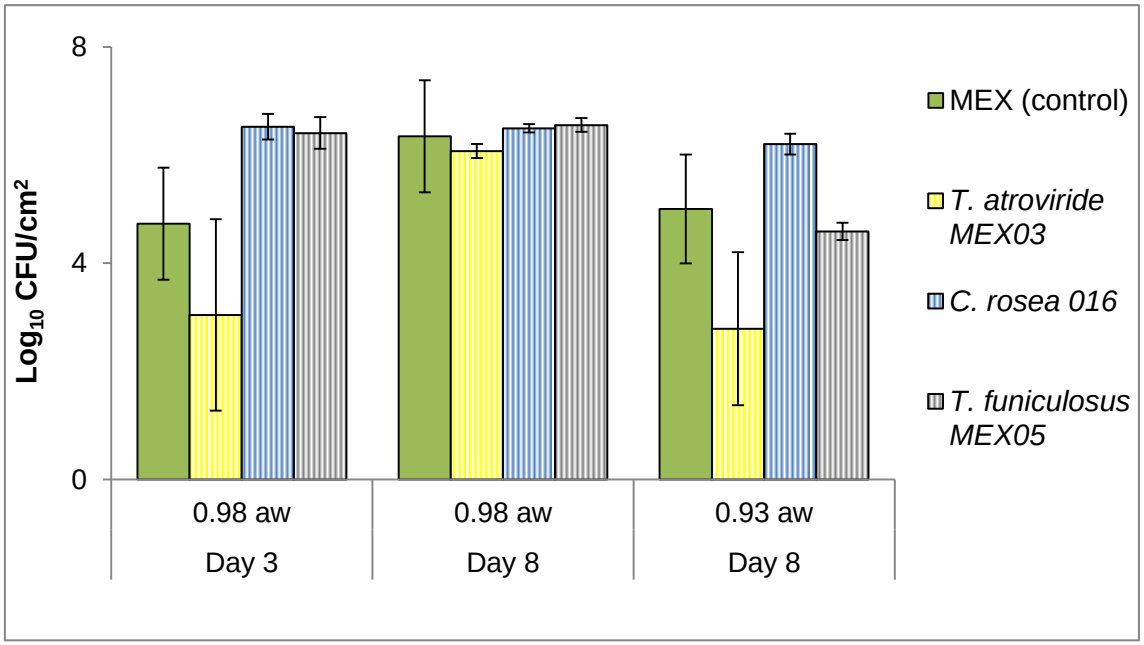


Figure 4.4 Viable conidial populations of the toxigenic *A. flavus* MEX01 strain log_{10} CFU/cm^2 of leaf, co-inoculated with different BCAs on MEA$^+$ for 3 and 8 days at 0.98 and 0.93 a_w at 25°C. Data are means of four replicates per treatment with standard error.

To differentiate between the toxigenic *A. flavus* MEX01 and the atoxigenic Afl$^-$ MEX02, the conidial populations were assessed on CAM. Figure 4.5 shows an example of the serial dilution medium. This shows that the toxigenic strain on the CAM medium produce a distinctive yellow sporulation within a thin white colony contour. The strain displayed a blue fluorescent ring under UV light, positive for aflatoxin production. The atoxigenic Afl$^-$ MEX02 colonies were mainly circular white and fluffy with a lighter yellow pigmentation and did not fluoresce under UV light. This allowed both strains to be quantified.

Figure 4.6 compares toxigenic and atoxigenic viable conidial production based on isolation from the senescent maize leaf segments. On day 3, at 0.98 a_w the atoxigenic strain (alone) produced significantly more viable CFUs (log 6.9) than the toxigenic strain (log 4.7). As water stress was imposed the viable populations of conidia became similar. Overall, there was no significant effect on the conidial production by the toxigenic strain.

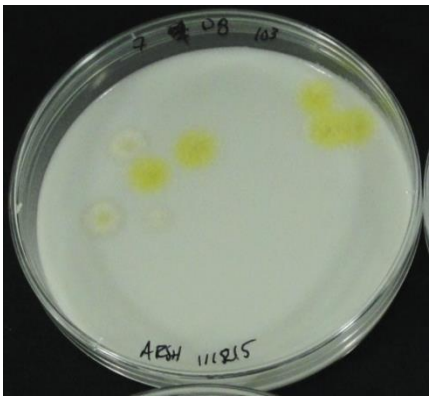


Figure 4.5 *A. flavus* inoculated on CAM incubated at 25°C. Toxigenic *A. flavus* MEX01 bright yellow colonies and Afl$^-$ MEX02 white colonies.

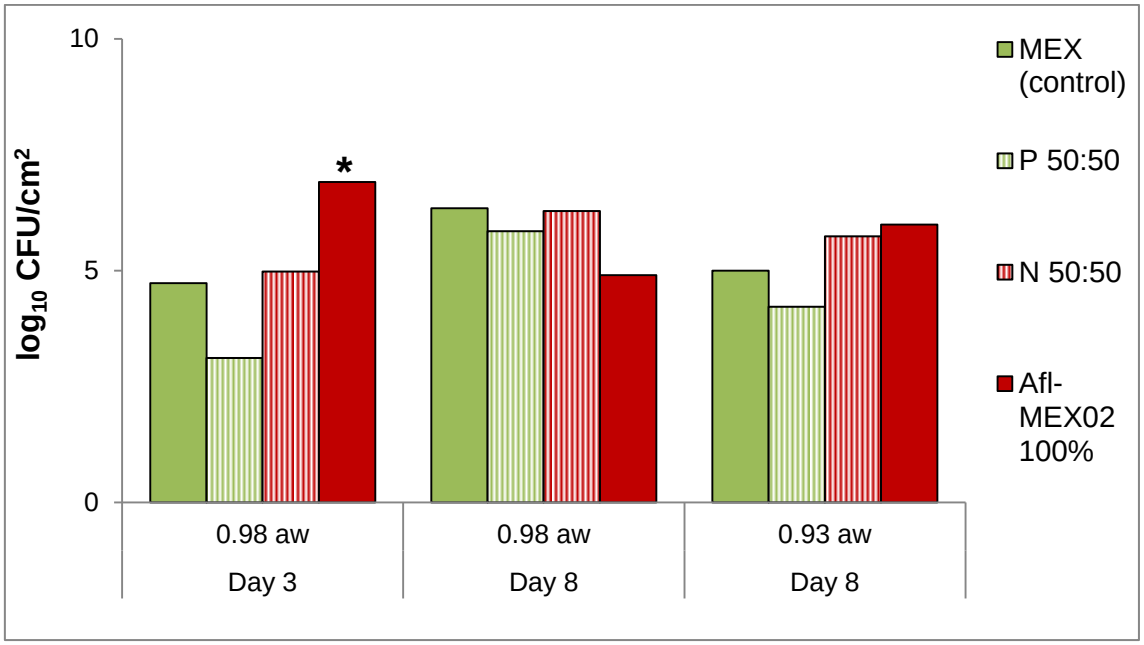


Figure 4.6 *A. flavus* log_{10} CFU/cm^2 of leaf, MEX01 and Afl^- MEX02 co-inoculated on CAM for 3 and 8 days at 0.98 and 0.93 a_w at 25°C. P=positive for aflatoxin production, N=negative for aflatoxin production. Mean of four replicates, means of the same treatment with an asterisk are significantly different from the control ($p<0.05$).

4.4 Discussion

Maize debris is a key reservoir for toxigenic *A. flavus* strains during its life cycle. Thus, the control of *A. flavus* inoculum potential at this stage has implications as a strategy to reduce aflatoxin contamination in groundnuts and maize (Bandyopadhyay *et al.*, 2016). However, few studies have examined whether the mechanism of action of atoxigenic strains mixtures or other BCAs is partially due to a reduction in *A. flavus* conidial sporulation on crop residue.

The present study has attempted to examine this in more detail for the first time. As *A. flavus* is xerophilic, BCAs must be able to compete in the environmental niches, due to ability of the toxigenic *A. flavus* strains to survive, become active and subsequently infect ripening maize as well as groundnuts under water stress conditions. The present study has shown that at both 0.98 and 0.93 a_w, during co-inoculation of potential BCAs with the toxigenic strain, there was very little control of the conidial production by the toxigenic *A. flavus* strain. Of particular interest was the fact that with three of the candidate BCAs, the sporulation by the toxigenic strain was actually stimulated. This does suggest that BCAs need to have the right environmental resilience to the temperature and a_w changes to effectively minimise sporulation capacity of toxigenic *A. flavus* strains. Previously, Giorni *et al.* (2012) analysed *A. flavus* temporal sclerotial sporulation on Czapek Dox Agar (CZ) and maize stalks, at different temperature and a_w conditions. They found that there was a significant difference between sporulation from sclerotia on day 3 and day 8, with a maximum occurring on the latter day. *A. flavus* sporulation on maize stalks was reduced with ≤0.90 a_w and with the maximum sporulation at 0.97 a_w. Battilani *et al.* (2013) utilised this information as part of a predictive model of *A. flavus* infection. The data that they used to develop the model included germination and sporulation rates on crop residue with a minimal a_w for sporulation rate of 0.90 and for spore germination of 0.85 a_w.

In the examination of the viable populations of the BCAs and the toxigenic strain it was important to understand whether the differences in total viable populations were due to the interactions between pathogen:antagonist or

whether dominance was influenced by the BCAs presence under different water stress conditions. At 0.98 a_w the atoxigenic strain had a higher population than the toxigenic strain at 0.98 a_w after 3 days incubation. From an ecological point of view the fact that all the other BCAs could not be recovered at 0.93 a_w suggests that they cannot compete effectively under water stress. This shows how important an understanding of the ecology of the BCAs is to try and identify those which will be effective in the niche where the pathogen may be active (Mohale *et al*., 2013a; Samsudin *et al*., 2016). *T. atroviride* MEX03 and *C. rosea* 016 have the ability to grow fast on decaying tissue, and produce conidia. However, these studies were mainly carried out at 0.995 a_w (Schubert *et al.* 2008; Zhang *et al.*, 2008). Also, sporulation could be influenced by the conidial number even if low conidial populations induces germination (Leeder *et al.,* 2011). It has been shown previously that some pathogens are able to germinate more effectively on leaf sheaths of cereal straw, and over a wider water activity range than other soil-borne fungi. Thus *Fusarium* and *Penicillium* species were able to effectively germinate over a wider range of a_w levels than other fungi which colonise crop residue such as *Trichoderma* and *Gliocladium* species (Magan, 1988).

In the present study, it was noticeable that the presence of some of the candidate BCAs stimulated asexual sporulation by the toxigenic *A. flavus* strain. This could be due to the *A. flavus* able to utilise breakdown products from hydrolytic enzyme activity of the BCAs releasing nutrients. However, previous studies to understand the mechanism of action of both atoxigenic strains of *A. flavus* in controlling AFB_1 production and *C. rosea* 016 in controlling fumonisin production in maize did not find any differences in Carbon utilisation patterns between the BCAs and pathogens (Mohale *et al*., 2013a; Samsudin *et al*., 2016).

4.5 Conclusions

This study suggests that it is very difficult for some BCAs to effectively reduce the inoculum potential of toxigenic *A. flavus* strains in crop debris. Indeed, the competitor fungi should effectively compete under the range of environmental conditions in which *A. flavus* is able to survive and grow when conditions become conducive. Many of the BCAs appear not to be xerophilic in nature and thus may only be able to compete over a narrower range of water availability and temperature conditions. This may explain why biocontrol strains used at present in West and East Africa consist of mixtures of atoxigenic strains to try and overcome this specific problem (Bandyopadhyay *et al.*, 2016). This present study suggests that the candidate BCAs are not effective at reducing the inoculum potential in this phase of the life cycle of *A. flavus*.

Chapter 5. Efficacy of the best biocontrol agents on aflatoxin B_1 production in stored maize grain under different a_w x temperature conditions

5.1 Introduction

Maize can be contaminated by *A. flavus* during its development, especially under drought stress and during insect attack. This can result in AFB_1 contamination continuing during poor post-harvest storage. This can represent a food safety risk to the consumer during downstream processing (Magan *et al.*, 2003). The grain quality will also be affected reducing the nutritional value of the grain. To prevent AFB_1 contamination, atoxigenic *A. flavus* strains are being used as BCAs to try and reduce the toxigenic inoculum load pre-harvest and apparently also during storage (Atehnkeng *et al.*, 2014). Previous studies have shown that control of mycotoxigenic fungi and mycotoxin production is influenced by environmental conditions, inoculum ratio of antagonist:pathogen and nutritional resources (Mohale *et al*., 2013a; Mohale *et al.*, 2013b; Al-Saad *et al.*, 2016; Samsudin *et al.*, 2016). It is thus critical that the chosen BCAs are able to adapt to the environmental conditions and effectively utilise the resources and displace the toxigenic strains. Thus in the present work it was important to examine the efficacy of the best candidate BCAs on stored maize grain under different antagonist:pathogen inoculum ratios and in different interacting environmental conditions of a_w levels.

The objectives of this Chapter were to:

a) Develop a moisture adsorption curve for gamma irradiated maize grains.
b) Compare the effect of different inoculum ratios of the best BCAs + toxigenic *A. flavus* strains (NRRL 3357; MEX01) on relative gene expression of *aflD* (structural) and *aflR* (regulatory) genes by *A. flavus* at 0.98 and 0.93 a_w at 30°C on stored maize grains for 10 days.
c) Evaluate the effect of the BCAs on AFB_1 production by the two toxigenic *A. flavus* strains (NRRL 3357 and MEX01).

5.2 Materials and Methods

5.2.1 Fungal strains

The two toxigenic *A. flavus* strains used in this study, and the two BCAs (*C.rosea* 016 and atoxigenic *A. flavus*) have been detailed in Chapter 3.

5.2.2 Inoculum preparation

The spore inoculum was obtained from seven-day old cultures on MMA (3%, 0.98 a_w) and the spore inoculum prepared as detailed previously in Section 3.2.2. Spore suspensions were diluted with sterile water to achieve the target concentration of $1x10^4$ spores/mL.

5.2.3 Irradiated maize grains moisture content

Under sterile conditions 5 g of the gamma irradiated maize grain (12kGys, Synergyhealth; Swindon, UK) were transferred to 25 mL Universal bottles. Known amounts of sterile water were added in the range of 0.5-3.5 mL. The samples were sealed, shaken and left to equilibrate overnight at 4°C. After equilibration, the samples were allowed to stabilize at 25°C and the a_w measured for three replicates of each sample using an AQUALAB® Series 3TE (Decagon Devices Inc., Pullman, Washington, USA). The samples were then dried overnight at 80°C to obtain the moisture content (MC). A moisture adsorption curve was obtained by plotting the a_w against the MC. An additional curve was plotted of the amount of added water against the a_w (Figure 5.1), to determine the amount of water needed to modify the grain to the target values for the experiment (0.98; 0.93 a_w). During the equilibration of the maize grain to the treatment a_w levels, the addition of 0.2 mL of the spore suspension were taken into account.

