CRANFIELD UNIVERSITY

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FUNGAL DIVERSITY, PEST DAMAGE AND BIOCONTROL OF AFLATOXINS IN GM AND CONVENTIONAL BRAZILIAN MAIZE CULTIVARS UNDER EXISTING AND FUTURE CLIMATE CHANGE SCENARIOS

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Ph.D. THESIS
Academic Year: 2015 - 2018

Supervisor: Prof. Naresh Magan, DSc
Associate Supervisor: Dr Angel Medina
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ABSTRACT

This study (a) evaluated the fungal biodiversity, toxigenic mycobiota and mycotoxin profiles associated with conventional (non-GM) and genetically modified (GM) isogenic maize cultivars (cvs) from Brazil, (b) studied the ecology of the isolated strains of toxigenic *Aspergillus flavus* using non-GM and the isogenic GM cv as substrates under different water activity(a_w) and temperature interactions *in vitro*, (c) screened mycobiota for potential biocontrol agents (BCAs) and compared the interactions between atoxigenic (AFL^-) and toxigenic (AFL^+) *A. flavus* strains and other antagonistic species for *in vitro* control of aflatoxins (AFs) using different spore inoculum ratios, (d) examined the best potential BCAs to apply *in situ* in stored GM and non-GM isogenic maize cvs on AFs production and related expression of structural (*afl*D) and regulatory (*afl*R) toxin biosynthetic genes, and (e) examined the resilience of the biocontrol efficacy under simulated pest damage and climate change (CC) scenarios.

The majority of the GM and isogenic non-GM cvs analysed (20 samples; 10 each type) had moisture content (%MC) and a_w levels within the safety range for safe storage (<0.70 a_w). *Fusarium* and *Penicillium* spp were the predominant genera identified with a low percentage of isolation of *A. flavus* strains in the maize cultivars examined. There was no significant difference ($p<0.05$) in the frequency of isolation between non-GM and GM cvs. A total of 22 *A. flavus* strains were isolated, of which 15 were non-aflatoxin producers, and 7 were aflatoxin B_1 (AFB_1) producers. Six of these strains were from non-GM maize cvs. Six pairs of isogenic GM- and non-GM cvs (n=12) out of the 20 used in this work were selected and analysed in more detail using LC-MS/MS. The mycotoxin profiles showed 29 compounds present, with higher amounts of *Fusarium* toxins than any other, which paralleled the high isolation frequency of *Fusarium* spp. AFs were not detected, while Fumonisins (B_1 or B_2) were present in 10 out of the 12 cvs, with only 2 non-GM cultivars having contamination levels above the EU legislative limits (4000 µg kg^{-1}). The distribution of the mycotoxins indicated differences between the non-GM and GM cvs ($p<0.05$) with the latter having lower overall concentrations of mycotoxins.

Subsequently, from the 22 isolated strains of *A. flavus* 4 were selected (3 toxigenic and 1 atoxigenic) for ecological studies using 3 pairs of GM- and non-GM maize cvs as substrate. The strains were able to colonize and grow on maize-based nutritional matrices from both GM (two pesticide and one herbicide + pesticide resistant) and non-GM cvs. The type of cvs did not have a significant effect on the growth of *A. flavus*, however temperature and a_w had a significant effect ($p<0.05$) on the fungal development. The optimal conditions for growth were slightly different from those for AFB_1 production. Optimal growth occurred at 30-35^oC and 0.99 a_w, whereas AFB_1 production was optimal at 25-35^oC and 0.99 a_w. Each strain showed a different pattern of AFB_1 production and there was a shift in the optimal conditions depending on the combination of a_w × T^oC × maize cv.

In vitro a total of 8 atoxigenic (AFL^-) and 8 other strains from different genera were tested as BCAs against 5 toxigenic strains (AFL^+). This showed that *A. flavus* was highly dominant *in vitro*. One yeast strain (Y6) was able to compete against *A. flavus* on malt extract agar (MEA) at 0.98 a_w but when it was co-cultivated in milled-maize agar (MMA) against the toxigenic AFL^+ strain resulted in an increase in AFB_1 when compared to the control. The interaction of the toxigenic AFL^+ × atoxigenic AFL^- strains were mutual intermingling on both MEA and MMA. On MMA for co-cultivation of different inoculum ratios the screening was only done against 3 AFL^+ toxigenic strains to examine effects on AFB_1 control. The overall control of AFB_1 ranged from 29 to 100%. The most effective ratio of spores of the atoxigenic vs toxigenic strains was found to be a mixture of 50:50 mixed conidial inoculum of each strain.

Based on the *in vitro* screening for potential BCAs, the atoxigenic *A. flavus* strains were examined to determine whether they had a deletion in biosynthetic genes involved in AFs and cyclopiazonic acid (CPA) production using multiplex PCR. Five atoxigenic strains (AFL^-) were found to have large deletions of genes in the AFs cluster. While 3 atoxigenic strains amplified most of the markers in the AF cluster, however they were still unable to produce AFs. The strain selected for *in situ* biocontrol studies (*Af*53H – $AFL4^-$) had a large deletion of AF markers but

had all the CPA markers. The AFL4^{-} was able to significantly reduce AFB_1 when paired with toxigenic strains in a 50:50 spore ratio in stored GM and non-GM maize cvs. The relative gene expression of *afl*D and *afl*R in one of the toxigenic strains (AFLb^{+}) used as pathogen was significantly inhibited by the chosen BCA. The correlation of gene expression $\times$ AFB_1 was positive indicating that suppression in the gene expression pathway contributed to the lower toxin levels. The overall biocontrol action seems to have been most effective when used in stored GM maize cultivars.

Different levels of simulated pest damage (0, 5 and 15%) showed that AFB_1 production did not increase with a higher level of damage regardless of whether pesticide resistance or herbicide + pesticide resistance cvs were compared with non-GM isogenic ones. The toxin production in 15% damaged maize grain was lower or equal to that with no or 5% damage. The gene expression of *afl*R and *afl*D involved in AFs biosynthesis showed differences between the maize cvs. However, the correlation of gene expression × AFB_1 was not significantly positive.

The BCA showed resilience under T^{o}C × CO_2 × a_w × simulated pest damage conditions with similar control levels of AFB_1 which was achieved under existing environmental conditions. The use of a GM cvs showed better results for biocontrol under water stress (0.95 a_w) and elevated CO_2 at 35^{o}C when the kernels were undamaged. However, biocontrol in conventional maize was better when there were damaged kernels at 0.95 a_w × 35^{o}C ×1000 ppm CO_2

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LIST OF ABBREVIATIONS

∅	Diameter
Δ	Delta
λ_{em}	Emission wavelengths
λ_{ex}	Fluorescence excitation wavelengths
Ωm	Ohm-meter
%IF	Percentage fungal isolation
%MC	Percentage of moisture content
AF	Aflatoxin
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFL	*Aspergillus flavus*
ANOVA	Analysis of Variance
AV	Average
a_w	Water activity
BCA	Biological control agent
bp	Base pair
Bt	*Bacillus thuringiensis*
CAM	Coconut cream agar
CC	Climate change
cDNA	Complementary DNA
CFU	Colony forming unit
CO_2	Carbon dioxide
CON	Conventional
CPA	Cyclopiazonic acid

Cq	Quantification cycle
CTAB	Cetyl trimethylammonium bromide
CV/CVs	Cultivar/cultivars
DG18	Dicloran -18- glycerol agar
DON	Deoxynivalenol
EC	European Commission
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
et al.	*et alii*
EU	European Union
FA	Fatty acid
FDA	Food and Drug Administration
FLD	Fluorescence detector
FUM	Fumonisin
g	Gram
GM	Genetically modified
h	hour
HPLC	High Pressure Liquid Chromatography
HT	Herbicide tolerance
IAC	Immunoaffinity columns
I_D	Index of dominance
IPCC	Intergovernmental Panel on Climate Change
IR	Insect resistance
kb	Kilobase
kcal	Kilocalorie
kg	Kilogram
kGy	KiloGray
LC-MC/MS	Liquid chromatography– tandem mass spectrometry

LOD	Limit of detection
Log_{10}	Logarithm to the base 10
Log_2	Logarithm to the base 2
LPM	Litre per minute
MC	Moisture content
MEA	Malt extract agar
mg	Milligram
min	Minute
mL	Mililitre
mm	Millimetre
MMA	Milled-maize agar
N_2	Liquid nitrogen
NA	Nutrient agar
NaCl	Sodium chloride
ng	Nanogram
nM	Nanomolar
NOAA	National Oceanic and Atmospheric Administration
non-GM	non-Genetically modified (conventional)
^oC	Degree Celsius
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
ppm	Parts per million
RNA	Ribonucleic acid
RPM	Rotation per minute
RT-qPCR	Real time quantitative PCR
s	second
SD	Standard deviation
SD-	No surface disinfection

SD+	Surface disinfection
SE	Standard error
TFA	Trifluoracetic acid
T^{o}C	Temperature
UNEO	United Nations Environment Organization
UNEP	United Nations Environment Programme
US/USA	United Stated of America
USDA	United States Department of Agriculture
UV	ultraviolet
v/v	Volume per volume
VGC	Vegetative compatibility groups
w/v	Weight per volume
x g	Times gravity
YES	Yeast extract agar
ZEA	Zearalenone
µg	Microgram
µL	Microlitre
µm	Microlitre
µmol/mM	Micromolar
v/v	Volume per volume
SPE	Solid Phase extraction

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CHAPTER 1

GENERAL INTRODUCTION, LITERATURE REVIEW AND RESEARCH OBJECTIVES

CHAPTER 1. GENERAL INTRODUCTION, LITERATURE REVIEW AND RESEARCH OBJECTIVES

1.1 General Introduction

Maize is cultivated worldwide, and in Brazil, it is a staple food source for consumers in both urban and rural areas. Maize is disposed to infection by *Aspergillus flavus* and contamination with aflatoxins (AFs) during ripening and poor post-harvest storage. These are carcinogenic compounds and there is legislation in many countries to minimise the exposure of consumers to this group of mycotoxins (van Egmond et al., 2007).

AFs contamination at levels that can cause acute aflatoxicosis in humans rarely occurs in well-developed countries (FDA, 2012). However, about 4.5 billion people in developing countries are exposed to uncontrolled and unmonitored amounts of AFs (Williams et al., 2004). Recent examples of acute aflatoxicosis occurred in India where the consumption of mouldy maize contaminated with aflatoxin resulted in 100+ children dying and 400+ hospitalised (Probst et al., 2007), and in Tanzania in 2016 where over 20+ children died (Kamala et al., 2018). A recent UNEP report on issues of environmental concern has focussed on AFs accumulation in the context of climate change concerns (UNEP, 2016). In fact a study on AFs occurrence in Serbian maize showed that AFs contamination in maize harvested in 2012 was the result of prolonged warm weather and extreme drought conditions (Kos et al., 2013).

Climate change (CC) models projected decline in summer precipitation and increases in temperature in different regions of the world, associated with drought stress episodes. The environment in which crops will be grown in the next 10-20 years may change significantly with atmospheric CO_2 concentrations, which at present is about 400 ppm, but expected to increase to 800-1200 ppm in the next 25 years (Medina et al., 2014). The global temperature is expected to rise at the rate of $0.03^{o}C\ year^{1-}$ due to CO_2 and other greenhouse gases increase. In addition, changes in rainfall patterns with extreme wet and drought periods are

expected to occur. Thus, there are three-way interacting factors (temperature x elevated CO_2 x drought stress) which will significantly impact on crop yield and quality (Magan et al., 2011).

Magan et al. (2011) and Medina et al. (2014; 2015) suggested that significant impacts would occur due to interactions between elevated CO_2 levels and temperature/water availability on growth and mycotoxin production of key mycotoxigenic fungal species and also on nutritional quality of staple grains. Maize is particularly prone to drought stress (Lobell et al., 2011), which can predispose it to increased disease susceptibility and pest damage impacting on yield and quality (Pandey et al., 2017). This could influence the amounts of contamination with AFs and may also influence the production of other mycotoxins produced by *A. flavus* and related species, such as cyclopiazonic acid (CPA). A study by Medina et al. (2015) showed that *in vitro* the effect of these three-way interactions between these CC factors (2-3 x ambient CO_2), temperature (+ 2-4^{o}C) and drought stress (water stress) did not affect growth of *A. flavus* but significantly stimulated aflatoxin B_1 (AFB_1) production and some of the structural and regulatory genes involved in the biosynthesis of the toxin.

In Brazil today most of the maize cultivated is genetically modified (GM). The GM trait, which is most commonly cultivated in both winter and summer crops, is a combination of insect-resistance (*Bt*) and herbicide-tolerance (HT) (ISAAA, 2017). The use of GM crops with HT traits may represent a significant decrease in the use of pesticides and a reduction in the farming costs. While the GM crops with *Bt* traits are essentially to control insects, mainly in the order Lepidoptera, that are common problems in tropical and sub-tropical areas such as Brazil. The environmental variations caused by CC may have impacts on the number of insects contaminating maize crops in Brazil and pest damage of *Bt* crops.

In summary, there is little information on how such factors might affect the susceptibility of GM maize and non-GM maize both pre- and post-harvest. In Brazil, where GM maize is commonly the main crop grown there is little information on whether CC might predispose maize to more or less AFs. This

may be important in understanding the future risks from these carcinogenic toxins and might have an impact on control strategies, including biocontrol resilience (Medina et al., 2017a).

1.2 Literature review

1.2.1 Maize

Maize (*Zea mays* ssp. *mays*) was domesticated from the wild ancestor teosinte (*Zea mays* ssp. *Parviglumis)* approximately 10 000 years ago in Mexico (van Heerwaarden et al., 2011). However, the evolution from teosinte to modern maize is considered to be very complex. Whereas teosinte produces only 6 to 12 kernels with a hard-outer covered, modern maize produces a cob of 20 rows or more with exposed kernels (Tian, Stevens and Buckler IV, 2009) (Figure 1.1).

Originating in the Andean region of Central America and initially a tropical plant, maize is grown today worldwide, even in regions with temperate climate (Ranum et al., 2014). Maize is produced in both Northern and Southern hemispheres due to additional stabilization bonus as the world harvests are alternated throughout the year (Abbassian, 2006). Different types of maize are grown throughout the world. Maize kernels can be different colours ranging from white to yellow to red to black (Ranum et al., 2014). Along with rice (*Oryza* sativa) and wheat (*Triticum* spp.), maize is one of the most significant crops both for human and animal consumption (Farfan et al., 2015). The nutritional quality and integrity of maize kernels can be influence by genetic background, environment, and kernel processing (Nuss and Tanumihardjo, 2010).

Macronutrient combinations within kernels contribute to overall energy density. Maize contains starch, protein and lipids supplying an energy density of about 365 kcal/100 g (USDA - National Nutrient Database, n.d.; Table 1.1). Starch represents 73% of the kernel's total weight, and genes involved in its synthesis are among the most important for grain production, critical to yield and quality (Tian, Stevens and Buckler IV, 2009). Maize also contains vitamin C, vitamin E, vitamin K, vitamin B_1 (thiamine), vitamin B_2 (niacin), vitamin B_3 (riboflavin), vitamin B_5 (pantothenic acid), vitamin B_6 (pyridoxine), folic acid, selenium, N-p-coumaryl tryptamine and N-ferrulyl tryptamine (Kumar and Jhariya, 2013).

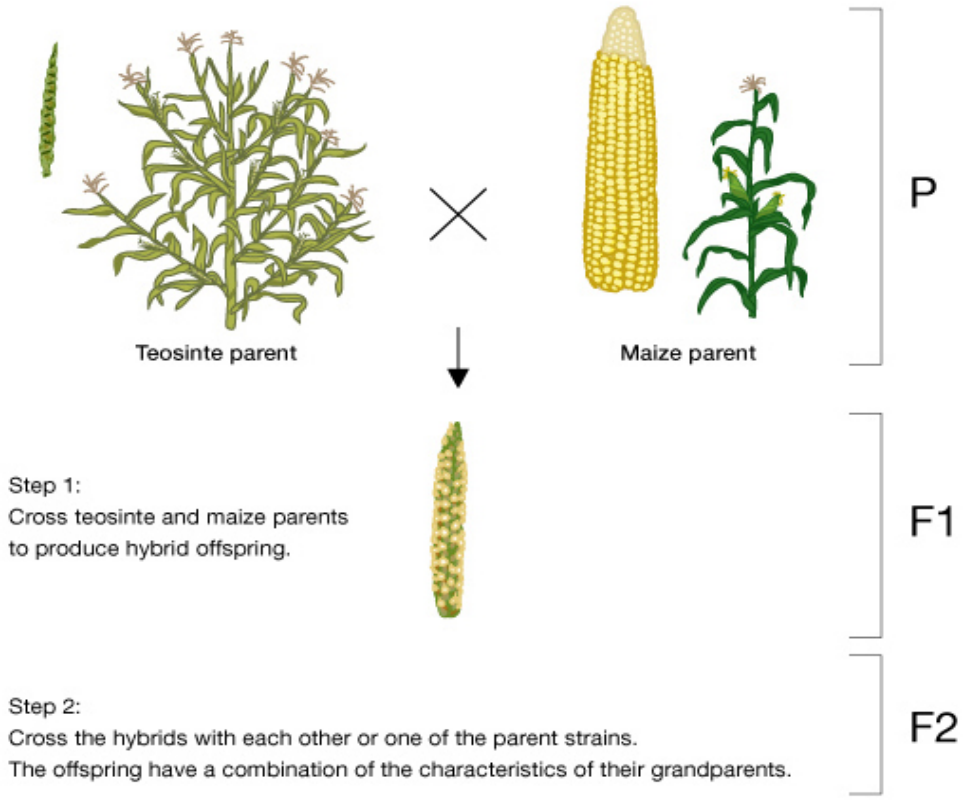

Figure 1.1. Evolution of modern maize from its ancestor teosinte. Source https://learn.genetics.utah.edu/content/selection/corn/.

Table 1.1. Composition per 100 g of edible portion of maize (Rouf Shah, Prasad and Kumar, 2016)

Compound	Amount in 100g
Carbohydrate	71.88 g
Protein	8.84 g
Fat	4.57 g
Fibre	2.15 g
Ash	2.33 g
Moisture	10.23 g
Phosphorus	348 mg
Sodium	15.9 mg
Sulphur	114 mg
Riboflavin	0.10 mg
Amino acids	1.78 mg
Minerals	1.5 g
Calcium	10 mg
Iron	2.3 mg
Potassium	286 mg
Thiamine	0.42 mg
Vitamin C	0.12 mg
Magnesium	139 mg
Copper	0.14 mg

1.2.2 World maize production

The world maize production in 2017 was 1 billion tonnes (41,122 million bushels) from about 187 million hectares representing a 2% increase from the previous year. Globally, temperate Midwestern U.S and temperate regions world-wide are the most important and highest yielding production areas where the majority of investment in maize breeding has been focused (Schimmelpfennig et al., 2004). The USA provides about 37% of the world production. Other important producing countries are China, Brazil, Mexico, Argentina, South Africa, Canada, India and Ukraine (USDA, 2018).

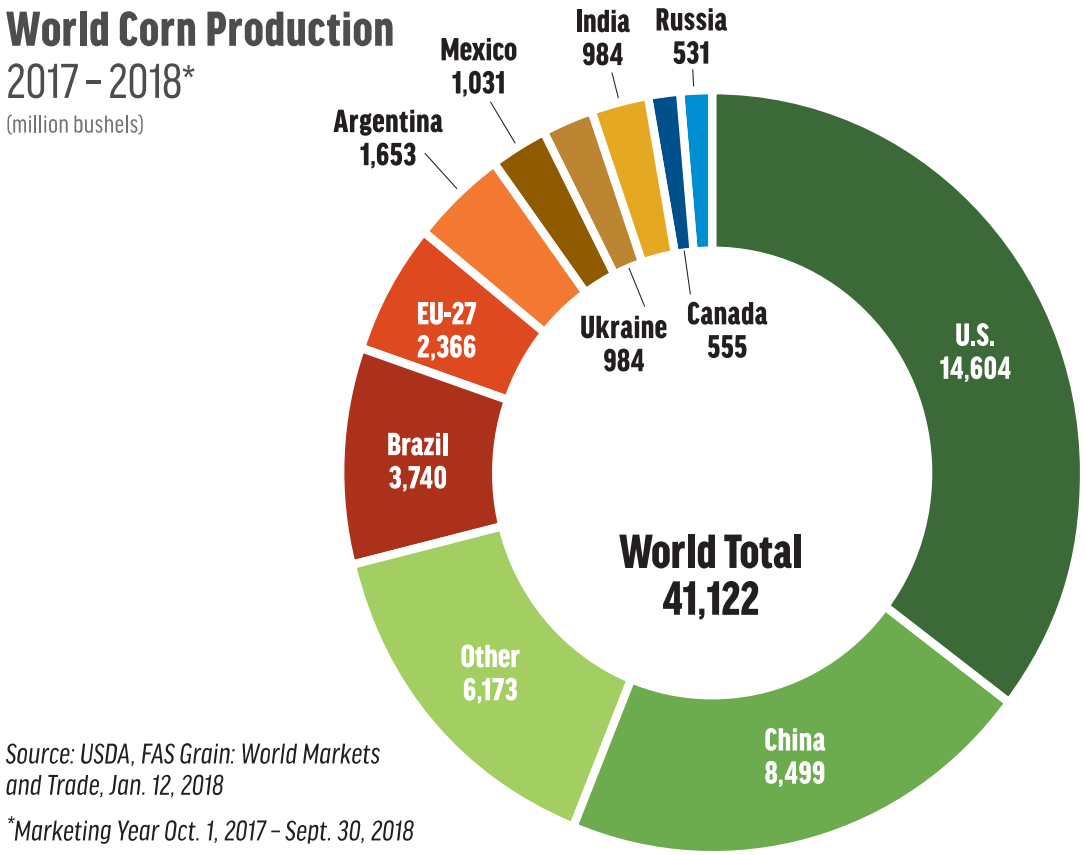

Figure 1.2. World maize production 2017 – 2018. Source USDA (2018).

Maize is at the centre of global food security as one of the most vital cereal crops in diets worldwide providing nutrients for humans and animals. It is one of the most widely traded agricultural commodities (Wu and Guclu, 2013). In many populations worldwide, maize is a dietary staple, representing the primary portion of calories consumed in many lower-income countries (Wu and Guclu, 2013). Several industrial processes are able to generate an extensive variety of maize products (Gwirtz and Garcia-Casal, 2014). Maize is converted into wide range of products: starch, sweeteners, oil, beverages, glue, industrial alcohol, and ethanol. In the decade, the use of maize for fuel production has increased taking approximately 40% of the production in the US. The ethanol industry absorbs a larger share of the maize production, causing an increase in prices intensifying demand and affecting the prices of the product for animal feed and human consumption (Ranum et al., 2014).

1.2.3 Biotechnology of maize

Domestication and plant breeding have been ongoing for millennia which has resulted in the evolution of wild cultivars into high yielding domesticated currently used (Hufford et al., 2012). Maize was transformed from its wild teosinte (Yamasaki, Wright and McMullen, 2007) resulting in a highly modified inflorescence and plant architecture (Doebley, 2004). Improvements after domestication have also resulted in significant improvements in yield, plant habit, biochemical composition, and other characteristics. At the genetic level, these phenotypic changes were the result of strong artificial selection of target genes (Wright et al., 2005). The remarkable increases in productivity over the last four decades demonstrate the success of modern maize breeding (Jiao et al., 2012).

One of the major technological innovations in global agriculture in the last few decades has been the development of genetic modifications (GM) in plant and animals (Popp and Lakner, 2013) through genetic engineering. A GM plant or animal is an organism that has been modified through the addition of a small number of genetic traits from other organisms through molecular techniques. The genetic traits incorporated are able to provide protection from pests, pathogens, tolerance to pesticides, or improve its quality (Bessin, 2003). Genetic engineering is accomplished in basic steps: isolation of DNA fragments from a donor organism, insertion of an isolated donor DNA fragment

into a vector genome and growth of a recombinant vector in an appropriate host (see Figure 1.3) (Greenaway, 1980; Nicholl, 2008).

Basically, GM crops are now divided into 3 generations with different features. The first generation refers to traits providing environmental stress and herbicide tolerance, and insect resistance. The second generation features value-added output traits such as nutrient-enhanced seeds for feed. The included traits in the third generation of GM crops would allow production of pharmaceuticals and products beyond traditional cultivation systems (Fernandez-Cornejo et al., 2014).

The first GM soybeans were harvested in 1996, since then the used of biotech crops and its adaptations became one of the most disputed and controversial topics (Adenle, 2011; Fedoroff, 2010). Genetic modification technology has had a significant positive impact on farmer income at a global level. In 2013, for example, the GM crops were responsible for adding about 5.5% to the global production of the four main cultivated crops: soybeans, maize, canola and cotton (Brookes and Barfoot, 2015).

1.2.4 Different types of Biotech in maize

The major GM traits used in maize crops today are able to provide herbicide tolerance (HT) and insect-resistance (IR). In 2017, 47% of the world's GM crops were engineered to be HT, 23.3% engineered to be IR, and 41% with a combination of both IR and HT (ISAAA, 2017). This means that considering together IR and HT stacked and HT single trait, 88% of all GM crops in the world are engineered to be tolerant to herbicides (Tourangeau and Slater, 2015).

In addition, since 2013 Monsanto has been commercializing a drought-tolerant (DroughtGard™) trait in maize crops. More recently, Monsanto submitted to FDA the maize cultivar MON 87403 that expresses a variant of the ATHB17 transcription factor protein from *Arabidopsis thaliana*, lacking the N-terminal 113 amino acids, designated ATHB17Δ113. This protein appears to increase yield by increasing ear biomass at the silking stage (Table 1.2).

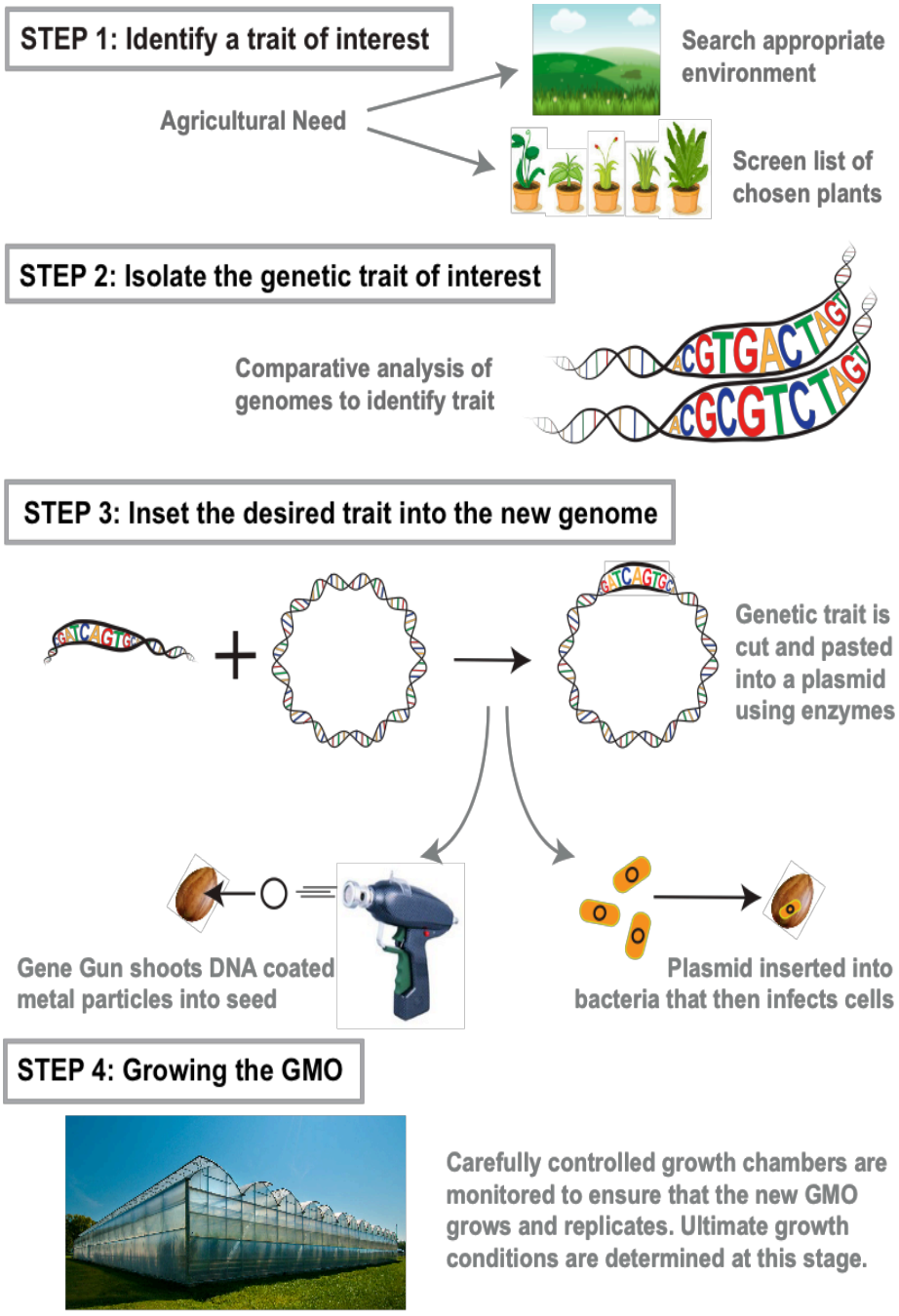

Figure 1.3. General steps to create a genetically modified organism. Source http://sitn.hms.harvard.edu/flash/2015/how-to-make-a-gmo.

In the case of DroughtGard™ Hybrids (event MON 87460), it is the first drought-tolerant biotechnology trait for maize available. It was designed to aid the plants to resist drought stress reducing the risk of failure in drought conditions (Monsanto, 2006). DroughtGard™ maize has the trait for “cold shock protein B” (cspB) isolated from a drought-tolerant gene found in *Bacillis subtilis*. The bacterial gene assists the plant to deal with environmental stress such. The action is based on disentangling RNA, which folds up abnormally when the plant is water-starved, then helping the plant cope with drought. Thus, the energy the plant would have spent fixing drought-entangled RNA could contribute to grain yield (Gilbert, 2010).

Herbicide-tolerant (HT) crops are engineered to resist applications of particular herbicides, which would otherwise kill the crop. Thus the herbicide can be applied on entire fields, killing the weeds but not the GM crop (James, 2014). The first HT trait commercialized was Monsanto’s GM Roundup Ready®, which are genetically engineered to tolerate applications of the glyphosate-based herbicide Roundup® (James, 2014). Glyphosate herbicide is one of the most commonly used broad-spectrum herbicides. It kills plants by blocking the EPSPS (5-enolpyruvylshikimate 3-phosphate) enzyme involved in the biosynthesis of aromatic amino acids, vitamins and many secondary plant metabolites (Li et al., 2015). The engineering of glyphosate-tolerant crops is based in introducing the EPSPS synthase variants derived from *Agrobacterium tumefaciens* CP4 and *Pseudomonas stutzeri* A1501 that are not inhibited by glyphosate (Funke et al., 2006). Crops resistant to Glyphosate are being cultivated in 18 countries, with substantial impacts in Brazil, Australia, Argentina and Paraguay (Gilbert, 2013).

The use of HT crops has been utilised by farmers because it can reduce the cost crop production, weed control should be easier and can result in increased farmer incomes (Brookes and Barfoot, 2015). Besides Roundup Ready, alternative GM crop with herbicide tolerance has been commercialized by the name of LibertyLink™ (Bayer CropScience, USA). LibertyLink™ is the only GM crop tolerant to both glyphosate and Glufosinate ammonium. Glufosinate tolerance is the result of insertion of the gene encoding the enzyme PAT (phosphinothricin-N-acetyltransferase) isolated from *Streptomyces viridochromogenes* that is the same organism from which Glufosinate was originally isolated. The PAT enzyme modifies and inactivates the herbicide and

confers tolerance to the plant. Toxic properties of PAT enzyme are not known (Biosafety Clearing-House, 2015).