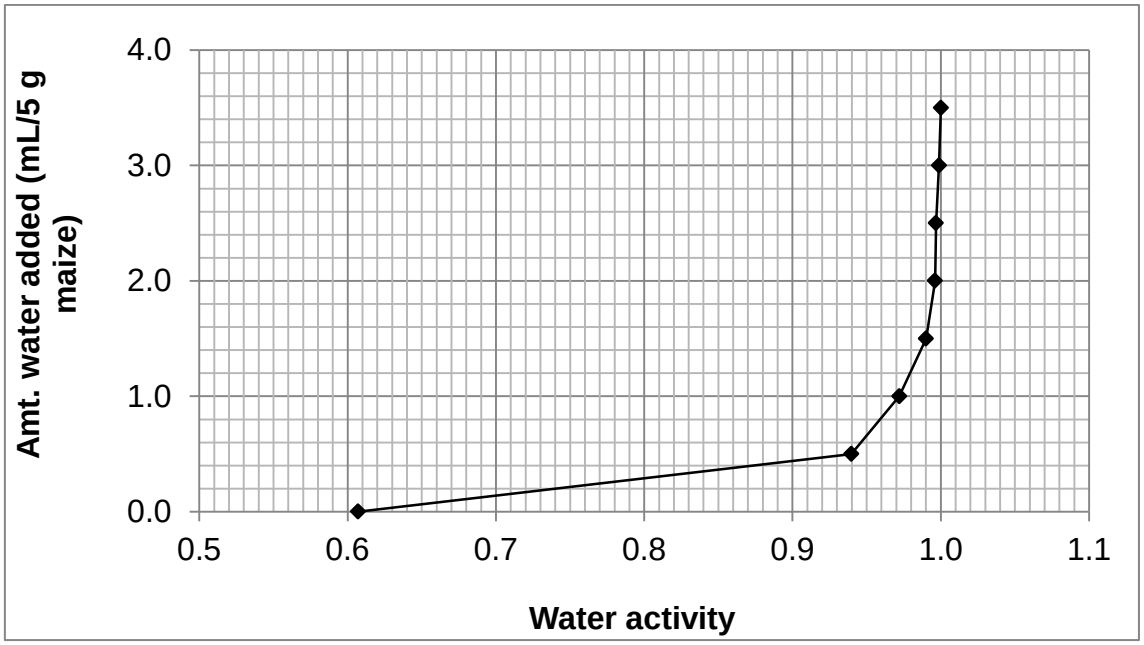


Figure 5.1 Adsorption curve for the amount of water needed to modify the maize grain water activity

5.2.4 Co-inoculation of BCAs with *A. flavus* at different inoculum ratios on irradiated maize grains

The best BCAs that affected AFB_1 production on MMA were selected for analysis *in vivo*, on irradiated maize grains, using different inoculum ratios. Spore suspension of each toxigenic *A. flavus* strain (NRRL 3357; MEX01) were co-inoculated with the two BCAs, Afl^- MEX02 and *C. rosea* 016, at different inoculum ratios (100:0; 75:25; 50:50; 25:75; 0:100; Samsudin and Magan 2016). Before inoculation the a_w of the maize grains was modified to 0.98 and 0.93 a_w, under sterile conditions, 10 g of maize grains were placed in sterilized glass culture vessels with microporous lids (Magenta™ Sigma-Aldrich, MO, USA) and the grains were left to stabilise overnight at 4°C. The spore suspension was prepared and counted as described in Section 3.2.2, vortexed and mixed at different inoculum ratios in 25 mL Universal bottles (Table 5.1). The maize grains (10 g) were equilibrated at 25°C and inoculated with 0.2 mL of the different inoculum ratios; the glass storage vessels were shaken to distribute the inoculum uniformly over the grain. Randomized block design was used; the treatments were 5 different inoculum ratios per each BCA and three

replicates of each treatment. The same a_w, toxigenic *A. flavus* strain and BCA were kept in the same plastic environmental chambers containing a 500 mL beaker with a water:glycerol solution to maintain the ERH at the same treatment level as the modified maize a_w. Three replicates per treatment were used and incubated at 30°C for 10 days. After this period 5 g of maize grain were dried for AFB_1 clean up using Immunoaffinity Colums (IAC) and quantification with HLPC-FLD. The other 5 g were frozen in liquid N_2 and stored at -80°C for subsequent RNA extraction and qPCR for the *aflD* and *aflR* gene expression.

Table 5.1 Summary of the different treatments and ratios of pathogen:antagonist used in these experiments at two a_w.

Treatment	*A. flavus* toxigenic (NRRL or MEX01)	BCAs	Concentration spores/mL
Positive Control	100	0	$1X10^4$:0
1	75	25	$7.5x10^3$:$2.5x10^3$
2	50	50	$5.0x10^3$:$5.0x10^3$
3	25	75	$2.5x10^3$:$7.5x10^3$
Negative control	0	100	0:$1X10^4$

5.2.5 Relative gene expression using qPCR

RNA extraction: two repetitions per treatment of 10 day were used, the positive controls and 50:50 mixture treatments only, as it has been done in the previous experiments. The negative control of Afl^- MEX02 was also extracted. Total RNA was extracted according to the Spectrum™ Plant Total RNA Kit protocol (Sigma-Aldrich, MO, USA) as described by the manufacturer and this was subsequently used for cDNA synthesis.

A contaminated maize grain was placed in a sterile Falcon tube (15 mL). It was resuspended in 1 mL of lysis solution previously supplemented with of β-mercaptoethanol 10 µL/mL. After vortexing the sample (30 s), the tubes were frozen in liquid N_2. Subsequently thawed, incubated at 56°C for 3 mins and centrifuged at maximum speed for a further 3 mins. The RNA extraction was done according to the procedure described in Section 3.2.4 with the DNase step added. For the On-Column DNase Digestion it was washed after binding the

RNA to it. Wash Solution 1 (300 µL) was added to the Binding Column and it was centrifuged at max speed for 1 min. The flow-through liquid was discarded. The RNase-Free DNase Set (Qiagen, Hilden, Germany) was used. A DNase I incubation mix was prepared beforehand according to the number of samples, containing per sample: 10 µL of reconstituted DNase I with 70 µL of Buffer RDD, mixed by hand and centrifuged. 80 µL of the DNase incubation mix were added directly to centre of the Binding Column, the cap closed and the column was incubated for 15 min at room 25°C. Then 500 µL of Wash Solution 1 were added to the Binding Column and centrifuge at max speed for 1 min. The flow-through liquid was discarded. 500 µL of Wash Solution 2 was added, the column centrifuged at maximum speed for 30 s, with the flow-through liquid discarded and the step repeated. The column was dried by centrifugation at a maximum speed for 1 min, and then the collection tube discarded. The column was transferred to a new labelled collection tube. The elution solution was pipetted (50 µL) into the centre of the column and centrifuged at maximum speed for 1 minute. The RNA quality, quantity and integrity were determined with 1.5 µL aliquot, following the protocol of the manual on the Experion™ Automated Electrophoresis System (Bio-Rad Laboratories Ltd., Hertfordshire, UK) with the Experion™ RNA StdSens Starter Kit which calculates the RNA Quality Indicator (RQI) and generates a virtual gel. RNA was stored at -80°C until cDNA synthesis.

The cDNA synthesis was done following the protocol with Oligo-dT primers of Omniscript® RT kit as previously described in Section 3.2.4. The RNA was diluted with RNase-free water to obtain 100 ng RNA in 20 µL of total cDNA reaction. Afterwards the qPCR was done using probes of aflatoxin genes labelled with the reporter molecule 6-carboxyfluorescein (FAM) at the 5’ end and the BlackHole Quencher 1 (BHQ1, Biolegio) at the 3’ end. The pair benTaq1, benTaq2 and benProbe were based on the *β-tubulin* gene. The benProbe was labelled with the reporter cyanine-5 (CY5) at 5’ end and the BlackHole Quencher 1 (BHQ1) at the 3’ end. The qPCR was done in the Thermal Cycler Bio-Rad CFX96 Real Time System C1000. The reaction mixture was prepared in an Eppendorf and afterwards distributed in individual PCR

tubes. The mix contained 1.5 µL of cDNA template (diluted 1:5) for a final volume of 12.5 µL. in the Thermal Cycler Bio-Rad CFX96 Real Time System C1000. The data analysis was made with the software Bio-Rad CFX Manager, Version 3.1 Relative quantification of the expression of *aflD* and *aflR* genes was performed with the reference gene *β-tubulin* as an endogenous control to normalise the quantification of the mRNA target for differences in the amount of total cDNA added to each reaction in the relative quantification assays and used for all treatments. The expression ratio was calculated as previously described by Livak and Schmittgen (2001). In this study, to analyse the effect of BCAs on target gene expression the calibrator corresponded to toxigenic *A. flavus* strains (NRRL 3357 and MEX01) grown singly on irradiated maize grains at different a_w levels. When the variability within technical replicates (n=2) of the *aflD* or *aflR* expression, was higher than 0.5 Ct , the data was not used (Nolan *et al.*, 2006).

5.2.6 Aflatoxin extraction, analysis with HPLC-FLD

a) Aflatoxins standard preparation

A seven-point calibration curve was made with working standards with different concentrations from 400-0.5 AFB_1 ng/mL, prepared as described in Section 2.2.5.1.

b) Aflatoxin extraction

AflaStar™ R - Immunoaffinity Columns (IAC, Romer Labs Inc., MO, USA), are used to extract and purify aflatoxins (B_1, B_2, G_1 and G_2) from feed and food using monoclonal antibodies. The column has a guaranteed retention capacity of 500 ng of total aflatoxins using PBS with 20% methanol, although the capacity can change using highly contaminated samples. The toxin extraction from irradiated maize grains was made following the protocol recommended by the manufacturer, with some modifications.

Solvents and buffers: the extraction solution chosen and prepared beforehand was methanol (HPLC grade): deionised water (60:40 v/v). The Phosphate Buffer Saline (PBS) was prepared following the Cold Spring Harbour Protocol. A stock solution of 1 M was prepared dissolving 4 g NaCl, 0.72 g Na_2HPO_4, 0.12 g

KH_2PO_4 and 0.1 g KCl adding deionised water to a volume of 500 mL. The PBS solution was autoclaved and stored at 25°C. To prepared the buffer recommended for the clean-up procedure of the IAC protocol, PBS 0.05 M/ 0.15 M NaCl with pH 7.4, 50 mL of PBS (1 M), 8.359 g of NaCl and deionised water were added for 1 L volume and mixed until the NaCl was dissolved. Afterwards the pH was checked with a pH meter and adjusted to 7.4 using NaOH or HCl.

Sample preparation: the maize grain samples were dried in the oven overnight at 80°C and ground with a Waring® Blender (2-speed 1 L, 8011ES, Waring® Laboratory Science, Torrington, CT, USA) and a blender container (Pulverizer, SS110). The ground samples were stored in a 50 mL Falcon tube, at room temperature protected from light until AFB_1 extraction.

Extraction: 4 g of the ground maize were added to the blender container with 2 g of NaCl and 16 mL of a solution of methanol:water (60/40 v/v), then blend for 3 minutes. The mix was filtered through qualitative filter paper (GE Healthcare Whatman, No.1, Dia. 90 mm, 1001-090, Fisher Scientific UK Ltd, Loughborough, UK) into a 100 mL Erlenmeyer flask. 3 mL of the filtrate were added to a 50 mL Falcon tube previously filled with 27 mL of PBS (PBS 0.05 M/ 0.15 M NaCl with pH 7.4) and shaken (if the filtrate was turbid it was centrifuged for 5 mins at RCF 4500), the pH (6-8) was checked with pH stripes. The rest of the extract was stored in a 15 mL Falcon tube and kept at -20˚C. 15 mL of the diluted filtrate were passed through the IAC, allowed to drip and the flow-through discarded. The IAC container was rinsed with 10 mL of deionised water and the column cleaned passing through 10 mL more. The column should not dry completely during the process. For the aflatoxins elution, the IAC was placed on top of a safe-lock Eppendorf and 2 mL of methanol (HPLC grade) passed through. The eluent was dried using a heat-block at 40°C (VWR® Analog Dry Block Heater, VWR International, LLC.) and N_2 (Figure 5.2). Between each treatment, the blender container and the lid were rinsed with sodium hypochlorite 1%, water and soap, tap water, deionised water, and dried with paper, then with air until complete dryness.

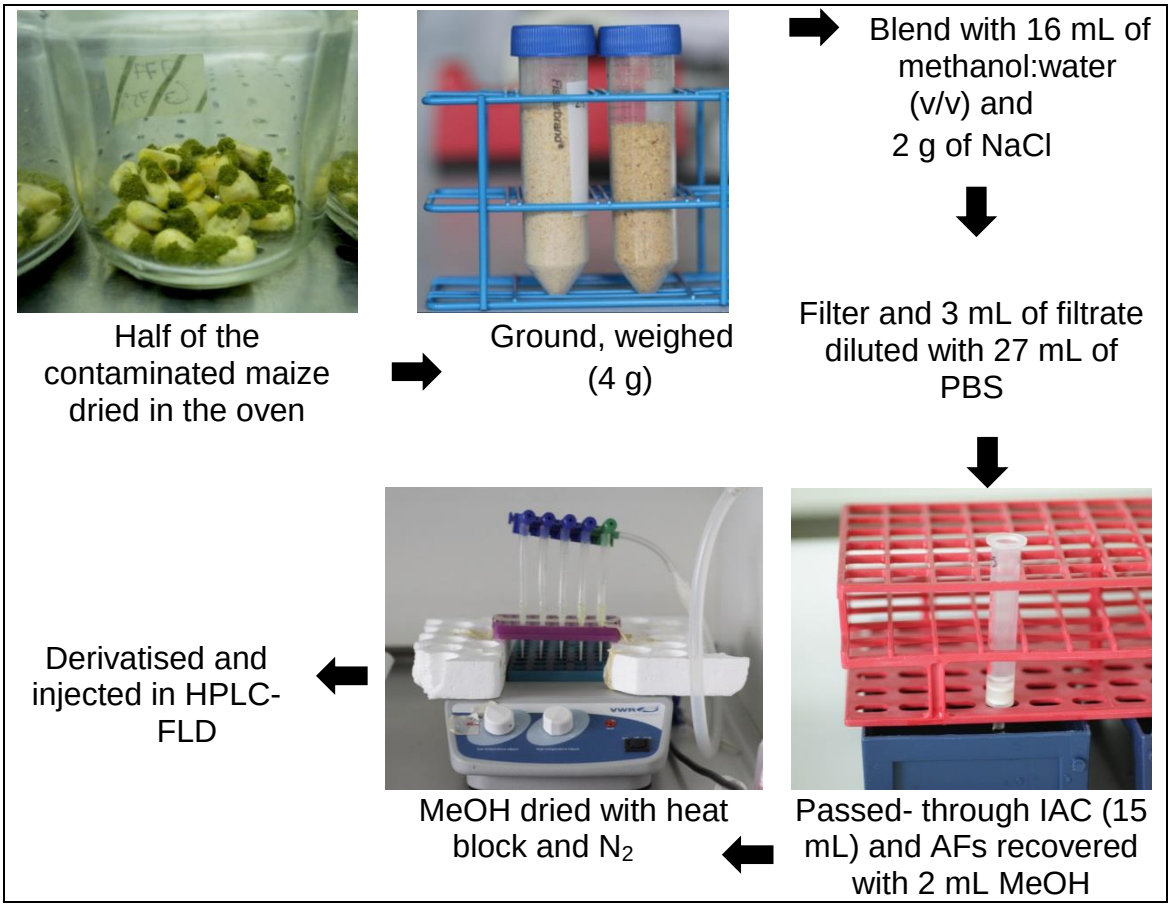


Figure 5.2 Diagram of aflatoxin purification from maize grain using IAC.

c) <u>Derivatisation and HPLC-FLD analysis</u>

Derivatisation and HPLC analysis were made as described previously in Section 2.2.5.1 with slight modifications. In this case, the analytical column was a Zorbax, Eclipse plus (Zorbax, Eclipse plus C_{18}, 4.6 x 100 mm, 3.5 µm, Agilent, USA). The injection volume 10 µL and the run time 12.5 min per sample. At the beginning of each run the standards were injected, one standard between every 10 samples and at the end of the run. The solvents used were HPLC grade. The standard curve was made in Excel (Microsoft® Excel®) plotting the area obtained with the HPLC software (ChemStation for LC Systems Rev.B.04.02 SP1 (208), Agilent Technologies 2001-2010) against the AFB_1 standard concentration injected. The standard curve was done to find the correlation using linear regression (Figure 5.3). The AFB_1 produced was quantified as ng/g of maize grain (dry weight).