Insect-resistance GM crops involves the introduction of genes from *Bacillus thuringiensis* (*Bt* toxin) producing plants able to resist to certain pest species (Qaim, 2014). The *Bt* toxin is known as a harmless protoxin in its free form. It is activated only in the stomach and intestinal tract of target insects by the action of enzymes. Once activated, the *Bt* toxin causes cell rupture and leaking by attaching itself to the membrane in the stomach and intestinal canal that no longer function properly (Schnepf et al., 1998).

The *Bt* traits are most widely used in maize and cotton. According to Qaim (2014) if the insect pests are not effectively controlled through chemical pesticides, the main effect of switching to *Bt* crops is a reduction in the chemical pesticide usage. There are indications that *Bt* crops, which provide resistance to some Lepidopteran and Coleopteran insect pest species, have helped reduce chemical pesticide use and increase effective yield (Krishna and Qaim, 2007; Carpenter, 2010; Gassmann et al., 2014). Klümper and Qaim (2014) cite as results of the GM technology adoption an increased crop yields by 22% and farmer profits by 68%, and reduction in chemical pesticide use by 37%. The yield increases, and pesticide reductions were larger for IR than for HT crops. Also, the yield and profit gains are shown to be superior in developing countries. However, the main concern about *Bt* crops is that pest species may develop resistance or that non-*Bt* targets insects may become a new problem (Krishna and Qaim, 2012).

The GM *Bt* traits have mostly impacted in higher incomes through improved yields. Farmers, especially in developed countries, have also benefited from decrease in production costs (Brookes and Barfoot, 2015). Similarly, the first maize drought tolerant (DroughtGard™), planted in the US in 2013 on 50,000 hectares area increased over 5 fold to 275,000 hectares in 2014 (James, 2014).

Table 1.2. Inventory of varieties of GM maize under Biotechnology consultation on food by FDA – Food and Drug Admiration, USA.

BNF number	Trait	Event designation unique identifier	FDA letter date
148	Herbicide tolerance	MON 87419 MON-87419-8	Mar 11, 2016
151	Insect resistance and herbicide tolerance	MZIR098 SYN-ØØØ98-3	Apr 29, 2016
17	Insect Resistance	Bt11 SYN-BTØ11-1	May 22, 1996
18	Insect Resistance	MON 801 MON-ØØ8Ø1	Jul 24, 1996
24	Insect Resistance	176 SYN-EV176-9	Jul 14, 1995
28	Herbicide Tolerance	DLL25 DKB-8979Ø-5	Mar 8, 1996
29	Herbicide Tolerance	T14 ACS-ZMØØ2-1	Dec 14, 1995
29	Herbicide Tolerance	T25 ACS-ZMØØ3-2	Dec 14, 1995
31	Herbicide Tolerance and Altered Fertility	MS3 ACS-ZMØØ1-9	Jun 7, 1996
34	Insect Resistance	MON 810 MON-ØØ81Ø-6	Sep 25, 1996
34	Insect Resistance	MON 809 PH-MON-8Ø9-2	Sep 25, 1996
35	Insect Resistance and Herbicide Tolerance	MON 802 MON-8Ø2ØØ-7	Nov 5, 1996
35	Insect Resistance and Herbicide Tolerance	MON 805	Nov 5, 1996
35	Herbicide Tolerance	MON 830	Nov 5, 1996
35	Herbicide Tolerance	MON 831	Nov 5, 1996
35	Herbicide Tolerance	MON 832	Nov 5, 1996
36	Herbicide Tolerance	676 PH-ØØØ676-7	Dec 24, 1998
36	Herbicide Tolerance	678 PH-ØØØ678-9	Dec 24, 1998
36	Herbicide Tolerance	680 PH-ØØØ68Ø-2	Dec 24, 1998
40	Insect Resistance	DBT418 DKB-89614-9	Mar 11, 1997
41	Insect Resistance	CBH35-1 ACS-ZMØØ4-3	May 29, 1998

Continues next page…

51	Herbicide Tolerance	GA21 MON-ØØØ21-9	Feb 13, 1998
66	Herbicide Tolerance and Altered Fertility	MS6 ACS-ZMØØ5-4	Apr 4, 2000
71	Herbicide Tolerance	NK603 MON-ØØ6Ø3-6	Oct 18, 2000
73	Insect Resistance	1507 DAS-Ø15Ø7-1	May 18, 2001
75	Insect Resistance	MON 863 MON-ØØ863-5	Dec 31, 2001
81	Insect Resistance	DAS-59122-7 DAS-59122-7	Oct 4, 2004
87	Change in Composition (other)	REN-ØØØ38-3 REN-ØØØ38-3	Oct 5, 2005
93	Insect Resistance	Event TC6275 DAS-06275-8	Jun 30, 2004
95	Change in Composition (other)	Event 3272 SYN-E3272-5	Aug 7, 2007
97	Insect Resistance	MON 88017 MON-88Ø17-3	Jan 12, 2005
99	Insect Resistance	MIR604 SYN-IR6Ø4-5	Jan 30, 2007
106	Herbicide Tolerance	HCEM485	Jul 31, 2012
107	Insect Resistance	MON 89034 MON-89034-3	Aug 8, 2007
111	Herbicide Tolerance	Event 98140 DP-Ø9814Ø-6	Sep 9, 2008
113	Insect Resistance	MIR162 SYN-IR162-4	Dec 9, 2008
116	Altered Growth Properties	MON 87460 MON-8746Ø-4	Dec 10, 2010
120	Herbicide Tolerance	DAS-40278-9 DAS-40278-9	Apr 13, 2011
126	Herbicide Tolerance	MON 87427 MON-87427-7	Apr 13, 2012
128	Insect Resistance	Event 5307 SYN-Ø53Ø7-1	Feb 29, 2012
136	Insect Resistance and Herbicide Tolerance	Event 4114 DP-004114-3	Mar 25, 2013
137	Herbicide Tolerance	VCO-Ø1981-5 VCO-Ø1981-5	May 7, 2013
145	Insect Resistance and Herbicide Tolerance	MON 87411 MON-87411-9	Oct 17, 2014
147	Altered Growth Properties	MON 87403 MON-87403-1	Jun 19, 2015
150	Herbicide tolerance	MZHGOJG SYN-ØØØJG-2	Feb 23, 2016

Source: https://www.accessdata.fda.gov/. Accessed on 01/10/

1.2.5 GM maize production worldwide

The global area of GM crops has increased from 1.7 million hectares in 1996 to almost 190 million hectares in 2017 representing more than 100-fold increase (ISAAA, 2017). From 1996 to 2013, biotech crops are said to have caused economic gains of US$133.3 billion globally at farm level (Brookes and Barfoot, 2015). Soybeans, maize, cotton and canola consist in the most produced biotech area globally. In 2017, GM soybeans accounted for the largest area (50%), followed by maize (31%), cotton (13%) and canola (5%) whereas other GM crops such as sugar beet, alfalfa, papaya, squash, poplar, tomato, sweet pepper, and eggplant represented about 1% (ISAAA, 2017).

The USA was the first country to adopt GM crops and it is still the largest cultivator, accounting for 40% of the global area under GM crops in 2017. Brazil grows 26% and Argentina 12%. Together, these top three countries grow over 78% of the world's GM crops. India and Canada contribute for 13% of the total global GM hectarage. Although China is significant producer of maize, GM crops account for only 1% of the total. This total is similar in countries such as Paraguay, South Africa, Pakistan and Uruguay. These countries represent 98% of the total global GM hectarage in 2017 (ISAAA, 2017) (Figure 1.2).

In the EU, the use of genetically modified plants is still concentrated on the cultivation of GM maize MON810 (Cressey, 2013) mainly in Spain and Portugal. The GM varieties provided 30% of the national *Bt* maize production in Spain and about 10 % in Portugal in 2014 (James, 2014).

Table 1.3. Global area of biotech (GM) crops cultivated in 2016 and 2017 by country in million hectares (ISAAA, 2017).

	Country	2016	%	2017	%	+/–	%
1	USA*	72.9	39	75.0	40%	2.1	3%
2	Brazil*	49.1	27	50.2	26%	1.1	2%
3	Argentina*	23.8	13	23.6	12%	-0.2	-1%
4	Canada*	11.1	6	13.1	7%	1.5	18%
5	India*	10.8	6	11.4	6%	0.6	6%
6	Paraguay*	3.6	2	3.0	2%	-0.6	-18%
7	Pakistan*	2.9	2	3.0	2%	0.1	3%
8	China*	2.8	2	2.8	1%	0.0	0
9	South Africa*	2.7	1	2.7	1%	0.0	0
10	Bolivia*	1.2	1	1.3	1%	0.1	7%

+/- indicates increase or reductions comparing the previous year

1.2.6 Brazilian GM production

In Brazil, maize represents an important economic and social product in both family farming and agribusiness (Vidal et al., 2015). Brazil is the second largest biotech crop producer, after the USA, and is emerging as a global leader in this sector. Soybean is the most produced crop, followed by maize. Brazil continued to be the top developing country, planting 69 biotech approved traits: 11 traits for soybean, 39 for maize, 15 for cotton, 1 for bean, 1 for eucalyptus and 1 for sugarcane (Céleres©, 2017; ISAAA, 2017).

Brazil is the 3rd biggest producer of maize. In 2017 about 98.5 million tonnes were produced, representing a 13% increase compared to the previous year (USDA, 2018). Furthermore, from the total planted area in 2017 around 89% of the maize was genetically modified (GM), making Brazil the 2nd largest producer of biotech maize after the USA. The 15.6 million hectares of GM maize produced comprised of about 21% insect resistant (IR), 4% herbicide tolerance (HT) and 75% stacked trait maize IR/HT (ISAAA, 2017). The production in the country is used mainly for animal feed (82%), particularly for poultry and pig production (de Lourdes Mendes de Souza et al., 2013).

GM maize cultivation was authorized in Brazil in 2007. The first event allowed for commercialization was MON810 (Vidal et al., 2015), a maize cv produced by the Monsanto Company known commercially as YieldGard®. It is characterized by the inserted gene Cry1Ab, derived from *B. thuringiensis*. The product of the expression of the Cry1Ab gene is the Cry1Ab protein, producing the *Bt* toxin, which is toxic to insects in the order Lepidoptera, including two very common species in tropical areas: *Spodoptera frugiperda* and *Helicoverpa zea* (Mosanto, 2002).

Biotech crop adoption continues to grow in Brazil with significant investment in the technology with a view to having access to increased yields and greater operating performance (Jame s, 2014b). Maize is cultivated in the summer and winter months in Brazil, with many differences between the management during the cropping season. All three categories of GM-type maize, IR, HT, and the stack of IR/HT, are cultivated

in both summer and winter maize. The total area planted with GM traits may reach 15.6 million hectares in 2017 (Table 1.4).

Table 1.4. Area planted and percentage of trait in Brazilian maize in 2016 and 2017 (ISAAA, 2017)

	Area Planted (MHa)		% Trait Hectares	
	2016	2017	2016	2017
Total Maize	17.73	17.55		
IR	3.67	3.26	23.4%	20.9%
HT	0.68	0.66	4.3%	4.2%
IR/HT	11.32	11.69	72.2%	74.9%
Total Biotech Maize	**15.67**	**15.60**	**88.4%***	**88.9%***

*adoption rate; IR – insect resistant; HT – herbicide tolerant; MHa: million hectares

1.2.7 Pests in maize

Insects are the most diverse species of animals worldwide. Predominantly in the tropics and sub-tropics, where the climate offers favourable environment for a wide range of insects, herbivorous insects could be responsible for destroying 20% of the annual world's total crop production. (Sallam, 1999).

Pests able to invade maize in the field can cause extensive damage significantly reducing the yield. The main order causing losses during the growing season are Lepidoptera (see Figure 1.4). The larvae of many Lepidoptera can cause severe damage to agricultural crops (Sallam, 1999). Eggs of lepidopterans are laid on the leaves and stems of host plants, and the caterpillars (larvae) feed on the developing seeds and fruits. The larvae are avid plant feeders and, when the number in a plantation is high, can defoliate host plant rapidly (Russell, 2013).

For example, *Ostrinia nubilalis* larvae are reported to reduce maize yield by tunnelling into the ear shank causing ears to drop before harvest and tunnelling into stems reducing water and nutrient transfer (CABI, n.d.). Whereas *Diabrotica virgifera virgifera* is one of the most serious insect pests of maize. Its larvae damage maize roots reducing the ability of the plant to absorb water and nutrients from the soil causing plant lodging. In addition, adult feeding on silks interferes with pollination causing estimated losses in yield of around 15% (Tinsley, Estes and Gray, 2013).

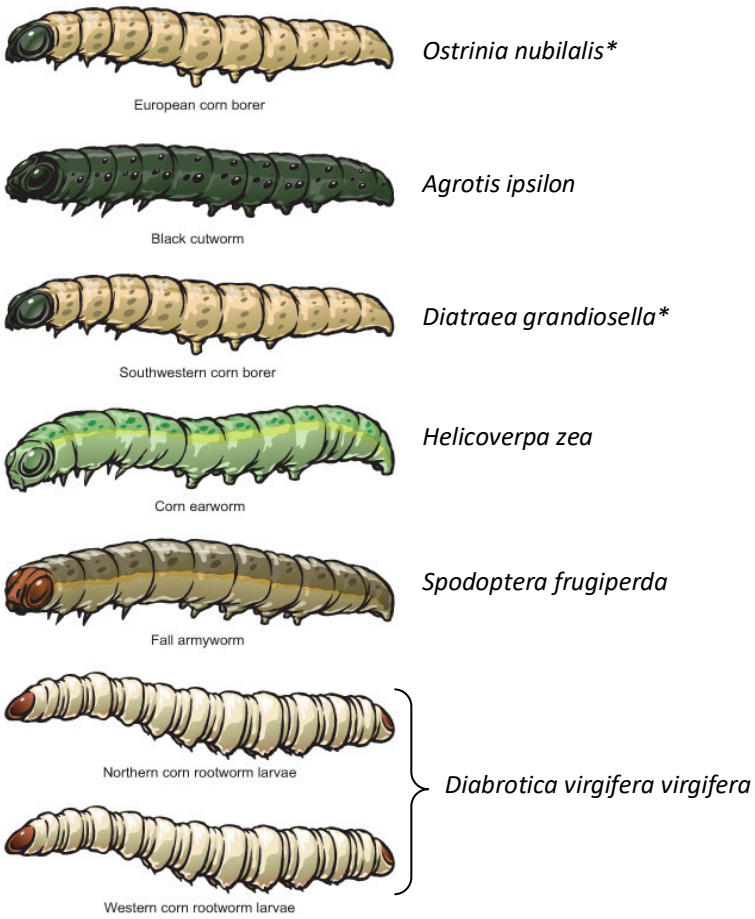

Figure 1.4. Main pests able to invade maize on field and targeted by cultivars with *Bt* toxin. Source: http://www.dowagro.com/en-us/usag/product-solution-finder/traits/smartstax. *incidence not registered in Brazil

Postharvest pests, on the other hand, include species from the orders Coleoptera and Lepidoptera, which are distributed worldwide, causing yield and quality losses of grains and by-products causing significant economic impacts (García-Lara, Chuck-Hernández and Serna-Saldivar, 2013). Species such as *Sitophilus zeamais*, *Prostephanus truncatus*, *Sitotroga cerealella*, *Rhyzopertha dominica* and *Tribolium castaneum* are considered the major pests and cause serious concerns in global agriculture (Figure 1.5) (López-Castillo et al., 2018).

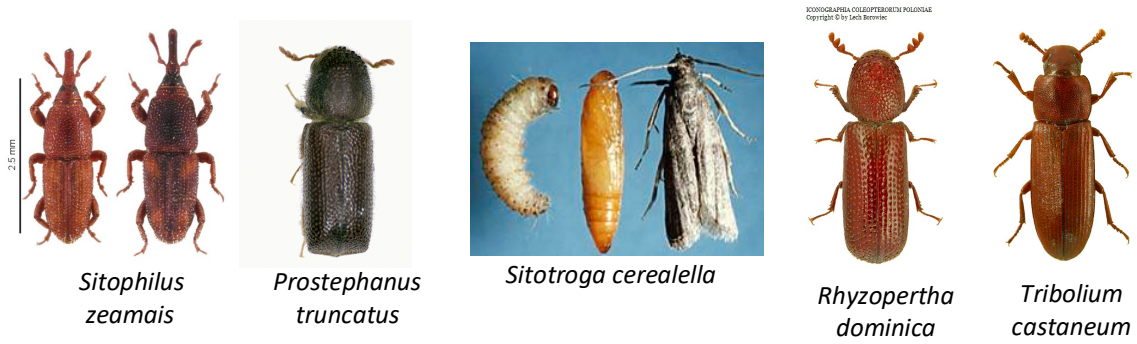

Figure 1.5. Main pests able to invade maize during storage

Primary storage insect pests such as the maize weevil (*S. zeamais* - Coleoptera: Curculionidae), and the larger grain borer (*P. truncates* - Coleoptera: Bostrichidae) often start the infection in the field before harvest when the eggs are laid and continues through storage. Both larvae and adults bore into the grains through neat round holes and feed on the grain resulting in average losses of 30% in stored maize (Taruvinga, Mejia and Sanz Alvarez, 2014). The maize weevil is a major stored grain pest of maize in Brazil where high amount of insecticides are used for its control, whereas the larger grain borer is not commonly present (Santos, 2006). The grain moth *Sitotroga cereallela* (Lepidoptera: Gelechiidae) is able to attack the crops during ripening in the field and is usually transported inside the grain to the storage facilities. The larvae, after hatching, bore into a grain and complete their development entirely within a single grain. Infestation produces abundant heat and moisture that may encourage fungal infection and invasion by secondary pests (Taruvinga, Mejia and Sanz Alvarez, 2014).

In the case of the lesser grain borer *R. dominica* (Coleoptera: Bostrichidae) both adult and larval stages cause heavy damage. Both adults and larvae bore into the kernels to feed on the germ and endosperm creating fine powder, shells of bran and particles of faeces (Agri-Facts, 2014). Differently, infestation by the red floor beetle *T. castaneum* (Coleoptera: Tenebrionidae) is categorized as a secondary pest because the adult and immature forms feed on pre-cracked or broken grains, which were damaged by primary pests (Johnson, 2009; White, 1982)

The GM maize with *Bt* toxin were developed against herbivorous lepidopteran pests attacking leaves, but the toxin also affects Lepidoptera attacking the kernels. However, coleopteran maize weevils were not harmfully affected by transgenic *Bt* maize (Cry1Ab toxin) in their development characteristics (development time, body mass), and females emerging from transgenic maize kernels were larger (Hansen, Lövei and Székács, 2013). On the other hand, *Bt* toxins were efficient to control *S. cerealella*. Furthermore, the spectrum and efficacy of *Bt* cultivars against stored grain insects is predicted to reduce the damage by storage pests (Bushra and Aslam, 2014).

The correlation of pest invasion and fungal infection have been extensively reported (Battilani et al., 2013; Blandino et al., 2014; Tirado et al., 2010; Dowd, 2000). Insect damage is one factor that predisposes maize to mycotoxin contamination. The insect creates wounds facilitating fungal colonization, serving as vectors for fungal spores (Munkvold and Hellmich, 1999; Cast, 2003). Thus, the relationship between pest resistant GM- and non-GM crops becomes of interest in relation to potential for mycotoxin contamination.

1.2.8 Fungal contamination in maize

Maize can be infected by several fungal pathogens during the growing season, harvest and storage (Figure 1.6). Besides pre-harvest fungal foliar pathogens such as rusts, leaf spots, rhizoctonia, anthracnose, eyespot, downy mildews and ergot, the primary genera which can contaminate maize with mycotoxins come from the genera *Fusarium*, *Aspergillus* and *Penicillium* (Battilani et al., 2013). The most common and economically important diseases caused by such fungi are *Fusarium* ear rot (caused by *F. verticillioides*, *F. proliferatum*, and *F. subglutinans*), *Gibberella* ear rot (caused by *F. graminearum*), and *Aspergillus* ear or kernel rot (caused by *A. flavus*) (Munkvold, 2014). These species can survive in crop residues, especially if residues are left on the soil surface and the same crop is grown in the same field continuously (Abbas et al., 2009).

The main impacts of such fungal spoilage are grain discoloration, decrease in the commercial value, decrease in the germinative capacity, dry matter loss, heating, unpleasant odours, chemical and nutritional changes (Abramson, Sinha and Mills, 1984). Recent studies suggest that very small losses in dry matter can result in

fumonisin contamination of maize kernels by *F. verticillioides* (Mylona et al., 2014). Besides such quality losses, contamination by toxic secondary metabolites (mycotoxins) produced by these fungi have serious effects on human and animal health, and can cause cancer (Wu, 2014), even in low concentrations (e.g. aflatoxins; Bennett and Klich, 2003).

The Fusarium species can be found in most bioclimatic regions of the world including tropical and temperate grasslands (Leslie and Summerell, 2008). *F. verticillioides* which has a narrow host range (only maize) and *F. proliferatum* which has a very wide plant host range produce fumonisins which have been linked to oesophageal cancer in some populations in Southern Africa and in China where maize is the staple food (IARC, 2002; Kimanya et al., 2008). Fumonisin B_1, B_2 and B_3 in particular can accumulate in maize kernels (Pascale, Visconti and Chelkowski, 2002).

In tropical and sub-tropical areas of maize cultivation the main concern is the colonisation of ripening cobs during silking by *A. flavus* and *A. parasiticus*, which produce the carcinogenic group of AFs (Perrone, Gallo and Logrieco, 2014; Singh et al., 2014). Recent work in Europe, especially northern Italy has shown that *A. flavus* has become predominant in feed maize and superseded *Fusarium* species under very hot and dry conditions, indicators of CC factors (Giorni et al., 2007; Perrone, Gallo and Logrieco, 2014). Although the occurrence of *A. parasiticus* has been less common (Rodrigues et al., 2011), other new aflatoxigenic species such as *A. mottae*, *A. sergii* and *A. transmontanensis* have been isolated from maize and almonds in Portugal (Soares et al., 2012). *Aspergillus* spp. in section *Nigri* also have been reported as often occurring on maize kernels as opportunistic pathogens (Abarca et al., 2004).

Despite the constant advances on genetic modifications in maize the impacts of gene insertion on the microbial community have not been completely investigated. While the reduction of mycotoxins, mainly produced by *Fusarium* spp. have been associated with the use of GM maize cvs (Pellegrino et al., 2018), information on fungal diversity are less evident. The use of GM crops could have impacts on microbial development. Silva et al. (2014) analysed the endophytic bacterial community of GM maize cvs comparing them with the near-isogenic non-GM line and reported differences between the types of maize that could be associated with the insertion of gene encoding the protein PAT. However, Verbruggen et al. (2012) investigated the arbuscular

mycorrhizal fungal community on GM and non-GM maize using molecular fingerprinting and RNA-based pyrosequencing and did not detect consistent changes. Similar results were reported by Cotta et al. (2013). Gatch and Munkvold (2002) conducted field experiment for 3 years, and reported that *F. verticillioides, F. proliferatum*, and *F. subglutinans* were significantly lower in GM IR hybrids than in non-GM hybrids in 2 of the 3 years studied. However, *Giberella zeae* was higher in some GM samples. Reduced infection of GM IR maize stalks by *F. verticillioides* may provide an unoccupied niche that can be filled by other pathogens (Gatch and Munkvold, 2002). Furthermore, investigation of changes on mycotoxigenic fungal community, especially *A. flavus* when GM cvs are used, or even if there are differences between IR and HT might become important.

Aspergillus ear rot (*Aspergillus flavus)*

Diplodia ear rot (*Stenocarpella maydis)*

Gibberella ear rot (*Gibberella zeae)*

Fusarium ear rot (*Fusarium sp.)*

Figure 1.6. Diseases in maize caused by fungal invasion in the field.

1.2.9 Mycotoxin contamination

Mycotoxins are secondary metabolites produced by fungi that may be injurious to humans and animals upon ingestion, inhalation, or skin contact. Mycotoxins are considered to be the most toxic natural contaminants of food which are produced by a specific range of filamentous fungal species from the *Aspergillus, Fusarium*, *Penicillium* and *Alternaria* genera (Bennett and Klich, 2003). They are very heat stable and thus once they have been produced in raw food commodity, they are difficult to destroy during downstream processing. Thus, both food and feed can be affected. The

metabolism of ingested mycotoxins in food can result in accumulation in different organs or tissues, and then enter the processed food chain through meat, milk, or eggs (Marin et al., 2013).

According to the Food and Agriculture Organization (FAO), more than 25% of the worlds' agricultural production is contaminated with mycotoxins. Thus, several actions have been initiated worldwide aimed at trying to control mycotoxin contamination (Malachová et al., 2014). These include specific regulatory limits, sampling and analytical procedures, decontamination, diversion to less risky uses for contaminated product (Park and Troxell, 2002), and initiatives to control either fungal growth or mycotoxin production.

Some mycotoxins are predominantly produced pre-harvest (e.g. dexoynivalenol by *F. graminearum*) while others are specifically produced at post-harvest (e.g. ochratoxin A by *Penicillium verrucosum*), both in small grain cereals. However, in other crops such as maize, tree nuts and groundnuts, contamination can occur pre-harvest, during harvesting and drying or during post-harvest storage, especially in sub-tropical and tropical regions. Many of the oil-based commodities are very hygroscopic and can adsorb moisture easily. Thus, drying and safe storage become critical factors to minimise mycotoxin contamination (Magan and Aldred, 2005).

The fungi that colonize grains can be divided into three groups. These are so called field fungi, storage fungi and those which can occur both pre- and post-harvest because of their ecology (Magan and Lacey, 1988). The field fungi are present in the phyllosphere of ripening crops and also can colonise seeds either during development or after maturity in the field. Storage fungi contaminate raw commodities during harvesting and can survive saprophytically on dying, dead, or naturally dried crop debris. This group can colonise the highly nutritional food matrices when drying or storage has been poor, especially under intermediate moisture conditions. These include predominantly mycotoxigenic species from the *Aspergillus* and *Penicillium* genera and non-mycotoxigenic spoilage moulds such as the *Eurotium* group of species *(=Aspergillus glaucus* group*).* The intermediate group include these xerotolerant and xerophytic species which can colonise ripening crops under dry conditions and continue to grow post-harvest (Agarwal and Sinclair, 1996; Magan and Aldred, 2007).

Overall, there are a very large number of secondary metabolites which can be toxic produced by different fungal species. Studies by Berthiller et al. (2007) showed that there are probably 300-400 metabolites produced. However, the *Aspergillus*, *Penicillium* and *Fusarium* genera are considered the most important mycotoxin producers. The most known and studied mycotoxins besides aflatoxins are ochratoxin A, trichothecenes type A (T-2, HT-2 toxin); trichothecenes type B (DON, Nivalenol, 3- and 15-acetyl-DON, and DAS), zearalenone, fumonisins, moniliformin, PR toxin and patulin (Bennett and Klich, 2003).

The effects of these mycotoxins have been extensively studied. Ochratoxin A is a nephrotoxin to all animal species studied to date and is most likely toxic to humans (Creppy, 1999) and known as liver toxin, immune suppressant, potent teratogen, and carcinogen (Kuiper-Goodman and Scott, 1989). Within the trichothecene type B group, DON have been reported to induce toxicologic and immunotoxic effects in a variety of cell systems and animal species (Rotter et al., 1996), while NIV have been linked to inhibition on proliferation of human lymphocytes (Severino et al., 2006). T-2 toxin can precipitate reproductive disturbances in sows, and zearalenone and its derivatives are known for its oestrogenic properties (Placinta et al.,1999). Fumonisins, on the other hand, have been associated with equine leukoencephalomalacia and porcine pulmonary oedema (David Miller, 2008; Wu and Munkvold, 2008).

1.2.10 The life cycle of *Aspergillus flavus* in maize

Aspergillus flavus is an opportunistic fungal pathogen able to infect developing maize cobs during silking, especially if the plants have been weakened by drought stress (Dolezal et al., 2014), and damaged by insects (Chulze, 2010). Once *A. flavus* is present in plant tissue, it can continue to colonise the kernels and produce AFs. At harvest, the moisture content of maize cobs will allow colonisation by *A. flavus*. Thus, if not dried efficiently, AFs levels may increase during storage, especially in tropical and sub-tropical environments. Because of its xerophilic nature, *A. flavus* and related species are very aggressive and able to cause storage rots rapidly and increase contamination with AFs (Scheidegger and Payne, 2003a). Environmental conditions can play an important role in disease development. Interactions between intrinsic and

extrinsic factors also have an influence on *A. flavus* growth and AFs production in stored commodities (Magan and Aldred, 2004; 2007).

Figure 1.7 summarises the life cycle of *A. flavus* in maize. The species is capable of surviving and overwintering in plant residues as mycelium or sclerotia (Battilani et al., 2012; Abbas et al., 2009). The conidia can be transmitted by air or insects to serve as new inoculum on host plants or debris in the field to germinate and start the infection cycle (Abbas et al., 2009). Colonisation of the ripening kernels is most aggressive on maize plants that have been exposed to heat or drought stress (Scheidegger and Payne, 2003a). Unlike most fungi *A. flavus* is favoured by hot dry conditions. It has a wide range of temperature tolerance (19–35°C) with about 28°C optimum for growth and 28–30°C for AFs production. Some strains of *A. flavus* can grow in very dry environments (e.g. 0.73 a_w) and produce AFs at 0.85 a_w (Sanchis and Magan, 2004).

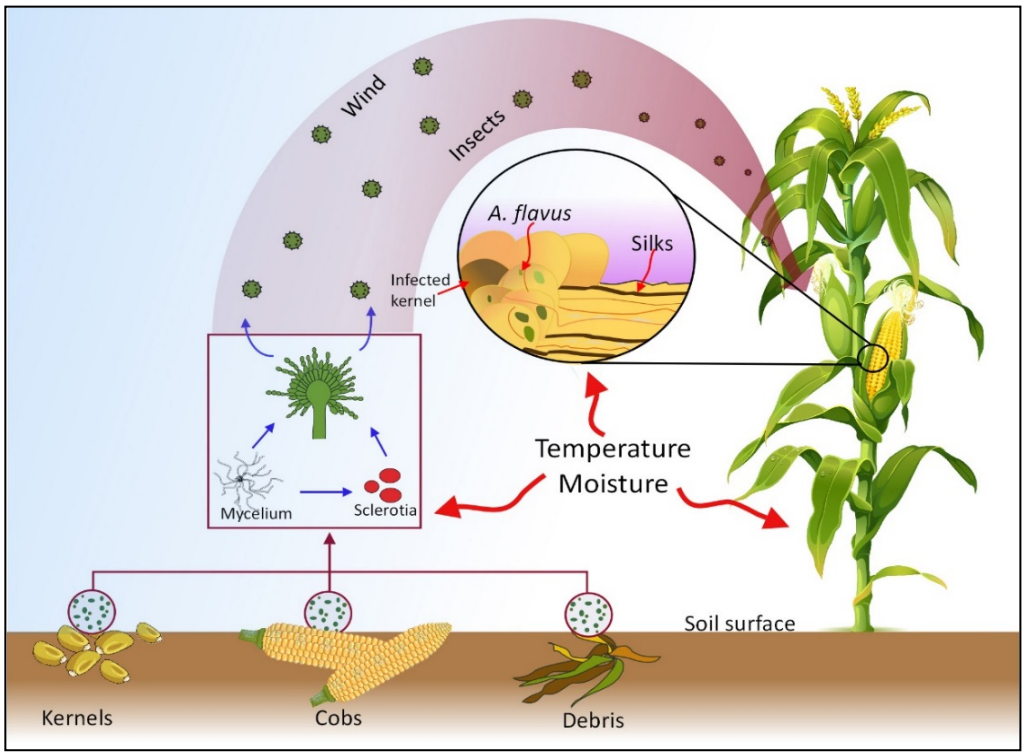

Figure 1.7. Summary of the infection cycle of *A. flavus* in maize. Adapted from Scheidegger and Payne (2003). Sclerotia and conidia produced by *A. flavus* growing on crop debris and in the soil, serve as primary inoculum. Secondary inoculum is provided by conidia produced on crop debris or on infected plants when environmental conditions are favourable for disease development.