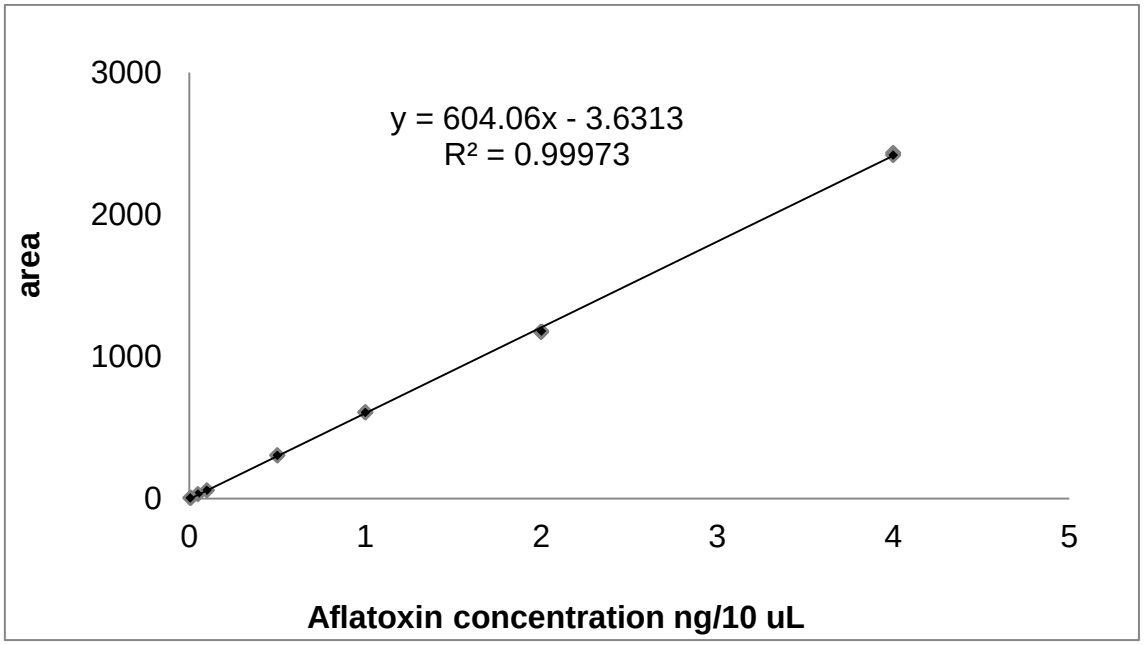


Figure 5.3 AFB_1 standard curve for quantify the AFB_1 in the samples.

5.2.7 Statistical Analysis

All treatments were replicated at least three times. AFB_1 production data was transformed using (log_{10} + 1) before statistical analysis. The relative gene expression data was normalized against the reference gene, with $2^{-\Delta\Delta CT}$ method, that allows the calculation of the expression ratio between the sample and the calibrator gene (Livak and Schmittgen, 2001). Shapiro-Wilk test and Levene's test were used to assess normality and variance homogeneity, respectively. When any of the assumptions was not achieved, the data was analysed with non-parametric tests, Kruskal-Wallis to analyse if there was difference between treatments and Mann-Whitney to compare the treatments with the control. Otherwise ANOVA analysis was applied and post-hoc Tukey's Honest Significant Difference (HSD) to compare the significant differences between treatments. R version 3.1.2 (2014-10-31) -- "Pumpkin Helmet". Copyright (C) 2014 The R Foundation for Statistical Computing.

5.3 Results

5.3.1 Interaction between BCAs and *A. flavus* on stored maize grain

a) *Effect of different inoculum ratios of toxigenic BCAs on* A. flavus *relative gene expression of aflatoxin biosynthetic genes*

Figure 5.4 shows the effect of interactions between the BCAs and the toxigenic *A. flavus* strains on *aflD* and *aflR* expression in the 50:50 ratio treatments. The relative expression of these genes by the toxigenic strains NRRL 3357 and MEX01 (100:0 ratio) was used as a positive control and calibrator. Figure 5.4a shows the effect of the atoxigenic Afl^- MEX02 on *A. flavus* NRRL 3357 *aflD* expression at 0.93 a_w. This atoxigenic strain almost completely inhibits *aflD* expression of the toxigenic strain during co-inoculation. Furthermore, the relative expression of the atoxigenic strain in the negative control (0:100 ratio; pathogen:BCA) expression was lower compared to the toxigenic strain. With more available water (0.98 a_w) *C. rosea* 016 down-regulated the toxigenic strain (MEX01) *aflD* expression (Figure 5.4b). The atoxigenic Afl^- MEX02 strain also decreased *aflR* expression (Figure 5.4d). Under water stress the atoxigenic Afl^- MEX02 strain (0:100, negative control) had *aflD* down regulation when compared to the toxigenic *A. flavus* MEX01 strain. Unfortunately, in some of the other treatments and replicates the variability in the *aflD* and *aflR* expression was higher than the 0.5 Ct within biological and technical replicates.

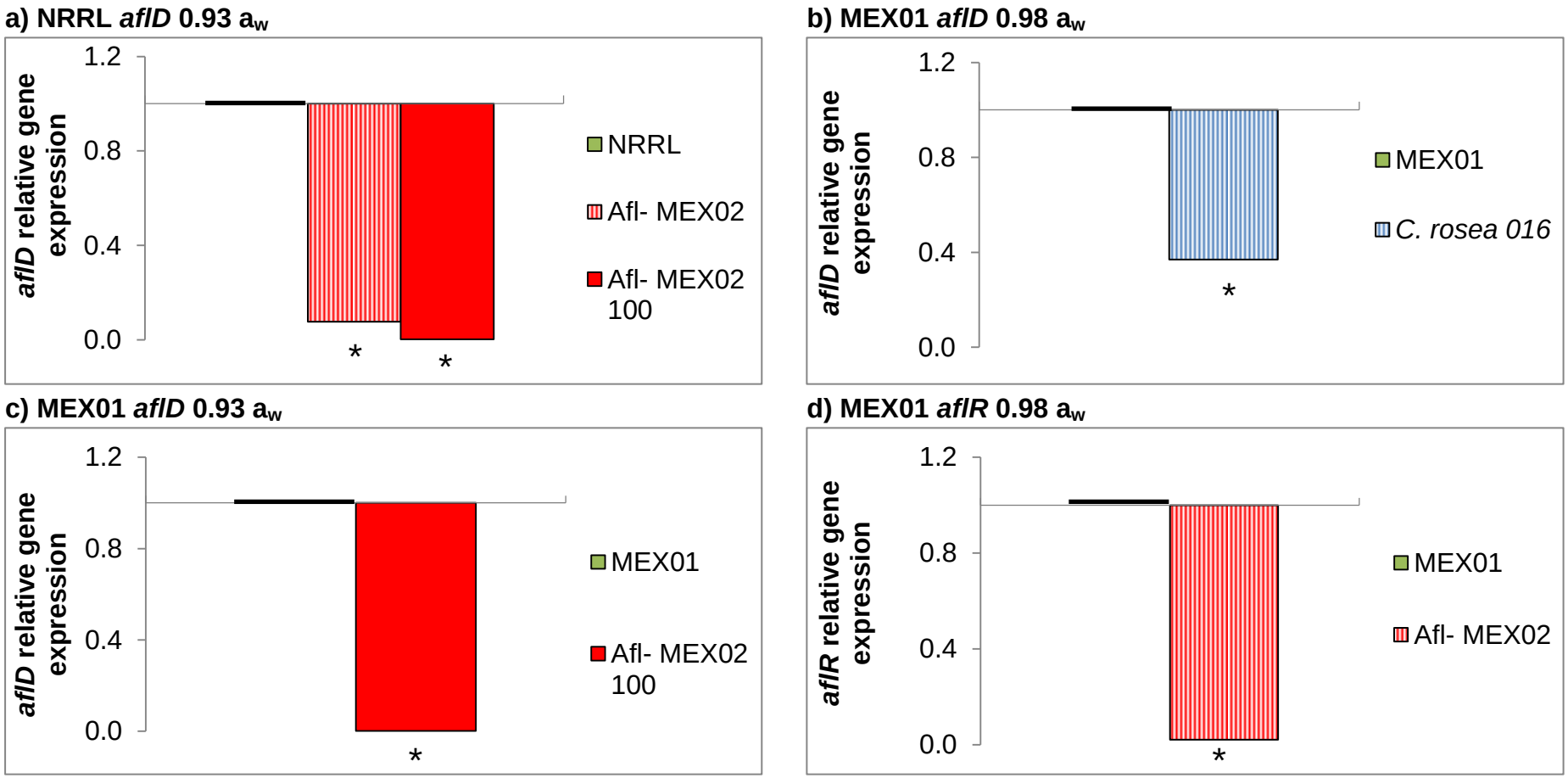


Figure 5.4 Relative gene expression values of *aflD* and *aflR* in *A. flavus* toxigenic strains (NRRL 3357 and MEX01) inoculated with the BCAs (50:50 ratio) on irradiated maize grain incubated at 30°C for 10 days at 0.98 and 0.93 a_w. Toxigenic strain (100:0) was used as the calibrator =1. Means of the same BCA followed by asterisk are significantly different compared to the control ($p<0.05$).

b) *Effect of different inoculum ratios of BCAs on AFB_1 production by* A. flavus *toxigenic strains*

Table 5.2 shows the effect of the atoxigenic Afl^- MEX02 and *C. rosea* 016 strains during co-inoculation with both toxigenic strains on AFB_1 production at different inoculum ratios on the stored maize grains at 0.98 and 0.93 a_w incubated at 30°C for 10 days. With more freely available water both BCAs reduced significantly AFB_1 production by >60% ($p<0.05$) at 50:50 and 25:75 inoculum ratios compared to the control and different inoculum ratios from same BCA and a_w. Also at 0.93 a_w, the atoxigenic *A. flavus* strain was able to decrease significantly the production >90% ($p<0.05$) at the same inoculum ratios (50:50 and 25:75). Regardless of the a_w level the atoxigenic strain Afl^- MEX02 (0:100, negative control) produced significantly lower amounts of AFB_1 when compared to the toxigenic strain. AFB_1 production by NRRL 3357 at 0.93 a_w was unaffected by the presence of *C. rosea* 016 BCA regardless of the inoculum ratio.

Table 5.3 shows the effect of the BCAs inoculated with toxigenic *A. flavus* MEX01 on AFB_1 production. With more freely available water the atoxigenic Afl^- MEX02 decreased AFB_1 production >80% at high inoculum concentration (25:75, $2.5x10^3$:$7.5x10^3$ spores/mL, toxigenic MEX01:Afl^- MEX02). Under water stress AFB_1 production by the toxigenic *A. flavus* MEX01 strain was decreased >85% when co-inoculated with the atoxigenic Afl^- MEX02 strain at 50:50 or higher inoculum ratios (50 and 75%). *C. rosea* 016 was not effective at inhibiting AFB_1 production by the toxigenic *A. flavus* MEX01, regardless of the inoculum ratio or a_w level at 30°C.

Table 5.2 AFB_1 production by type strain NRRL 3357 in mixed inoculum with BCAs at different ratios incubated for 10 days at 30°C on irradiated maize grains modified to 0.98 and 0.93 a_w.

		AFB_1 (ng/g) ± SE		
a_w	**%**	**NRRL 3357 (control)**	**Afl⁻ MEX02**	***C. rosea* 016**
0.98	100:0	45548.69 ± 2561.01 [a]		
	75:25		82870.59 ± 19527.51 [a]	38545.57 ± 17007.93 [ab]
	50:50		**15223.95 ± 843.30 [b]**	**11449.62 ± 3803.46 [b]**
	25:75		**6377.38 ± 1648.90 [c]**	**15909.38 ± 11398.62 [b]**
	0:100		**5.49 ± 0.80 [d]**	-
0.93	100:0	61930.29 ± 19823.38 [a]		
	75:25		9658.12 ± 2995.09 [ab]	76262.67 ± 10193.64 [a]
	50:50		**5562.96 ± 3690.37 [b]**	23642.16 ± 7413.17 [a]
	25:75		**2905.71 ± 1024.33 [b]**	69372.92 ± 19270.99 [a]
	0:100		**7.48 ± 1.02 [c]**	-

Data are means of triplicates ± standard error. Means of the same treatment (BCA and a_w) followed by different letter are significantly different ($p<0.05$). Bolded and underlined data means reduction of AFB_1 production compared to the control.

Table 5.3 AFB_1 production by isolate strain MEX01 in mixed inoculum with BCAs at different ratios incubated for 10 days at 30°C on irradiated maize grains modified to 0.98 and 0.93 a_w.

		AFB_1 (ng/g) ± SE		
a_w	%	MEX01 (control)	Afl$^-$ MEX02	*C. rosea* 016
0.98	100:0	45996.12 ± 16006.38 [a]		
	75:25		10694.28 ± 8645.15 [ab]	39558.86 ± 4409.98 [a]
	50:50		8953.60 ± 3891.26 [ab]	32442.52 ± 7112.37 [a]
	25:75		**4929.27 ± 3977.21 [b]**	29599.38 ± 2436.87 [a]
	0:100		**5.49 ± 0.80 [c]**	-
0.93	100:0	27787.85 ± 5271.57 [a]		
	75:25		10241.95 ± 406.14 [ab]	33623.01 ± 3006.49 [a]
	50:50		**3855.11 ± 1441.09 [bc]**	28508.95 ± 1263.22 [a]
	25:75		**3552.33 ± 1894.00 [c]**	49133.30 ± 17918.31 [a]
	0:100		**7.48 ± 1.02 [d]**	-

Data are means of triplicates ± standard error. Means of the same treatment (BCA and a_w) followed by different letter are significantly different ($p<0.05$). Bolded and underlined data means reduction of AFB_1 production compared to the control.

5.4 Discussion

The stored maize experiments were designed to examine whether the results *in vitro* on maize-based media were consistent in stored maize grain. Both results, the qPCR for *aflD* and *aflR* and AFB_1 production suggest that the BCAs at 50:50 (antagonist:pathogen) or higher ratios inhibited toxin production significantly. Previously, Dolezal *et al.* (2013) found that *A. flavus* is capable of colonising maize grain with different gene clusters being over expressed during pathogenesis.The impact on *aflD* and *aflR* expression was previously demonstrated for 50:50 ratio of potential bacterial cells and *A. flavus* spores on maize-based media under different a_w levels (Al-Saad *et al.*, 2016). They suggested that this was a good indication of efficacy for AFB_1 control. Verheecke *et al*. (2015) found that five out of six Streptomyces strains could down-regulate aflatoxin biosynthetic genes, with one bacterial strain stimulating the expression of some of the key structural and regulatory genes. This suggests variability between bacterial strains in efficacy in inhibiting the biosynthetic genes involved in mycotoxin production, in range between 7.7-fold to 100-fold.

AFB_1 production by the toxigenic type strain (NRRL 3357) was higher under water stress at 0.93 a_w than with more available water at 0.98 a_w. in stored maize grain than in previous *in vitro* studies (see Chapter 4). For the toxigenic MEX01 strain toxin production was higher on colonised maize grain at 0.98 a_w. This may partially be due to the relative nutritional status of maize grain vs maize-based media. Overall, the atoxigenic Afl^- MEX02 strain was the most effective BCA in reducing AFB_1 production by both toxigenic strains tested. In most cases at 50:50 or 75:25 ratio in favour of the BCA, there were significant reductions in AFB_1 contamination on stored maize grain. In most cases this achieved >60% control. Previously, Mohale *et al.* (2013b) tested atoxigenic strains from Lesotho, southern Africa, found significant reduction of AFB_1 in stored maize grain with >70%. The *C. rosea* 016 was only effective at 0.98 a_w. At 0.93 a_w it was not able to compete effectively against the toxigenic *A. flavus* strain and displace it or influence toxin production. This suggests that the ability

to adapt and colonize is different from the atoxigenic strain of *A. flavus*. The former is not a xerophile and thus unable to compete effectively under water stress conditions. In contrast, the atoxigenic *A. flavus* may be at an advantage and able to effectively compete with the toxigenic strains. It has also been suggested that it is important to use native strains from the same region in which you want to control the pathogen. The atoxigenic strain was obtained from Mexican white maize. It was more effective than the *C. rosea* 016 which was isolated from crop debris in Europe. This has previously been suggested by others (Mohale *et al.*, 2013b; Bandyopadhyay *et al*., 2016). Giorni *et al*. (2009) and Mohale *et al*. (2013a) also studied the growth, carbon source (CS) utilisation patterns and Niche Overlap Indices (NOI) of toxigenic and atoxigenic *A. flavus* strains, at different a_w and temperatures. Giorni *et al*. (2009) and Mohale *et al*. (2013a) analysed 24 CS that represent the principal components of maize grains. Amylopectin represents 60% of the content of the maize grain dry matter and is consumed by *A. flavus* at 30°C. Mohale *et al*. (2013a) suggested that the CS utilization patterns were similar for both toxigenic and atoxigenic strains. It is possible that there is competition for nutrients when mixed inocula are colonizing maize grains.