1.2.11 Aflatoxins (AFs)

Aflatoxins are difuranocoumarin derivatives produced by a polyketide pathway by members of the *Aspergillus* section *Flavi* (Klich, 2007). They have been shown to be produced by *A. flavus, A. parasiticus* (Codner, Sargeant and Yeo, 1963)*, A. nomius* (Kurtzman, Horn and Hesseltine, 1987)*, A. pseudotamarii (Goto, Wicklow and Ito, 1996), A. bombycis* (Peterson et al., 2001*), A. toxicarius, A. parvisclerotigenus* (Frisvad et al., 2005*), A. minisclerotigenes, A. arachidicola* (Pildain et al., 2008) *and A. pseudonomius and A. pseudocaelatus* (Varga et al., 2011). The most abundant aflatoxin-producing species associated with maize is *A. flavus* (Abbas, Zablotowicz and Locke, 2004). Aflatoxin is predominantly produced by *A. flavus* and *A. parasiticus*. Toxigenic *A. flavus* mainly produces aflatoxin B_1 and B_2, whereas A. parasiticus is well known for producing all the four aflatoxins (B_1, B_2, G_1 and G_2) (Yogendrarajah et al., 2015). However, Frisvad et al. (2019) reported that strains of *A. flavus* isolated from South Korea were able to also produce analogues G_1 and G_2.

Approximately 18 AF analogues are known, and the most toxic analogues are B_1, B_2, G_1 and G_2 (Figure 1.8). Although all aflatoxins have carcinogenic, teratogenic, mutagenic and immunosuppressive effects (Pitt and Hocking, 2006), the International Agency for Research on Cancer (IARC) has classified aflatoxin B_1 as a class 1A carcinogen with hepatotoxicity and cumulative effects (IARC, 2002). In addition to liver cancer, it has been associated with acute poisoning, immune-system dysfunction and stunted growth in children (Wu, 2014). The presence of AFs is estimated to cause up to 28 % of the total worldwide cases of hepatocellular carcinomas (HCC), the most common form of liver cancer (Liu and Wu, 2010). The correlation between naturally occurring food contaminants and cancer is not known by the majority of people (Wu, 2014). AFs are considered to be the group of mycotoxins of greatest concern from a global perspective (Streit et al., 2012). They are heat stable and difficult to destroy during processing. Thus exposure, both acute and chronic, can have significant impacts on vulnerable groups, especially babies and children (Rodríguez et al., 2014).

The frequency and levels of *A. flavus* colonization and aflatoxin contamination variates both seasonally and geographically (Kaaya, Kyamuhangire and Kyamanywa, 2006), depending on differences in the fungal strains (Ehrlich, 2014; Yin et al., 2008), and

interactions with other organisms and environmental conditions. AF contamination appears at both pre- and post-harvest and is highly dependent on biological (biotic) and environmental (abiotic) factors. At post-harvest the invasion can occur when the crop is harvested in wet periods or any unseasonal rains, followed by inappropriate storage conditions. In addition to this, mechanical or insect and bird damage, drought stress can increased contamination in the pre-harvest and result in more AF production (Bhatnagar-Mathur et al., 2015).

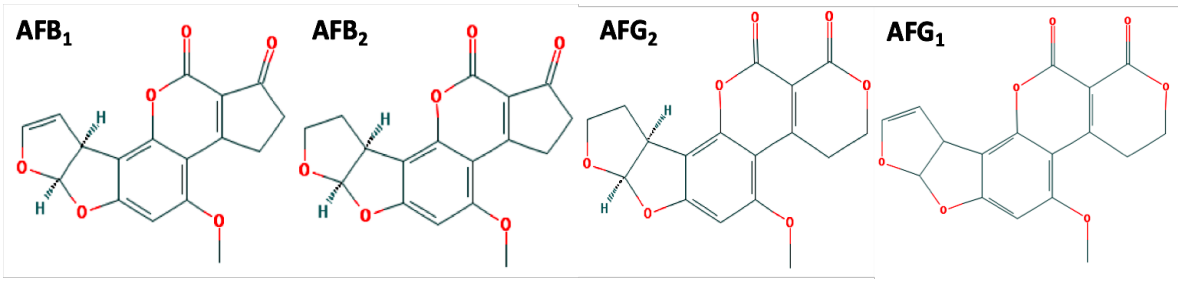

Figure 1.8. Chemical structure of the four main aflatoxins produced by *Aspergillus* section *Flavi:* aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2). Source: http://pubchem.ncbi.nlm.nih.gov.

1.2.12 Biosynthetic pathway of aflatoxin production

Aflatoxin biosynthesis has been proposed to involve at least 23 enzymatic reactions (Yu and Cleveland, 2007) and at least 34 genes (Yu et al., 2004). Thus far, at least 15 structurally intermediates have been identified in the biosynthetic pathway (Yu et al., 2004). The genes involved in AF biosynthesis are in a 70 kb gene cluster. They encode a DNA-binding protein functioning in the pathway gene regulation, and other enzymes such as cytochrome P450-type monooxygenases, dehydrogenases, methyltransferases, and polyketide and fatty acid synthases (Bhatnagar, Ehrlich and Cleveland, 2003).

Bennett, Chang and Bhatnagar (1997) proposed a scheme for pathway transformation of aflatoxin. In this scheme, norsolorinic acid is the decaketide product of a polyketide synthase and the first stable precursor in aflatoxin biosynthesis. After norsolorinic acid (*nor*), the polyketide undergoes to an estimated 12 to 17 enzymatic transformations, leading through a series of pathway intermediates, including averantin (AVN),

averufanin (AVNN), averufin (AVF), versiconol hemiacetal acetate (VHA), versiconol (VAL*)*, versicolorins A (*ver*A) and B (*ver*B, demethylsterigmatocystin (DMST), and sterigmatocystin (ST), to the final product, aflatoxin B_1. More recently Cleveland et al. (2009) illustrate the aflatoxin biosynthesis pathway (Figure 1.9).

Several functions in the biosynthetic pathway have been elucidated. For example, the AF regulatory gene (*aflR*) encodes a positive regulator (AFLR) which activates a pathway gene transcription (Chang et al., 1995b), whereas the gene *aflS* (*afl*J) encodes for a protein factor (AFLS) which was found to be involved in the regulation of transcription. Disruption of *afl*S results in the failure to produce aflatoxin in *A. flavus* (Meyers et al., 1998). Additionally, the gene *afl*D (*nor*-1) encodes an enzyme (norsolorinic acid ketoreductase) known to catalyse the conversion of the first stable AF biosynthesis intermediate, norsolorinic acid, to averantin. Thus, *afl*D is a key structural gene (Zhou and Linz, 1999). Abdel-Hadi et al. (2011) presented results showing that *afl*D expression is a reliable marker to discriminate between toxigenic and non-aflatoxigenic strains.

1.2.13 Fungal colonisation and mycotoxin contamination of Brazilian maize

The tropical and sub-tropical climatic regions in Brazil are favourable for fungal colonisation of a wider range of food matrices. Contamination of maize with aflatoxins and fumonisins has been frequently reported in Brazil (Baquião et al., 2012; Kawashima and Valente Soares, 2006; Moreno et al., 2009). Brazilian maize production is destined mainly for animal feed (82%) particularly for poultry and pig production (de Lourdes Mendes de Souza et al., 2013). According to Salay and Zerlotti-Mercadante (2002) the incidence of AFs, ochratoxin A, and zearalenone in maize can vary significantly in different regions of Brazil. However, the incidence of fumonisins is very widespread all over Brazil at very high levels (Caldas and Oliveira, 2012).

Previously, Caldas and Silva (2007) warned that the results of the mycotoxin exposure assessment conducted at the local and national level showed that certain sub-groups of the population, especially children and elderly people who might eat large amounts

of maize products might be at greater risk in Brazil. In a study by Moreno et al. (2009) of 300 maize samples analysed, about 96% had aflatoxin contamination.

In a more recent study, Oliveira et al. (2017) found fumonisin B_1 and B_2 in all the maize samples analysed. Zearalenone and deoxynivalenol were the second most common mycotoxin, followed by aflatoxins B_1 and G_1 at levels above Brazilian legislative limits (20 $\mu g.kg^{-1}$ – ANVISA, 2011). The study also analysed non-regulated mycotoxins and reported fusarin C in more than 50% of samples. Considering the potential risks associated with each mycotoxin individually, the co-occurrence of these toxic compounds could increase the likelihood of morbidity and mortality in both humans and animals (Oliveira et al., 2017).

The establishment of regulatory limits and the implementation of monitoring programmes are important to help keep mycotoxin contamination under control in the food supply chain. The first Brazilian legislation on aflatoxins was published in 1976, setting a maximum level of 30 $\mu g\,kg^{-1}$ for aflatoxins $B_1 + G_1$ for all food and feed commodities. In 2011, a maximum level of 20 $\mu g\,kg^{-1}$ for sum of aflatoxin ($B_1 + B_2 + G_1 + G_2$) in maize and maize-based products was stablished in Brazil by the Resolution RDC No.7/2011 and for the MERCOSUL countries according to the Resolution GMC No. 25/02 (ANVISA, 2011). Similar maximum levels are adopted by U.S. Food and Drug Administration with a limit of 20 $\mu g\,kg^{-1}$ for total aflatoxins. On the other hand, the European Union (EU) has the most stringent regulatory mycotoxin limits in foods. In the EU, the maximum levels for aflatoxin B_1 is 5.0 $\mu g\,kg^{-1}$ and the sum AFs is 10 $\mu g\,kg^{-1}$ in maize which may be processed and used for human consumption or used as an ingredient in foodstuffs. The limits are laid down in Commission Regulation (EC) No 1881/2006.

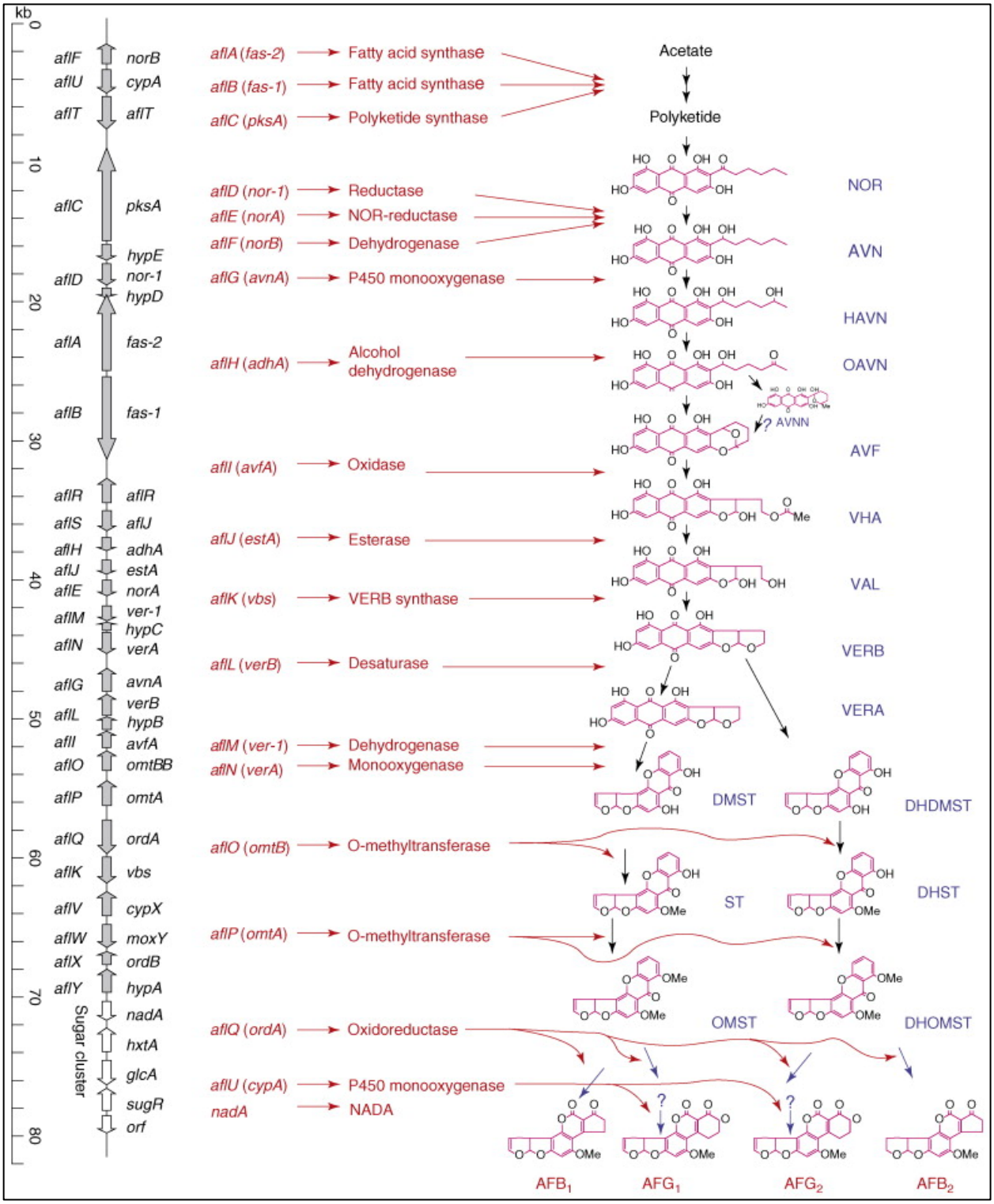

Figure 1.9. Aflatoxin biosynthetic pathway and clustered genes (Cleveland et al., 2009).

The genes with their new and old names are shown on the left, and the generally accepted pathway for aflatoxin biosynthesis is presented on the right. The vertical line represents the 82kb aflatoxin biosynthetic pathway gene cluster and sugar utilization gene cluster in *A. parasiticus* and *A. flavus*. Abbreviations: NOR, norsolorinic acid; AVN, averantin; HAVN, 5′-hydroxy-averantin; OAVN, oxoaverantin; AVNN, averufanin; AVF, averufin; VHA, versiconal hemiacetal acetate; VAL (VHOH), versiconal; VERB, versicolorin B; VERA, versicolorin A; DMST, demethylsterigmatocystin; DHDMST, dihydrodemethylsterigmatocystin; ST, sterigmatocystin; DHST, dihydrosterigmatocystin; OMST, *O*-methylsterigmatocystin; DHOMST, dihydro-*O*-methylsterigmatocystin; AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; and AFG_2, aflatoxin G_2.

1.2.14 Contamination of GM- and non-GM maize with aflatoxins

The problem of mycotoxin contamination requires integrated strategies of Good Agricultural Practice (GAP) during harvesting and storage, to processing and utilization (Munkvold, 2014) to reduce risks of high contamination. Processes such as rotation and tillage can impact on fungal dispersion and can result in mycotoxin contamination, most significantly deoxynivalenol and zearalenone, or even fumonisins and aflatoxins (Munkvold, 2014). Additionally, the planting date can considerably affect fungal infection because the infection depend on the co-occurrence of a susceptible plant developmental stage and favourable environmental conditions (Munkvold, 2014). Drought-stress could increase maize vulnerability to aflatoxin and fumonisin contamination (Abbas et al., 2009).

In addition, pest management is also important approach for reducing the mycotoxin risk (Alma et al., 2005). Mainly Lepidopteran species serve as vectors for fungal spores creating infection sites for toxigenic fungi (Alma et al., 2005). GM insect resistance genes (IR- *Bt*) are suggested as most effective way to manage Lepidopteran insects and reduce the associated with mycotoxin risk (Munkvold et al., 1999; Wu, 2006). The *Bt* toxin attaches itself to the cell membrane in the stomach and intestinal canal of the insects causing cell rupture and leaking killing insects (Schnepf et al., 1998), and as result its population is reduced during the cultivation.

Wu (2006) cited that the benefits of GM *Bt* maize for reduction of mycotoxin damage has been practically ignored. As adoption of GM crops continues to increase on a global scale. Thus, policy makers worldwide need to consider the economic and health impacts of GM crops as a secondary benefit. In some cases, the reduction of mycotoxins offered by GM maize can impact economically. In less developed countries where certain mycotoxins are significant contaminants of food, GM maize adoption may even improve human and animal health (Wu, 2006).

Windham et al. (1999) described that using a GM maize (*Bt*11) more than 75% of AF was reduced compared with its conventional isogenic when the plants

where infested by Southwestern corn borers. When plants were infested with Southwestern corn borers and inoculated with *A. flavus*, AF concentrations in the GM hybrid were about 50% lower. Field studies conducted by Munkvold and Hellmich (1999) showed reduced kernel infection by *A. flavus* and lower aflatoxin concentrations using Bt11 and MON810 hybrids compared with their non- GM *Bt* counterparts. Although the results described by the authors support the utility of *Bt* hybrids for management of *Fusarium* and *Aspergillus* and fumonisin and aflatoxin, this might not be enough when conditions are very favourable for disease. The limitation of GM *Bt* maize hybrids is their spectrum of activity. Damage to ears of *Bt* hybrids by other insects probably leads to fungal infection and mycotoxin contamination. Clements et al. (2003) described the inefficiency of GM *Bt* hybrids against corn earworms, and thus did not significantly reduce mycotoxin contamination associated with such insects.

On the other hand, no studies have compared the influence of GM crops with either herbicide-tolerance or herbicide-tolerance + insect-resistance on colonisation by *A. flavus* and AF contamination, in relation to non-GM maize cultivars. In addition, Wani (2010) suggested that the development of transgenic varieties with anti-fungal traits that confer resistance to aflatoxin-producing fungi would be extremely valuable. However, developing fungal resistant crops to combat pre-harvest infection and resulting contamination has remained a challenge.

In fact, the first study to report the possibility to create an aflatoxin-free transgenic maize using host induced gene silencing was done by Thakare et al. (2017) creating a possibility for a new generation of GM maize cultivars. The authors transformed maize plants with a kernel-specific RNA interference (RNAi) gene cassette targeting the *afl*C gene, which encodes an enzyme in the *Aspergillus* aflatoxin biosynthetic pathway. After pathogen infection, aflatoxin could not be detected in kernels from these RNAi transgenic maize plants (Thakare et al., 2017). Meanwhile, biocontrol has been largely investigated as potential tools to reduce aflatoxin contamination of crops. The predominant studies have focused

on atoxigenic *A. flavus* strains being used to control toxigenic strains in cotton, maize and groundnuts commercially (Bhatnagar-Mathur et al., 2015).

1.2.15 Biocontrol of *Aspergillus flavus* and aflatoxins in maize

Contamination of cereal commodities by fungi and mycotoxins results in dry matter, quality, and nutritional losses and represents a significant hazard to the food chain (Magan and Aldred, 2007a). The impacts of mycotoxins, and in particular, aflatoxin imposes an vast socio-economic cost (Wu and Khlangwiset, 2010). In industrial countries, the estimated cost of aflatoxins contamination are clearer, because the costs are primarily market related (Wu, 2004). In less developed countries, however, estimating the total socio-economic cost of aflatoxins is more complex and difficult (Wu and Khlangwiset, 2010).

Approaches for prevention, elimination, and decontamination or inactivation aiming minimize the risk of mycotoxin in the pre-harvest, at harvest and post-harvest have been explored (Choudhary and Kumari, 2010). Interventions to reduce aflatoxin-induced illness could be grouped into three categories: dietary, clinical and agricultural. Dietary and clinical can be considered ‘secondary’ interventions. Agricultural interventions are methods or technologies applied either in the pre-harvest and post-harvest stages and are considered ‘primary’ interventions, because they directly reduce aflatoxin contamination in food (Wu and Khlangwiset, 2010).

Biological control, which is the use of non-chemical means of controlling both pests and diseases has been considered among the most promising technologies for sustainable agriculture (Tracy, 2014). Biocontrol technologies using competitive exclusion of toxigenic *A. flavus* strains by non-toxigenic ones have been demonstrated to significantly reduce the contamination of maize (Dorner, 2009), peanuts (Dorner et al., 2009) and cotton (Cotty, 1994) with aflatoxins. Indeed there are commercial products such as “aflaguard” and “aflasafe” which are been used in different agroecosystems in the USA and also in West and East Africa (Cotty et al., 2007).

Biocontrol broadly refers to the use of organisms to reduce the incidence of pests, diseases, or toxins (Pitt and Hocking, 2006). Bacterial species such as *Bacillus subtilis*, *Lactobacillis* spp., *Pseudomonas* spp., *Ralstonia* spp. and *Burkholderia* spp. have been shown to inhibit growth of *A. flavus* and consequent production of aflatoxins (Palumbo, Baker and Mahoney, 2006). Several strains of *B. subtilis* and *P. solanacearum* isolated from the non-rhizosphere maize soil inhibited aflatoxin accumulation (Nesci, Bluma and Etcheverry, 2005). However, the use of these microorganism are less effective under field conditions because of their requirement for water films to colonise the rhizosphere and compete effectively with *A. flavus* at the infection sites, especially under drought stress conditions (Dorner, 2005, 2004). Similarly, some saprophytic yeast species such as *Pichia anomala* and *Candida krusei* have shown potential as biocontrol agents of *A. flavus* in vitro (Masoud and Kaltoft, 2006). Other species which have been screened include *Trichoderma* spp., *Pseudomonas aeruginosa*, and *Streptomyces cavourensis* (Waliyar et al., 2013). Nevertheless, translating *in vitro* experiments into field efficacy has not always been effective because environmental screens to identify stress tolerant strains have not often been used (Mohale et al., 2013). Also, often effects on growth of *A. flavus* have been addressed, without focusing directly on inhibition of aflatoxin contamination (Bhatnagar-Mathur et al., 2015).

Other potential options for *A. flavus* management in the field has been mostly focused on the use of atoxigenic isolates of *A. flavus* which may be able to compete with the toxigenic strains displacing them resulting in less aflatoxin contamination. This approach has been very successful in cotton in the USA (Cotty et al., 2008; Dorner, 2004). Cotty et al. (2007) studied the biocontrol strategy referring to field application of atoxigenic *A. flavus* strain AF36. The authors found significant reductions in aflatoxin contamination in cottonseed with AF36 applications in Arizona, Texas, and California, USA. In the applications, wheat seeds were coated with conidia of the AF36, and these seeds were applied to cotton fields at a strategic time so that the atoxigenic strains competitively exclude toxigenic strains from colonizing crops and thereby reduce aflatoxin concentration. Atehnkeng et al. (2008) inoculated atoxigenic strains into wounds

in maize ears together with an aflatoxin strain producer. They found that the atoxigenic strains competed effectively reducing the aflatoxin production both pre-harvest and post-harvest in maize production in Nigeria.

The effectiveness of biocontrol using atoxigenic *A. flavus* strains is based on the fact that these are predominantly asexual, genetically stable and aggressive as competitors coupled with their inability to recombine with native toxigenic strains (Abbas et al., 2011a; Ehrlich and Cotty, 2004). Although the mechanism by which a non-aflatoxigenic strain interferes with aflatoxin accumulation of toxigenic strains has not been definitively elucidated (Huang et al., 2011). Hruska et al. (2014) supported the theory of competitive exclusion through robust propagation and fast colonization by the non-aflatoxigenic strain which was correlated with depression of the aflatoxigenic strains. However, this study also pointed out concerns regarding the long-term use of non-aflatoxigenic fungi for suppression of native toxigenic strains in biocontrol strategies. Studies to compare the C-source utilisation patterns by atoxigenic and toxigenic strains of *A. flavus* showed that, regardless of environmental conditions, the utilisation rates of maize related nutrients were relatively similar for these two types of strains (Mohale et al., 2013). Thus, the competitive exclusion theory may not be the comprehensive mechanism by which control is achieved.

Available studies suggest that biocontrol strains are capable of reducing aflatoxin-producing populations only by four- to five-folds (Ehrlich, 2014). Two commercial products based on atoxigenic *A. flavus* strains have been approved in the U.S. by the Environmental Protection Agency for biological prevention of aflatoxin: afla-guard® commercialized by Syngenta is for use on peanuts and maize, and AF36® developed in Arizona, USA is for use in cottonseed. Other promising *A. flavus* biocontrol candidate strains include TX9-8 (Chang and Hua, 2007), AF051 (Jiang, Yan and Ma, 2009) and TOϕ (Degola, Berni and Restivo, 2011). The use of native atoxigenic isolates may reduce some concerns about safety and environmental impacts (Atehnkeng et al., 2008). However, few studies have been made to examine the efficacy of atoxigenic *A. flavus* strains in GM- and isogenic non-GM maize cultivars in terms of aflatoxin control.

1.2.16 Climate change: knowledge of impacts on fungal contamination and mycotoxin production

The potential effect of climate change (CC) scenarios on food production and food security has become a very important problem worldwide. Available evidence and predictions suggest overall negative effects on agricultural production as a result of CC, especially when more food is required by an ever increasing population (Dwivedi et al., 2013). According to United Nations' projections (USDA/DESA, 2011) the world population would reach 9,3 billion by 2050 and the climate will be warmer by 2°C due the increase in the CO_2 and ozone concentrations (Jaggard, Qi and Ober, 2010). The concentration of CO_2 in the atmosphere has continuously increased from pre-industrial levels at 280 ppm to 412 ppm at present (NOAA and Climate.gov, 2018 - Figure 1.10), and is expected to reach above 800 ppm by the end of the century (IPCC, 2014).

The increase in CO_2 and ozone concentrations, and the resulting changes in temperature and precipitation patterns, will affect plant physiology with possible significant effects on the sensitivity of plants to environmental stresses such as drought, waterlogging or heat, and plant diseases caused by pathogenic microorganisms (Mikkelsen et al., 2015). These abiotic and biotic stresses are a major cause of yield and quality loss and are significant limitations for plant production globally (Mikkelsen et al., 2015). Also, Boote et al. (2005) and Schlenker and Roberts (2009) suggest crop yield decline at temperatures above 30°C. Lobell et al. (2011) indicated a decline of 3.8% in the global maize production, as a result of warming during the period from 1980 to 2008.

Magan et al. (2003) cited climate as a crucial factor for fungal colonization and mycotoxin production. The effect of CC on mycotoxin production is very complex and difficult to predict completely because many of the factors will interact with each other (Dwivedi et al., 2013). The largest effects on mycotoxins from CC has been suggested to include so called hot spots of southern Europe, parts of the USA and South America. Crops when subjected to drought and high temperature, especially during the reproductive phase, are under greater risk of aflatoxin

contamination by *A. flavus* and *A. parasiticus*, with perhaps also a greater risk of increasing aflatoxin contamination during storage (Paterson and Lima, 2011).

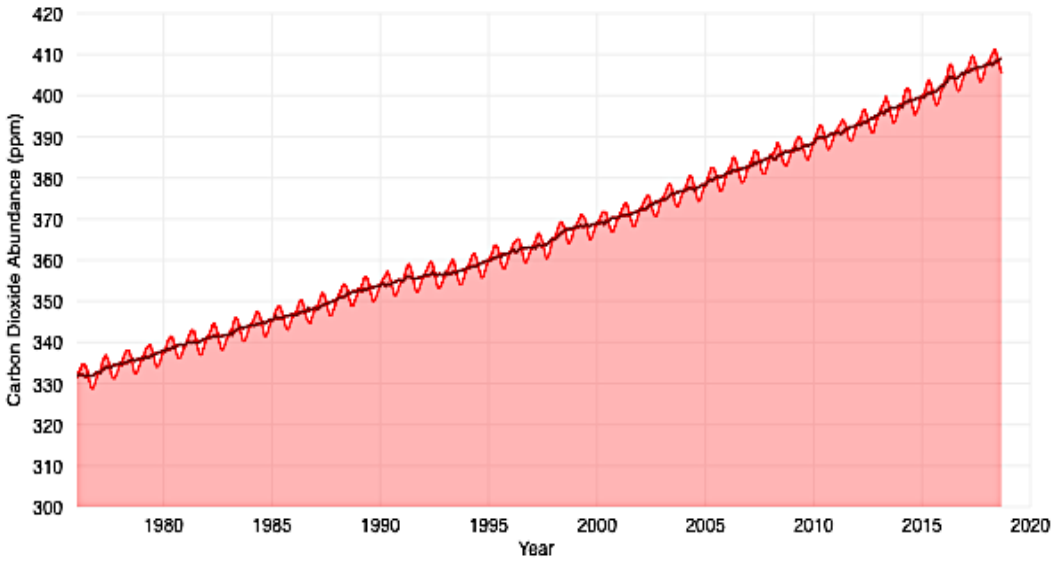

Figure 1.10. Carbon dioxide (CO_2) abundance in parts per million (ppm) at NOAA's Mauna Loa Observatory on Hawai'i (NOAA and Climate.gov, 2018). The bright red line shows monthly average CO_2. The dark red line shows the annual trend, calculated as a 12-month rolling average.

Water activity (a_w) and temperature, and their interactions with *A. flavus* growth, genes in the aflatoxin biosynthetic cluster pathway, and toxin production have been shown to significantly influence the amounts of aflatoxin produced (Magan, Medina and Aldred, 2011; Miraglia et al., 2009). However, these need to be examined when interacting with elevated CO_2 to get a true picture of the potential impacts which might occur on fungal colonisation and toxin production. This would help in providing the necessary data to develop more accurate models of the potential impacts on mycotoxigenic fungi (Medina et al., 2014). Indeed, no information exists on how infection of GM-maize by *A. flavus* and aflatoxin production may be affected by different CC *scenarios* and how this may differ from non-GM cultivars.

Magan et al. (2011) and Medina et al. (2014) examined that a_w × temperature stress effects on changes in mycotoxin production when the temperature is changed by +3 and +5°C. In most cases when temperature was changed, and

drought stress imposed the mycotoxin production was unchanged or slightly reduced. However, these studies did not include CO_2 as another interacting abiotic factor. Recently, Vaughan et al. (2014) showed that twice the existing CO_2 concentrations increased the susceptibility of maize to *Fusarium verticillioides* proliferation although fumonisin B_1 mycotoxin production was not affected.

Medina et al. (2015), conducted the first study which examined the effect of three-way interacting climate change factors (water stress × temperature × elevated CO_2) on *A. flavus* and aflatoxin production. This study showed that *in vitro* essays, while growth was relatively unaffected, the expression of key genes such as the *aflR* and *aflD* were significantly increased together with the production of aflatoxin B_1. More recently, Gilbert et al. (2018) using RNA-Sequencing demonstrated that AFB_1 production on stored maize grain was altered by a_w × temperature × elevated CO_2. Also, several genes involved in the biosynthesis of secondary metabolites, exhibit different responses to a_w or temperature stress depending on the atmospheric CO_2 content. At 37°C and 1000 ppm CO_2 the transcription factor *afl*R was decreased. After 10 days incubation the expression of biosynthetic genes in maize stored at 30°C generally decreased. However, the effects of high CO_2 (1000 ppm) and water stress (0.91 a_w) showed decreased values, possibly in response to elevated AFB_1 levels (Gilbert et al., 2018).

The effects of CC on mycotoxins in the pre-harvest phase may be via the fungi, the hosts and hosts–fungi interactions. Nonetheless, short-term seasonal fluctuations may mask the long-term effects environmental change on mycotoxin production (Paterson and Lima, 2010). Up to the point of harvest, the plant will play a key role in determining the degree of mycotoxin contamination. While during storage, the condition of the grain is a major factor in its stability. If CC alters the nature of the conditions in both pre- and post-harvest the stability of the crop between harvesting and marketing will be affected (Tirado et al., 2010).

According with EFSA projections, the aflatoxin contamination in maize will change in distinct CC scenarios. With +2°C higher levels of contamination are expected in the areas where maize is currently grown (Battilani et al., 2013).

Whereas in the +5°C scenario the levels of contamination are predicted to be lower, but risks are expected towards northern EU countries. In the +5 °C scenario the projections estimated constant advance in flowering and harvesting dates. (Battilani et al., 2013). Reduction in season length, and advance in flowering and harvest dates could allow an expansion of the crop growing areas, inferring possible changes in the maize vulnerability to fungal colonization and subsequent AF production in the field (Battilani et al., 2013, 2016).