In the present study, *C. rosea* 016 was not as effective against *A. flavus* as was found against *Fusarium* spp., in contrast to the results obtained by Luongo *et al*. (2005), Palazzini *et al*. (2013) and Samsudin and Magan (2015). Luongo *et al.* (2005) analysed *C. rosea* against *Fusarium* spp. on wheat straw, maize stubble and ears. On wheat straw and maize stubble, it was effective in decreasing sporulation of the pathogen. On maize ears, it reduced the colonization by 50%. Palazzini *et al.* (2013) used *C. rosea* against *Fusarium* on wheat stalks, under field conditions. They suggested that the success of *C. rosea* was due to its ability to colonize decaying tissue faster than *Fusarium*. The previous analysis on senescent maize leaves under different environmental conditions (Chapter 4) showed that *C. rosea* 016 was not able to grow faster that *A. flavus*. Samsudin (2015) showed that at 0.98 a_w *C. rosea* 016 inhibit fumonisin B_1 production by >50%. However, under water stress it stimulated toxin production at 50 and 75%. Samsudin *et al*. (2016) analysed the NOI and the CS

consumption of *C. rosea* 016. The BCA was able to use 10 CS at 30°C regardless of the a_w compared to the 21 CS that *F. verticilloides* FV1 could use. *C. rosea* 016 consumed the carbohydrates before the amino acids and preferred amylose over amylopectin. If *C. rosea* 016 is not able to outcompete *A. flavus* and obtain the nutrients from the grain it would not be effective as a BCA for *A. flavus* control. *A. flavus* can influence metabolic changes in the maize grain during its infection and might cause the up-regulation of sucrose hydrolysing enzymes to obtain glucose (Dolezal *et al.*, 2014)

5.5 Conclusions

This study showed the importance of analyzing the effect of BCAs using different inoculum ratios and environmental conditions on AFB_1 production by toxigenic *A. flavus strains* on stored maize grain. There were differences in efficacy *in vitro* on maize-based media and on stored maize grain in reducing AFB_1. The relative gene expression of the structural *aflD* and the regulatory gene *aflR* were down-regulated in the presence of the BCAs. The most effective BCA was the atoxigenic Afl^- MEX02 which was capable of reducing AFB_1 production by both toxigenic strains (NRRL 3357 and MEX01) by >60% on stored maize grain at 50% and 75% antagonist:pathogen inoculum ratios, under all conditions tested. While *C. rosea* 016 was able to grow on the maize grain regardless the condition, it was not successful at reducing AFB_1 production. The atoxigenic Afl^- MEX02 strain was successful against both toxigenic strains *in vitro* and in stored maize grain. The next phase was to examine whether control can be achieved in maize cobs of different ripening stages which represent different nutritional levels and also naturally different a_w levels.

Chapter 6. Impact of the best two biocontrol agents on control of aflatoxin B_1 on maize cobs of different ripening stages.

6.1 Introduction

Aspergillus flavus is a pathogen that can colonise maize cobs during any reproductive stage, from silk (R1) to maturity (R6). During silking, insect damage and drought stress can increase the risk of infection by providing entry points to the fungal spores. During silking the nutritional status of the ripening maize grains changes from free amino acids to protein, and soluble sugars to starch accumulation. During this process the nutrients move from the endosperm to the embryo which influences the moisture content and thus water availability from 90% to <25% at maturation (Ingle *et al.*, 1965; Picot *et al*., 2011). This will influence fungal infection and represents conditions under which *A. flavus* colonisation and AFB_1 contamination can occur (Magan and Aldred, 2007b).

Previous studies have analysed *A. flavus* and/or maize gene expression during infection using microarrays (Georgianna *et al*., 2010; Reese *et al*., 2011; Dolezal *et al*., 2014) or maize defense genes during co-inoculation of *A. flavus* with an atoxigenic strain (Lanubile *et al*., 2017). The latter studies were not focused specifically on the genes related to aflatoxin biosynthetic genes or the biocontrol agent (BCA). The effect of the BCAs on aflatoxin biosynthetic gene expression and concomitant AFB_1 production by *A. flavus* needs to be quantified under the natural water activity conditions of ripening maize cobs of different maturity. Recently, Samsudin *et al.* (2017) examined the potential for control of *F. verticillioides* and fumonisin B_1 production in different ripening stages of maize cobs. This suggested that colonisation and biocontrol were influenced by ripening stage of the maize cobs. It is surprising that no such similar studies have been focused on examining potential biocontrol of *A. flavus* and AFB_1 production in maize cobs of different ripening stages.

The objectives of this Chapter were:

a) Measure the a_w of maize cobs at different ripening stages (Milk R3, Dough R4 and Dent R5).
b) Examine the efficacy of the atoxigenic *A. flavus* strain (Afl^- MEX02) and *C. rosea* 016 on control of AFB_1 production by toxigenic *A. flavus* strain (MEX01) when co-inoculated in 50:50 conidial inoculum ratios in maize cobs of different ripening stages (R3, R4 and R5) at 30°C.
c) Evaluate the effect of the BCAs on the relative toxigenic *A. flavus* strain gene expression of *aflD* and *aflR* in the different ripening stages of the maize cobs in (b).
d) Quantify the effect of the two BCAs on AFB_1 production by toxigenic *A. flavus* strain.

6.2 Materials and Methods

6.2.1 Fungal strains

The toxigenic *A. flavus* strain (MEX01), the atoxigenic *A. flavus* strain (Afl^- MEX02) and *C. rosea* 016 were used in this study.

6.2.2 Inoculation preparation

The fungal strains were point inoculated individually with a sterile needle, making 3 points on 3% MMA (0.98 a_w) and incubated at 25°C for 7 days and the conidial spore suspensions made as described in Section 3.2.2. The spore suspensions were diluted as required to obtain a concentration of $1x10^4$ spores/mL.

6.2.3 Maize cobs sampling at different ripening stages

The maize cobs were collected at different ripening stages from the National Institute of Agriculture and Botany (NIAB, Cambridge, UK). The type of maize was ES Regain (Euralis Semences, used for feed).

Table 6.1 shows the three reproductive stages which were Milk (R3), Dough (R4) and Dent (R5) and their main characteristics. The cobs were taken at the different ripening stages taken to the laboratory and snap frozen with liquid nitrogen and stored at -20°C until use (Samsudin *et al.*, 2017). Sub-samples were used to detach some maize kernels. The kernels were placed in a water activity meter container and placed in the AQUALAB® Series 3TE (Decagon Devices Inc., Pullman, Washington, USA) to measure the water activity at 25°C.

Table 6.1 Maize cob characteristics and appearance at three different growth stages (R3, R4 and R5; Ritchie *et al*., 1992 ; Nielsen, 2001*; Picot et al*., 2011).

Growth Stages	
Milk (R3) The grains contain a “milky” white fluid. The starch is accumulating in the endosperm. The moisture content is between 80-70%.	
Dough (R4) The fluid is changing consistency due to starch accumulation. The moisture content is between 70-60%.	
Dent (R5) The milk line appears, dividing the milky fluid from the starch. The starch accumulation continues and the line moves to the tip. The moisture content is between 50-40%.	

6.2.4 Maize cob preparation for fungal inoculation

The flash-frozen cobs were thawed at 4°C for 24 h. Subsequently under sterile conditions the husks were removed, the cobs were cut in three pieces (approx. 5-6 cm each piece) and distributed in three different environmental chambers according to the ripening stage. The cob pieces were left to stabilise for three hours at 25°C until inoculation. Spore suspensions were made as detailed in Section 6.1.2. The control treatment was the toxigenic *A. flavus* MEX01 in the 100:0 ratio ($1x10^4$:0 spores/mL) with the BCA and the other treatments

consisted of a 50%:50% pathogen:antagonist ratio of conidia ($5x10^3$:$5x10^3$ spores/mL). The cob pieces were inoculated with 100 µL of the treatments and incubated at 30°C for 10 days. R3 and R4 stages had three replicates (a cob piece per replicate) and R5 stage had four. The bottom of each chamber was covered with aluminium foil and contained a beaker (250 mL) with 200 mL of a sterile solution of glycerol/water to maintain the ERH at the same level as the cob a_w (Figure 6.1). The colony diameter was measured at the end of the incubation period (10 days). For *aflD* and *aflR* gene expression, under sterile conditions, a dozen contaminated kernels were removed with forceps and spatula, frozen in liquid N_2 and kept at -80°C for subsequent RNA extraction and qPCR. The rest of the cob was kept for AFB_1 extraction, clean-up by IAC and quantification with HPLC-FLD.

Figure 6.1 Illustration of the cob pieces distribution (R4 stage) for inoculation to analyse the effect of the BCAs on AFB_1 production and gene expression (*alfR* and *aflD*) by *A. flavus* MEX01.

6.2.5 Fungal colonisation of maize cobs

The fungal growth on maize cobs at different ripening stages was measured after 10 days incubation. The colony extension was measured in two directions at right angles to each other to calculate the radial colonisation. The colony radius was measured from the centre to the edge following the perimeter of the cob, with a cellophane strip due to its flexibility as shown in Figure 6.2. Then the strip was marked and compared with a ruler. The results were expressed as colony diameter (mm) following the formula: $d = r\ x\ 2$.

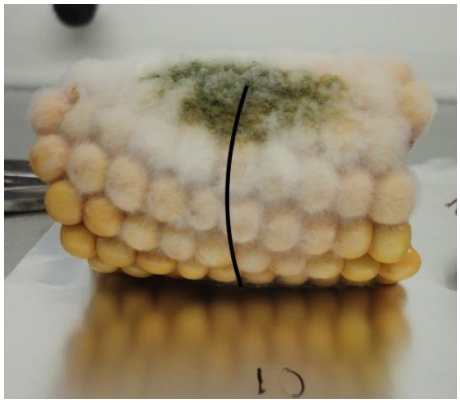


Figure 6.2 Illustration of fungal growth measurement on the maize cob. The black line represents the radius measurement taken with the cellophane strip.

6.2.6 Relative gene expression

RNA extraction: the 10 day treatments were used in triplicate (biological repetitions) including the positive controls and 50:50 mixture. Total RNA was extracted according to the Spectrum™ Plant Total RNA Kit protocol as described by the manufacturer and then used for cDNA synthesis.

Three maize grains with symptoms were ground into a fine powder with liquid N_2, in a pre-frozen mortar and pestle previously sterilized with 70% IPA. Liquid N_2 was added as needed to keep the sample frozen. Afterwards, approx. 50 mg of the sample were transferred to a frozen sterile 0.2 mL graduated skirted tube with EasyGrip screw cap (STARLAB Ltd, Milton Keynes, UK). One mL of lysis solution previously supplemented with β-mercaptoethanol 10 µl/mL was added, vortexed (30 s) and frozen at -80°C until use. Afterwards, the samples were thawed on ice, incubated at 25°C for 3 mins and centrifuged at maximum speed for a further 3 mins. The RNA extraction was done as described in Section 3.2.4 with the On-Column DNase Digestion (Section 5.2.4). The RNA was eluted with 50 µL of elution buffer, the quantity, quality and integrity done with a 1.5 µL aliquot with Experion™ Automated Electrophoresis System and Experion™ RNA StdSens Starter Kit. The RNA was stored at -80°C until cDNA synthesis.

For cDNA synthesis, the reverse transcription protocol was done with Oligo-dT primers of Omniscript® RT kit as previously described in Section 3.2.4. Then the qPCR was made to analyse the expression of the structural gene *aflD* and the

regulatory gene *aflR* of the aflatoxin biosynthetic pathway. The qPCR was done in the Thermal Cycler Bio-Rad CFX96 Real Time System C1000 as previously described in Section 5.2.5. The reaction mixtures were prepared in duplicate for each biological sample per gene and two non-template controls (NTC) were added in each run. Relative quantification of the expression of *aflD* and *aflR* genes was performed with the reference gene *β-tubulin* as an endogenous control to normalise the quantification of the mRNA target. In this study, to analyse the effect of the biocontrol agent on the target gene expression of the toxigenic *A. flavus* strain, MEX01 was grown on cobs at different ripening stage as the calibrator.

6.2.7 Aflatoxin extraction, analysis with HPLC-FLD

Aflatoxins standard preparation, aflatoxin extraction from the sample and clean up with IAC following the protocol previously described in Section 5.2.6 with some modifications.

a) Aflatoxin extraction

The whole cob was placed in a beaker, dried in the oven at 60°C for 4 days, cooled, and stored in a desiccator protected from light until use. All the dried grains were removed in the safety cabinet and ground with the Waring® blender container and blender. The ground samples were stored in a 50 mL Falcon tube at 25°C protected from light until AFB_1 extraction. For the extraction 5 g of the ground maize were added to the blender container with 2 g of NaCl and 20 mL of methanol:water solution (60/40 v/v) and blend for 3 minutes. The mix was filtered through qualitative filter paper and the process previously described in Section 5.2.6b was followed.

b) Derivatisation and HPLC-FLD analysis

After the sample was passed through the IAC, the methanol was evaporated and derivatised following the procedure previously described in Section 2.2.5.1 Likewise, the aflatoxins were quantified in the HPLC-FLD with the modification mentioned in Section 5.2.6. The analytical column was ZORBAX Eclipse plus

C_{18} column (4.6 x 100 mm, 3.5 µm, Agilent, USA). The injection volume was 10 µL and the run time 12.5 min per sample. The standard curve was made in Excel plotting the area obtained with the HPLC software against the AFB_1 standard concentration injected. The AFB_1 analysed was quantified as ng/g of maize grain (dry weight).

6.3 Statistical analysis

The treatments had three or four replicates. Before the statistical analysis the relative gene expression data was normalised with the reference gene and the expression of the treatments compared to the control. AFB_1 production was transformed ($\log_{10}$ + 1). To assess normality and variance homogeneity the tests were Shapiro-Wilk and Levene. If the assumptions were met, the data was analysed with t-test or ANOVA and Tukey's Honest Significant Difference (HSD) as post-hoc. When the data did not achieve the assumptions, Kruskal-Wallis was used and Mann-Whitney to compare the treatments. The analyses were done with R version 3.1.2 (2014-10-31) -- "Pumpkin Helmet". Copyright (C) 2014 The R Foundation for Statistical Computing.

6.4 Results

6.4.1 Interaction between the BCAs and toxigenic *A. flavus* MEX01 on maize cobs of different ripening stages

a) Effect of the BCAs on fungal growth of the toxigenic A. flavus *strain*

The a_w of the different developing stages were Milk (R3) 0.985 a_w, Dough (R4) 0.976 a_w and Dent (R5) 0.958 a_w. Figure 6.3 shows the effect of the atoxigenic Afl^- MEX02 and *C. rosea* 016 on the toxigenic *A. flavus* MEX01 growth on maize cobs of different ripening stages. The BCAs did not affect the colonisation of the cobs by the toxigenic strain (MEX01) when compared to the control. Also, the ripening stage had no effect on the colonisation by the toxigenic strain. The colony morphology of *A. flavus* MEX01 growth (control) during co-inoculation with BCAs, had a similar appearance within treatments. In the R3 and R4 cobs the colonies were white and cottony with light green sporulation at the centre. In the most mature cobs (R5; 0.958 a_w) morphologically there appeared to be higher conidial production than in the R3 and R4 maize ripening stages. The growth of colonies on maize cobs at stage R5 was significantly bigger compared to the growth of colonies on R3 ($p \leq 0.05$).

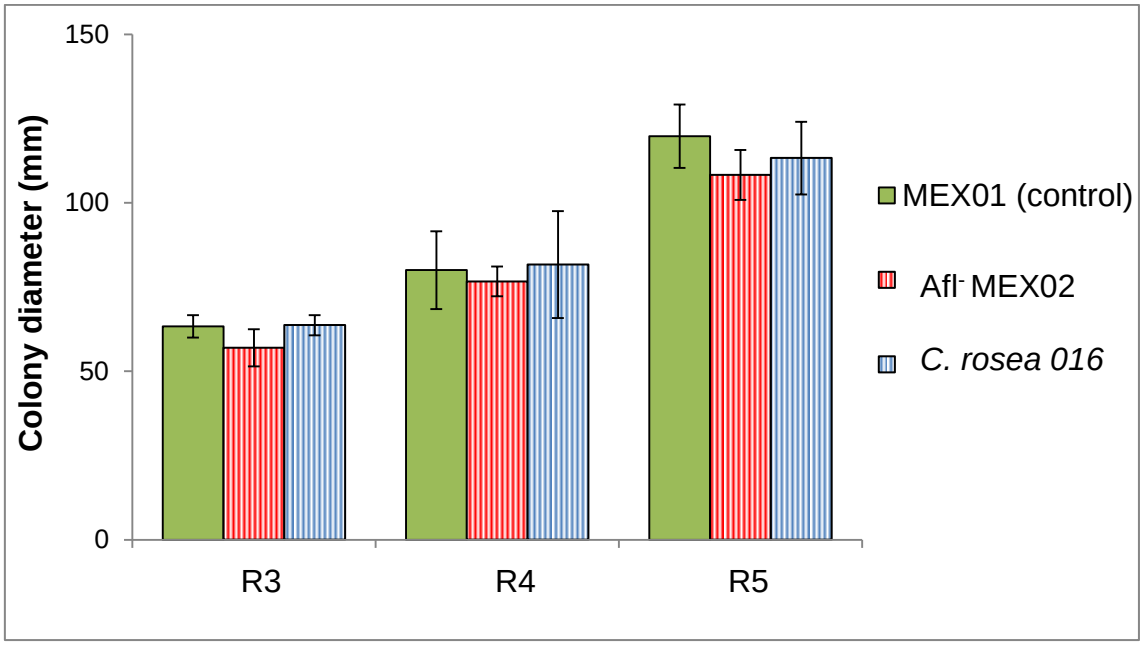


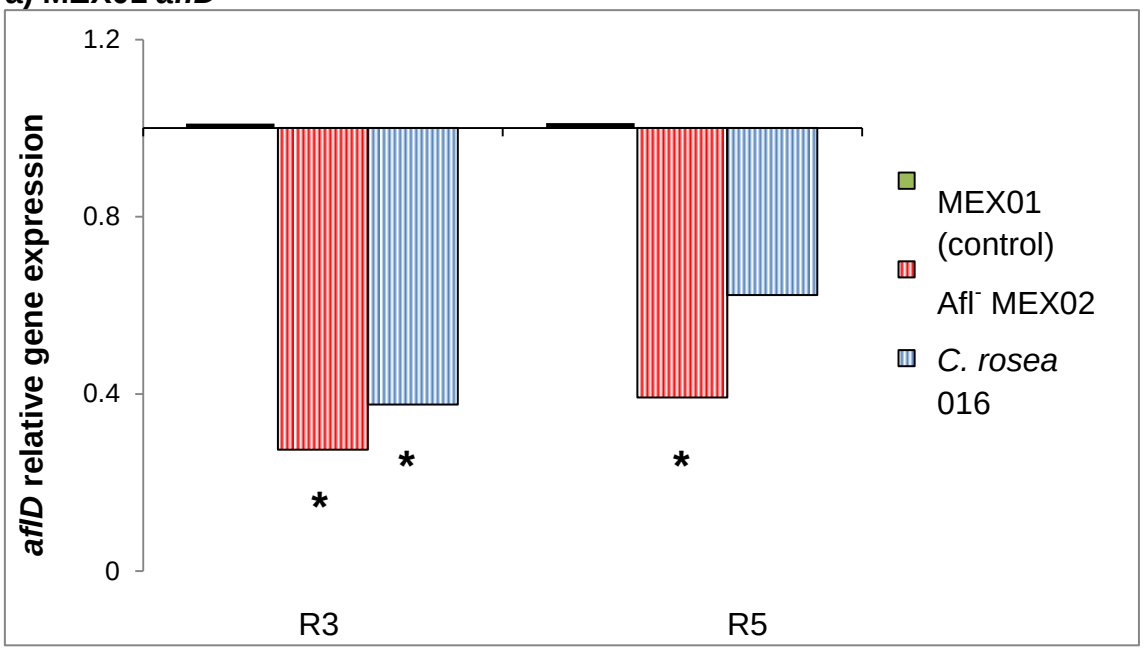
Figure 6.3 Colony diameter (mm) of toxigenic *A. flavus* MEX01, + atoxigenic Afl$^-$ MEX02, and + *C. rosea* 016 strains, inoculated in a 50:50 conidial ratio on maize cobs of different ripening stages (R3, 0.985 a_w; R4, 0.976 a_w; and R5, 0.958 a_w) incubated at 30°C for 10 days. Data are means of three replicates for R3 and R4 cobs and four replicates for R5 ± standard error. The graph shows the comparison within rippening stages.

b) *Effect of BCAs on relative gene expression of the two aflatoxin biosynthetic genes by the toxigenic* A. flavus *MEX01 strain*

Figure 6.4 shows the effect of the two BCAs on the relative gene expression of *aflD* (structural) and *aflR* (regulatory) genes by the toxigenic *A. flavus* MEX01. At R3 stage (0.985 a_w) both BCAs decreased significantly the expression of the structural gene (*aflD*) in >70 and >60% ($p<0.05$). In the R5 (0.958 a_w) treatment the atoxigenic Afl$^-$ MEX02 strain significantly inhibited the *aflD* gene expression of the toxigenic strain by >60% with a $p<0.05$ (Figure 6.4a). Figure 6.4b compares *aflR* gene expression by the toxigenic strain at the three different ripening stages when co-inoculated with the two BCAs. In the R3 ripening stage, there was a decrease in *aflR* expression in the presence of the atoxigenic strains by >80% and with the *C. rosea* 016 by >50%. At R4 stage

(0.976 a_w) the atoxigenic BCA strain was also able to inhibit significantly ($p<0.05$) *aflR* expression by >85%.

a) MEX01 *aflD*



b) MEX01 *aflR*

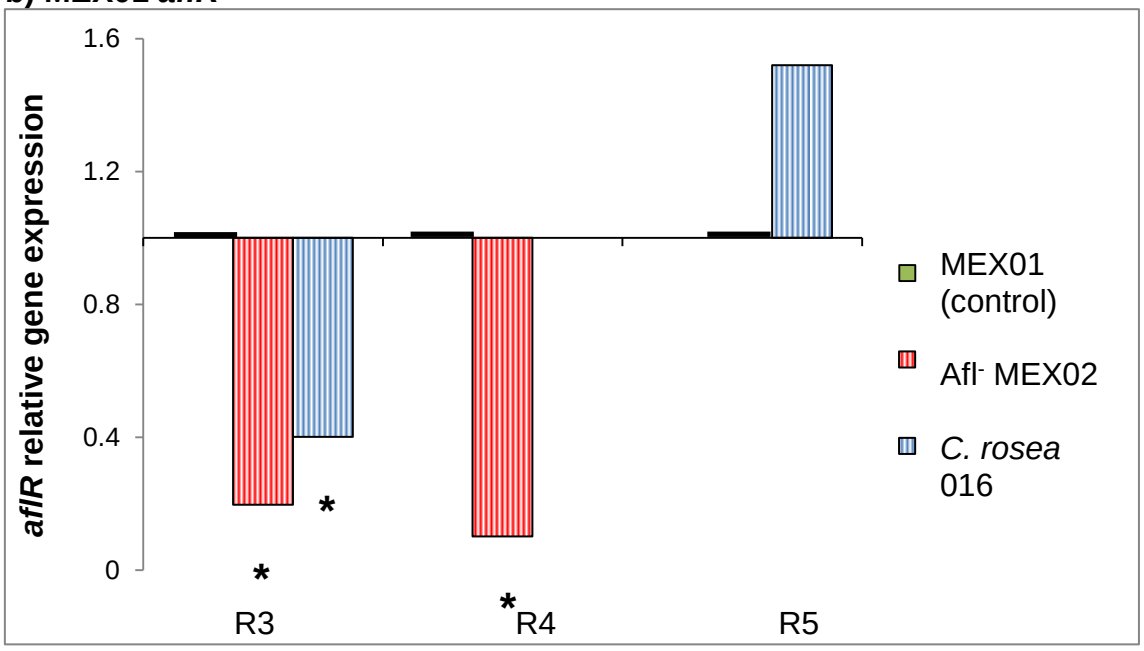


Figure 6.4 Relative gene expression values of *aflD* and *aflR* by the toxigenic *A. flavus* MEX01 strain co-inoculated with BCAs (50:50 ratio) on maize cobs at different ripening stages (R3, R4, R5) incubated at 30°C for 10 days. The control (100:0 ratio) was used as the calibrator (1). Data are means of triplicates ± standard error. Means of the same treatment with an asterisk are significantly different from the control ($p<0.05$).

c) *Effect of the two BCAs on AFB_1 production by* A. flavus *MEX01*

Figure 6.5 shows the effect of the atoxigenic *A. flavus* strain and *C. rosea* 016 on AFB_1 production by the toxigenic *A. flavus* strain in the three different maize cob ripening stages. Overall, AFB_1 production was unaffected by the presence of the BCAs, regardless of the ripening stage.

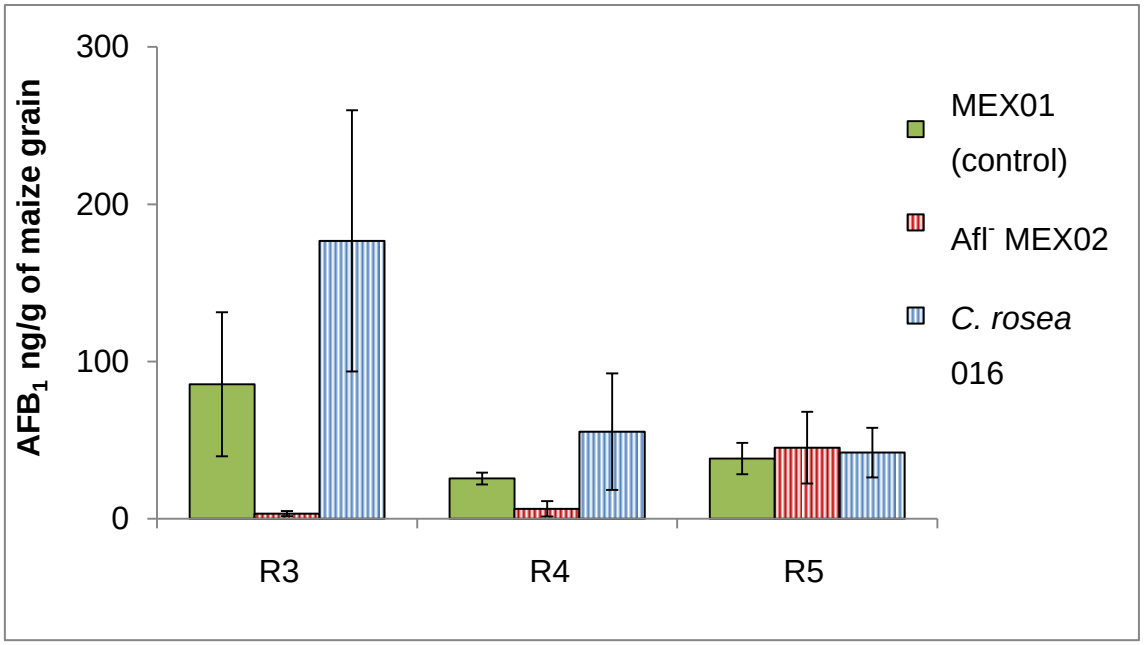


Figure 6.5 Aflatoxin B_1 production by the toxigenic *A. flavus* MEX01 strain when inoculated in 50:50 ratio of conidia on maize cobs at three different ripening stages incubated for 10 days at 30°C. Data are means ± standard error.

6.5 Discussion

This Chapter analysed the efficacy of the candidate BCAs when co-inoculated with the toxigenic *A. flavus* strain on maize cobs of different ripening stages. The stages represented different a_w levels and nutritional compositions during the silking process. However, the ripening stage did not affect the ability of the toxigenic strain to colonise the cobs. While it was able to colonise the maize cobs at all ripening stages, the growth was significantly bigger at R5. This suggests that *A. flavus* is able to colonise maize cobs rapidly during silking, if entry points are available for infection. The temperature used in this study represents optimum conditions for the colonisation of maize by *A. flavus* (Battilani *et al.*, 2013). Indeed, many studies have suggested that 0.98-0.99 a_w is also optimum for growth of *A. flavus* on both synthetic media and on ripened maize grain (Abdel-Hadi *et al*., 2012; Mohale *et al*., 2013a). Virulent strains of *A. flavus*, like the MEX01 strain used in this study appeared to be able to colonise the cob ripening stages even if the kernels were not directly damaged, causing physiological damage characterised by browning of the kernels. Previously it has been suggested that *A. flavus* is adapted to the ripening stages of maize expressing specific genes to utlise the CS available (Reese *et al*., 2011;Dolezal *et al*., 2014). *C. rosea* 016 may not be suitable as a BCA on maize cobs at higher temperature, as its growth was negatively affected by the temperature and by the nutrient availability. This was previously shown by Samsudin *et al*. (2017). They analysed the relative utilisation patterns of C-sources in maize by *C. rosea* 016 and *F. verticillioides* (FV1), both in vitro and co-inoculated on maize cobs at different ripening stages (R3, R4 and R5). At 30°C in the R3 ripening stage no growth of the mixed inoculum (FV1 and *C. rosea* 016) occurred when compared to the growth measured in the R4 and R5 stages. FV1 grew faster on the cobs at the 3 ripening stages when incubated at 25°C.

The capability of the BCAs to affect the gene expression of the structural (*aflD*) and regulatory gene (*aflR*) suggested some effects on the toxigenic *A. flavus* strain used. This certainly indicated a decrease in both the structural *aflD* and regulatory *aflR* genes. This was >50-60% overall, depending on maize ripening

stage. The results in the R3 ripening stage of the maize cobs were similar to those obtained in the 0.98 a_w treatments on stored maize grain (see Chapter 5). Verheecke *et al.* (2015) studied the efficacy of *Streptomyces* strains against toxigenic *A. flavus* on synthetic media. They examined 5 different AFB_1 biosynthetic genes. In their study the *aflR* expression was decreased by a *Streptomyces* strain, but *aflD* expression was unaffected by any strain. However, there studies did not include the impact of a_w level which may have affected the relative control. In the study by Al-Saad *et al*. (2016) examining efficacy of bacterial strains on *aflD* and *aflR* relative expression, they found significant decreases in these genes in the presence of the BCAs, however this was not always clear from relative toxin control.

AFB_1 production by toxigenic *A. flavus* MEX01 was not significantly different in the three ripening stages examined. The two candidate BCAs were not effective at decreasing AFB_1 production despite the results obtained with the gene expression. Thus these results are different from those obtained in stored mature maize grain where efficacy was clear at both 0.98 and 0.93 a_w at 30°C (see Chapter 5). However, in the present study only 50:50 ratio of antagonist:pathogen was used. In the previous study different ratios were used, including 50:50 ratio. It is possible that the relative threshold inoculum level of the antagonists necessary for AFB_1 control were not reached in the present study on the different ripening stages. The actual ratio when mixed represented 50 µL each BCA ($1x10^3$ spores/mL) of each antagonist and pathogen. This may be too low to have any effect on growth and toxin production by *A. flavus*. Samsudin *et al.* (2017) used a higher concentration of spores, 100 µL each BCA ($1x10^6$ spores/mL) of the antagonist and pathogen when examine the control on *F. verticillioides* and fumonisin B_1 production on maize at different ripening stages. Other studies with BCAs suggest an inoculum level of the antagonist of at least $1x10^{5-6}$ spores/mL is required (Mohale *et al*., 2013b; Samsudin *et al.,* 2017). Thus, perhaps the threshold concentrations of the BCAs were not reached to effectively control AFB_1 production. This also suggests that similar studies as those completed in Chapter 5 with different ratios may help to

identify what the threshold of antagonists are needed for controlling AFB_1 on ripening maize cobs.