Furthermore, higher temperatures due to climate warming could create new challenges related to alter insect population growth rates, increasing insect overwintering and voltinism (number of generations of an organism in a year), altering crop-pest synchrony and geographical ranges of important pest species. Changes in the insect feeding patterns caused by CC will require adaptation of insect management practices (Wu et al., 2011). All these factors could lead to increase the damage in the kernels and allow more rapid entry of fungal contamination, and by extension, increase mycotoxins levels. Thus, the use of GM *Bt* crops could be a strong ally under climate change conditions where problems associated with insects may increase, however there is little knowledge about this correlation under several climatic variations in maize.

1.3 Research Objectives

The overall aim of this study was to investigate and understand the impacts of climate change (CC) on the colonization of conventional and GM maize by *Aspergillus flavus* and the production of AFs. This requires a good understanding of the ecophysiology of *A. flavus*, the key genes involved in the biosynthetic pathway of aflatoxin formation and how gene traits inserted in the maize provide insect resistance or herbicide tolerance which might impact on future risks and control strategies, including biocontrol.

The present work was divided into five sequential Phases each with its own aims and objectives:

The **first Phase** was to establish the fungal biodiversity on GM and non-GM maize isogenic cvs from Brazil in two seasons (2015 and 2016) in terms of isolation frequency (% IF) and relative fungal populations (CFUs/g dry weight) for *A. flavus* and other maize mycobiota. The ability to produce aflatoxins (AFs) by *Aspergillus* section *Flavi* strains isolated and full mycotoxin contamination profiles of the maize samples were examined. Their moisture content and water availability were measured to try and correlate the relationship between microbial load and the quality of the maize samples.

The **second phase** was to study the effect of different interacting environmental conditions on growth and AFB_1 production *in vitro* (3% milled-maize agar) using different GM and the isogenic non-GM maize cvs. This phase was designed to understand the patterns and optimal conditions for growth and AFB_1 production of the strains isolated from Brazilian maize and whether using GM cvs can affect the colonisation behaviour in terms of growth and AFB_1 production when compared with non-GM maize.

The **third Phase** was designed to isolate and identify possible candidate antagonistic microorganisms from Brazilian maize (bacteria, yeasts, filamentous fungi) which were antagonistic to the pathogen *A. flavus*. These studies were done in dual-culture assays. Antagonism was assessed from interaction scores

in dual-culture assays, Index of Dominance, and by quantifying the inhibition of *A. flavus* growth rate/colony development. This enabled the identification of some potential candidates for further studies. The combined effects of varying inoculum ratios of selected biocontrol agents (BCAs) on AFB_1 production by *A. flavus in vitro* (3% milled-maize agar) under different a_w levels were then studied.

The **fourth Phase** was designed to monitor the deletions of key biosynthetic genes in the AFs and cyclopiazonic acid (CPA) cluster in order to select the most viable BCA amongst the atoxigenic strains used in the third phase. A strain with important deletions in the AF cluster was studied as a BCA in the storage environment using kernels from conventional and GM maize. The efficacy of atoxigenic *A. flavus* strains as BCAs was measured under different a_w conditions on the relative control of AFB_1, and on relative expression of a structural (*alf*D) and regulatory (*afl*R) gene.

The **fifth Phase** studied the effect of simulated pest damage in irradiated maize grain on the biosynthetic pathway genes involved in AFB_1 production**.** Cultivars of GM maize and isogenic non-GM were selected and artificially damaged and studied under different a_w levels at optimal temperature for AFB_1 expression and production. Subsequently, the effect of climate change scenarios on gene expression of key biosynthetic genes and on AFB_1 production associated with simulated pest damage was investigated under different a_w conditions, temperature and CO_2 levels. The resilience of the BCAs were tested under these conditions.

The present study was divided into 5 phases. Each phase and the specific aims are described below:

Phase 1. Fungal diversity and mycotoxin profiles of conventional and GM isogenic maize lines from Brazil

a. To compare the fungal diversity of 10 pairs of GM and non-GM maize cvs from Brazil from the harvest seasons 2015 and 2016

b. To determine the isolation frequency (% IF) of the dominant fungi and total fungal load (CFUs/g dry weight) in the maize cvs
c. Comparison of the mycotoxin profiles (e.g., aflatoxins, ochratoxins trichothecenes, fumonisins) present in the non-GM and their respective isogenic GM cvs using LC-MS/MS
d. To isolate *A. flavus* strains or other fungal species for use in later biocontrol screening studies in this project
e. To examine the *A. flavus* isolates for their ability to produce aflatoxins using HPLC-FLD

Phase 2. Ecological studies on growth and aflatoxin B_1 production by *Aspergillus flavus* in conventional and GM maize-based matrices

a) To examine the effects of different interacting environmental factors (*i.e.*, different a_w x temperature levels) on growth rate and aflatoxin B_1 (AFB_1) production by the isolated *A. flavus* strains on maize-based media using non-GM cultivars and their respective isogenic GM lines

Phase 3. Screening for potential biocontrol agents (BCAs) for control of *Aspergillus flavus* and aflatoxin B_1 production using different inoculum ratios and water activity regimes *in vitro*

a) To determine the interactions, competitiveness and Index of Dominance (I_D) between the isolated *A. flavus* strains and the type strain of *A. flavus* NRRL 3357 with competitors (N=16) using a colony-based interaction approach
b) To examine the best potential BCAs to control AFB_1 production *in vitro* by using different ratios of pathogen: antagonist (100:0; 25:75; 50:50; 25:75; 0:100) on milled-maize agar modified to 0.98 and 0.95 a_w using a conventional maize cultivar and its respective isogenic GM line as nutritional substrates.

Phase 4. Monitoring deletions in the aflatoxin gene cluster of *Aspergillus flavus* strains with potential biocontrol action and effects on biosynthetic toxin gene expression and aflatoxin B_1 production

a) To select atoxigenic *A. flavus* strains with potential biocontrol action and examine the deletion of key structural and regulatory genes involved in aflatoxin biosynthesis using Multiplex PCR
b) To examine the best atoxigenic/toxigenic strain ratio in stored maize kernels of two conventional (non-GM) and their respective isogenic genetically modified (GM) cultivars under different a_w conditions on relative control of AFB_1, and on relative expression of a structural (*alf*D) and regulatory (*afl*R) gene in the aflatoxin biosynthetic pathway

Phase 5. Biocontrol resilience under simulated pest damage and climate change scenarios in conventional and GM Brazilian maize

a) To simulate three different levels of pest damage (0, 5 and 15%) in gamma-irradiated maize kernels of conventional (non-GM) and genetically modified (GM) cultivars at 0.98 and 0.95 a_w and 30^{o}C to examine effects on the temporal aflatoxin B_1 (AFB_1) production (10 and 20 days), and on the relative expression of the *aflD* and *aflR* biosynthetic genes
b) To examine the effects of interacting CC conditions using the optimal temperature and +5^{o}C at 400 vs 1000 ppm CO_2 on *A. flavus* colonisation, aflatoxin B_1 production and on the relative expression of the *aflD* and *aflR* biosynthetic genes using one GM maize cultivar and its respective isogenic non-GM line
c) To examine the relative effectiveness of the best BCA under CC scenarios and with simulated pest damage on AFs control.

The flow diagram of the thesis is presented in Figure 1.11.

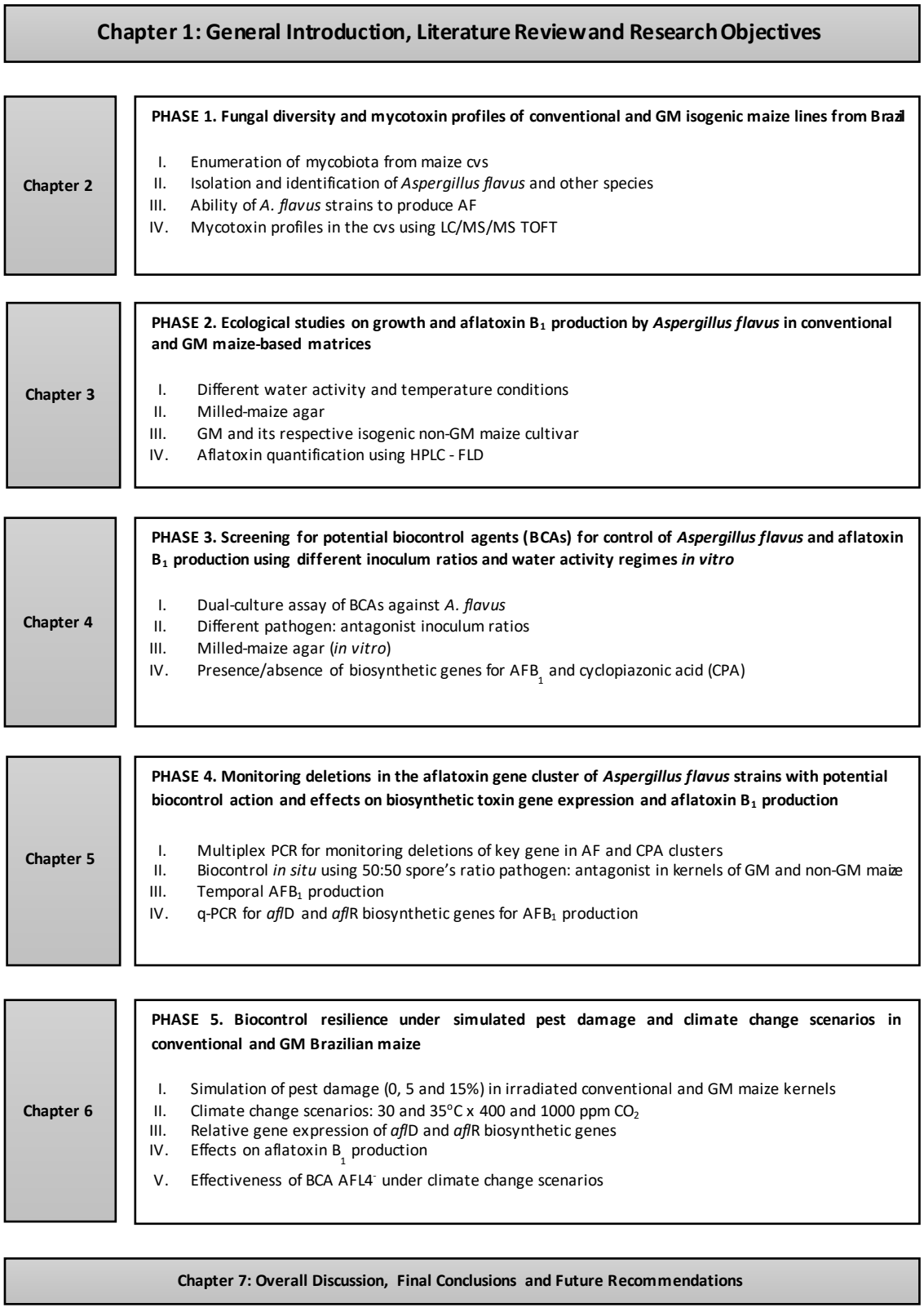

Figure 1.11. Flow diagram of experimental work and thesis arrangement

CHAPTER 2

FUNGAL DIVERSITY AND SECONDARY METABOLITE PROFILES OF CONVENTIONAL AND GM ISOGENIC MAIZE LINES FROM BRAZIL

CHAPTER 2. FUNGAL DIVERSITY AND MYCOTOXIN PROFILES IN CONVENTIONAL AND GM ISOGENIC MAIZE LINES FROM BRAZIL

2.1 Introduction

Fungal pathogens of maize are important as they can cause significant economic losses in quality and yield pre-harvest, during harvesting and post-harvest storage. Besides foliar fungal pathogens such as rusts, leaf spots, *Rhizoctonia*, anthracnose, eyespot, downy mildews and ergot, the primary genera which can contaminate maize with mycotoxins come from the genera *Aspergillus, Fusarium* and *Penicillium* (Battilani et al., 2013). The most common and economically important diseases caused by such fungi are *Aspergillus* ear or kernel rot (caused by *A. flavus*) and *Fusarium* ear rot (caused by *F. verticillioides*, *F. proliferatum*, and *F. subglutinans*), *Gibberella* ear rot (caused by *F. graminearum*) (Munkvold, 2014).

Currently more than 300 types of mycotoxins with different chemical structures and toxic effects are known. However only some of them are considered important in the food safety context, among them aflatoxins, ochratoxin A, fumonisins, zearalenone, and some trichothecenes (deoxynivalenol and nivalenol) (Arisseto-Bragotto, Feltes and Block, 2017). Aflatoxins are the most highly toxic secondary metabolites, therefore, there is great demand for aflatoxins research to develop suitable methods for their quantification, precise detection and control to ensure the safety of consumers' health (Kumar et al., 2016).

The tropical and sub-tropical climatic regions in Brazil are favourable for fungal colonisation of a wider range of food matrices. Contamination of maize with aflatoxins and fumonisins has been frequently reported in Brazil (Baquião et al., 2012; Caldas and Oliveira, 2012; Kawashima and Valente Soares, 2006; Moreno et al., 2009; Salay and Zerlotti Mercadante, 2002).

The adoption of GM crops continues to increase on a global scale and the effects on mycotoxin contamination has been to a large extent ignored (Wu, 2006). It has been reported that when plants were infested with Southwestern corn borers, a GM (*Bt*11) hybrid had >75% reduction in aflatoxins when compared with its non-Bt counterpart (Windham et al., 1999). In Brazil, despite the large GM maize production, few surveys have investigated the correlation and the effects of the cultivation of GM crops with mycotoxin contamination compared with non-GM maize cultivars. Furthermore, no studies have compared the influence of GM crops which have either herbicide-tolerance, insect resistance or both herbicide-tolerance + insect-resistance on colonisation by dominant mycobiota and mycotoxin contamination and comparisons with the original non-GM isogenic cvs.

2.2 Objectives

a. To compare the fungal diversity of 10 pairs of GM and non-GM maize cvs from Brazil from the harvest seasons 2015 and 2016
b. To determine the isolation frequency (% IF) of the dominant fungi and total fungal load (CFUs/g dry weight) in the maize cvs
c. Comparison of the mycotoxin profiles (e.g., aflatoxins, ochratoxins trichothecenes, fumonisins) present in the non-GM and their respective isogenic GM cvs using LC-MS/MS
d. To isolate *A. flavus* strains or other fungal species for use in later biocontrol screening studies in this project
e. To examine the *A. flavus* isolates for their ability to produce aflatoxins using HPLC-FLD

2.3 Materials and Methods

2.3.1 Maize samples

The maize samples were harvested in Brazil in the 2015 and 2016 seasons in two different States (location 1 – Paraná, location 2- Mato Grosso; Figure 2.1). Location 1 is a humid subtropical zone with average temperature ≥25°C in the harvest season. Location 2 has a tropical climate with mean temperatures ≥28°C

in the harvest season. One sample (approximately 1 kg) of each maize cv (total of 10 pairs) were obtained after harvest and drying, and stored at 4°C. The details of the different Brazilian cvs examined are shown in Table 2.1.

2.3.2 Measurement of moisture content (%MC) and of water activity (a_w) of the maize samples

Three 10-g sub-samples of each cultivar were weighed and placed in glass vials. These were dried in an oven at 110°C for 24h. Thereafter, they were placed in a desiccator jar containing silica gel and left to cool and the final dry weight obtained. The percentage moisture content (%MC) was then calculated on a wet weight basis using the equation below:

$$\%\mathrm{MC} = \frac{\text{wet weight of sample} - \text{dry weight of sample}}{\text{wet weight of sample}} \times 100$$

Equation 2.1. Formula to calculate the percent of moisture content (%MC)

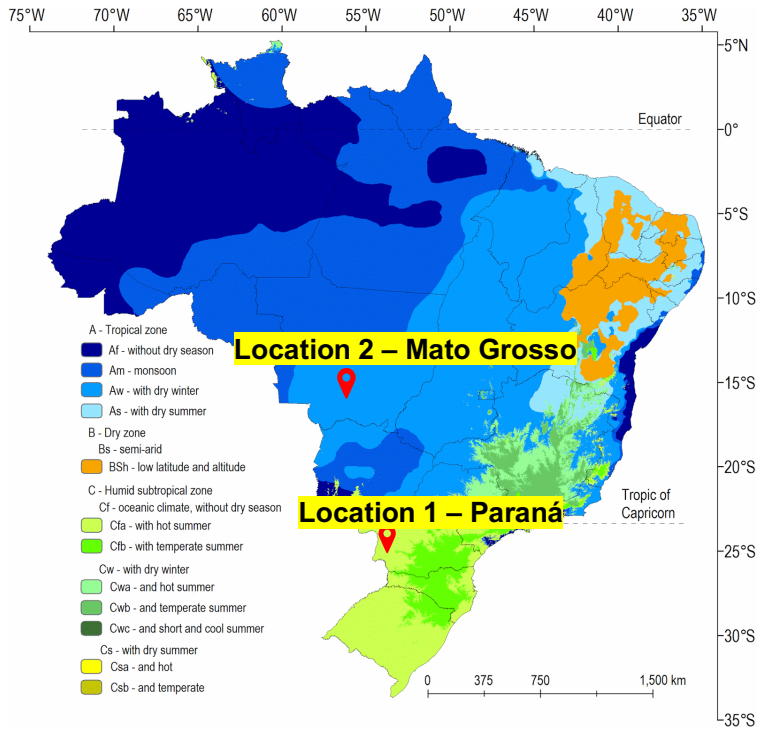

Figure 2.1. Brazil map of climate classification (Köppen and Geiger, 1936; Alvares et al., 2013) indicating the two locations from where the maize samples originated: Location 1 - Paraná and Location 2 - Mato Grosso.

Table 2.1 Characteristics of the GM and non-GM cultivars of maize grain used in this study.

Conventional cultivars (non-GM)	GM cultivar	Traits tolerance present in the GM cvs
AS 1555 CON	AS 1555 PRO®	Pesticide tolerant
P30F53 CON	P30F53 H®	HT - Glufosinate ammonium Antibiotic resistance IR - Lepidopteran
P2530 CON	P2530 Hx®	IR - Lepidopteran
BM-709 CON	BM-709 PRO_2®	HT - Glyphosate IR - Lepidopteran
M20-A78 CON	M20-A78 PW®	HT - Glyphosate HT - Glufosinate ammonium IR - Lepidopteran
CD-384 CON	CD-384 PW®	HT - Glyphosate HT - Glufosinate ammonium IR - Lepidopteran
AS 1556 CON	AS 1556 PRO_2®	HT - Glyphosate IR - Lepidopteran
nd	2B587 Hx®	Glyphosate HT - Glufosinate ammonium IR - Lepidopteran
nd	Hybrid YH®	IR - Lepidopteran
nd	AG 9030 PRO_3®	HT - Glyphosate IR - Lepidopteran
Landrace -Yellow kernel	nd	-
Landrace -Round white kernel	nd	-
Landrace -Red kernel	nd	-

IR- insect resistance; HT – herbicide tolerance; nd indicates the isogenic line of the cv was not used in the study. More information about the cultivars used are showed in the Appendix A.

Moisture content alone is not very useful in determining whether the maize could be prone to spoilage by fungi. Water activity (a_w), on the other hand, is an accurate measurement of the availability of water for microbial activity (Magan, 2007). The a_w of the different maize samples was measured using the AquaLab® 4TE DUO (Decagon Devices, USA). Separate sub-samples of the maize (5 g) cultivars were brought to room temperature for equilibrium (20-25^{o}C). Prior to aw measurements, the AquaLab® was calibrated with calibration standard solutions at 2 levels of a_w: 0.760 and 0.984. The maize kernels were transferred into plastic measurement vials and placed in the machine. Three replicates of each sample were measured.

2.3.3 Mycological examination

Preparation of growth media for microbial cultivation: Fungal and bacterial cultivation were achieved using different types of growth media. Commercial defined media, Malt Extract agar (MEA), Nutrient agar (NA) and Dichloran-Glycerol 18% agar (DG18) were obtained from Oxoid (Basingstoke, U.K.). MEA and NA are non-selective media which supports the growth of a wide range of fungi and bacteria respectively. DG18 is a selective medium which supports growth of xerotolerant/xerophilic yeasts and filamentous fungi from dried and semi-dry foods (Samson et al., 2018). NA is commonly used for the routine cultivation, maintenance and enumeration of non-fastidious bacteria (*i.e.*, bacteria not having a complex nutritional requirement), and MEA for yeasts and filamentous fungi. The preparation of each growth media was done according to the manufacturers' instructions using deionised water (15Ωm). The media was autoclaved at 121°C for 15 minutes at 15 psig. Cyclohexamide was used as an antifungal agent to NA, and chloramphenicol as an antibacterial agent post- and pre-autoclaving respectively.

Enumeration of fungi and bacteria in maize samples*:* The enumeration of fungi and bacteria was performed by the serial dilution technique. Sub-samples of maize (10 g) were soaked for 3 h in 90 ml of sterile distilled water supplemented with 0.05% (w/v) technical agar (Oxoid, Basingstoake, U.K.) and 0.025% (w/v)

Tween 80. The samples were homogenised for 5 min in a stomacher bag (Lab-Blender 400; Seward Medical, U.K.), then serial dilutions (10^{-2} to 10^{-4}) were performed and aliquots of 100 µL from each dilution spread plated in triplicate on DG18 and MEA for fungal populations, and on NA for bacterial populations. The Petri plates (9 cm∅) were incubated at 25°C for 7 days for fungi, and at 30°C for 48 h for bacteria. The colonies growing on the plates in a range of 10 to 300 colonies were counted and their numbers expressed as Log_{10} of colony forming units per gram of dry mass of sample (Log_{10}CFU.g dry mass^{-1}) using the Equation 2.2. To obtain the actual fungal load, the calculated CFUs were adjusted based on the actual dry weight of the maize kernels after drying and reported as CFUs/g dry weight (Mohale et al., 2013):

$$\boldsymbol{a})\ CFU = \frac{number\ of\ colonies}{dilution\ counted\ \times\ volume\ of\ aliquot}$$

$$\boldsymbol{b})\ Log_{10}CFU.g\ dry\ weight^{-1} = Log_{10}(2 \times CFU + 1)$$

Equation 2.2. Formulas used to calculate colony forming units.

Frequency of isolation of fungi: From each sample, 100 maize kernels were sub-sampled. Fifty (50) kernels from the sub-sample were first surface-disinfected (+SD) with sodium hypochlorite 0.4 % v/v (NaOCl) for 2 min and left to dry then plated. The remain kernels were plated without surface disinfection (-SD). The kernels were directly plated (twenty-five kernels per medium; five kernels per plate) on DG18 and MEA media. The plates were incubated at 25°C for 7 days then inspected visually for fungal growth.

The fungal occurrence, i.e., number of maize grains from which *Aspergillus* sections *Flavi*, *Nigri*, and *Circumdati*, *Penicillium* and *Fusarium*, were identified. Subsequently, *Aspergillus flavus* strains were isolated and a monosporic culture obtained. The strains were tested for mycotoxin production capacity. To obtain the isolation frequency, all fungal colonies growing from directly plated kernels on MEA and DG18 were recorded and calculated according to Equation 2.3:

$$\%\ isolation\ frequency = \frac{number\ of\ maize\ kernels\ colonized\ by\ a\ species}{total\ number\ or\ maize\ kernels\ per\ plate} \times 100$$

Equation 2.3. Formula to calculate isolation frequency.

Screening for the ability to produce aflatoxin: The strains of *A. flavus* isolated from the maize samples were sub-cultured in MEA and after 7 days a spore suspension was made using sterile distilled water supplemented with 0.05% (w/v) technical agar and 0.025% (w/v) Tween 80 to prevent the formation of stray colonies on Petri plates. The suspensions were centrally inoculated on Petri plates of 50% Coconut Cream agar medium (CAM; Davis, Lyer and Diener, 1987). An Aflatoxin-positive type strain (*A. flavus* NRRL 3357) obtained from Prof. D. Bhatnagar of the Southern Regional Research Centre, New Orleans, LA, USA was used as a positive control. The plates were incubated at 25°C in the dark for 5-7 days then the reverse of the plates was observed under UV light (365 nm) for formation of blue fluorescence, indicating the ability to produce aflatoxins B_1 and B_2.

2.3.4 Aflatoxin analysis

Extraction of aflatoxins from cultures: For confirmation of the ability to produce aflatoxins, isolated strains of *A. flavus* were inoculated on aflatoxin-conducive Yeast Extract Sucrose agar (YES: 2 % yeast extract, 15 % sucrose, 0.05% $MgSO_4.7H_2O$) at 25°C for 10 days. The experiment was performed in triplicate. Six agar plugs (approx. 0.3 g) were taken from the agar medium across each plate with a 5 mm-diameter sterile cork-borer. These plugs were placed into pre-weighed 2-mL Eppendorf tubes and re-weighed. Aflatoxins were extracted from the agar plugs by adding 1 mL of chloroform in the 2-mL tube and shaking for 1h. A volume of 800 µL from the tube of chloroform + biomass was transferred to a new tube and the solvent evaporated to dryness overnight. The derivatization was performed afterwards.

Derivatization of aflatoxins: Into the 2-ml Eppendorf tube with the residue of each sample, 200 µL of hexane were added, vortexed for 30 s and followed by the addition of 50 µL trifluoracetic acid (TFA, Fisher Scientific, UK). The mixture was vortexed for 30 s and left for 5 min. Thereafter, 950 µL water: acetonitrile (9:1, v/v) was added and the entire contents of the tube vortexed for 30 s then the mixture left for 10 min to allow thorough separation of the layers. The hexane (upper layer) was discarded, and the aqueous portion filtered through nylon filters (13 mm × 0.22 µm) using a 1 mL syringe directly into amber silanized 2-mL vials (Agilent Technologies, USA) for HPLC analysis. All the analytical reagents used were HPLC grade.

Preparation of standards: A stock solution of 200 µL of aflatoxin mixed solution (Romer Labs® UK Ltd) comprising of 0.2 µg. mL^{-1} of aflatoxin B_1 (AFB_1) and aflatoxin G_1 (AFG_1) and 0.06 µg. mL^{-1} each of aflatoxin B_2 (AFB_2) and aflatoxin G_2 (AFG_2) was prepared. The stock solution was transferred into 2-mL tubes, left to evaporate to dryness overnight inside a fume cupboard and, thereafter, derivatized as described above (section: *Derivatization of aflatoxins*).

HPLC analysis: The quantification of the ability of strains of *A. flavus* to produce AFs was performed by reversed-phase HPLC. The HPLC system used was an Agilent 1200 series with fluorescence detector (λ_{exc} 360 nm; λ_{em} 440 nm). A C_{18} column (Agilent Poroshell® 120 EC-18, 4.6 x 100 mm, 2.7 µm particle size preceded by a Phenomenex® Gemini C18 column, 3mm, 3µm guard cartridge; Phenomenex, CA, USA) was used for separation. Followed by isocratic elution with methanol: water: acetonitrile (30:60:10, v/v/v) and mobile phase at flow rate of 1.0 $mL.min^{-1}$. The injection volume was 10-50 µL according to each set of samples. A set of standards was injected (0.05–4 ng of aflatoxins per injection) and standard curves generated by plotting the peaks area against the amounts of aflatoxins. Only AFB_1 and AFB_2 were detected in the samples. The respective R^2 values for the calibration curves were: 0.991 and 0.989.

2.3.5 Identification of mycotoxin profiles in the GM and non-GM maize cultivars using LC-MS/MS

A multi-targeted metabolomics approach was also used to identify the mycotoxins present in the maize samples. For these studies, because of the limited amount of maize available, two sub-samples of 6 out of 7 pairs of GM and isogenic non-GM cvs were analyzed (Table 2.2). The analysis was performed by Dr. Michael Sulyok in BOKU, Tulln, Austria. The milled sub-samples (5g) of maize were extracted using 20 mL extraction solvent (acetonitrile: water: acetic 79:20:1 (v/v/v) followed by a 1 + 1 dilution using acetonitrile: water: acetic 20:79:1 (v/v/v). Five µL of the diluted extract was directly injected into the sampling port for LC-MS/MS in the equipment for analysis (Malachová et al., 2014).

A QTrap 5500 LC-MS/MS System (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray electrospray ionization (ESI) source and a 1290 Series HPLC System (Agilent, Waldbronn, Germany). Chromatographic separation was performed at 25°C on a Gemini® C_{18}-column, 150 x 4.6 mm i.d., 5 µm particle size, equipped with a C_{18} 4 x 3 mm i.d. security guard cartridge (all from Phenomenex, Torrance, CA, US). The chromatographic method as well as the chromatographic and mass spectrometric parameters were previously described (Malachová et al., 2014). ESI-MS/MS was performed in the time-scheduled multiple reaction monitoring (MRM) mode both in positive and negative polarities in two separate chromatographic runs per sample by scanning two fragmentation reactions per analyte.

Quantification was performed via external calibration using serial dilutions of a multi-analyte stock solution. The limit of detection for each mycotoxin is demonstrated in the Appendix H. The accuracy of the method has been verified on a continuous basis by regular participation in proficiency testing schemes (Malachová et al., 2014, 2015). This approach has recently applied to targeted metabolomics in cereal samples (Garcia-Cela et al., 2018).

Table 2.2. The six GM and isogenic non-GM maize samples selected for the targeted mycotoxin analyses by LC MS/MS.

non-GM maize cultivars	Isogenic GM line
AS 1555 CON	AS 1555 PRO®
P30F53 CON	P30F53 H®
P2530 CON	P2530 Hx®
CD-384 CON	CD-384 PW®
M20-A78 CON	M20-A78 PW®
BM-709 CON	BM-709 PRO_2®

2.3.6 Statistical analysis

Datasets were subjected to Shapiro-Wilk tests to determine normality and Levene's test to assess the equality of variance. Water activity (a_w) and percentage moisture content (%MC) satisfied the two assumptions after transformation to cube root. Afterwards, one-way analysis of variance (ANOVA) was performed. The colony forming unit (CFU), percentage of fungal isolation and secondary metabolites data violated the two assumptions of ANOVA even after transformations and consequently non-parametric tests (Wilcoxon /Kruskal-Wallis; p=0.05) were used for analyses (Chan and Walmsley, 1997). Where there was significance after the Kruskal-Wallis test, median comparisons for each pair of the different cultivars were made using the Wilcoxon - Each Pair test (p=0.05) while significance in ANOVA was done by comparisons of the means using Tukey HSD (p=0.05). The statistical package JMP®14 (SAS Institute Inc., 2018, Cary NC, USA) was used to perform the analyses.

2.4 Results

2.4.1 Water activity (a_w) and moisture content (M.C)

The a_w of the maize samples used in this work varied between 0.60 and 0.90 and the moisture content (%MC) between 11 and 22% (Figure 2.2). The recommended conditions for safe storage of maize are MC <15% and $a_W \leq 0.70$ (Magan and Aldred, 2007). Most of the samples were within the safety levels for storage for these parameters. However, the cultivar 2B587 Hx had a much higher MC of 22% = 0.90 aw, suggesting that poor drying was carried out for this particular sample. Significant differences at Tukey-Kramer HSD ($p<0.05$) were found for 3 of the 20 cultivars for both %MC and a_w.

2.4.2 Enumeration of fungi and bacteria

The samples used in this work had a high contamination with populations of fungi which was supported by both serial dilution and the frequency of isolation results. The overall population of micro-organisms (log_{10}CFU g^{-1} dry sample) of all the cultivars for fungi and bacteria was <8.60 and <6.8, respectively (Figure 2.3). There was not significant difference in the populations (CFUs) between the samples at the 5% of significance level for either bacteria, fungi (MEA) or fungi only (DG18). Bacteria were not detected in two cultivars: BM-709 PRO2 and M20-A79 CON.

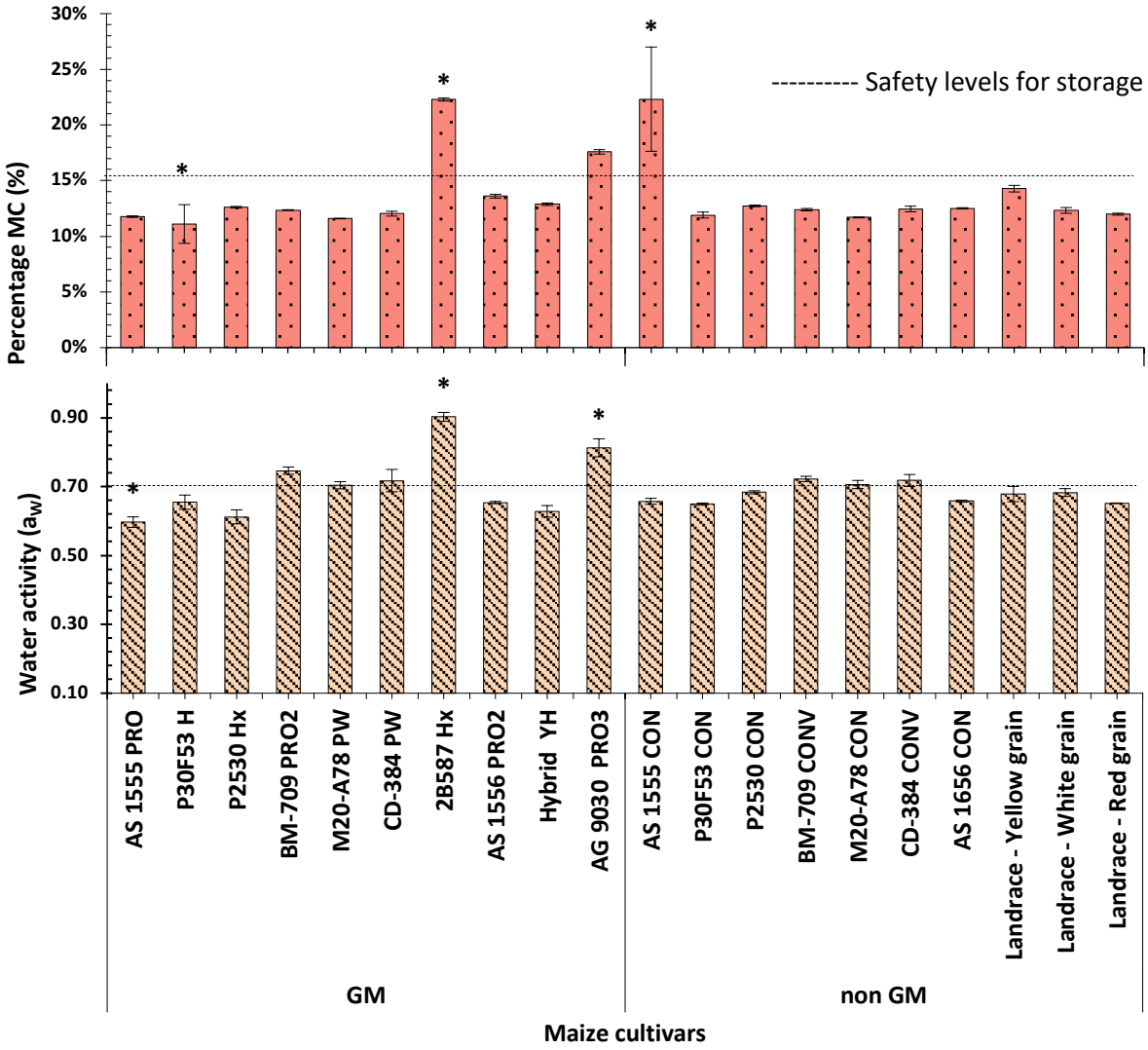

Figure 2.2.Values of moisture content (M.C. %) and water activity (a_w) of the maize samples; bars+ SE. * indicates most significant differences in the values compared to the other samples using the Tukey-Kramer HSD ($p<0.05$).

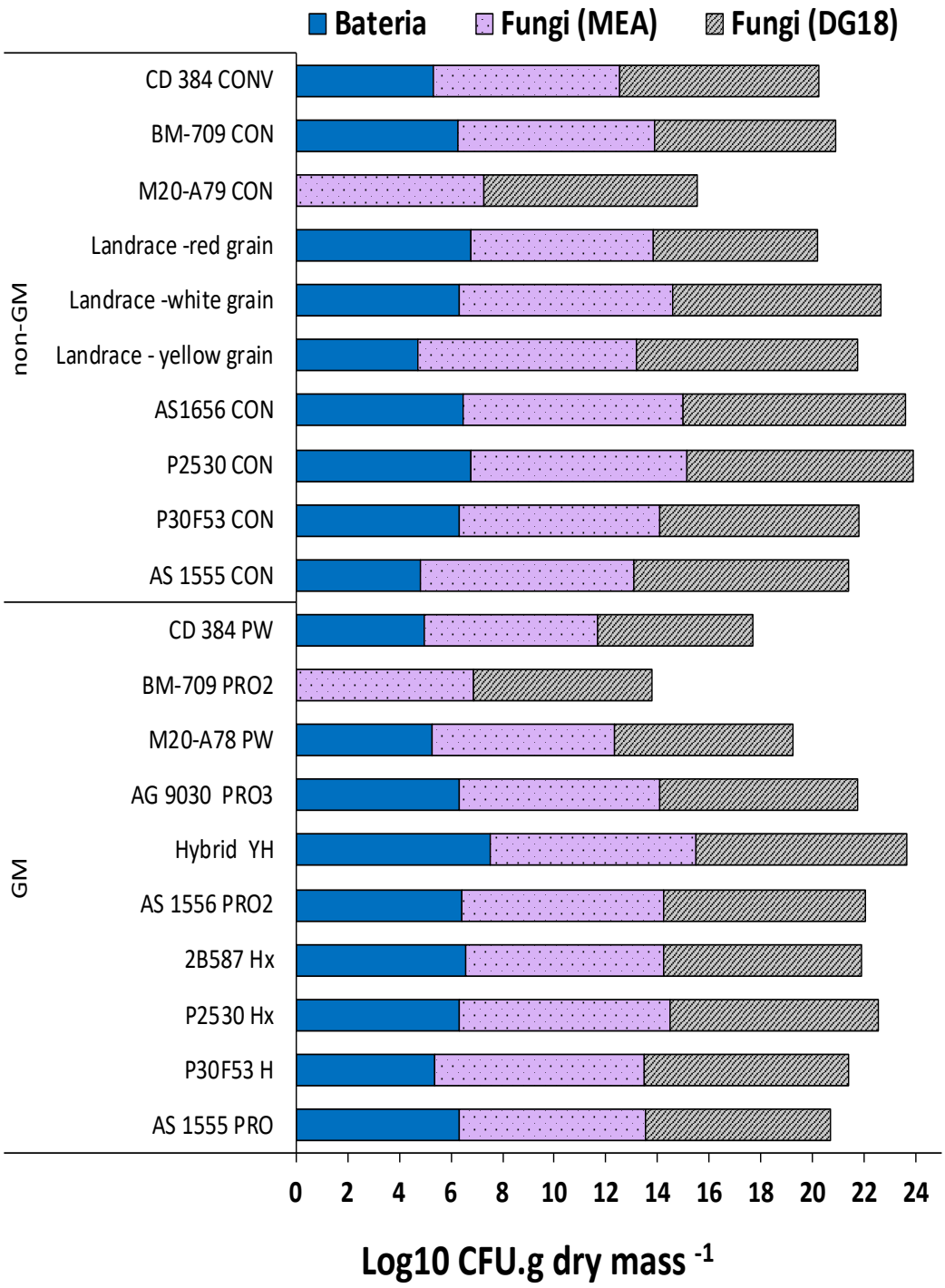

Figure 2.3. Overall populations of micro-organisms in the maize cultivars expressed as Log10 CFUs g dry weight^{-1}.

2.4.3 Frequency of isolation of fungi and identification

Figures 2.4 and 2.5 show the relative frequency of isolation of different fungi from the different cultivars when plated with or without disinfection. There was a higher frequency of isolation in the non-disinfected plated maize kernels than in those which were disinfected. The surface disinfection allowed the isolation of internally colonising fungi from the maize kernels directly plated on the different media to recover the fungi actually growing in the grain. The mycological analysis also showed that *Fusarium*, *Penicillium* and *Eurotium* were the principal contaminants of maize kernels from both GM and non-GM cultivar samples. *A. flavus* was isolated from 12 out of 20 cv samples (see Figure 2.5).There was no significant difference (p = 0.05) in the frequency of isolation of the different fungi on the two-culture media (DG18 x MEA) used or between non-GM and GM cultivars (see Figure 2.4).

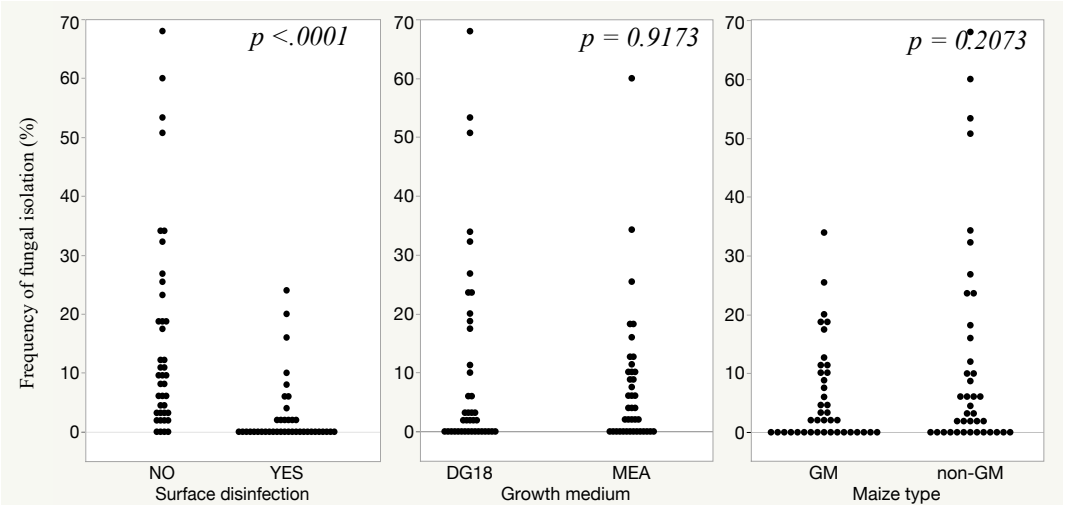

Figure 2.4. Distribution of frequency of fungal isolation (%) comparing surface disinfection (YES) no surface disinfection (NO), growth medium (DG18 - Dichloran 18% Glycerol; MEA – malt extract agar) and maize cultivar (GM and non-GM). p<0.05 indicates significant difference between levels.

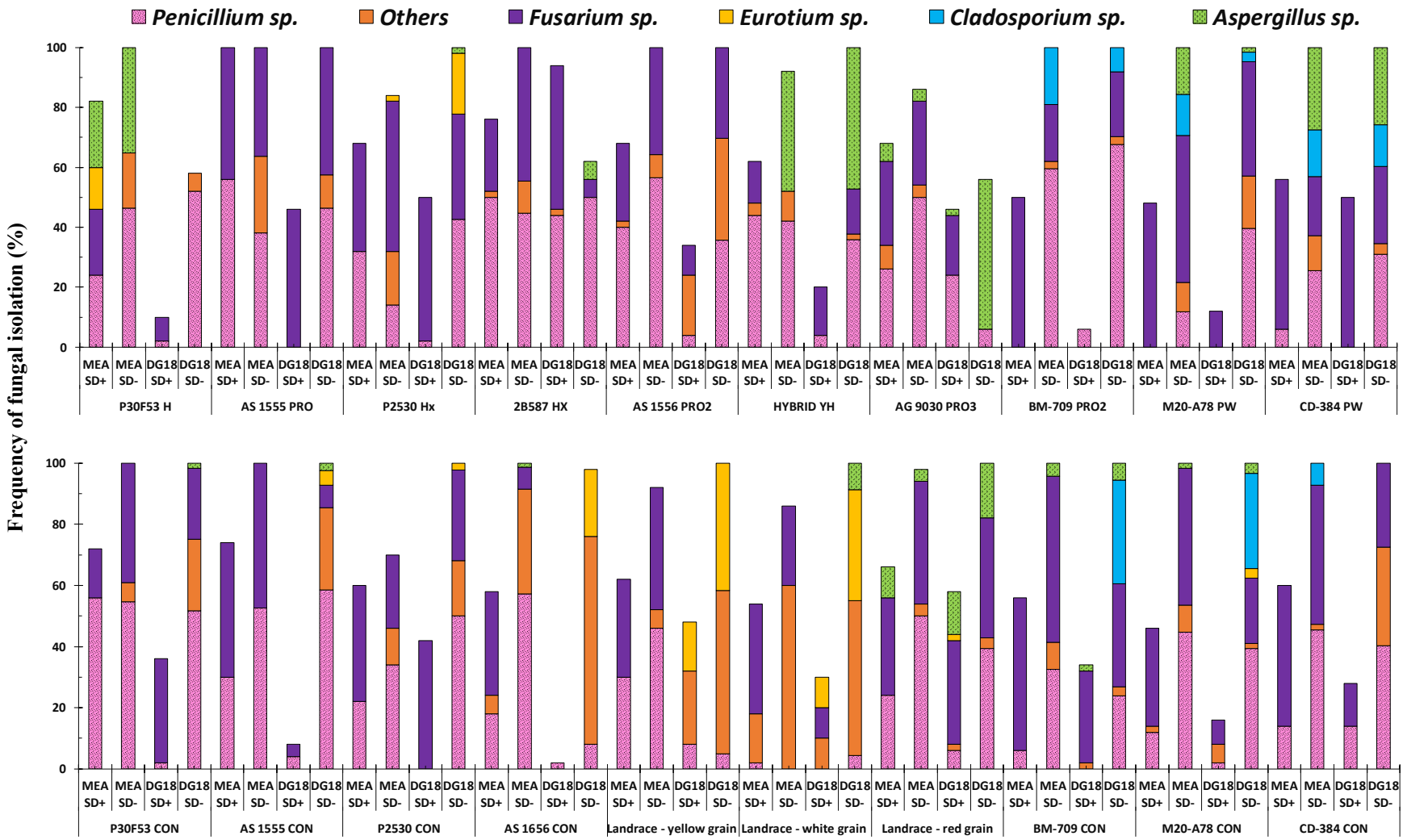

Figure 2.5. Frequency of fungal isolation (%) in maize and the identified species. "Others" sp. includes: *Rhyzopus, Epicocum, Cladosporium, Mucor, Alternaria, Wallemia and Trhichoderma*. SD+ surface disinfected/ SD- non-surface disinfected. MEA: Malt Extract agar; DG18: Dichloran-glycerol 18 % agar.

2.4.4 Aflatoxin B_1 production

Strains of *A. flavus* identified through mycological analyses were isolated, purified through monosporic culture and assessed for aflatoxins production using coconut cream agar (CAM) (Figure 2.6) and, subsequently the aflatoxin production was confirmed by HPLC-FLD.

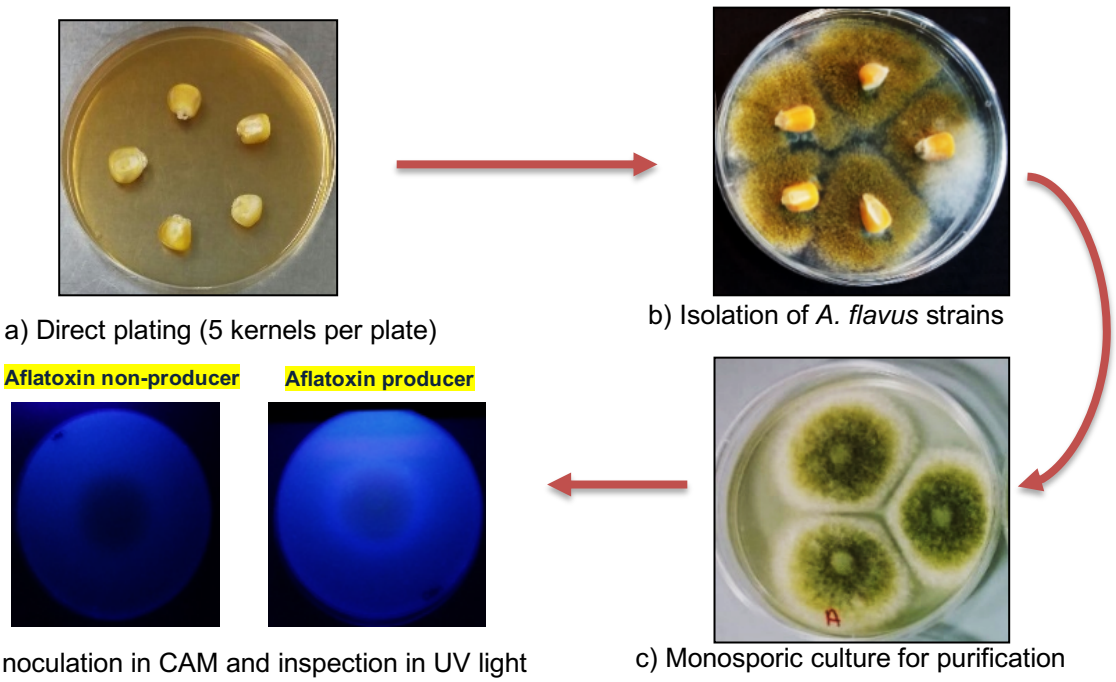

Figure 2.6. Process of direct plating (a), isolation of *A. flavus* (b), purification through monosporic culture (c) and detection of fluorescence under UV light (d)

Although the AFB_1 production by the strains of *A. flavus* was confirmed by HPLC analyses only for 7 out of 22 strains, the CAM method was a good indicator of whether the strain was aflatoxin producer or not. The fluorescence production indicated that only one of the strains isolated from GM samples was AFB_1 producer, while 6 out of 7 producers' strains were isolated from non-GM maize. The strains isolated and the capacity for aflatoxin production are shown in Table 2.3. The toxigenic strains were confirmed through the HPLC analysis to be *A. flavus* since only AFB_1 and AFB_2 were detected. The strains *AfLRG-a* and *AfLRG-*

b, isolated from the same landrace cultivar but from different replicate plates, were the highest producers of both aflatoxin B_1 and B_2.

Table 2.3. Aflatoxin B_1 and B_2 production by the *A. flavus* strains isolated from the GM and non-GM maize cultivars.

Strain	Type	Cultivar of origin	Fluorescence on CAM	Aflatoxin (ng. g^{-1}) by HPLC	
				AFB_1	AFB_2
AfASC-a	non-GM	AS 1555 CON	+	3.3 ± 0.9	n.d
AfASC-b		AS 1555 CON	+	1.7 ± 0.6	n.d
AfAS1C		AS 1556 CON	-	n.d	n.d
AfBMC-a		BM-709 CON	-	n.d	n.d
AfBMC-b		BM-709 CON	-	n.d	n.d
AfBMC-c		BM-709 CON	-	n.d	n.d
AfP30C-a		P30F53 CON	+	0.3 ± 0.6	n.d
AfP30C-a		P30F53 CON	+	0.2 ± 0.0	n.d
AfM20C		M20-A78 CON	-	n.d	n.d
AfFRC		non - GM FR[1]	-	n.d	n.d
AfLRG-a		Landrace – Red grain	+	106.7 ± 21.6	1.2 ± 0.3
AfLRG-b		Landrace – Red grain	+	28.4 ± 3.9	0.8 ± .01
AfCDPW-a	GM	CD-384 PW®	-	n.d	n.d
AfCDPW-b		CD-384 PW®	-	n.d	n.d
AfCDPW-c		CD-384 PW®	-	n.d	n.d
AfYH-a		Hybrid YH®	-	n.d	n.d
AfYH-b		Hybrid YH®	-	n.d	n.d
AfM20-a		M20-A78 PW®	-	n.d	n.d
AfM20-b		M20-A78 PW®	-	n.d	n.d
AfM20-c		M20-A78 PW®	+	0.5 ± 0.1	n.d
AfPHx		P2530 Hx®	-	n.d	n.d
AfF53H		*P30F53 H*®	-	n.d	n.d

Aflatoxin **+** producer/ **-** non-producer. AFB_1 - Aflatoxin B_1; AFB_2 - Aflatoxin B_2; [1] maize sample used only for isolation; n.d – not detected; values correspond to average ± SD (n=3)

2.4.5 Identification of mycotoxins and related compounds in the GM and isogenic GM maize cultivars by LC-MS/MS

The LC MS/MS analysis of the 6 GM and their isogenic non-GM maize cvs showed a higher presence of mycotoxins related to *Fusarium* and *Penicillium* spp. while toxins produced by *A. flavus* were largely absent. A total of 29 secondary metabolites were present in the samples (Table 2.4).

An overall comparison of metabolites detected in the samples indicates that although there was no significant difference in the percentage fungal isolation, the presence of mycotoxins was higher in the non-GM cultivars (Figure 2.7). Two regulated toxins (fumonisin B_1 and B_2) were detected in higher amounts in the non-GM cultivars. Comparing each cultivar individually it was possible to identify marked differences between the GM and its isogenic non-GM line (p<0.05) (see Table 2.4). The non-GM cultivar CD-384 CON had the highest levels of fumonisin B_1 (6480.5 µg g^{-1}) while for its GM line (CD-384 PW®) the same toxin was not detected.

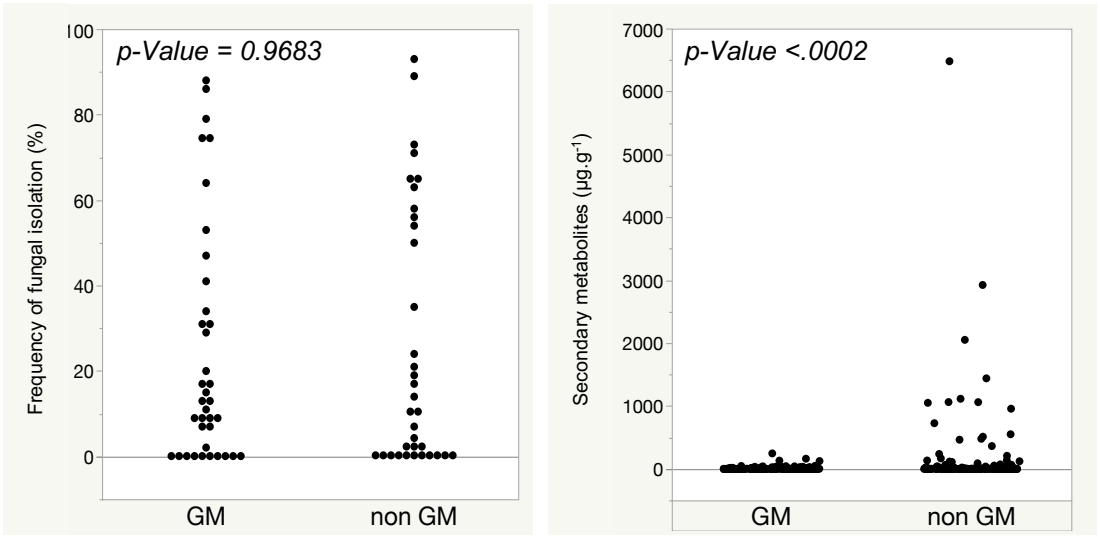

Figure 2.7. Distribution of frequency of isolation (%), and mycotoxin and related compounds identified from the 6 selected GM and non-GM maize samples. p<0.05 indicates significant difference between GM and non-GM cultivars.

Table 2.4. Mycotoxins and related compounds identified by LC MS/MS in Brazilian GM and isogenic non-GM cultivars.

		non-GM maize cultivars						GM maize cultivars					
Secondary metabolites (µg.kg^{-1})	**Group**	**1555 AS CON**	**BM-709 CON**	**CD-384 CON**	**M20-A78 CON**	**P2530 CON**	**P30F53 CON**	**1555 AS PRO**	**BM-709 PRO$_2$**	**CD-384 PW**	**M20-A78 PW**	**P2530 Hx**	**P30F53 H**
♦Fumonisin B_1		110.4	1062.7	6480.5	167.6	<LOD	2924.3	<LOD	124.7	<LOD	<LOD	24.7	162.2
♦Fumonisin B_2		26.4	464.0	2051.7	53.3	16.3	1439.6	<LOD	46.8	<LOD	16.9	<LOD	47.7
Fumonisin B_3		21.4	82.5	1061.3	<LOD	<LOD	363.2	<LOD	<LOD	<LOD	<LOD	<LOD	34.5
Fumonisin B_4		<LOD	137.1	1115.1	25.1	11.5	511.8	<LOD	19.7	<LOD	<LOD	<LOD	40.6
H. Fumonisin B_1	A	5.5	<LOD	133.5	<LOD	<LOD	37.3	<LOD	<LOD	<LOD	<LOD	<LOD	33.7
Fusarin C		26.1	<LOD	1050.6	<LOD	<LOD	237.2	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Bikaverin		21.8	17.0	957.1	<LOD	<LOD	116.9	<LOD	<LOD	<LOD	<LOD	<LOD	26.4
Beauvericin		<LOD	<LOD	<LOD	<LOD	<LOD	4.2	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Fusaric acid		<LOD	<LOD	63.4	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Fusarinolic acid		<LOD	<LOD	726.7	<LOD	<LOD	208.0	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Equisetin		<LOD	0.8	<LOD	<LOD	<LOD	2.9	<LOD	3.2	<LOD	26.8	1.2	<LOD
Berkedrimane B		3.6	<LOD	1.2	<LOD	<LOD	21.6	<LOD	<LOD	<LOD	<LOD	<LOD	11.2
Chrodrimanin		<LOD	<LOD	65.7	<LOD	<LOD	550.9	<LOD	<LOD	<LOD	<LOD	<LOD	246.7
Demethylsulochrin		1.7	<LOD	3.9	<LOD	<LOD	2.5	<LOD	<LOD	<LOD	<LOD	<LOD	8.8
Penicillide		8.3	<LOD	26.1	<LOD	<LOD	36.4	<LOD	<LOD	<LOD	<LOD	<LOD	28.7
Pinselin	B	<LOD	<LOD	<LOD	<LOD	<LOD	1.8	<LOD	<LOD	<LOD	<LOD	<LOD	6.8
Purpactin A		5.5	<LOD	3.4	<LOD	<LOD	22.1	1.0	<LOD	1.0	<LOD	<LOD	43.3
Questiomycin A		121.7	10.1	89.3	7.5	478.9	71.4	29.3	<LOD	31.5	4.7	12.6	40.5
Rugulovasine A		17.7	2.3	7.0	<LOD	5.4	9.9	2.5	<LOD	33.7	8.0	2.5	5.9
Dehydroaustinol		<LOD	<LOD	5.6	<LOD	<LOD	40.1	2.7	<LOD	<LOD	<LOD	<LOD	16.1
Asperphenamate		53.5	0.5	0.6	0.1	<LOD	0.2	132.0	<LOD	10.0	<LOD	<LOD	9.5
Cyclo(L-Pro-L-Tyr)		3.1	3.8	<LOD	1.9	1.0	1.1	2.6	2.2	1.8	5.4	2.4	4.0
Emodin		0.3	<LOD	0.4	<LOD	<LOD	0.4	<LOD	<LOD	<LOD	<LOD	<LOD	0.6
Iso-Rhodoptilometrin	C	0.5	<LOD	1.3	<LOD	<LOD	0.8	0.2	<LOD	0.1	<LOD	<LOD	1.4
NBP		4.0	<LOD	<LOD	<LOD	<LOD	<LOD	8.8	<LOD	<LOD	<LOD	<LOD	3.1
Tryptophol		27.9	10.5	24.9	<LOD	<LOD	19.4	16.8	15.7	10.4	8.2	14.2	14.5
Alternariol	D	<LOD	<LOD	2.9	<LOD	<LOD	8.2	<LOD	<LOD	<LOD	<LOD	<LOD	8.5
Alternariolmethylether		1.9	<LOD	1.6	<LOD	<LOD	3.3	<LOD	<LOD	0.2	<LOD	<LOD	1.8
Asperglaucide	E	22.6	0.1	1.6	0.6	<LOD	0.3	21.3	<LOD	16.6	<LOD	<LOD	53.2

♦ Indicates mycotoxins regulated by Legislation (sum Fumonisin B_1 + B_2 = 4000 µg.kg^{-1}); Group: A – *Fusarium* sp. metabolites, B – *Penicillium* sp. metabolites, C – Unknown sp. metabolites, D – *Alternaria* sp. metabolites, E – other species. <LOD values lower than the limit of detection of the equipment; CON indicates the term "conventional"; H. Fumonisin B_1 - Hydrolysed fumonisin B1; NBP - N-Benzoyl-Phenylalanine.

2.5 Discussion

The majority of the samples analysed in this study had moisture content (%MC) and water activity (a_w) levels within the safety range for storage without fungal spoilage. There was no significant difference ($p = 0.05$) in the frequency of isolation of the different fungi on the two-culture media (DG18 x MEA). *Fusarium* and *Penicillium* spp were isolated in the highest frequency from the 20 cultivars examined. There were no differences in the dominant species when comparing GM and non-GM cvs. Samples without surface disinfection (SD-) had a higher overall significantly higher contamination suggesting contamination from field and harvesting operations. *A. flavus* was only present in 50% of samples in lower frequency and a total of 22 strains were isolated and 15 classified as non-aflatoxigenic, whereas 7 were AFB_1 producers. Interestingly, the majority of the strains isolated from non-GM cultivars were AFB_1 producers and the majority of strains from GM cultivars were atoxigenic.

Both field and storage fungi were detected in the maize cultivar samples. Field fungi that can colonize the ripening grains during silking prior to harvesting. Most of the field fungal species do not infect the crops after harvest. However, *A. flavus* and *Fusarium* species often infect ripening maize during ripening, especially when episodes of pest damage which can allow entry into the ripening cobs. (Lacey, 1989; Battilani et al., 2011). *Fusarium* was the main species from the field isolated in all the samples, while other field fungal species like *Cladosporium* and *Alternaria* were present but less frequent.

During drying and storage some fungi become more active and depending on the management post-harvest can colonise the stored cereals and initiate colonisation by spoilage and mycotoxigenic fungi under conducive environmental conditions. Storage fungi mainly include species of the genera *Aspergillus* and *Penicillium* (Miller, 1995). *Penicillium* spp were identified in a higher frequency in all the samples. *Aspergillus* section *Nigri* and *Flavi* were isolated but less frequently, with *A. flavus* being isolated from 12 cultivars.

Although *A. flavus* was isolated from both GM and non-GM maize, aflatoxin was not detected by LC MS/MS analysis. This fact might be due to proper storage conditions after harvest reducing the chances for fungal development, since secondary metabolism is commonly associated with sporulation processes (Calvo et al., 2002). The main regions where maize is grown in Brazil are sub-tropical or tropical climatic zones which are very conducive to fungal colonization. Where good agricultural practices (GAP) is not followed, and poor hygienic practices occur will of course lead to a higher risk of contamination with aflatoxins (Sabino et al., 1989).

Environmental factors such as a_w and %MC are important parameters to determine the quality of kernels during storage. Water activity is only one of the environmental factors influencing colonisation of cereals by fungi (Abdullah, Nawawi and Othman, 2000). Poor post-harvest management can lead to rapid deterioration in nutritional quality of seeds. Microbial activity can cause undesirable effects in grains including discoloration, contribute to heating and losses in dry matter (Magan and Aldred, 2007b). It is important in temperate cereals that the moisture content of 14.5–15% (wet weight basis a_w= 0.70) is not exceeded to ensure stable medium-term storage without spoilage and any post-harvest mycotoxin contamination (Garcia-Cela et al., 2018). For maize, which is often harvested at >18-19% MC then effective and rapid drying to 15% MC is essential to minimise mycotoxin contamination. It has previously been shown that leaving maize at harvest for even a few hrs before drying can lead to significant increase in mycotoxin contamination (Blandino et al., 2004).

The presence of mycotoxins produced by *Fusarium* sp. in the samples used in this study may be due to some species such as *F. verticillioides* can infect maize systemically and also survive as in an endophytic phase which may contribute to the contamination with fumonisins found in these cultivars. It was interesting to note that the number and types of secondary metabolites identified was higher in non-GM samples, including concentrations of fumonisin B_1 + B_2 above the legislative limits. There are no previous studies which have examined the secondary metabolite profiles of GM and non-GM maize.