It may also be that because *A. flavus* is a xerophilic fungus able to colonise maize of the whole range of ripening stages that for effective control much higher BCA inoculum levels are needed for effective control. The approach used by Bandyopadhyay *et al*. (2016) is to use atoxigenic strains which are grown on sorghum grain and apply these to the soil during early growth of maize crops. These atoxigenic strains appear to reduce the inoculum potential of the toxigenic strains and thus less inoculum is available for infection of maize cobs during silking. However, the survival of the atoxigenic strains, especially on crop debris has not been examined previously. The present study (see Chapter 4) certainly suggested that it is difficult to reduce incoculum potential of toxigenic *A. flavus* strains for asexual conidial reproduction under a range of water availability conditions. This area needs more focus, as well as the relationship between pest control and *A. flavus* control which can be intimately related because of the damage that pests can cause (e.g. corn borers).

6.6 Conclusions

The present study demonstrated the importance of analysing the effect BCAs directly on maize cobs at different ripening stages, on potential for control of toxigenic *A. flavus* strains. This study certainly showed that toxigenic strains of *A. flavus* are able colonise the maize cobs regardless of the ripening stage. In this present study, with the 50:50 ratio of BCA:pathogen both *aflD* and *aflR* relative expression was down-regulated by the atoxigenic *A. flavus* and the *C. rosea* 016 strain in all ripening stages and R3 for the latter. However, because this was not translated into control/inhibition of AFB_1 the inoculum threshold of the BCAs may not have been reached for effective control. More studies are required to compare different ratios of BCA:pathogen on different ripening stages of maize to identify the optimum levels necessary and whether this would be economically feasible.

Chapter 7. Potential effects of climate change scenarios on the biocontrol agents and on control of aflatoxin B_1 contamination of maize cobs of different ripening stages

7.1 Introduction

It has been suggested that interacting climate change (CC) environmental factors will have a significant impact on food security of staple commodities. Indeed, it terms of fungal pathogens and pests of cereals it has been suggested that the diversity of pathogens/pests will increase under CC scenarios and that they are predicted to be moving at up to 7 km/year towards the poles (Bebber *et al*., 2013, 2014). Magan *et al*. (2011) suggested that perhaps under extreme CC conditions (interacting conditions of increased temperature, elevated CO_2 and water stress) mycotoxigenic xerophilic fungi such as *A. flavus* and other species such as *Wallemia sebi* may become more important. However, few studies have examined the effect of these three interacting CC factors on such mycotoxigenic fungi although recent studies have been done on *F. verticillioides* in maize and *F. graminearum* in wheat (Vaughan *et al.*, 2014; Váry *et al.*, 2015). However, practically no studies have examined the interaction between BCAs and pathogens under CC conditions.

Recently, Medina *et al.* (2015) made the first study of the impact of changing CC environmental conditions (water stress, high temperature and high CO_2 levels) on a toxigenic *A. flavus* strain. This study showed that while CC interacting factors did not appear to affect growth of this strain, there was stimulation of AFB_1 production in both 650 and a 1000 ppm CO_2 at 37°C when compared to that at 30 and 34°C. This was supported by molecular analyses of the structural *aflD* and regulatory *aflR* genes which both increased significantly. A study of the effects of high CO_2 levels on maize and *F. verticillioides* infection showed that under CC conditions the maize plant was more susceptible to *F. verticillioides* contamination, but fumonisin B_1 production was not increased (Vaughan *et al.*, 2014). However, drought stress was not included in this study. Váry *et al.* (2015) showed that repeated acclimatisation of strains of both *F.*

graminearum and *Septoria tritici* for 20 and 10 generations respectively resulted in increased pathogenicity and symptoms of Fusarium head blight and leaf disease under CC CO_2 levels. However, the potential impact on Deoxynivalenol was not investigated. The effect of CC factors on pathogen:antagonist interaction needs to be addressed, as the strains used as a BCA may need to adapt to the environmental conditions to compete against the toxigenic fungal pathogen under CC scenarios (Atehnkeng *et al*., 2008; Ehrlich, 2014; Bandyopadhyay *et al*., 2016). Such studies are very scarce. Recently, Borisade and Magan (2015) examined CC effects on relative efficacy of entomopathogens for control of pests under different RH/Temperature and CO_2 regimes. They found that pest control efficacy was reduced under CC scenarios.

The objectives of this study were:

a) Evaluate the impact of interactions between a_w, temperature (30 and 37°C) and CO_2 (400 and 5000 ppm) on the atoxigenic *A. flavus* (Afl^- MEX02) strain and *C. rosea* 016 BCAs and efficacy against the growth of toxigenic *A. flavus* MEX01 strain when inoculated in 50:50 inoculum ratios during co-inoculation on maize cobs at different ripening stages (R3, R4 and R5) under different environmental conditions specified.
b) Examine the influence of CC factors in (a) and BCAs on relative expression of two key genes of aflatoxin biosynthetic pathway (*aflD* and *aflR*) in the toxigenic *A. flavus* strain.
c) Quantify the effect of the two BCAs on AFB_1 production by the toxigenic *A. flavus* strain under normal and extreme CC conditions in (a).

7.2 Materials and Methods

7.2.1 Fungal strains and inoculum preparation

The strains used were the toxigenic *A. flavus* MEX01 and two BCAs Afl$^-$ MEX02 and *C. rosea* 016, described in Section 3.2.1. To prepare the spore suspension the fungi were point inoculated on 3% MMA (0.98 a_w) as previously described in Section 3.2.2. The spore suspension was diluted to reach a concentration of $1x10^4$ spores/mL.

7.2.2 Maize cobs sampling at different ripening stages

The maize cobs used for the Milky ripe stage (R3) were a Sweetcorn variety (use for food) purchased from a supermarket. The cobs were taken to the laboratory and stored at 4°C overnight. The sampling of the maize cobs at R4 and R5 stages was previously described in Section 6.1.3. The a_w was measured by detaching grains from the cobs and measured in the laboratory at 25°C using the AQUALAB® Series 3TE (Decagon Devices Inc., Pullman, Washington, USA).

7.2.3 Preparation of maize cobs for inoculation

Cobs at R3 stage were stored at 4°C overnight. The flash-frozen cobs at R4 and R5 stage were thawed at 4°C for 24 h. Subsequently the cobs of the three stages (R3, R4 and R5) were stabilised at 25°C. Afterwards, under sterile conditions, the husks were removed; the cobs were cut in three pieces (approx. 5-6 cm) and distributed in 6 environmental chambers according to ripening stage and temperature. The bottom of the chambers was covered with aluminium foil. Two beakers (250 mL and 500 mL) were placed inside the chamber, containing a sterile solution of glycerol:water (200 mL and 500 mL, respectively) to maintain the ERH as the a_w of the cob. The spore suspensions were prepared as described in Section 7.2.1 to achieve the concentration of $1x10^4$ spores/mL.

a) R3 inoculation

A grain was punctured with a 0.4 cm sterile cork-borer and inoculated with 10 µL of spore suspension. The toxigenic MEX01 *A. flavus* strain was used as a positive control and the 50:50% treatments ($5x10^3$:$5x10^3$ spores/mL) with the BCAs were the atoxigenic *A. flavus* strain Afl^- MEX02 and *C. rosea* 016. In all cases four replicates per treatment were used.

b) R4 and R5 inoculation

A grain was pin-inoculated with a sterile inoculation needle, previously dipped in the spore suspension. The treatments were as detailed previously with a 50:50 spore ratio ($5x10^3$:$5x10^3$ spores/mL) of antagonist to pathogen used for inoculation. For the R4 cobs there were four replicates per treatment and for the R5 cobs there were 4-5 replicates.

After inoculation, the cobs were incubated for 10 days at two different environmental conditions 30°C/400 ppm CO_2 and the other simulating extreme CC conditions 37°C/5000 ppm CO_2. R3 had 4 treatments, positive control and two BCAs. R4 and R5 had two treatments, positive control and 50:50% with AFl^- MEX02. The treatments were separated per a_w and temperature/CO_2. The CO_2 treatments were incubated in a Sanyo CO_2 incubator (MCO-18 IAC, Electric Biomedical Co., Ltd). The atmospheric ERH in the incubator was maintained with 1.5 L of glycerol:water solution in the base of the chamber which was replaced after 5 days. Fungal growth was measured after 5 and 10 days as described previously (Section 6.1.5). On day 10 the mycelium was removed with sterile pre-frozen forceps and spatula. It was immediately frozen in liquid N_2 and kept at -80°C for subsequent RNA extraction and qPCR to analyse the expression of the structural gene (*aflD*) and regulatory gene (*aflR*). The cobs were kept at -20°C until AFB_1 extraction and quantification with HPLC-FLD.

7.2.4 Relative gene expression

RNA extraction: the 10 day treatments of the R4 and R5 cobs were used in triplicate. Total RNA was extracted following the protocol of the Spectrum™ Plant Total RNA Kit and afterwards used for cDNA synthesis.

The fungal biomass was ground into a fine powder with liquid N_2 in pre-frozen mortar and pestle previously sterilized with 70% IPA, adding liquid N_2 as needed. Then approx. 50 mg of the sample were placed in an autoclaved and pre-frozen 2.0 mL graduated skirted tube with EasyGrip screw cap. The sample was resuspended in 0.75 mL of lysis solution supplemented with β-mercaptoethanol 10 µL/mL. Immediately the mix was vortexed for 30 s, frozen in liquid N_2 and kept at -80°C until use. The samples were thawed in ice, incubated at 56°C for 3 mins then centrifuged at maximum speed for a further 3 mins. RNA extraction was done as the protocol described in Section 6.1.6. Quantity, quality and integrity were analysed with Experion RNA StdSens analysis kits and Experion™ Automated Electrophoresis System. The RNA was stored at -80°C until cDNA synthesis.

The cDNA synthesis, reverse transcription was made with the protocol of the manufacturer and Oligo-dT primers of Omniscript® RT kit. Afterwards the qPCR was done with the cDNA to analyse the relative gene expression of *aflD* and *aflR*, as previously described in Section 3.2.5. *A. flavus* MEX01 grown singly on the maize cobs at different ripening stages was the calibrator.

7.3 Aflatoxin extraction, analysis with HPLC-FLD

AFB_1 standards were prepared to obtain a seven-point calibration curve with different concentrations (400-0.5 AFB_1 ng/mL) as described in Section 2.2.5.1.

a) Aflatoxin extraction

For aflatoxin extraction, the cobs were placed inside a beaker and dried in the oven at 60°C until complete dryness. The dried grains of R3 stage were removed from a 16 cm^2 area around the wounded grain, due to limited colony growth. For R4 and R5: All the dried kernels were removed from the cob inside

the safety cabinet. The grains obtained from the different ripening stages were ground in a Waring® blender container and blender. The following steps for aflatoxin extraction and clean up with the IAC were previously described in Section 5.2.6b.

b) Derivatisation and HPLC-FLD analysis

The methanol was evaporated after recovering the toxin from the IAC. The samples were derivatised with TFA as described in Section 2.2.6. AFB_1 quantification was done with HPLC-FLD as explained in Section 6.1.7

7.4 Statistical analysis

R3 had 3 treatments with 4 replicates. R4 and R5 had 2 treatments with 3 and 4 replicates, respectively. The gene expression data was normalised with the reference gene and the expression of the treatments compared to the control. AFB_1 production was transformed logarithmically. Normality was assessed with Shapiro-Wilk and Levene for variance homogeneity. Depending on the data characteristics it was analysed with t-test or one and two-way ANOVA and Tukey's Honest Significant Difference (HSD) as post-hoc or Kruskal-Wallis with Mann-Whitney to compare treatments. When the data did not come from a normal distribution, the analysis done was pairwise comparing each condition (different a_w and different temperature/CO_2). The analysis was done with R version 3.1.2 (2014-10-31) -- "Pumpkin Helmet". Copyright (C) 2014 The R Foundation for Statistical Computing.

7.5 Results

7.5.1 Interaction of the BCAs with the toxigenic *A flavus* MEX01 during co-inoculation on maize cobs of different ripening stages

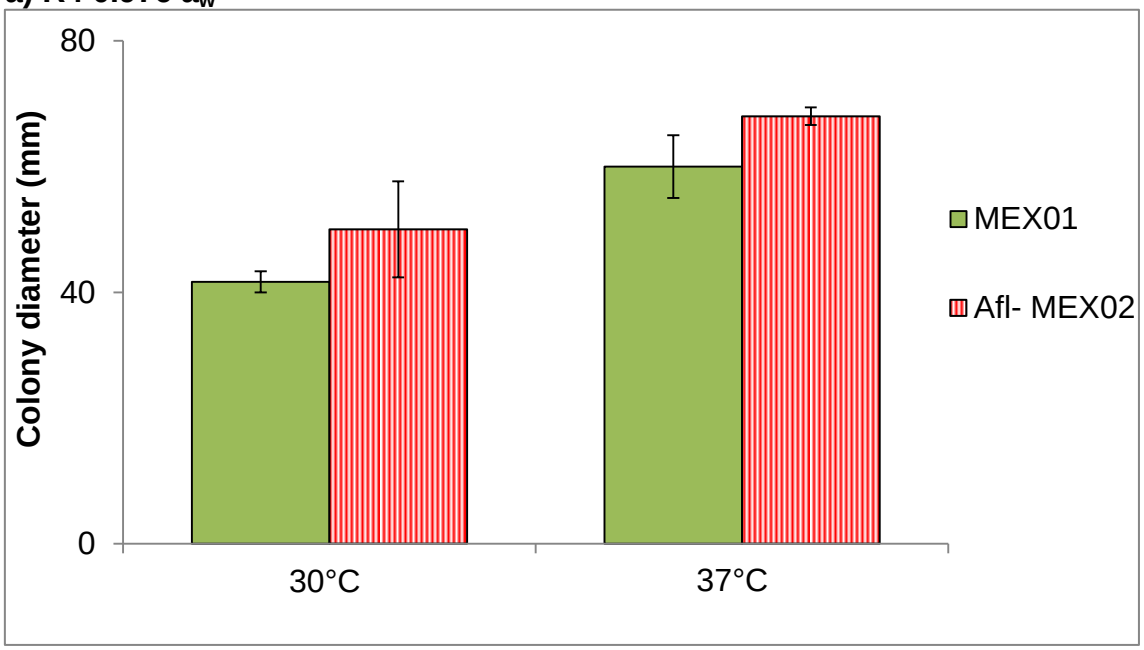
a) *Effect of the BCAs and climate change environmental factors on toxigenic* A. flavus *strain fungal growth*

The cobs at the R3 Milk stage were from a different cultivar than the ones previously used (Chapter 6). The data obtained from a_w measurement of R3 was 0.965 a_w, the other ripening stages (R4 and R5) cobs were 0.976 and 0.958 a_w, respectively. Figure 7.1 shows the colony diameter on day 5 of the toxigenic *A. flavus* strain during co-inoculation with the atoxigenic *A. flavus* strain under the different environmental conditions (30°C/400 ppm CO_2 and 37°C/ 5,000 ppm CO_2). At R4 there was no significant difference on fungal growth despite the BCA presence and regardless temperature/CO_2 level (Figure 7.1a). Figure 7.1b compares colony diameter on the R5 cobs. Similar colony sizes were measured in the control and treatment under normal environmental conditions. Growth on R5 cobs at day 5 under extreme CO_2 levels could not be assessed due to contamination with *Aspergillus* section *Nigri* species.