The fumonisins are a group of mycotoxins produced primarily by *Fusarium verticillioides* and *F. proliferatum* (Abbas et al., 2013), although a few other *Fusarium* species also may produce them. The primary health concerns associated with fumonisins are acute toxic effects in horses and swine, and carcinogenic properties. Fumonisins are hepatotoxic, nephrotoxic, atherogenic, immunosuppressive and embryotoxic in experimental animal systems (Nair, 1998). Fumonisin B_1 has been often detected in Brazilian maize (Bordin et al., 2015; Peluque et al., 2014) and even in sub products such as beer (Kawashima and Valente Soares, 2006; Piacentini et al., 2017).

Despite the higher frequency of isolation of *Penicillium* spp in the maize cultivars, no regulated mycotoxins were detected which are produced by this genus. However, a considerable number of emerging toxic secondary metabolites were present. The co-occurrence of such potential mycotoxins with others with known toxicity needs further examination. Indeed, the potential impact of multiple mycotoxins is now being assessed by EFSA (Battilani et al., 2016). Non-regulated secondary metabolites from *Fusarium* species, including beauvericin, fusaric acid and fusarin C were found in at least 50% of the samples analyzed by (Oliveira et al., 2017) from Brazilian maize. Fusarin C has demonstrated mutagenic activity and several immunosuppressive effects comparable to those of AFB_1 and sterigmatocystin intoxications (Cantalejo et al., 1999).

It is worthwhile highlighting the differences in mycotoxin profile between GM and non-GM maize found in this study. This is in contrast to the frequency of isolation of fungi which was similar across the cultivars examined. Some studies have suggested that there are lower mycotoxins in GM maize when compared with non-GM cultivars due the reduction of insects that represent an important vector of invasion of the grains. Associations between insect pests and toxigenic fungi are well known. Mainly Lepidopteran species act as vectors for fungal spores as well as damaging the ripening maize kernels, allowing entry of *A. flavus* and other spoilage moulds to infect the cobs (Alma et al., 2005). An effective way to manage *Lepidopteran* insects and reduce the associated mycotoxin risk is with GM insect resistance genes (Bt) (Munkvold et al., 1999; Wu, 2006). Pellegrino et al. (2018)

observed mycotoxin contamination in relation to plants expressing resistance to Lepidoptera (GM *Bt*) and this suggested that for all stacked *Cry1*Ab hybrids contained significant less fumonisins and trichothecenes.

Nineteen of 23 studies were compared in a review by (Ostry et al., 2010) on GM (*Bt*) maize concluded that *Bt* maize is less contaminated with *Fusarium* mycotoxins (FUM, DON, ZEA) than the conventional control cultivars in each case. However, Naef et. al (2006) suggested that Cry1Ab protein in maize residues has no direct effect on *F. graminearum* and *Trichoderma atroviride* but some corresponding Bt/non-Bt maize hybrids differed more in composition than Cry protein content alone, which can affect the saprophytic survival of mycotoxigenic fungi on crop residues.

Barroso et al. (2017) assessed incidence of *F. verticillioides* and the concentration of fumonisins in GM (*Bt*) and isogenic non-GM hybrids. The GM samples had a lower *F. verticillioides* frequency than non-GM. However, there was no statistical difference between fumonisin contamination when GM *Bt* and non-GM samples were compared. The results suggest that other environmental parameters could possibly trigger fumonisin production during plant development in the field.

Morphological characteristics can affect susceptibility to mycotoxin-producing fungi, either directly or indirectly. Hybrids with a thicker kernel pericarp are usually more resistant than those with a thinner pericarp, which can also contribute to resistance. To reduce mycotoxin risk, hybrid selection criteria should include partial resistance to ear rot diseases, appropriate maturity range, husk coverage characteristics, and adaptation to local conditions of abiotic stress (Munkvold, 2014).

In summary, this is the first study comparing the fungal contamination and internal infection of GM and non-GM maize cultivars from Brazil containing genetic traits for both insect resistance and/or herbicide tolerance, not only GM *Bt*. In addition, it was possible to obtain full secondary metabolite analyses of these cultivars for the first time. There was a strong trend indicating that the GM maize could be a

factor for reducing the content of fumonisins. However, the same cannot be concluded regarding aflatoxins since it was not detected in the samples due to overall good storage of the cultivars examined in this study. Understanding the potential implications of genetic traits inserted in the maize cultivars in relation to *A. flavus* colonization and aflatoxin production requires further investigation.

2.6 Conclusions

- *A. flavus* strains were isolated from both non-GM and GM maize, however the majority of toxigenic strains were from non-GM samples (6 strains) suggesting that perhaps GM cvs are perhaps more naturally resistant to colonisation by these strains
- There was no difference ($p<0.05$) in the fungal diversity comparing non-GM and GM maize cultivars
- The analysis of mycotoxins and related compounds by LC-MS/MS showed higher amounts of *Fusarium* metabolites which paralleled the high isolation frequency of *Fusarium* spp.
- The distribution of mycotoxins and related compounds indicated differences between non-GM and GM cultivars ($p<0.05$). GM maize had both lower fungal populations and concentrations of different toxins

CHAPTER 3

ECOLOGICAL STUDIES ON GROWTH AND AFLATOXIN B_1 PRODUCTION BY *ASPERGILLUS FLAVUS* IN CONVENTIONAL AND GM MAIZE-BASED MATRICES

CHAPTER 3. ECOLOGICAL STUDIES ON GROWTH AND AFLATOXIN B_1 PRODUCTION BY *ASPERGILLUS FLAVUS* IN CONVENTIONAL AND GM MAIZE-BASED MATRICES

3.1 Introduction

Aspergillus flavus is an opportunistic fungal pathogen able to infect developing maize cobs during silking, especially if the plants have been weakened by drought stress (Dolezal et al., 2014), or damaged by insects (Chulze, 2010). Once *A. flavus* is present in plant tissue, it can continue to colonise the kernels and produce aflatoxins. At harvest, the moisture content of maize cobs will allow colonisation by *A. flavus*. Thus, if not dried efficiently, aflatoxin levels may increase during storage, especially in tropical and sub-tropical environments. Due to its xerotolerant nature, *A. flavus* and related species are very aggressive and are able to cause storage rots rapidly and increase contamination with aflatoxins (Scheidegger and Payne, 2003a). Environmental conditions can play an important role in disease development. Interactions between intrinsic and extrinsic factors also have an influence on *A. flavus* growth and aflatoxin production in stored commodities (Magan and Aldred, 2004; 2007).

A. flavus is capable of surviving and overwintering in plant residues as mycelium or sclerotia (Battilani et al., 2012; Abbas et al., 2009). Crop residue is thus a primary source of the fungus to allow conidia to be transmitted by air or insects to serve as new inoculum to the host plants to start a new infection cycle (Abbas et al., 2009). The germination and colonization is most aggressive on maize plants that have been subjected to heat or drought stress (Scheidegger and Payne, 2003a). Unlike most fungi, *A. flavus* is favoured by hot dry conditions. It has a wide range of temperature tolerance (19–35°C) with about 28°C being optimum for growth and 28–30°C for aflatoxin production. Some strains of *A. flavus* can grow in very dry environments (e.g. 0.73 a_w) and produce aflatoxins at 0.85 a_w (Sanchis and Magan, 2004).

The problem of mycotoxin contamination requires integrated strategies of Good Agricultural Practice (GAP) during harvesting and storage, to processing and utilization (Munkvold, 2014) to reduce risks of high contamination. Hybrids with thicker pericarp are usually more resistant to fungal invasion. Process like rotation and tillage can impact on fungal dispersion and by result on mycotoxin contamination, most significantly deoxynivalenol and zearalenone, or even fumonisin and aflatoxin (Munkvold, 2014). Additionally, the planting date can considerably affect fungal infection because the infection depend on the co-occurrence of a susceptible plant developmental stage and favourable environmental conditions (Munkvold, 2014). Drought-stress could increase maize vulnerability to aflatoxin and fumonisin contamination (Abbas et al., 2009).

Insect-resistance (IR) crops involves the introduction of genes from *Bacillus thuringiensis* (*Bt* toxin) that make the plants resistant to certain pest species (Qaim, 2014). In its free form, the *Bt* toxin is known as a harmless protoxin. The activation occurs only in the stomach and intestinal tract of certain insects when the protoxin becomes an active toxin by the action of enzymes (Schnepf et al., 1998). Similarly, GM crops with herbicide-tolerance (HT) have been successfully used by farmers for weed control.

The adoption of GM HT maize has resulted in lower production costs and has increased farmer incomes (Brookes and Barfoot, 2015). The first GM HT to be developed was Roundup Ready® (Monsanto, USA) providing tolerance only against the weed killer glyphosate, followed by LibertyLink™ (Bayer CropScience, USA) that is able to provide tolerance to both glyphosate and Glufosinate ammonium. Glyphosate tolerance is the result of CP4.EPSPS gene from *Agrobacterium tumefaciens* strain CP4, whereas Glufosinate tolerance is the result of introducing a gene encoding the enzyme PAT (phosphinothricin-N-acetyltransferase) isolated from the same organism from which Glufosinate was originally isolated, *Streptomyces viridochromogenes* (Biosafety Clearing-House, 2015). Nowadays, most of the cultivars have stacked technologies to provide IR and HT at the same time. The effects of GM IR on fungal reduction and, as result,

decrease in mycotoxin content has been described previously (Alma et al., 2005; Schnepf et al., 1998; Windham, Williams and Davis, 1999; Wu, 2006).

No previous studies have compared the ability of *A. flavus* to colonise and produce AFs when grown on milled maize from GM (pesticide resistant or pesticide + herbicide tolerance) and their isogenic non-GM cultivars.

3.2 Objectives

To examine the effects of different interacting environmental factors (*i.e.*, different a_w x temperature levels) on growth rate and aflatoxin B_1 (AFB_1) production by the isolated *A. flavus* strains on maize-based media using the three GM and non-GM cvs as nutritional media

3.3 Materials and methods

3.3.1 Ecophysiology of *Aspergillus flavus* strains

Media preparation: Three cultivars of non-GM maize and their respective isogenic GM lines (Table 3.1) were chosen as the nutritional basal maize meal extract agar (MMEA). The cultivars from Brazil where selected from the list described previously in Section 2.3.2 – Table 2.1. The medium was prepared by mixing milled maize (3% milled maize, 2% technical agar number 3 – Oxoid, Basingstoke, U.K) in a solution of water with the water availability adjusted to 0.99, 0.95, 0.90 using glycerol due its stability over the experimental temperature range for modifying a_w. The media was autoclaved at 121°C for 15 min. The accuracy of the modifications in the a_w was confirmed using an Aqualab 4TE instrument (Decagon, Pullman, WA, USA).

Inoculation and mycelial growth measurements: Three toxigenic and one non-toxigenic strains were chosen for these studies based on AFB_1 production in the screening experiments and the atoxigenic strain as a representative of those which were unable to produce AFB_1. The toxigenic strains used were *AfLRG-a*, *AfASC-a*, *AfP30C-a*; and the atoxigenic strain was *AfPHx.*

Spore suspensions at 10^6 spores/mL of test strains were spread plated on MEA (Malt Extract Agar, Oxoid, UK) and incubated at 25°C for 24 h. These Petri plates were used to provide inoculum for the experiments. Agar plugs (3 mm Ø) were taken from these inoculation plates with a sterile cork borer and transferred in the centre of MMEA plates (9 cm Ø) for each treatment. The plates were sealed in polyethylene bags and incubated at 25, 30 and 35°C for 10 days. The diameter of the colonies was measured in two perpendicular directions (Figure 3.1a) daily for this period.

The growth rate of each strain under the distinct set of environmental factors was calculated by plotting the diameter of the colonies (mm) against time (days). From the regression lines made of the time points, which represented the linear phase of the growth curves using Microsoft Excel® was determined the growth rate (mm. day^{-1}). Experiments were carried out with three replicates per treatment and the experiment was repeated twice.

Table 3.1. Maize cultivars (non-GM and GM) selected for the ecophysiological studies as substrate for *A. flavus* growth

Conventional cultivar (non-GM)	Isogenic line (GM)	Traits tolerance present in the GM cvs
AS 1555 CON	AS 1555 PRO®	IR - Lepidopteran
P30F53 CON	P30F53 H®	Antibiotic resistance HT - Glufosinate ammonium and glyphosate IR - Lepidopteran
P2530 CON	P2530 Hx®	IR - Lepidopteran

IR- insect resistance; HT – herbicide tolerance;

3.3.2 Aflatoxin analysis

Extraction of aflatoxins from cultures: After 10 days incubation 6 agar plugs (approx. 0.3 g) were taken from the agar medium across the plates with the aid of a 5 mm-diameter sterile cork borer. The points from where each plug was taken

was to ensure that all the growth stages of the colonies were included: from the oldest (centre) to the youngest point (border of the plate; Figure 3.1b). These plugs were placed into pre-weighed 2-mL Eppendorf tubes and re-weighed. Aflatoxins were extracted from the agar plugs by adding 1 mL of chloroform in the 2-mL tube and shaking for 1h. A volume of 800 µL from the chloroform extract was transferred to a new tube and the solvent evaporated to dryness overnight. The derivatization, preparation of standards of aflatoxin and HPLC analyses was carried out as described in the CHAPTER 2 – section 2.3.4. Only AFB_1 was detected in the samples. The R^2 value for the calibration curve was $R^2 = 0.995$.

3.3.3 Statistical analysis

Statistical analysis was performed using the package JMP® Pro 14 (SAS Institute Inc., USA). Shapiro-Wilk tests was used to determine normality and Levene's test to assess the equality of variance. Both data from growth rate and AFB_1 production failed the requirements for analysis of variance therefore the nonparametric (Wilcoxon /Kruskal-Wallis; p=0.05) were used for analyses (Chan and Walmsley, 1997). Where there was significance after the Kruskal-Wallis test, median comparisons for each pair of the different cultivars were made using the Wilcoxon - Each Pair test (p=0.05)

Forward stepwise regression was used to obtain factorial to degree equations for AFB_1 (ng. g^{-1}) for conditions of toxin production (a_w, T^oC, $a_w \times T^oC$) for each strain per maize cultivar. Contour maps were built in JMP® Pro 14 using 5000 simulation data from the predicted formula.

3.4 Results

3.4.1 Effects of maize cultivar nutritional source and environmental factors on growth of toxigenic and atoxigenic *A. flavus* strains

For the ecophysiological studies 3 non-GM cultivars (AS 1555 CON, P30F53 CON and P2530 CON) cultivars and their isogenic GM lines (AS 1555 PRO®,

P30F53 H® and P2530 Hx®) were used as substrate sources for *A. flavus* development. Three isolated strains of *A. flavus* which were AFB_1 producers (*AfLRG-a*, *AfASC-a* and *AfP30C-a*) and one non-producer (*AfPHx*) were used in these studies.

The overall growth rate for all the strains shows optimal conditions at 30-35°C and 0.99 a_w for both substrates (non-GM and GM maize). There was no significant difference in the growth rate at 30 and 35°C ($p = 0.05$). The slowest growth was at 0.90 a_w in all the temperatures tested. There were no differences with regard to using milled maize as the nutritional source between the GM and non-GM maize treatments (see Figure 3.3).

The strain *Af*PHx had the highest growth rate (17.2 mm day^{-1}) with AS 1555 Pro, followed by *Af*LRG-a (16.9 mm day^{-1}) when it was grown with AS 1555 CON at 35°C/0.99 a_w. Conversely when *Af*LRG-a was cultivated on P2530 CON and its isogenic GM line P2530 Hx® (Figure 3.2), the development was significantly reduced ($p<0.05$) in all the temperatures at 0.99 a_w. The same did not appear at 0.90 or 0.95 a_w. The growth rates for all the strains are shown in Figure 3.4.

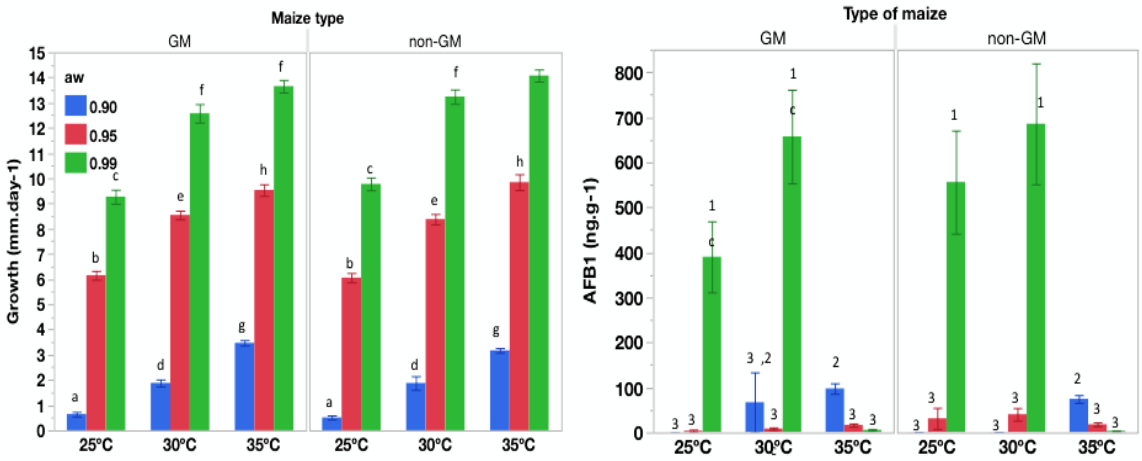

Figure 3.3. Overall values for growth rate (mm. day^{-1}) and AFB_1 (ng. g^{-1}) at temperatures (25, 30 and 35°C) and water activity (a_w = 0.99, 0.95 and 0.90) comparing the types of maize (GM and non-GM) used in the ecophysiological study. The same letter or numbers on the bars indicates no significant differences ($p<0.05$) using nonparametric test Wilcoxon for each pair. Bars represent AV±SE.

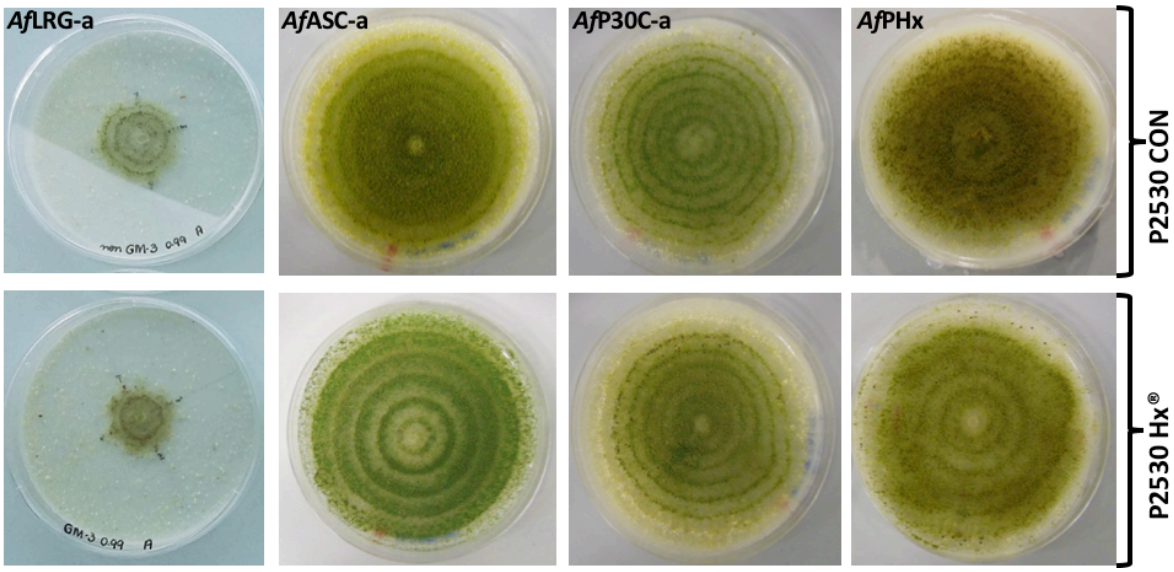

Figure 3.2. Plates from ecophysiology studies of *A. flavus* when using the maize cultivars P2530 CON (non-GM, upper section) and P2530 Hx® (GM, lower section) as substrate on day 10 at 30°C and 0.99 a_w for the toxigenic strains *Af*LRG-a, *Af*ASC-a, *Af*P30C-a and atoxigenic *Af*PHx.

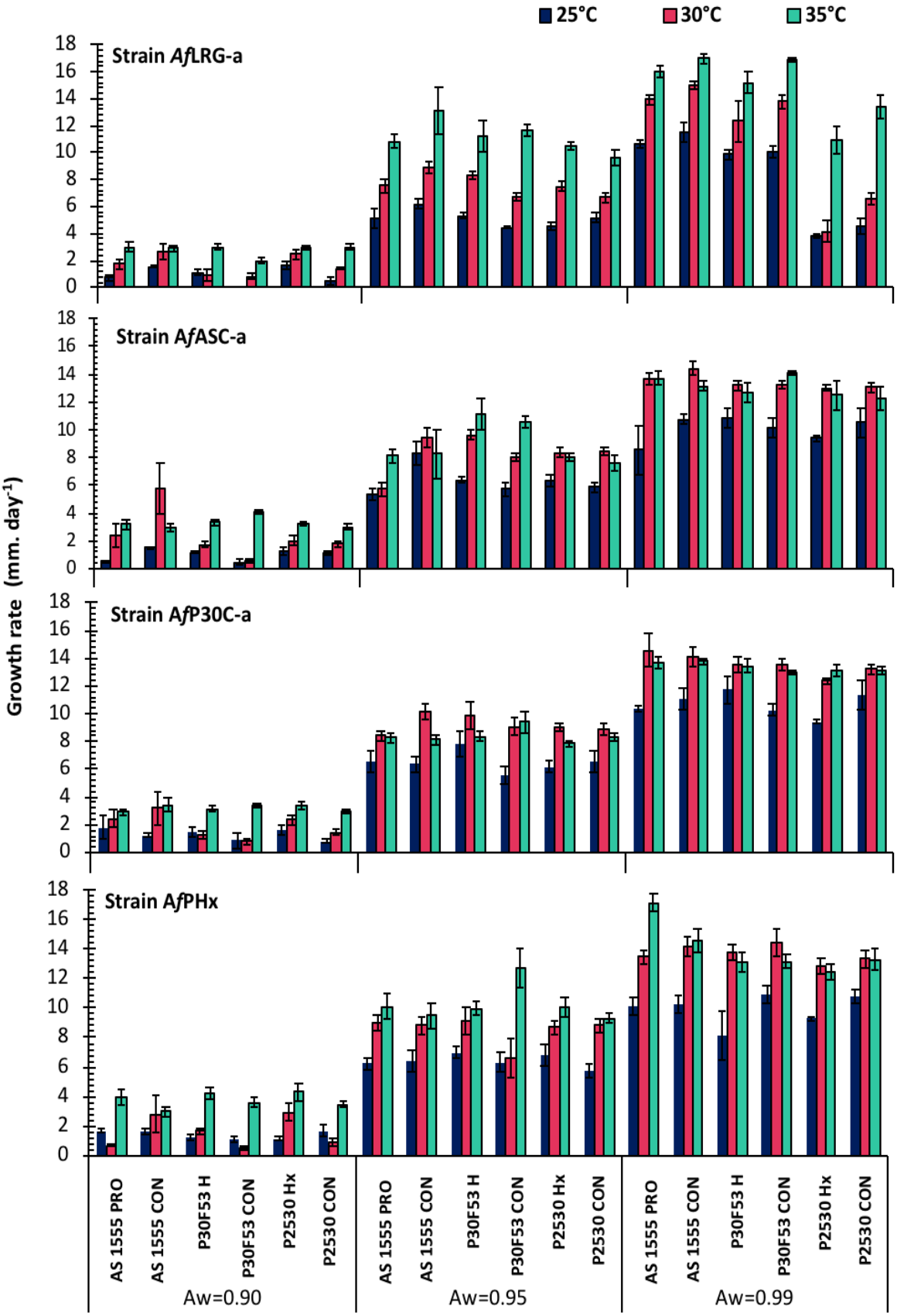

Figure 3.4 Growth rate (mm day^{-1}) for the *A. flavus* strains *Af*LRG-a, *Af*ASC-a, *Af*P30C-a and *Af*PHx on milled maize agar using the non-GM maize cultivars (AS 1555 CON, P30F53 CON and P2530 CON) and their respective isogenic GM lines (AS 1555 PRO®, P30F53 H® and P2530 Hx®) as substrate. Bars represent AV±SE.

3.4.2 Effect of maize nutritional source and environmental factors on aflatoxin B_1 production by of *A. flavus*

The production of AFB_1 was determined using HPLC-FLD from agar plugs collect on day 10. This showed that AFB_1 was produced on maize-based media of all the cultivars, regardless of whether they were isogenic GM or non-GM cultivars. There were however some differences in AFB_1 production between the strains of *A. flavus* tested. A_w was an important factor influencing the production of AFB_1 when comparing 0.90 and 0.99 a_w in the overall AFB_1 production (Figure 3.3). However, there was no significant difference (p=0.05) at 25 and 30^{o}C. All the toxigenic strains used in this study were able to produce AFB_1 in all the maize cultivars used as the nutritional media. Overall, observing the maize cultivars, each strain had a distinct behaviour depending on the combination of maize cv × a_w × T ^{o}C.

The strain *Af*ASC-a produced more AFB_1 on non-GM cv AS 1555 CON (≥ 800 ng. g^{-1}) followed by the isogenic AS 1555 PRO® (≥400 ng. g^{-1}). The optimal condition for this strain to produce toxin in most of the cultivars used as substrate was > 0.98 a_w at 30^{o}C (Figure 3.5). However, there was a peak of AFB_1 production at 35^{o}C and 0.90 a_w when the strain was cultivated in the non-GM cv P30F53 CON (≥100 ng. g^{-1}; Figure 3.5-a). The strain *Af*LRG-a was the highest AFB_1 producer among all the strains when the cultivar P30F53 CON was used as a substrate (≥6000 ng. g^{-1}; Figure 3.6-a). Interestingly, the toxin production on the cultivar P2530 CON was about 60-fold lower (≥100 ng. g^{-1}; Figure 3.6-c). In 5 out of 6 maize cultivars used, this strain had optimal condition for AFB_1 production at 30^{o}C and >0.98 a_w. However, the opposite occurred when the strain was grown in the cultivar P2530 Hx®, where more toxin was produced under stress conditions: <30^{o}C and 0.90 a_w (Figure 3.6-f). The strain *Af*P30C-a also produced more AFB_1 when cultivated using the maize sample P30F53 CON as a substrate (≥1000 ng. g^{-1}; Figure 3.7a) and lower levels with the cultivar P2530 CON (≥80 ng. g^{-1}; Figure 3.7c). The optimal conditions for this strain also were modified with the maize P2530 Hx® and more toxin was produced at 35^{o}C and 0.98 a_w (≥300 ng. g^{-1}; Figure 3.7f).

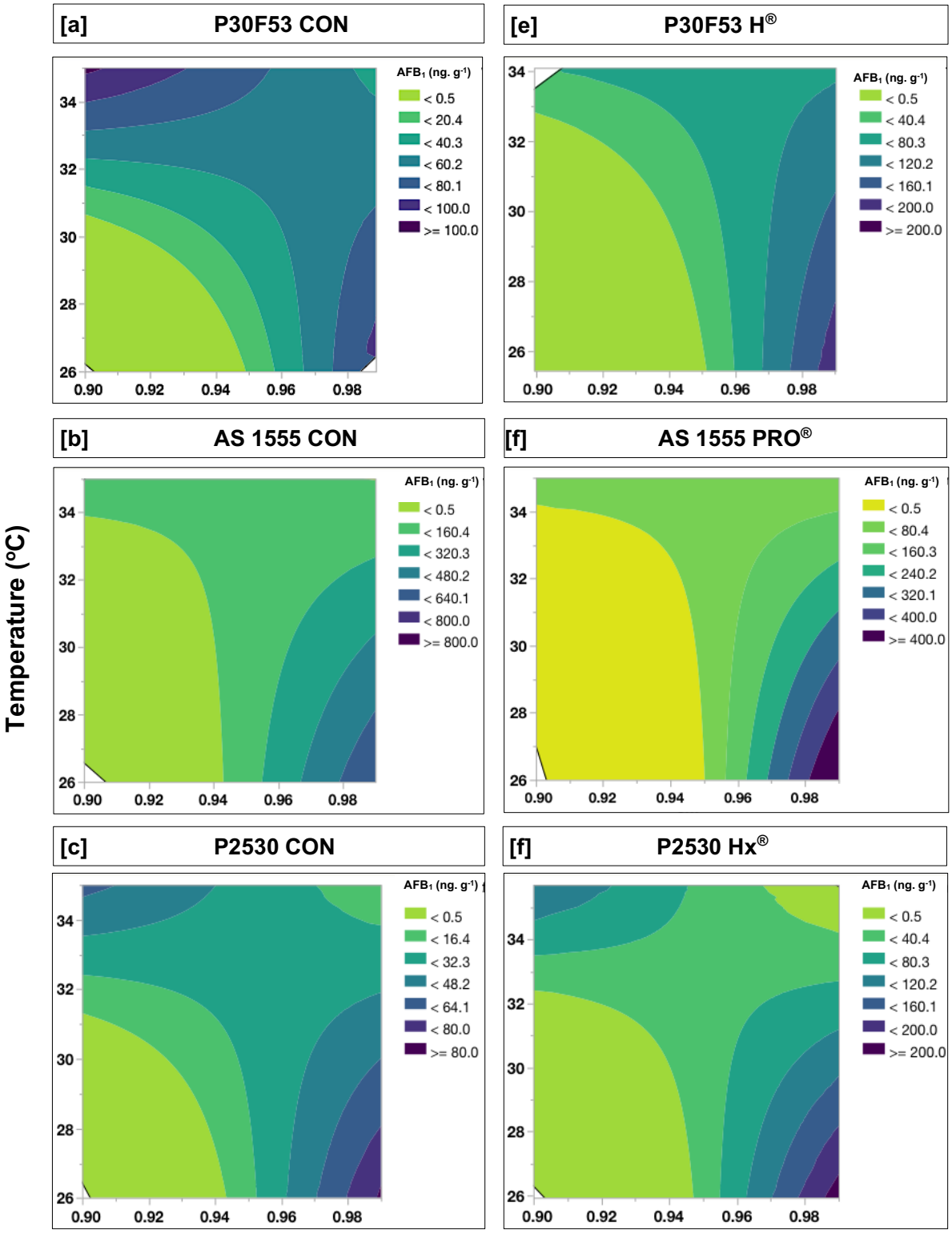

Figure 3.5. Contour maps for AFB_1 ($ng.g^{-1}$) production by the *A. flavus* strain AfASC-a on milled maize agar using the non-GM maize cultivars [a] AS 1555 CON, [b] P30F53 CON and [c] P2530 CON and, their respective isogenic GM lines [d] AS 1555 PRO®, [e] P30F53 H® and [f] P2530 Hx® as substrates.

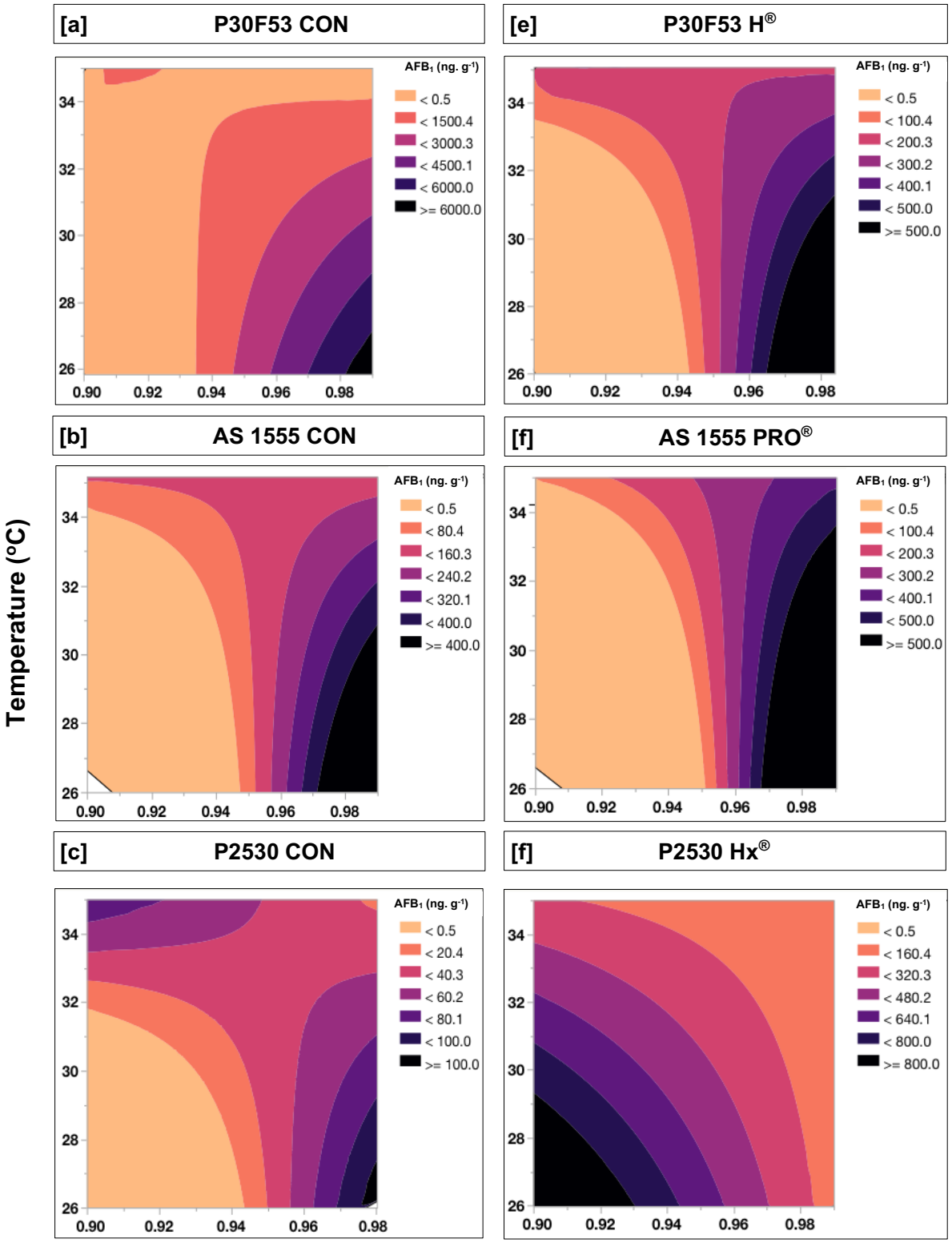

Figure 3.6. Contour maps for AFB_1 ($ng.g^{-1}$) production by the *A. flavus* strain AfLRG-a on milled maize agar using the non-GM maize cultivars [a] AS 1555 CON, [b] P30F53 CON and [c] P2530 CON and, their respective isogenic GM lines [d] AS 1555 PRO®, [e] P30F53 H® and [f] P2530 Hx® as substrates.

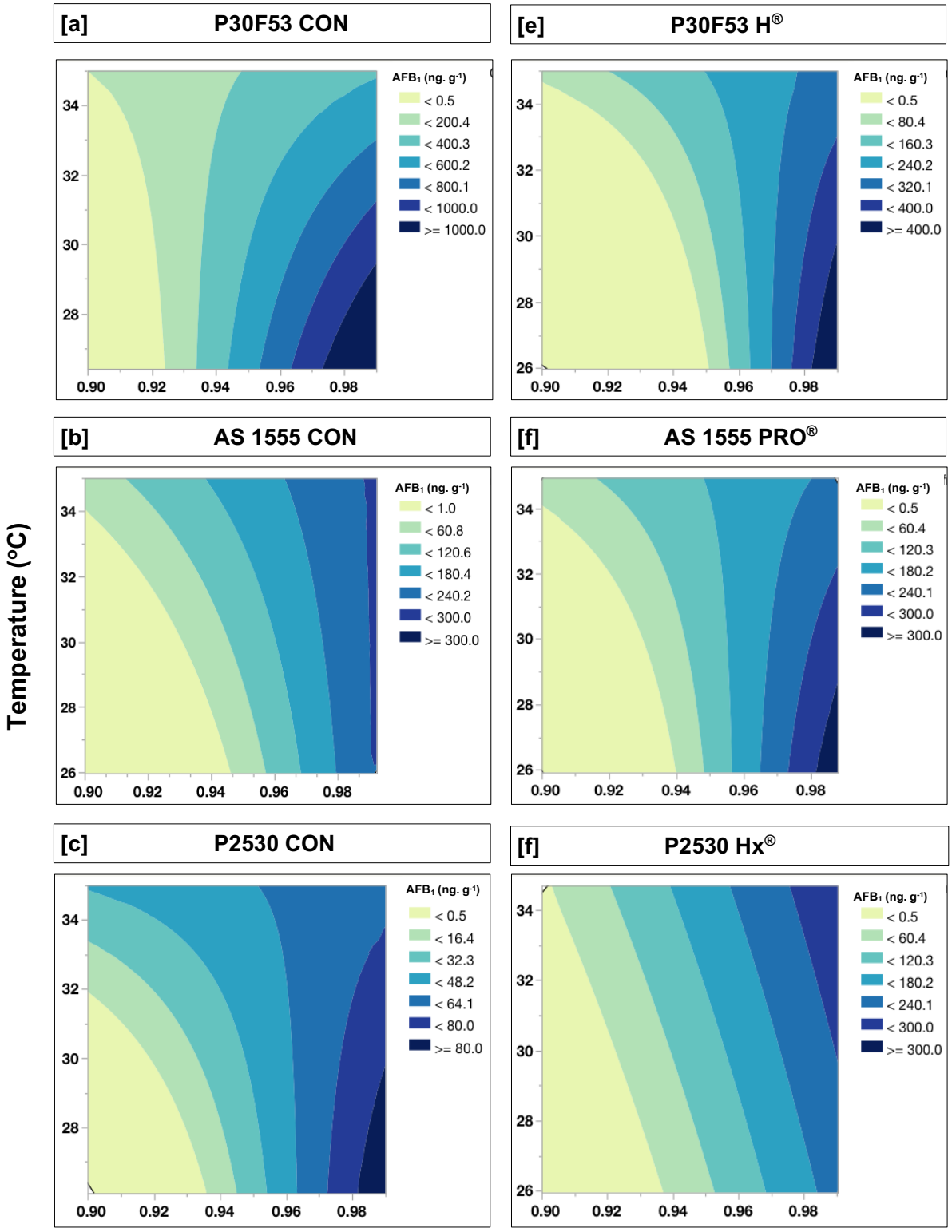

Figure 3.7. Contour maps for AFB_1 ($ng.g^{-1}$) production by the *A. flavus* strain *Af*P30C-a on milled maize agar using the non-GM maize cultivars [a] AS 1555 CON, [b] P30F53 CON and [c] P2530 CON and, their respective isogenic GM lines [d] AS 1555 PRO®, [e] P30F53 H® and [f] P2530 Hx® as substrates.

3.5 Discussion

In the present study, 3 GM maize cultivars (AS 1555 Pro®, P30F53 H® and P2530 Hx®) and their respective isogenic non-GM lines (AS 1555 CON, P30F53 CON and P2530 CON) were used as nutritional substrates for *A. flavus* growth to examine whether there were differences in colonisation or AFB_1 production. The results showed that all the strains were able to grow, and toxigenic strains were able to produce AFB_1 in both the GM and non-GM matrices. The environmental factors, a_w and temperature, and their interactions had a significant effect on the ecology of the strains examined.

The optimal conditions for growth and AFB_1 production found in this work were distinct, and this difference has previously been shown by Medina et al. (2014) who described growth a_w × temperature boundary conditions for toxin production being slightly different from that for growth. The production of secondary metabolites can sometimes be increased under environmental stress condition at lowered water availability and elevated temperatures. According to Bhatnagar et al. (2006, 2018), aflatoxin biosynthesis in *A. flavus* is optimal at temperatures between 29 and 30°C, but it is significantly decreased at temperatures <25 and >37°C. Unlike most fungi, *A. flavus* is favoured by hot dry conditions. Some strains of *A. flavus* can grow in very dry environments (e.g. 0.73 a_w) and produce aflatoxins at 0.85 a_w (Sanchis and Magan, 2004). In addition, the water availability and temperature, and their interactions with biosynthetic gene cluster has been shown to have a significant effect on aflatoxin biosynthesis (Magan et al., 2011; Miraglia et al., 2009).

Furthermore, associated with the environmental effects, the nutritional substrates of the different maize cultivars suggested differences in the patterns of AFB_1 production. The same strain produced different amounts of AFB_1 in each maize cultivar which suggests that nutritional make-up of the cultivars may be slightly different. However, while toxin production was influenced, no effects on colonisation were discernible.

The concentration of AFB_1 was not consistently higher in only one type of maize (GM or non-GM) indicating that each cultivar itself results in more or less toxin despite the genetic modification. When the cultivar P30F53 CON was used, for example, the AFB_1 content was significantly higher than its isogenic GM line for 2 strains (*Af*LRG-a and *Af*P30C-a). However, the same did not happen for the strain *Af*ASC-a where the production of AFB_1 using P30F53 CON and P30F53 H® was similar. Nevertheless, the conditions for the maximum production were at opposite sides in the contour map: 0.90 a_w at 35ºC and 0.99 a_w at 25ºC, for P30F53 CON and P30F53 H® respectively. One hypothesis for these differences could be the nutritional content of each hybrid that could be responsible for the distinct toxin amount since it could affect the metabolism of *A. flavus* strain although there were no significant changes in the colonisation patterns. However, the nutritional characteristics of the maize samples used in this work were not accessed.

Bakan et al. (2002) hypothesized that the biochemical composition of GM maize (MON 810 Bt) and the near isogenic line could be responsible for the differences in the fumonisins content found. However, it has been reported that the content of proteins, soluble nitrogen, starch and sugars did not differ between GM hybrids and their near-isogenic lines (Betz, Hammond and Fuchs, 2000; Masoero et al., 1999). Therefore, from the view of a macro-nutritional assessment, the genetically modified maize can be regarded as substantially equivalent to the parental maize line (Reuter et al., 2002).

A second hypothesis links the distinct AFB_1 production by the strains perhaps being related to the fatty acid (FAs) contents in maize. Levels of free FAs increase in response to various stresses and play a pivotal role in plant–microbe interactions (Walley et al., 2013). Seed FAs composition is also suggested to be a component of pathogen susceptibility and seed colonisation (Dall'Asta et al., 2012). As free FAs, linoleic acid levels partly regulate development, seed colonisation and mycotoxin production by *Aspergillus* spp (Calvo et al., 1999; Scarpari et al., 2014).

Using GC-MS analyses, Jiménez et al. (2009) obtained profiles of the major and minor fatty acids in maize with insect-resistant (*Cry1*Ab gene) indicating a high similarity when compared with its isogenic line grown in the same conditions. Frank et al. (2012) also using GC-MS profiling to investigate the impact of genetic modifications of insect-resistant maize (DKC78-15B, TXP 138F) and herbicide-tolerant maize (DKC78-35R) *versus* environmental influences, reported that the majority of differences observed were related to environmental factors rather than to the genetic modifications. Among them, location and season were predominant factors on the variability of metabolite profiles.

Battilani et al. (2018), on the other hand, presented one of the first studies exploring the Oxylipins: a newly emerging group of signals that serve as defence roles or promote virulence, to identify specific host and fungal genes and oxylipins governing the interactions between maize and *Fusarium verticillioides*. The pathways related to the oxidative stress and oxylipins production are involved both in the process of host-recognition and in the pathogenic phase in some species of filamentous fungi. In *A. flavus*, the oxylipins act as signals for regulating the biosynthesis of AFs, conidiogenesis and formation of sclerotia (Scarpari et al., 2014). Scarpari et al. (2014) suggested that the presence of the fungus provokes the production of oxylipins in the plant, which, in turn might influence the development of the fungus itself by stimulating certain physiological processes such as production of conidia and the biosynthesis of the toxin. However, the way in which this process aids pathogenesis is not yet elucidated. Furthermore, no studies have reported comparisons of oxylipins in GM and non-GM cultivars and their effect on *A. flavus* development.

Manetti et al. (2004, 2006) reported the first attempt to identify the metabolomic fingerprint of GM maize seeds using Nuclear Magnetic Resonance spectroscopy (NMR) to differentiate GM from non-GM cultivars. NMR to profile the metabolome of insect-resistant GM maize containing *cry1A(b)* gene was also used by Piccioni et al. (2009). Amongst the 40 water-soluble metabolites identified in all samples, the metabolites responsible for discrimination between GM and non-GM were ethanol, citric acid, trehalose and glycine-betaine, which presented higher levels

in the GM maize samples (Piccioni et al., 2009). However, relation of modifications in the content of these metabolites and the genetic modification has not been elucidated (Simó et al., 2014).

Trehalose is a naturally occurring disaccharide comprised of two molecules of glucose, widespread in many species of plants and animals (Avonce et al., 2006). It has not been reported as a cause for the increase in aflatoxin production. Glycine-betaine is an amphoteric amine that works as a compatible solute in plants under several environmental stresses such as moisture, temperature and salinity (Sakamoto and Murata, 2002). Plant species vary in their capacity to produce it. However, glycine-betaine is not significantly produced in maize, thus enhancing glycine-betaine content might indirectly affect AF accumulation by alleviating rises in temperature and drought stress (Reddy et al., 2014).

Overall, based on the patterns of AFB_1 production using the different maize cultivars either GM or non-GM in this study associated with environmental conditions and their interaction will affect the development of *A. flavus*. Although this study does not explore the individual macro and micro composition of the maize hybrids it reveals the importance of understanding how the different types of maize can impact on AF content subsequently during post-harvest storage. Further studies should be done to explore in more detail the differences in each type of maize and its impacts on AF production.

3.6 Conclusions

- All the strains used in this study were able to colonize and grow on maize-based nutritional matrices using both GM and non-GM cultivars
- There was no significant difference in using GM and non-GM maize as substrate in the growth of the *A. flavus* strains
- Temperature and a_w had a significant effect ($p<0.05$) in the fungal development

- The optimal conditions for growth were slightly different than that for AFB_1 production. Optimal growth occurred at 30-35^oC and 0.99 a_w, whereas for AFB_1 the optimal was at 25-35^oC and 0.99 a_w
- Each strain showed a different pattern of AFB_1 production depending on the combination of a_w × T^oC × maize
- The highest AFB_1 production was detect for the strain *Af*LRG-a when grown in the cultivar P30F53 CON
- There was a shift of AFB_1 production when the cultivar P2530 CON and P2530 Hx® were used as substrate

CHAPTER 4

SCREENING FOR POTENTIAL BIOCONTROL AGENTS (BCAS) FOR CONTROL OF *ASPERGILLUS FLAVUS* AND AFLATOXIN B_1 PRODUCTION USING DIFFERENT INOCULUM RATIOS AND WATER REGIMES *IN VITRO*

CHAPTER 4. SCREENING FOR POTENTIAL BIOCONTROL AGENTS (BCAS) FOR CONTROL OF *ASPERGILLUS FLAVUS* AND AFLATOXIN B_1 PRODUCTION USING DIFFERENT INOCULUM RATIOS AND WATER REGIMES *IN VITRO*

4.1 Introduction

Biocontrol has been considered among the most promising technologies for sustainable agriculture (Tracy, 2014). It broadly refers to the use of organisms to reduce the incidence of pests, diseases, or toxins (Pitt and Hocking, 2006). However, translating *in vitro* experiments into field efficacy has not always been effective because environmental screens to identify stress tolerant strains have not often been used (Mohale et al., 2013).

A potential option for *A. flavus* management in the field, have largely been focused on the use of atoxigenic isolates of *A. flavus* which may be able to compete with the toxigenic strains by displacing them and reduce the levels of aflatoxin B_1 contamination (Cotty et al., 2008; Dorner, 2004). Available studies suggest that biocontrol strains are capable of reducing aflatoxin-producing populations only by four- to five-folds (Ehrlich, 2014). Two commercial products based on atoxigenic *A. flavus* strains have been approved in the USA, by the Environmental Protection Agency for biological prevention of aflatoxin: afla-guard® commercialized by Syngenta is for use on peanuts and maize, and AF36® developed in Arizona, USA is for use in cottonseed. In Africa, the product *aflasafe*™ based on local strain mixtures*,* have been successfully applied as biocontrol agents with a reduction of 60-96% in aflatoxin production pre- and post-harvest. Other promising *A. flavus* biocontrol candidate strains include TX9-8 (Chang and Hua, 2007), AF051 (Jiang, Yan and Ma, 2009) and TOϕ (Degola, Berni and Restivo, 2011).

In addition, the use of native atoxigenic isolates may reduce some concerns about safety and environmental impacts (Atehnkeng et al., 2008). The effectiveness of biocontrol using atoxigenic *A. flavus* strains is based on the fact that these are predominantly asexual, genetically stable and aggressive as competitors coupled with

their inability to recombine with native toxigenic strains (Abbas et al., 2011a; Ehrlich and Cotty, 2004).

Considering the impacts caused by *A. flavus* and the widespread maize production in Brazil, isolation of potential agents for biocontrol naturally adapted to use under Brazilian environment conditions could represent a viable alternative for *A. flavus* management and AFs reduction.

4.2 Objectives

a. To determine the interactions, competitiveness and Index of Dominance (I_D) between the isolated *A. flavus* strains and the type strain of *A. flavus* NRRL 3357 with competitors (N=16) using a colony-based interaction approach
b. To examine the best potential BCAs to control AFB_1 production *in vitro* by using different ratios of pathogen: antagonist (100:0; 25:75; 50:50; 25:75; 0:100) on milled-maize agar modified to 0.98 and 0.95 a_w using a conventional maize cultivar and its respective isogenic GM line as nutritional substrates.

4.3 Materials and methods

4.3.1 Interaction scores and overall Index of Dominance (I_D) for screening of biological control agents (BCAs) *in vitro*

Fungal strains: Diverse yeast and filamentous fungal strains isolated from the maize cultivars described in CHAPTER 2 were selected for these experiments. The list of all the strains used is shown in the Table 4.1. A type strain NRRL3357 provided by Prof. D. Bhatnagar of the Southern Regional Research Centre, New Orleans, LA, USA was included as a positive control for AFB_1 production.

Media and inoculum preparation: The selected strains were sub-cultured in MEA (malt extract agar, Oxoid, Basingstoke, U.K) for 7 days at 25˚C to obtain fresh inoculum. For the filamentous fungal strains, a spore suspension at 10^6 spores mL^{-1} (counting chamber: Neubauer improved, Paul Marienfield, GE) was prepared and 5 µL of this suspension was inoculated 3.0 cm from the periphery of a 9 cm Ø Petri plate and about 3.0 cm from the antagonist *A. flavus* inoculation point (Figure 4.1). For

yeasts, fresh cultures (36h) were inoculated as a single streak with a sterile loop at a point 3.0 cm from the periphery of the Petri plate. The water availability of MEA was modified to 0.95 and 0.98 a_w using glycerol. All experiments were carried out with three replicates per treatment. Controls consisted of three replicates of each AFL^- and BCA strain centrally inoculated in Petri plates under the same conditions at 30°C

***Experimental design and types of interactions between interacting strains and numerical Index of Dominance (I_D) scores*:** This approach used macroscopic interactions between growing colonies of the antagonist and target pathogens. Each interacting strain was given a numerical score based on the type of interactions observed. There were three replicates per interaction per a_w treatment for all antagonist vs pathogen treatments.

The Petri plates were examined periodically, and each interacting species was given an individual score according to Magan and Lacey (1984): mutual intermingling (1:1), mutual antagonism on contact (2:2), mutual antagonism at a distance (3:3), dominance of one species on contact (4:0) and dominance at a distance (5:0). The scores were added together for each interacting strain to obtain an overall Index of Dominance (I_D). The best BCAs were selected based on the I_D score for subsequent interaction ratios of pathogen: antagonist cells/spores for examination of impacts on AFB_1 production.

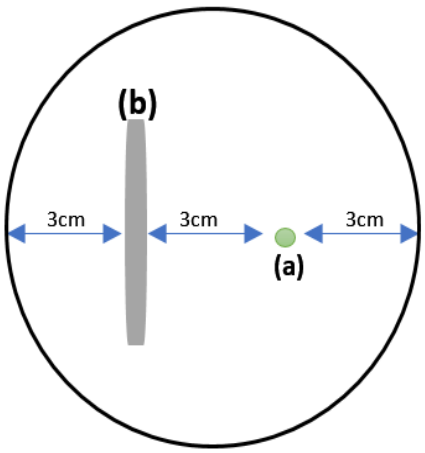

Figure 4.1. Plate schematic for the Interactions between antagonist and pathogen to obtain numerical scores for each interacting strain. (a) represents the *A. flavus strain,* and (b) represents the potential antagonist BCA inoculation point.

Table 4.1. Strains used for macroscopic interaction experiments

	Strain	Codes
Biocontrol agents	*Af*FRC	AFL1$^-$
	*Af*AS1C	AFL2$^-$
	*Af*PHx	AFL3$^-$
	*Af*F53H	AFL4$^-$
	*Af*BMC-b	AFL5$^-$
	*Af*M20a	AFL6$^-$
	*Af*M20C	AFL7$^-$
	*Af*CDPW-b	AFL8$^-$
	ISOL.F	i1
	ISOL. NGM-4	i2
	ISOL. H	i3
	Yeast B	i4
	Yeast 2	Y2
	Yeast 4	Y4
	Yeast 5	Y5
	Yeast 6	Y6
Pathogens	*Af*P30C-a	AFLa$^+$
	*Af*LRG-a	AFLb$^+$
	*Af*M20-*c*	AFLc$^+$
	AfASC-a	AFLd$^+$
	NRRL 3357	AFLe$^+$

4.3.2 Co-cultivation of different inoculum ratios of the best antagonists and toxigenic *A. flavus* strains on aflatoxin B_1 production

Fungal strains: Four atoxigenic *A. flavus* described in Section 4.3.1 were select as BCAs: *Af*FRC (AFL1$^-$), *Af*AS1C (AFL2$^-$), *Af*PHx (AFL3$^-$), *Af*F53H (AFL4$^-$) and Yeast 6 (Y6), while as pathogens with aflatoxin production the *A. flavus* strains AfASC-a (AFLd$^+$), AfLRG-a (AFLb$^+$) and type strain NRRL 3357 (AFLe$^+$) were used. The type strain was included as it is a positive control which has been used in other studies and for which the whole genome data is available (Mohale et al., 2013; Bhatnagar et al., 2018).

Media preparation: One cultivar of non-GM (P30F53 CON) maize and its respective isogenic GM line (P30F53 H® cv with insect resistance: gene Cry1F; and herbicide tolerance: gene PAT for Glufosinate ammonium) were chosen as the base-medium

for the milled maize meal agar (MMA). The medium was prepared by mixing milled maize (3% milled maize, 2% agar) in a solution with the water availability adjusted to 0.98 and 0.95 using glycerol due to its stability over the experimental temperature range for modifying a_w. The media was autoclaved at 121°C for 15 min. The accuracy of the modifications was confirmed using an Aqualab® 4TE instrument (Decagon, USA).

Inoculum: A spore suspension at 10^6 spores. mL^{-1} was determined using the counting chamber (Neubauer improved, Paul Marienfield, GE) for each *A. flavus* strain and different ratios of pathogen (toxigenic *A. flavus* strain) and BCA were prepared as shown in Table 4.2. Thereafter, 100 µL of the spore/cell suspensions was inoculated and spread plated on MMA and incubated at 30˚C for 14 days. Each treatment was done in triplicate. Agar plugs (n=6) were collected for AFB_1 quantification after 7 and 14 days.

Table 4.2. Inoculum ratios for pathogen: antagonist for biocontrol test *in vitro* (Mohale et al.; 2013)

SPECIES	Volume of spore suspension [number of spores. mL^{-1}]										TOTAL (mL)
A. flavus	6ml	[1.0×10^6]	4.5ml	[7.5×10^5]	1.5ml	[5.0×10^5]	3ml	[2.5×10^5]	0ml	[0]	15
BCA	0ml	[0]	1.5ml	[2.5×10^5]	4.5ml	[5.0×10^5]	3ml	[7.5×10^5]	6ml	[1.0×10^6]	15
Final ratio	**100:0**		**75:25**		**25:75**		**50:50**		**0:100**		

100:0 – control A. flavus toxigenic / 0:100 – control of Biocontrol agent (BCA)

4.3.3 Aflatoxin analysis

Six agar plugs (approx. 0.3 g) were taken from the agar medium across the plates with a 5 mm-diameter sterile cork borer. These plugs were placed into a pre-weighed 2-ml Eppendorf tube. Aflatoxins were extracted from the agar plugs by adding 1 mL of chloroform in the tube and shaken for 1h. A volume of 800 µL from the tube of chloroform + biomass was transferred to a new 2-mL Eppendorf tube and the chloroform was evaporated to dryness overnight. The derivatization was performed afterwards. The derivatization, preparation of standards of aflatoxin and HPLC analyses was carried out as described in the CHAPTER 2 – section 2.3.4. Only AFB_1 was detected in the samples. The R^2 value for the calibration curve was $R^2 = 0.990$.

4.3.4 Statistical analysis

The data from co-cultivation of different inoculum ratios of pathogen: antagonist and effects on aflatoxin B_1 production were subjected to Shapiro-Wilk tests to determine normality and Levene's test to assess the equality of variance. However, the data violated the two assumptions for ANOVA even after transformations and therefore non-parametric tests (Kruskal-Wallis/ Wilcoxon; p=0.05) were used for analyses (Chan and Walmsley, 1997). Where there was significance after the Kruskal-Wallis test, median comparisons for each pair were made using the Wilcoxon - Each Pair test (p=0.05). The statistical package JMP®14 (SAS Institute Inc., 2018, Cary NC, USA) was used to perform the analyses.

4.4 Results

4.4.1 Evaluation of interaction scores between antagonists and pathogen and overall Index of Dominance (I_D) in the *in vitro* screening tests

The scores for each interacting strain and the overall Index of Dominance (I_D) are shown in Table 4.3. The co-cultivation of the toxigenic *A. flavus* (AFL^+) with the atoxigenic (AFL^-) strains indicated the ability of both strains to mutually intermingle without inhibition, regardless of the water availability or substrate. In relation to the interaction between the toxigenic *A. flavus* and others microorganism, the former species was dominant (Figure 4.2). The only exception was the yeast Y6 that was mutually antagonistic on contact at 0.98 a_w on MEA. However, at 0.95 a_w the yeast could not grow effectively and the toxigenic *A. flavus* was dominant. The interaction of AFL^- x AFL^+ on MMA showed the same behaviour of the strains: mutual intermingling (Figure 4.3).

Table 4.3. Effect of water activity (a_w) on numerical scores for each antagonist strain and the total Index of Dominance (I_D) in relation to each AFL$^+$ strain on MEA after 10 days incubation at 30°C.

Interacting strains		a_w 0.95	a_w 0.98	I_D score
	AFLa$^+$	1:1	1:1	2:2
	AFLb$^+$	1:1	1:1	2:2
0:4	AFLc$^+$	1:1	1:1	2:2
	AFLd$^+$	1:1	1:1	2:2
	AFLe$^+$	1:1	1:1	2:2
I_D		1:1		10:10
	AFLa$^+$	1:1	1:1	2:2
	AFLb$^+$	1:1	1:1	2:2
AFL2$^-$	AFLc$^+$	1:1	1:1	2:2
	AFLd$^+$	1:1	1:1	2:2
	AFLe$^+$	1:1	1:1	2:2
I_D				10:10
	AFLa$^+$	1:1	1:1	2:2
	AFLb$^+$	1:1	1:1	2:2
AFL3$^-$	AFLc$^+$	1:1	1:1	2:2
	AFLd$^+$	1:1	1:1	2:2
	AFLe$^+$	1:1	1:1	2:2
I_D		1:1	1:1	10:10
	AFLa$^+$	1:1	1:1	2:2
	AFLb$^+$	1:1	1:1	2:2
AFL4$^-$	AFLc$^+$	1:1	1:1	2:2
	AFLd$^+$	1:1	1:1	2:2
	AFLe$^+$	1:1	1:1	2:2
I_D		1:1	1:1	10:10
	AFLa$^+$	1:1	1:1	2:2
	AFLb$^+$	1:1	1:1	2:2
AFL5$^-$	AFLc$^+$	1:1	1:1	2:2
	AFLd$^+$	1:1	1:1	2:2
	AFLe$^+$	1:1	1:1	2:2
I_D		1:1	1:1	10:10
	AFLa$^+$	1:1	1:1	2:2
	AFLb$^+$	1:1	1:1	2:2
AFL6$^-$	AFLc$^+$	1:1	1:1	2:2
	AFLd$^+$	1:1	1:1	2:2
	AFLe$^+$	1:1	1:1	2:2
I_D		1:1	1:1	10:10
	AFLa$^+$	1:1	1:1	2:2
	AFLb$^+$	1:1	1:1	2:2
AFL7$^-$	AFLc$^+$	1:1	1:1	2:2
	AFLd$^+$	1:1	1:1	2:2
	AFLe$^+$	1:1	1:1	2:2
I_D	AFLa$^+$	1:1	1:1	10:10
	AFLa$^+$	1:1	1:1	2:2
	AFLb$^+$	1:1	1:1	2:2
AFL8$^-$	AFLc$^+$	1:1	1:1	2:2
	AFLd$^+$	1:1	1:1	2:2
	AFLe$^+$	1:1	1:1	2:2
I_D				10:10

Interacting strains		a_w 0.95	a_w 0.98	I_D score
	AFLa$^+$	0:4	0:4	0:8
	AFLb$^+$	0:4	0:4	0:8
i1	AFLc$^+$	0:4	0:4	0:8
	AFLd$^+$	0:4	0:4	0:8
	AFLe$^+$	0:4	0:4	0:8
I_D				0:40
	AFLa$^+$	0:4	0:4	0:8
	AFLb$^+$	0:4	0:4	0:8
i2	AFLc$^+$	0:4	0:4	0:8
	AFLd$^+$	0:4	0:4	0:8
	AFLe$^+$	0:4	0:4	0:8
I_D				0:40
	AFLa$^+$	0:4	0:4	0:8
	AFLb$^+$	0:4	0:4	0:8
i3	AFLc$^+$	0:4	0:4	0:8
	AFLd$^+$	0:4	0:4	0:8
	AFLe$^+$	0:4	0:4	0:8
I_D				0:40
	AFLa$^+$	0:4	0:4	0:8
	AFLb$^+$	0:4	0:4	0:8
i4	AFLc$^+$	0:4	0:4	0:8
	AFLd$^+$	0:4	0:4	0:8
	AFLe$^+$	0:4	0:4	0:8
I_D				0:40
	AFLa$^+$	0:4	0:4	0:8
	AFLb$^+$	0:4	0:4	0:8
Y2	AFLc$^+$	0:4	0:4	0:8
	AFLd$^+$	0:4	0:4	0:8
	AFLe$^+$	0:4	0:4	0:8
I_D				0:40
	AFLa$^+$	0:4	0:4	0:8
	AFLb$^+$	0:4	0:4	0:8
Y4	AFLc$^+$	0:4	0:4	0:8
	AFLd$^+$	0:4	0:4	0:8
	AFLe$^+$	0:4	0:4	0:8
I_D				0:40
	AFLa$^+$	0:4	0:4	0:8
	AFLb$^+$	0:4	0:4	0:8
Y5	AFLc$^+$	0:4	0:4	0:8
	AFLd$^+$	0:4	0:4	0:8
	AFLe$^+$	0:4	0:4	0:8
I_D				0:40
	AFLa$^+$	0:4	2:2	2:6
	AFLb$^+$	0:4	2:2	2:6
Y6	AFLc$^+$	0:4	2:2	2:6
	AFLd$^+$	0:4	2:2	2:6
	AFLe$^+$	0:4	2:2	2:6
I_D				10:30

I_D: mutual intermingling (1:1), mutual antagonism on contact (2:2), mutual antagonism at a distance (3:3), dominance of one species on contact (4:0) and dominance at a distance (5:0)

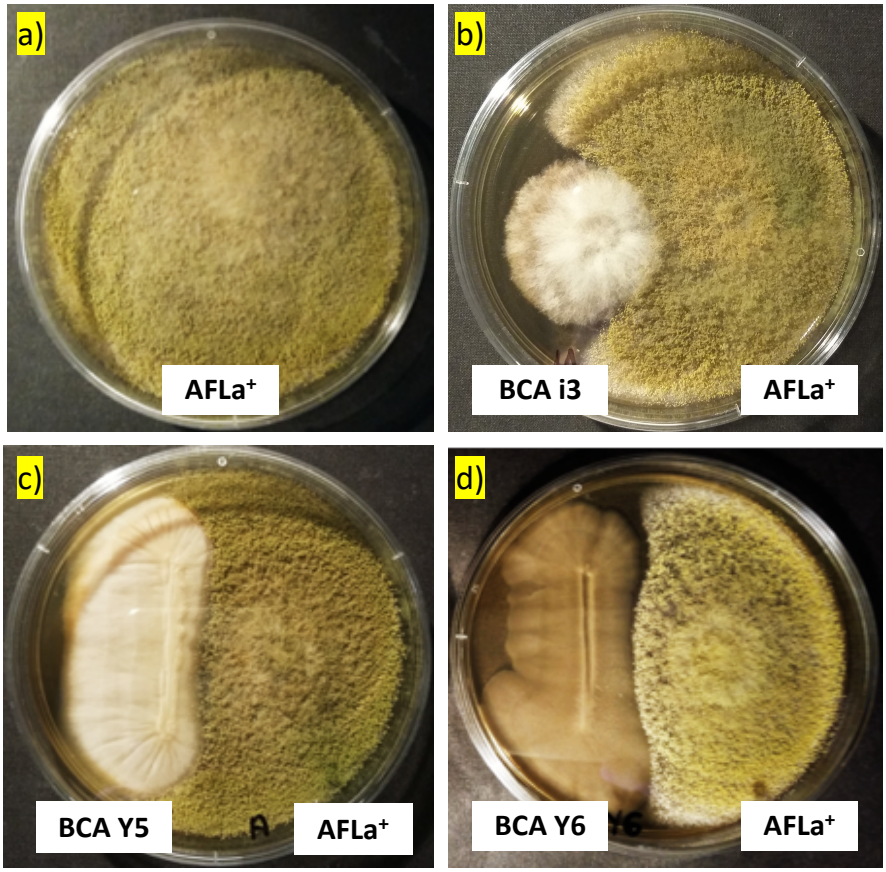

Figure 4.2. Examples of colony interactions of toxigenic *A. flavus* (AFL^+) and BCAs on MEA (0.98 a_w). Key: (a) control $AFLb^+$; (b) BCA i3 x $AFLb^+$ and (c) Y5 x $AFLb^+$: dominance of one specie on contact (0:4); (d) BCA Y6 x $AFLb^+$: mutual antagonism on contact (2:2).