Figure 7.1 shows the colonisation of the maize cobs at different ripening stages under normal conditions and extreme CC conditions. Very little fungal growth occurred on the R3 cobs at both temperatures (30 and 37°C). This was discrete and only present on the inoculated maize kernels. At 37°C/5,000 ppm CO_2 treatment the maize cobs were more contaminated by *Aspergillus* section *Flavi* and A. section *Nigri* species than at 30°C. On R4 cobs the colonisation had a cottony floccose white mycelial appearance with green conidial sporulation at the centre. At high temperature, the colony was heavily sporulating with a dusty appearance and completely covering the cobs. There was no difference between the controls and the BCA treatments. The aspect of the colony on R5 at 30°C was floccose with green sporulation, growing over the cob surface. The appearance of the colony at elevated temperature was dusty green and it was

scattered over the cob. At high temperature and extreme CO_2 conditions *Aspergillus* section *Nigri* contaminated more cobs than at 30°C.

a) R4 0.976 a_w



b) R5 0.958 a_w

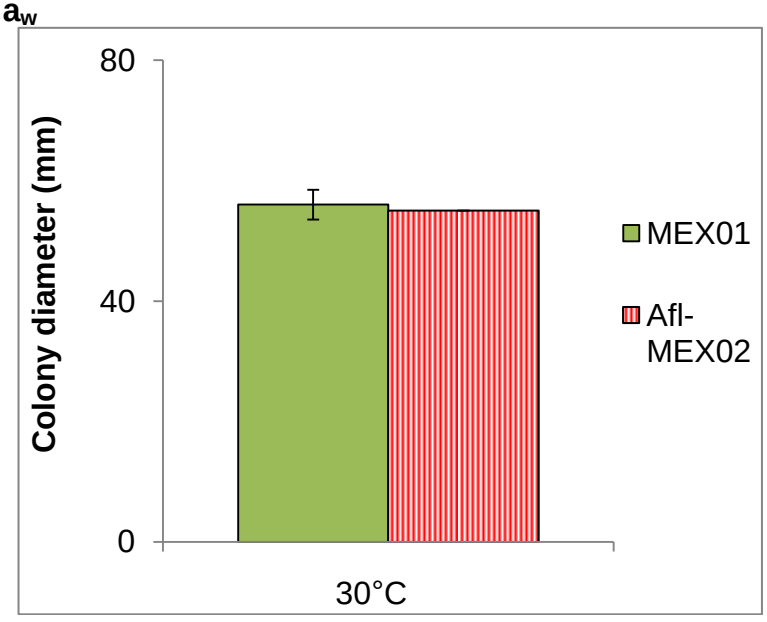


Figure 7.1 Fungal growth of MEX01 and in mixed inoculum with Afl$^-$ MEX02 inoculated on maize cobs (R4 and R5 stage) at different a_w x temperature x CO_2 conditions, after 5-day incubation. Data are means of three to five replicates ± standard error.

Figure 7.2 Fungal colonisation of maize cobs at R3, R4 and R5 incubated at 30°C/400 ppm CO_2 and 37°C/ 5000 ppm CO_2 for 10 days.

b) *Effect of the BCAs* on *relative gene expression of two aflatoxin biosynthetic genes and AFB_1 production by toxigenic* A. flavus *MEX01 strain*

Figure 7.3 compares the effect of the atoxigenic *A. flavus* strain (Afl^- MEX02) on *alfD* and *aflR* expression by the toxigenic *A. flavus* strain on the R4 and R5 cobs in two CO_2 levels (atmospheric and extreme levels). In R4 (0.976 a_w), *aflD* expression was slightly stimulated by the atoxigenic strain at both CO_2 conditions, but this was not significantly different from the control. At the R5 stage, less available water and high CO_2 levels, the atoxigenic strain

significantly decreased *aflD* expression by >90% (Figure 7.3b). The *aflR* expression at the R5 cob stage in both conditions was slightly stimulated in the presence of the atoxigenic BCA.

Figure 7.4 shows the effect of the two BCAs on AFB_1 production by the toxigenic *A. flavus* strain on the different ripening stages of the cobs in the different environmental treatments. At the R3 stage (0.965 a_w) under atmospheric CO_2 levels, the atoxigenic strain inhibit AFB_1 production by >90%. In the R3 cobs, under extreme CC conditions, AFB_1 production by the toxigenic *A. flavus* strain decreased significantly ($p<0.05$) regardless the BCAs presence, when compared to existing conditions (30°C/400 ppm CO_2). In the R4 cobs even though AFB_1 production was low, the atoxigenic strain significantly stimulated AFB_1 production (Figure 7.4c). At elevated temperature and CO_2 levels in the R4 cobs, AFB_1 production was significantly stimulated ($p<0.05$) but unaffected by the presence of the atoxigenic *A. flavus* BCA. In the R5 cob treatments where the a_w level was slightly reduced, the toxin production was increased significantly ($p<0.05$) compared to the other ripening stages. While the BCA x temperature/CO_2 interaction had no influence on aflatoxin production.

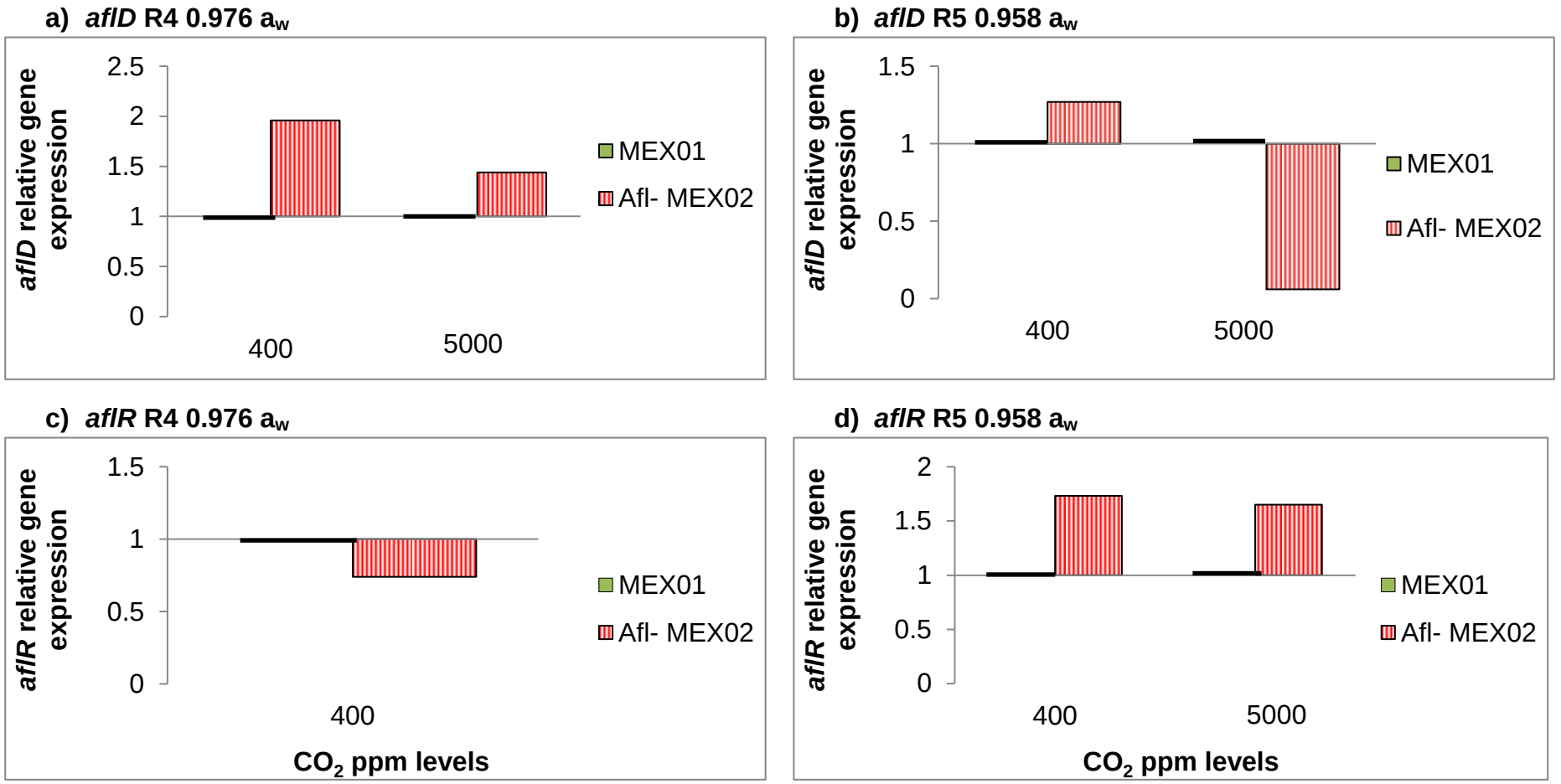
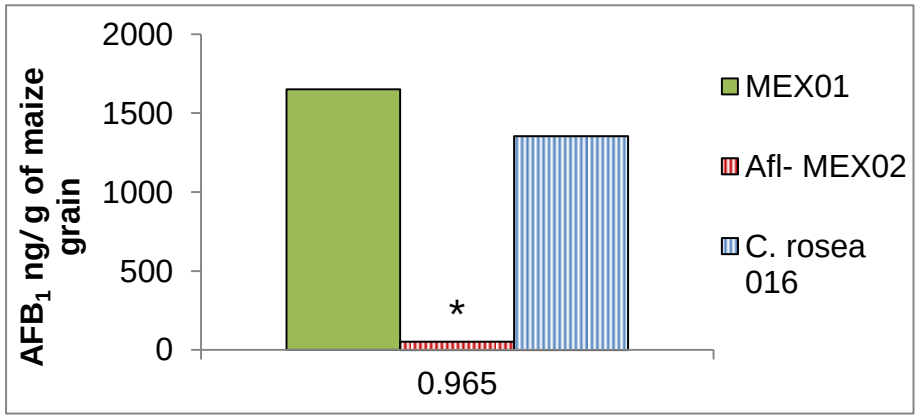
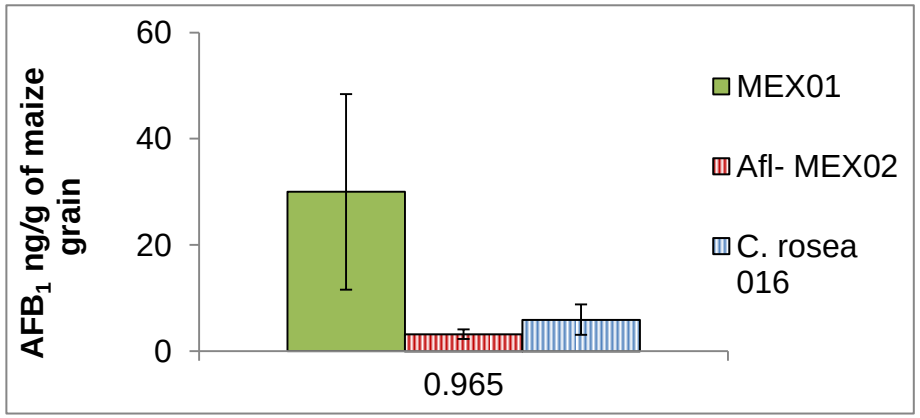


Figure 7.3 Relative gene expression of *aflD* and *aflR* by *A. flavus* MEX01 inoculated with Afl$^-$ MEX02 (50:50 ratio) on maize cobs at different ripening stages at 30 and 37°C with two CO_2 levels (400 and 10000 ppm) for 10 days. Toxigenic strain (100:0) was the calibrator =1. Means followed by asterisk are significantly different compared to the control ($p<0.05$).

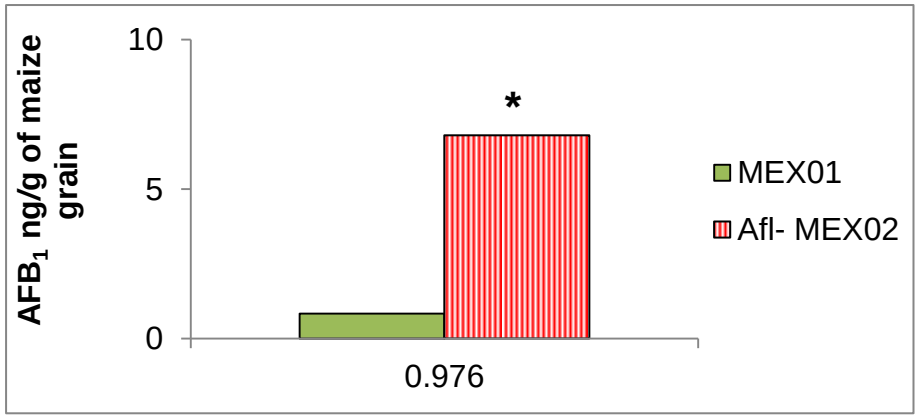
a) R3 30°C/400 ppm CO_2



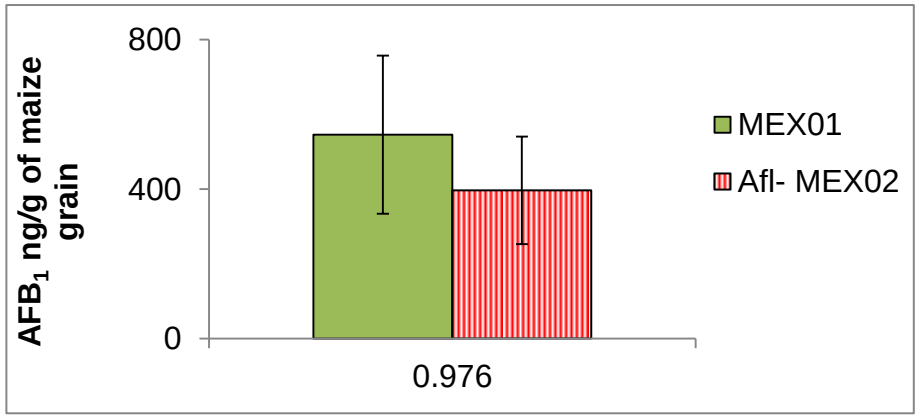
b) R3 37°C/5000 ppm CO_2



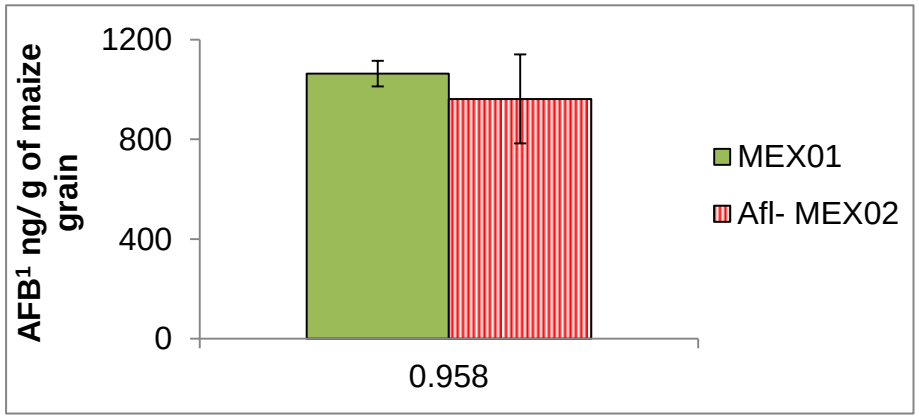
c) R4 30°C/400 ppm CO_2



d) R4 37°C/5000 ppm CO_2



e) R5 30°C/400 ppm CO_2



f) R5 37°C/5000 ppm CO_2

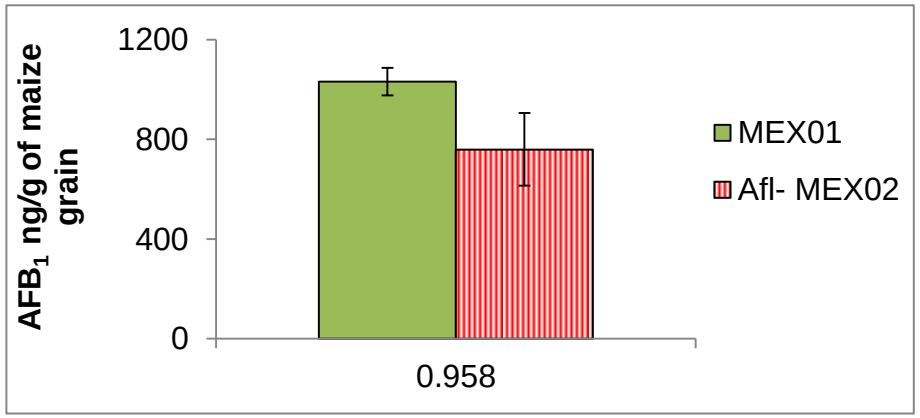


Figure 7.4 Aflatoxin B_1 production by the toxigenic *A. flavus* MEX01 *A. flavus* MEX01 inoculated with BCAs (50:50 ratio) on maize cobs at different ripening stages at 30 and 37°C with two CO_2 levels (400 and 5000 ppm) for 10 days. Data are means of three to five replicates ± standard error. Means followed by asterisk are significantly different compared to the control ($p<0.05$).

7.6 Discussion

This Chapter studied the impact of different a_w and temperature/CO_2 levels on the efficacy of two BCAs (atoxigenic *A. flavus* strain Afl^- MEX02; *C. rosea* 016) on a toxigenic *A. flavus* strain on cobs of different ripening stages, representing different natural a_w levels. On R3 cobs, *A. flavus* only grew inside the damaged grain, even if the temperature was optimal for growth (Abdel-Hadi *et al*., 2012). On R4 and R5 cobs there was no difference in colonisation by the toxigenic strain of *A. flavus* regardless of the BCA used and the environmental conditions examined. The lack of control of the toxigenic strain of *A. flavus* could be due to the use of 50:50 ratios ($5x10^3$:$5x10^3$ spores/mL) of BCA:pathogen which may not have reached an effective threshold for control. Also, the colonisation of the R3 cobs was very limited. These cobs could have been treated or very different from the other R4 and R5 cobs as they were obtained from a different source. Overall, the pin-inoculation method by damaging the kernels was more effective than the perforation method. Previously, the pin-bar approach has been used for infection of maize cobs during silking (Abbas *et al.*, 2006).

Based on the colonisation studies on the R3-R5 ripening stages A. *flavus* is able to effectively grow in all the ripening stages and in the CC environmental conditions examined. Previously, Medina *et al.* (2015) found little effect on growth of *A. flavus* on YES and on maize-based media. In the present study, because the cobs, especially R4 and R5 growth stage, were harvested and snap frozen, they still appeared to have some mycobiota. This was clear from the results at both 30-37°C where black *Aspergilli* grew on the cobs. The contamination could have been from the cobs or from the atmosphere during the experimental procedures. Overall, in this study the ratio (50:50) of conidial inoculum with a concentration of $5x10^3$ CFUs for BCA:pathogen. This may not have reached the threshold which was necessary for effective control of the toxigenic *A. flavus* colonisation. Recently Samsudin *et al*. (2017) examined the control of fumonisin B_1 production by antagonists on R3-R5 cobs including *C. rosea* 016. They used a higher 50:50 ratio (100 µL per BCA with $1x10^6$ CFU/mL) and found effective control of *F. verticilliodes* as well as toxin

production. However when they examined the impacts of CC scenarios (35°C /1000 ppm CO_2), *A. flavus* grew over the cob faster than *F. verticillioides* (Samsudin, 2015).

The impact of the BCAs on *A. flavus* gene expression was also analysed under different environmental conditions. At both the R4 and R5 cob stages, *aflD* gene expression at 30°C/400 ppm CO_2, was slightly stimulated. These results are different from the expression obtained at the same ripening stage in which the expression was down-regulated by the BCAs (see Chapter 6). Only *aflD* expression was down-regulated at 0.958 a_w, 37°C and 5000 ppm. The gene expression results obtained by Medina *et al*. (2015) at 0.97 and 0.95 a_w x 37°C x 1000 ppm CO_2, were different. They showed that *aflD* expression was not affected at 0.97 a_w but was stimulated 3.2-fold at 0.95 a_w. At 0.95 a_w the regulatory gene (*aflR*) was highly stimulated, with a 44-fold increase compared to the control. In this study, other factors that might affect the gene expression were the BCAs and nutritional content of the cobs at the different ripening stages.

In the R3 cobs, AFB_1 production by the toxigenic *A. flavus strain* was significantly decreased by the atoxigenic one at 30°C/400 ppm CO_2. Under extreme CC conditions the AFB_1 was lower *per se*. In all R4 cobs the atoxigenic strain stimulated toxin production at 30°C/400 ppm CO_2. In 37°C/ 5000 ppm CO_2 on R4 and R5 cobs, the AFB_1 production was high, stimulated by the temperature/CO_2 or by the a_w, and very different from studies where only the interaction between a_w x temperature was analysed. Analysing the two factors, the toxin production was decreased at 37°C (O'Brian *et al*., 2007; Schmidt-Heydt *et al*., 2009; Abdel-Hadi *et al*., 2012). Previously, Giorni *et al*. (2008) analysed the influence of modified atmosphere storage with CO_2 (up to 75% CO_2) under water stress on AFB_1 production by *A. flavus*. The toxin production decreased with such high CO_2 concentrations, especially on maize grain. However, these studies are unrelated to CC scenarios where we are examining much lower CO_2 levels (0.1%) and where interactions with temperature and water stress are included. Medina *et al.* (2015) showed that AFB_1 production

increased at 0.97 and 0.95 a_w at 37°C/1000 ppm CO_2 compared to the treatment with the same a_w and temperature with existing atmospheric CO_2 level. The present study showed that *A. flavus* growth was not significantly affected with the interaction of BCAs under different CC factors, but they can impact on gene expression and indeed may stimulate AFB_1 production by toxigenic *A. flavus* strains, under stress conditions.

7.7 Conclusions

The present study showed the impact of CC factors (a_w x temperature/CO_2) on efficacy of the chosen BCAs against a toxigenic *A. flavus* strain on maize cobs of different ripening stages for the first time. The R3 sweetcorn cultivar and the perforation technique were not useful to assess the effect of the BCAs and environmental conditions on *A. flavus*. The toxigenic *A. flavus* strain (MEX01) could colonise the maize cobs at R4 and R5 stages regardless of the environmental conditions and BCAs used. The pin-inoculation was more effective for toxigenic *A. flavus* growth, however the pathogen:antagonist ratio used may not have been high enough to reach a threshold for effective control to be achieved. Perhaps the 50:50 ratio or 75:25 ratio of antagonist to pathogen is necessary with at least Log5-6 required for control of *A. flavus* and AFB_1 contamination. More information is required on the impact of the three way interacting CC environmental factors on testing potential resileince of such BCAs for control of toxigenic fungal pathogens. Indeed, the pathogen may be more resilient and be more difficult to control under CC scenarios.

Chapter 8. General Discussion, Conclusions and Future work

8.1 General Discussion

Fungal contamination of maize, one of the most important staple cereal crops, can have impacts on human health because of contamination with mycotoxins and have significant economic impacts. Indeed, a recent report by the IARC suggests that in Low Middle Income Countries, exposure to aflatoxins via consumption of maize as a staple food is causing significant stunting in children and infants (Wild *et al.*, 2015). *A. flavus* is a ubiquitous fungus that can contaminate maize both pre- and post-harvest. The risk is higher during the silking stage and maize susceptibility increases with water stress and insect attack (Cotty and Mellon, 2006).

A key strategy to control *A. flavus* contamination is the use of biocontrol agents. Diverse organisms have been studied with this purpose but mainly atoxigenic *A. flavus* strains have appeared to be commercially successful in maize/groundnuts and in cotton (Abbas *et al.*, 2009). An important characteristic of the atoxigenic strains is the ability to compete effectively against the toxigenic strain in key components of the life cycle of the toxigenic species in maize. Also, the atoxigenic strains are more effective if they are isolated from the region of application. *A. flavus* growth and AFB_1 production are influenced by different environmental factors, for this reason, it is important to understand *A. flavus* ecophysiology.

The aim of this project was to understand better the interactions between *A. flavus* and other fungi present in maize and identify potential BCAs effective in reducing AFB_1 production. The study was focused on Mexican maize and potential of minimising AFB_1 contamination by using BCAs.

The isolation and identification of potential BCAs from four Mexican maize cultivars, showed the diverse mycobiota within regions, as two of the cultivars were from opposite parts of the country (north and south). The grains were

contaminated but the a_w and MC were very low and safe for long term storage because they were markedly below the 0.70 a_w and 14% MC, necessary for fungal spoilage (Magan and Aldred 2007a). *A. flavus* was present in three of the cultivars, and the strains were positive for AFB_1 production. This allowed the use of two isolates for the study, a toxigenic *A. flavus* strain MEX01 and a practically atoxigenic strain Afl^- MEX02 as a potential biocontrol agent. Other strains of *T. atroviride* and *T*. *funiculosus* were isolated and were considered as potential BCAs. These strains facilitated a screening study of potential efficacy of strains isolated from the Mexican maize to be evaluated for potential biocontrol use in the overall study.

After the isolation and molecular identification of the strains (toxigenic and potential BCAs), they were screened against the toxigenic *A. flavus* strains (NRRL 3357 type strain and MEX01 isolate) under different environmental conditions (a_w x temperature) on maize-based media made from Mexican maize flour. This helped identify and test 8 potential BCAs for antagonism of the toxigenic *A. flavus* using various criteria including a macroscopic colony level using the Index of Dominance approach. This narrowed down the potential BCAs which could be tested in mixed inocula (50:50 ratio) to determine the effects on AFB_1 production. It is important to use an environmental screen to identify potential control of mycotoxin production. This approach showed that 4 potential BCAs were promising. It is critical to identify what relative threshold levels of a BCA will be needed to effectively control a toxigenic fungal pathogen. This is important to understand whether the potential BCA can be economically produced and may be subsequently economically feasible. Thus, the approach was used to vary the inoculum ratio between the BCAs and the toxigenic *A. flavus* strains on both maize-media and on stored maize grain under different environmental conditions. Three of the four BCAs tested were from the Mexican maize samples because it has been suggested that it is important to utilise local strains than those from other climatic regions (Bandyopadhyay *et al.*, 2016).

The reduction of inoculum potential of *A. flavus* is an important part of the life cycle of *A. flavus* where efficacy could have significant implications for

subsequent infection of maize cobs during silking. Few studies have examined the potential for reduction of inoculum potential under different environmental regimes. The present study certainly suggested that regardless of BCA it was difficult to significantly reduce the inoculum potential of toxigenic *A. flavus* strains on crop residue. Indeed, some of the candidate BCAs stimulated asexual conidial production. Perhaps, more attention needs to be paid to the sclerotial survival on crop residue and in soil. If this is a major source of inoculum production then perhaps the focus should be on preventing or reducing the sclerotial number present. Giorni *et al.* (2012) showed that environmental factors certainly influenced sclerotial germination. Perhaps parasitic colonisation of the sclerotia to prevent germination in soil and on crop debris could have an impact on inoculum potential. Certainly, *Coniothyrium minitans* has been commercialised to parasitize sclerotia of *Rhizoctonia solani* in horticultural crops successfully (Whipps, 1997). The success of atoxigenic strains in West Africa has used sorghum grain as a carried of the biomass to facilitate colonisation of the soil and possibly crop residue. However, the mechanism of action has not been elucidated (Bandyopadhyay *et al.*, 2016). They suggest long term efficacy of their mixtures of atoxigenic strains pre-harvest and reducing the toxigenic strains presence.

This needs to be combined with studies on control of *A. flavus* colonisation of maize cobs during silking and in many tropical countries where drying and storage regimes are not adequate, control post-harvest. The present study has shown that certainly where different ratios were examined of BCAs very good control of AFB_1 was achieved by some candidates over environmental conditions examined. This provided useful information for examining efficacy of the best candidate BCAs on ripening cobs, both under existing and future CC scenarios for the first time. These studies were not very successful in demonstrating control of AFB_1 control by the best two candidate BCAs. It is possible that the threshold concentrations of the BCAs were not high enough to achieve the control of *A. flavus* growth and AFB_1 contamination. It was clear that no statistically significant reductions were observed in the R3, R4 and R5 stage cobs. More studies are required, perhaps using different spore ratios to

examine in more detail the potential efficacy of AFB_1 on maize cobs. The relative resilience of candidate BCAs and indeed on the toxigenic *A. flavus* in CC scenarios has not been examined previously on ripening maize cobs. This study suggests that it may be even more difficult to control AFB_1 contamination under such conditions because of the relative resilience of the toxigenic species. Indeed, Medina *et al.* (2015) showed that in stored maize kernels there is often a stimulation of AFB_1 production under three way interacting CC environmental factors.

8.2 Conclusions

The major findings were:

- A toxigenic *A. flavus* strain (MEX01) was isolated for the next studies also potential BCAs were isolated, including a low AFB_1 producer (Afl^- MEX02).
- The isolates were molecularly identified and also the toxin production was analysed.
- The screening for potential BCAs between 8 strains was successful; some were antagonists to the toxigenic *A. flavus* strains obtaining high I_D scores. The interactions were influenced by a_w and temperature.
- At 50:50 inoculum ratio, four potential BCAs reduced AFB_1 production atoxigenic Afl^- MEX02, *T. atroviride* MEX03, *T. funiculosus* MEX05 and *C. rosea* 016. The atoxigenic Afl^- MEX02 was the most effective, at different inoculum ratios, under water stress
- *T. atroviride* MEX03, *T. funiculosus* MEX05 and *C. rosea* 016 were effective against MEX01 at 0.98 a_w
- The BCAs did not decrease *A. flavus* sporulation, on the contrary, they stimulated the sporulation
- The effect of the BCAs on AFB_1 production was different in vitro than on stored maize grain
- The key genes (*aflD* and *aflR*) were down-regulated by the BCAs at 0.98 and 0.93 a_w.
- The atoxigenic strain Afl^- MEX02 was effective and reduced AFB_1 production at 50:50 and 75:25 ratios.
- *C. rosea* 016 was able to decrease AFB_1 production by NRRL 3357 at 0.98 a_w only.
- On maize cobs the toxigenic *A. flavus* strain MEX01 could colonise the maize cobs at all the ripening stages tested. Both BCAs down-regulated the relative gene expression of *aflR* and *aflD* at 50:50 ratio
- This was the first study to show the impact of CC factors (a_w, temperature/CO_2) on the BCAs against toxigenic *A. flavus* on maize cobs.

- The pin-inoculation technique was more useful to assess *A. flavus* growth than the perforation technique.
- The 50:50 ratio ($5x10^3$:$5x10^3$ spores/mL) might not have reached a threshold for effective control of *A. flavus* in the ripening maize cobs.

8.3 Future work

- Afl$^-$ MEX02 was the most effective biocontrol on MMA and maize grain, so it will be important to analyse whether it produces cyclopiazonic acid production.
- Test *C. rosea* 016 against *A. flavus* making the application in a higher inoculum ratio and before *A. flavus* inoculation to examine if *C. rosea* will be faster and effective on debris as other studies have demonstrated against *Fusarium*.
- The relation between relative gene expression and toxin production would be better understood with a temporal study of *A. flavus* relative gene expression (*aflD* and *aflR*) and AFB_1 production done with the most effective biocontrols.
- Test different inoculum ratio pathogen:antagonist with a higher concentration on maize cobs at different ripening stages, to achieve the inhibition threshold also under CC scenarios. Use different CO_2 concentrations in combinations with different temperatures and ripening stages, focusing mainly in R5 as the results showed that *A. flavus* grew more at that stage.

PUBLICATION AND TRAINING

1. Abstract submitted and poster presented at Focused Meeting 2015: International Meeting on The Invasive Fungus. Society for General Microbiology, Manchester, U.K., 7th - 9th September 2015.
2. Abstract submitted and oral presentation given at XIII Symposium of Mexican Students and Studies, UCL, London, U.K. 24th July 2015
3. Abstract submitted and oral presentation given at the ICFM 2016 Workshop “Current and Future Trends in Food Mycology – Methods, Taxonomy, and Emerging Problems”, Freising, Germany, 14th June 2106
4. ERASMUS INTENSIVE PROGRAMME (IP): Methods in Food Mycology and Mycotoxicology, Bragança, Portugal, 8th – 22th June 2014
5. New-to-teaching in laboratory-based subjects, Higher Education Academy (HEA), 2nd July 2015, Cardiff, Wales.
6. Modules attended “Food Mycology”, “Fungal Ecology II”, “Mathematical foundations”, “Climate change, food security, fungal spoilage and mycotoxins”, “Mould species identification using molecular techniques”.
7. Short courses from the Doctoral Training Centre (DTC) some of which were: “An Intro to the Statistical Treatment of Experimental Data”, “Getting Started in Your Research”, “Technical Writing Skills for Your Progress Reviews”, “Research Integrity and Being An Ethical Researcher”, “Assisting In The Supervision of MSc Students”.

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