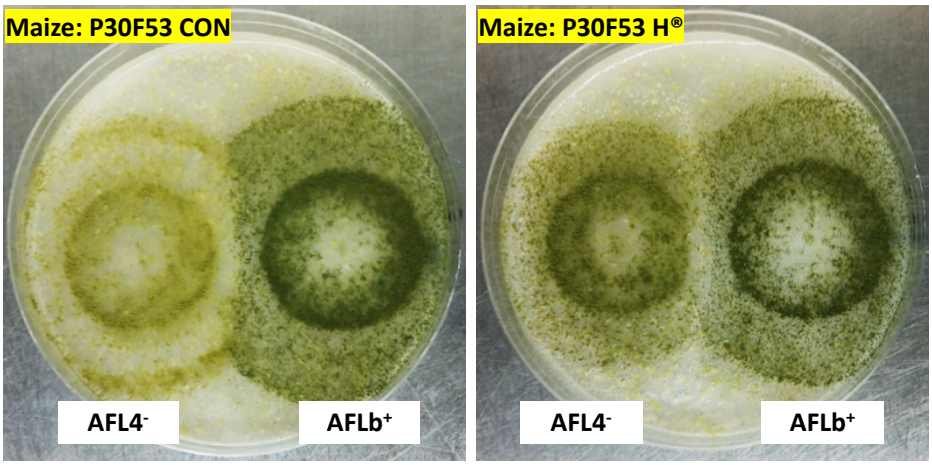

Figure 4.3. Plates from co-cultivation of *A. flavus* atoxigenic $AFL4^-$ against antagonist $AFLb^+$ in milled maize media (0.98 a_w) using the cultivars P30F53 CON (non-GM) and P30F53 H® (GM) as substrate. I_D = mutual intermingling (1:1).

4.4.2 Co-cultivation of different inoculum ratios of pathogen:antagonist and effects on aflatoxin B_1 production at different water activity levels

The *in vitro* mixture of atoxigenic and toxigenic strains on MMA in different ratios showed the ability to reduce AFB_1 content by all four AFL^- strains selected. Using 75 and 50% spores of the antagonistic *A. flavus* strains were more efficient for control of mycotoxins at both a_w levels. Although the ratio of 75:25 (AFL^+: AFL^-) was statistically different compared with the control, the reduction of AFB_1 was less effective. The overall AFB_1 content for the ratios AFL^-: AFL^+ are shown in Figure 4.4. It indicates difference between 0.98 and 0.95 a_w ($p<0.05$), however, the same did not occur between the maize cultivars (GM or non-GM) used as substrates for *A. flavus* development.

Toxigenic AFL^+ strains (b^+, d^+ and e^+) used as pathogens had a strong difference in the AFB_1 production pattern ($p<0.0001$ in Kruskal-Wallis test). Nonetheless all the BCAs were able to significantly reduce the toxin production. The type strain NRRL3357 ($AFLe^+$) was the highest producer in all the conditions (> 1000 ng. g^{-1}) and the relative control ranged from 61% to 100% (see Table 4.4). The lower control ability occurred when using the higher amount of pathogen inoculum (75:25 AFL^+: AFL^-) and under stress conditions: 0.95 a_w. In this scenario, the antagonistic strain $AFL4^-$ was not able to reduce the toxin content when paired with the native strain $AFLb^+$: no control of toxin content after 7 days was observed for the non-GM maize, and after 7 and 14 days when GM maize was used as matrix. Interestingly, in optimal conditions for growth and toxin production (0.98 a_w at 30°C) the control of AFB_1 was substantial even after 14 days, as shown in Figure 4.5.

In addition to the atoxigenic *A. flavus* strains used as potential BCAs, a yeast strain (Y6) was select as a BCA because of its ability to compete with *A. flavus* in mutual antagonism after contact (see Section 4.3.2- Figure 4.2). Since the water requirements for the growth of the yeast is distinct, only the higher water activity (0.98) was used in this experiment. Even though Y6 was able to have some effect when paired with toxigenic *A. flavus* on contact on MEA, the same effect did not occur with a mixture of AFL^+: Y6 in different inoculum ratios in MMA. The use of Y6 caused an increase in the AFB_1 production when compared with the control (see Figure 4.6).

Using Y6 as an antagonist even when lower amounts of the pathogen was inoculated, the toxin production increased significantly (p=0.05). The strongest effect occurred when Y6 was mixed with the strain AFLe^{+} at a ratio of 25:75. The toxin content increased by >20 folds on day 14 using both types of maize (GM and non-GM) as substrates.

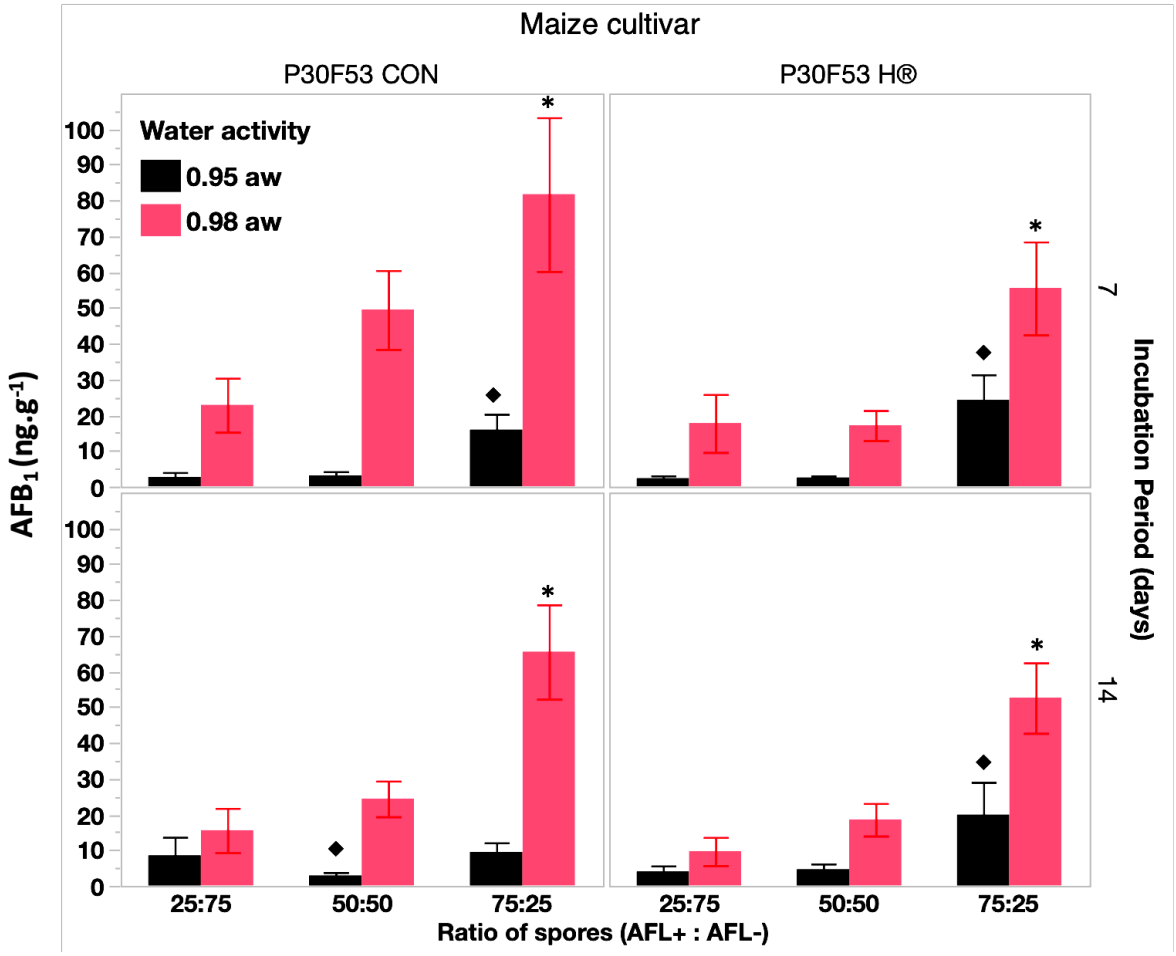

Figure 4.4. Overall AFB_1 content for all the strains tested by inoculum ratio pathogen: antagonist (AFL^{+}: AFL^{-}) on MMA using maize cultivars P30F53 CON (non-GM) and P30F53 H® (GM) as substrate.

The symbols (* for 0.98 a_w and ◆ for 0.95 a_w) indicate significant difference in nonparametric comparisons for each pair using Wilcoxon Method (p=0.05) for each type of maize. Bars represent AV± SE.

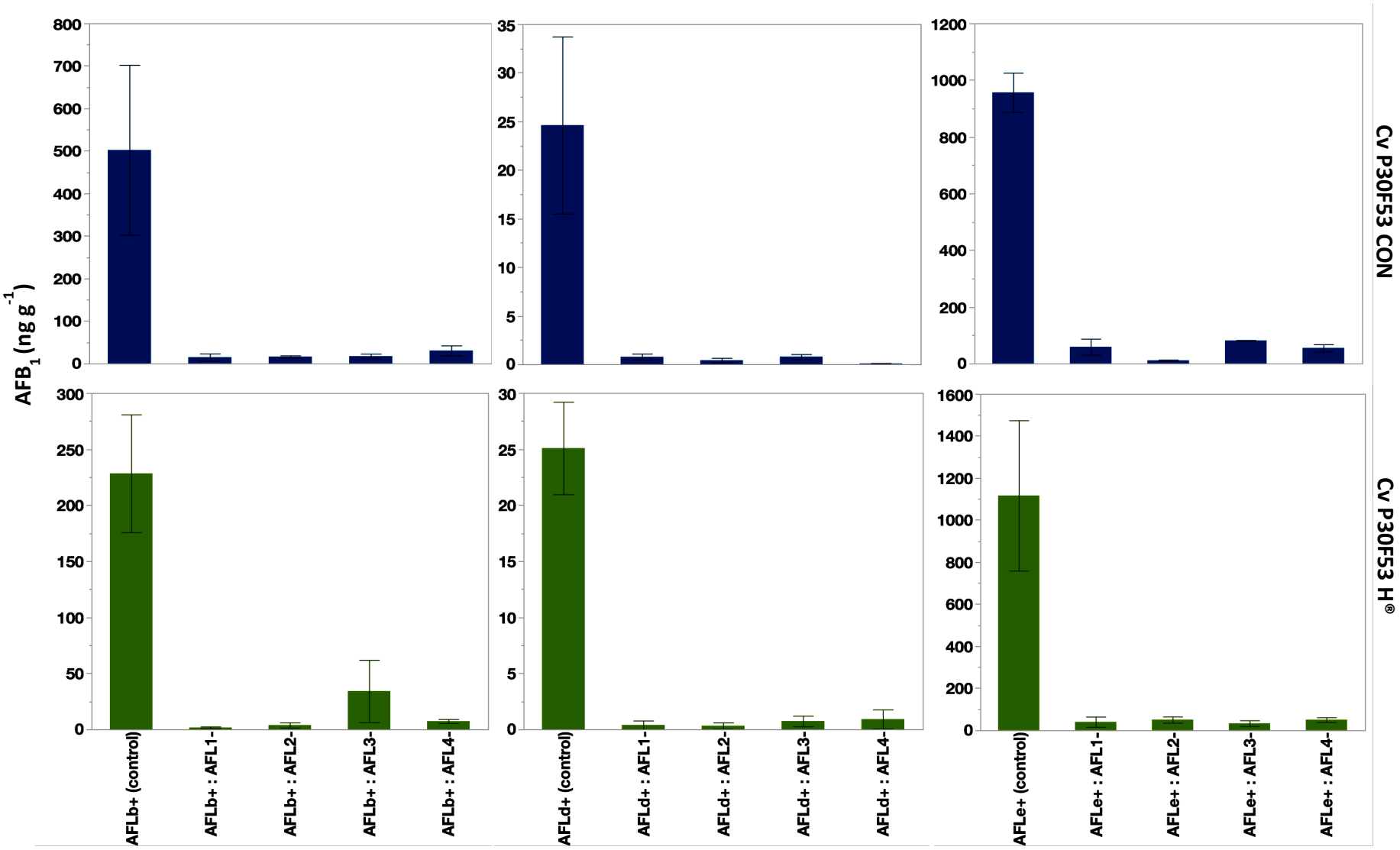

Figure 4.5. Control of AFB_1 by the BCAs $AFL1^-$, $AFL2^-$, $AFL3^-$ and $AFL4^-$ in the ratio 50:50 pathogen: antagonist at 0.98 a_w after 14 days incubation at 30°C using MMA with the maize cultivars P30F53 CON (non-GM) and P30F53 H® (GM) as substrate. Control correspond to the ratio 100:0 AFL+: AFL-; no AFB_1 was detect in 0:100 AFL+: AFL- (LOD <0.1 ng. g^{-1}). Bars represent AV ± SE.

Table 4.4. Relative control (%) of aflatoxin B_1 (AFB_1) production for the interaction pathogen: antagonist in different ratios of inoculum using GM and non-GM maize as media substrate and modified water availability (0.98 and 0.95 a_w) after 7- and 14-days incubation at 30°C.

Interaction			CV P30F53 CON (non-GM)				CV P30F53 H® (GM)			
			0.98 a_w		0.95 a_w		0.98 a_w		0.95 a_w	
P*	A*	Ratio	day 7	day 14	day 7	day 14	day 7	day 14	day 7	day 14
AFLb+	AFL1-	25:75	100	100	93	97	99	99	80	82
		50:50	99	97	83	92	99	99	72	91
		75:25	94	95	85	86	79	72	53	85
	AFL2-	25:75	100	100	93	97	98	98	96	100
		50:50	75	97	96	100	98	98	94	100
		75:25	87	97	89	95	84	78	90	98
	AFL3-	25:75	81	100	87	86	56	100	81	97
		50:50	77	96	86	100	92	85	77	89
		75:25	99	94	91	97	93	92	86	98
	AFL4-	25:75	99	99	69	54	99	99	46	89
		50:50	93	94	90	97	97	97	70	93
		75:25	65	71	-	35	79	77	-	-
AFLd+	AFL1-	25:75	100	100	100	99	100	100	99	100
		50:50	96	97	98	97	100	98	97	98
		75:25	74	91	73	29	84	80	91	87
	AFL2-	25:75	100	100	100	100	100	99	100	100
		50:50	98	98	100	100	98	99	100	100
		75:25	98	99	96	98	99	97	97	100
	AFL3-	25:75	96	99	91	100	97	94	98	100
		50:50	96	98	90	99	96	97	97	100
		75:25	75	93	89	97	37	59	97	100
	AFL4-	25:75	100	100	100	100	98	98	100	100
		50:50	100	100	100	98	98	96	97	99
		75:25	95	99	94	98	93	93	97	99
AFLe+	AFL1-	25:75	97	98	98	99	96	98	99	99
		50:50	88	94	100	100	95	96	100	99
		75:25	83	89	99	99	93	95	94	96
	AFL2-	25:75	97	98	100	98	96	96	100	100
		50:50	98	99	100	100	95	95	100	100
		75:25	95	93	95	98	90	90	96	100
	AFL3-	25:75	88	89	100	100	97	98	100	100
		50:50	88	91	100	100	95	97	100	100
		75:25	77	78	98	99	80	88	99	100
	AFL4-	25:75	97	97	99	100	98	99	100	100
		50:50	90	94	98	100	95	96	100	100
		75:25	61	81	93	100	78	88	97	100

P* = pathogen *A. flavus* toxigenic (AFL+); A* = Antagonist *A. flavus* atoxigenic (AFL-); (-) indicates no control of AFB_1 production. % of relative reduction >99.9 were considered as 100%.

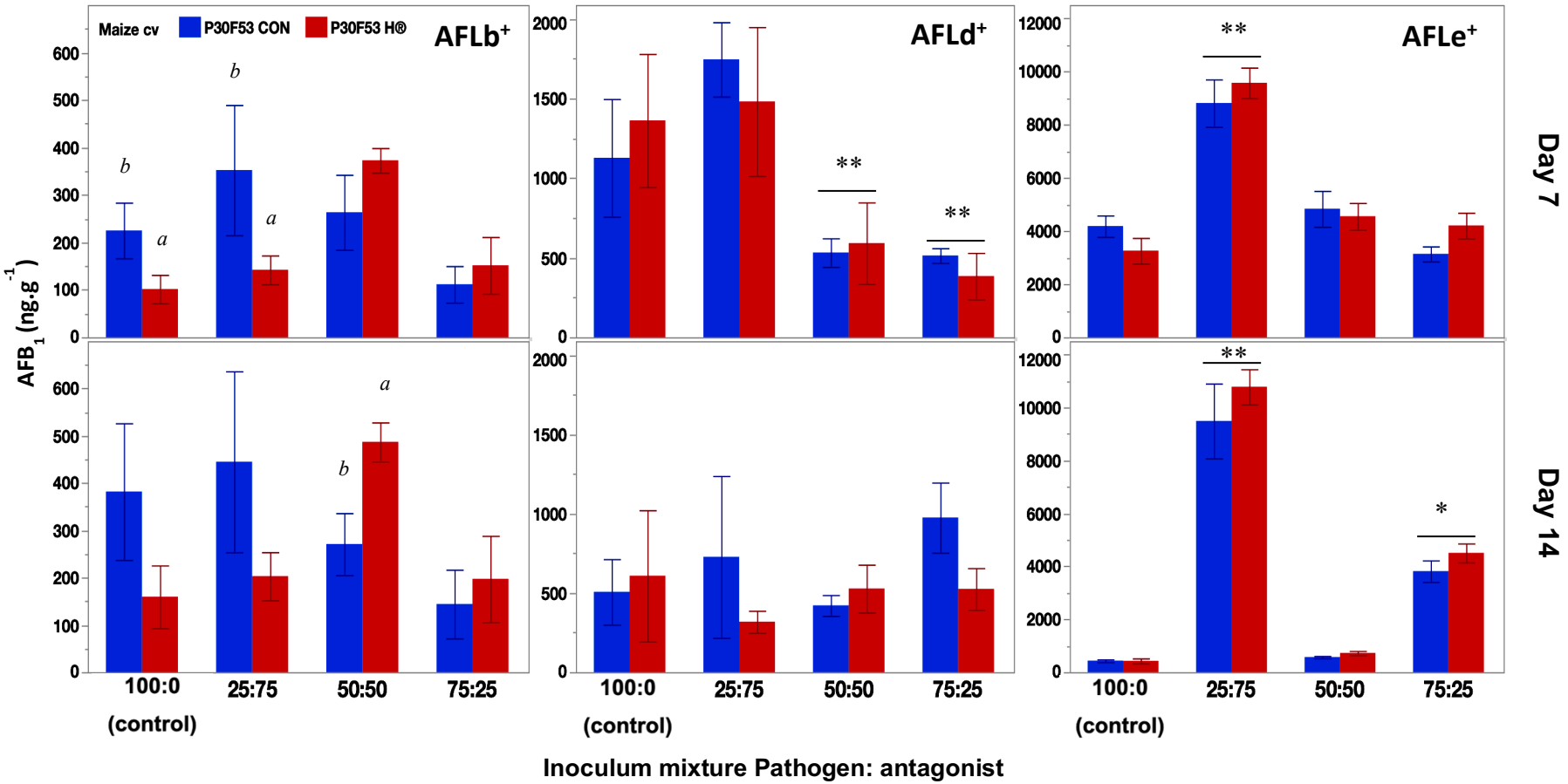

Figure 4.6. Effect of antagonist yeast (Y6) on AFB_1 production of toxigenic strains of *A. flavus* ($ALFb^+$, $AFLd^+$ and $AFLe^+$) with different spore's ratios of pathogen: antagonist on maize-based media using conventional (P30F53 CON) and GM (P30F53 H®) cultivars with water availability modified to 0.98 a_w after 7 and 14 days at 30°C. Bars represent AV±SE. Same symbol shows difference between the ratios for each toxigenic strain; same letter represents differences between GM and non-GM maize ($p<0.05$).

4.5 Discussion

This study selected 8 atoxigenic *A. flavus* strains and 8 other fungal strains from the mycobiota from the Brazilian maize cultivars to screening for potential BCAs using a challenge test between individual strains and to obtain interaction scores and an overall Index of Dominance (I_D) to help identify good competitors. This approach gave higher numerical scores to fungi able to dominate *in vitro* rather than being antagonistic. By adding the scores against different pathogenic *A. flavus* strains under different water availability conditions it was possible to obtain information on those that may be effective. Magan et al. (2003) developed his Index to assist with interpreting patterns of colonisation and dominance in stored grain ecosystems. The overall I_D scores are a good guide to the competitiveness of an individual strain or species (Mohale, 2010). This showed that *A. flavus* was highly dominant when paired with other fungal species selected as potential BCAs, with the exception of one yeast strain (Y6), where the interaction was mutually antagonistic on contact (2:2) at 0.98 a_w on MEA.

Subsequently, 4 out of the 8 atoxigenic AFL^- strains initially used plus the yeast Y6 were tested in different inoculum ratios of pathogen: antagonist. The pathogens (AFL^+) were 2 strains isolated from Brazilian maize in order to evaluate the ability to inhibit native strains, and one highly toxigenic type strain (NRRL3375). Although the interaction scores between AFL^+ and AFL^- were mutual intermingling, not affecting the growth, the production of AFB_1 can still be influenced. The production of mycotoxin depends on a wide range of environmental, epidemiological and genetic factors, therefore, growth inhibition is not a good indicator of whether a reduction in mycotoxin production will be achieved (Pfliegler et al., 2015; Medina et al., 2017).

Using the approach of different ratios (25:75, 50:50, 75:25, 0:100 and 100:0) of atoxigenic and toxigenic *A. flavus* strains it was possible to obtain some useful data on the effect of having more or less inoculum of a competitor on AFB_1 production. All the AFL^- strains used in this study were able to reduce the AFB_1 production at both water availability levels (0.95 and 0.98) examined after 7 and

14 days at 30°C. These results are a good method for effective screening of candidates for choosing the best strains for *in vivo* assays. In addition, by using the maize cultivars P30F53 CON (non-GM) and P30F53 H® (GM) as nutritional matrices showed no difference in the production or reduction of AFB_1.

This study considered that using a 50:50 ratio of AFL^-: AFL^+ would be the most effective approach to checking for biocontrol. However, studies suggested that increasing spore inoculum of atoxigenic strains relative to toxigenic ones would give better control of the aflatoxin production (Degola, Berni and Restivo, 2011; Pitt and Hocking, 2006) although it may not be cost effective. It has also been suggested that the use of indigenous strains may also be important to obtain effective control of AFB_1 contamination (Probst et al., 2011).

The used of non-mycotoxin-producing strains of fungi derived from the same species or taxonomic group that produces toxins has shown great promise for reducing mycotoxins in crops (Cleveland et al., 2003). The effectiveness of biocontrol using atoxigenic *A. flavus* strains is based on the fact that these are predominantly asexual, genetically stable and aggressive as competitors coupled with their inability to recombine with native toxigenic strains (Abbas et al., 2011a; Ehrlich and Cotty, 2004). Although the mechanism by which a non-aflatoxigenic strain interferes with aflatoxin accumulation of toxigenic strains has not been definitively elucidated (Huang et al., 2011). Abbas et al. (2011) and Hruska et al. (2014) supported the theory of exclusion in which the introduced atoxigenic strains have the ability to compete and prevent toxigenic strains from occupying the ecological niche.

However, Hruska et al. (2014) also pointed out concerns regarding the long-term use of non-aflatoxigenic fungi for suppression of native toxigenic strains in biocontrol strategies. Studies have also compared the Carbon-source utilisation patterns by atoxigenic and toxigenic strains of *A. flavus* and showed that, regardless of environmental conditions, the utilisation rates of maize related nutrients were relatively similar for these two types of strains (Mohale et al., 2013). Thus, the competitive exclusion theory may not be the comprehensive

mechanism by which control is achieved. Huang et al. (2011) hypothesised that an unknown signalling in the pathway could be initiated in the toxigenic strain by physical interaction triggering a downregulation or prevention of normal expression of AFs when the suitable biocontrol is applied. However, the exact mechanism of action of BCAs using atoxigenic strains of *A. flavus* is not well understood.

Additionally, in this study, a yeast (Y6) isolated from maize against toxigenic *A. flavus* strains was able to compete effectively suppressing the dominance of toxigenic AFL^+ strains on MEA (30°C, 0.98 a_w). However, the use of Y6 in this study was not effective for the reduction of AFB_1 using different ratios with the toxigenic AFL^+: yeast in MMA. In fact, the ratio of 25:75 (AFL^+: Y6) caused a substantial increase in the AFB_1 production in all the toxigenic strains used in the experiment. It may be linked to the stress caused by an elevate amount of yeast inoculum resulting in an increase in toxin production. Previously, Perez et al. (2015) found yeasts to be effective candidates for control of AFB_1 linked to their ability to produce metabolites that have a significant suppressing effect on the expression of genes related to mycotoxin biosynthesis and/or inhibiting the growth of filamentous fungi (Pfliegler, Pusztahelyi and Pócsi, 2015). However, growth of yeasts and moulds together on the same substrate can lead to positive or negative interactions (Masoud and Kaltoft, 2006).

In fact, Medina et al. (2017) described that there are also cases where in studies for selection of BCAs the interactions between BCAs and mycotoxigenic species can lead to a stimulation of mycotoxin. It has been suggested that there may be some signalling or trigger for the mycotoxigenic species to increase the production of secondary metabolites when under abiotic stress. In a study reported by Al-Saad et al. (2016), when 50:50 bacterial cells: *A. flavus* toxigenic was used, in some cases a stimulation of toxin production occurred relative to the controls. This suggests that the presence of the bacterial cells associated with environmental conditions (temperature × a_w) may result in a combined stress which may stimulate the biosynthesis of toxin as a defence response. Further studies could help to understand how the interactions of *A. flavus* and other

microorganisms can affect the production of toxins in the field or storage environment.

In summary, four atoxigenic *A. flavus* strains isolated from Brazilian conventional and GM maize showed potential for biocontrol when different ratios of inoculum AFL^+: AFL^- were used *in vitro* studies. Further tests are necessary in order to evaluate the ability of these strains to mitigate the AFB_1 production *in situ* system. Additionally, theses strains will be studied considering the presence/absence of biosynthetic genes for expression and production of aflatoxin and the emergent mycotoxin cyclopiazonic acid.

4.6 Conclusions

- 8 atoxigenic strains of *A. flavus* (AFL^-) and 8 other fungal strains from different genera were tested against 5 toxigenic *A. flavus* (AFL^+) and the interaction and total I_D scores for the fungal interaction calculated. This showed that toxigenic *A. flavus* strains were highly dominant *in vitro*
- No other fungal specie had the ability to reduce the growth of *A. flavus* on MEA
- A yeast strain (Y6) was able to compete against *A. flavus* in MEA but only at 0.98 a_w
- When Y6 was co-cultivated in MMA against AFL^+ it caused an increase in AFB_1 compared when compared to the control
- The interaction of AFL^+ x AFL^- was mutual intermingling on both MEA and MMA
- All the strains selected for the co-cultivation of different inoculum ratios AFL^+: AFL^- were able to reduce the AFB_1 of the 3 pathogenic strain, including the natives $AFLb^+$ and $AFLd^+$
- The overall control of AFB_1 *in vitro* ranged from 29 to 100 %
- When $AFL4^-$ was paired with the native strain $AFLb^+$ under abiotic stress (0.95 a_w at 30^{o}C) in a ratio of spores 75: 25 AFL^+: AFL- there was not control of toxin production after 7 days using non-GM maize, and after 7 and 14 days when GM maize was used as the nutritional matrix

CHAPTER 5

MONITORING DELETIONS IN THE AFLATOXIN GENE CLUSTER OF *ASPERGILLUS FLAVUS* STRAINS WITH POTENTIAL BIOCONTROL ACTION AND EFFECTS ON BIOSYNTHETIC TOXIN GENE EXPRESSION AND AFLATOXIN B_1 PRODUCTION

CHAPTER 5. MONITORING DELETIONS IN THE AFLATOXIN GENE CLUSTER OF *ASPERGILLUS FLAVUS* STRAINS WITH POTENTIAL BIOCONTROL ACTION AND EFFECTS ON BIOSYNTHETIC TOXIN GENE EXPRESSION AND AFLATOXIN B_1 PRODUCTION

5.1 Introduction

Biocontrol using competitive exclusion of toxigenic *A. flavus* strains by non-toxigenic ones have been demonstrated to significantly reduce the contamination of maize (Dorner, 2009), peanuts (Dorner et al., 2009) and cotton (Cotty, 1994) with AFs. This approach has been considered among the most promising technologies for sustainable agriculture (Tracy, 2014). The effectiveness of biocontrol using atoxigenic *A. flavus* strains is based on the fact that these are predominantly asexual, genetically stable and aggressive as competitors coupled with their inability to recombine with native toxigenic strains (Abbas et al., 2011a; Ehrlich and Cotty, 2004). Although the mechanism by which a non-aflatoxigenic strains interfere with aflatoxin biosynthesis in toxigenic strains has not been definitively elucidated (Huang et al., 2011).

Furthermore, the stability of biocontrol agents over the range of environmental factors necessary for effective control is also a source of concern (Medina et al., 2017a). In the case of *A. flavus*, these concerns are typically addressed by restricting the biocontrol agents to native atoxigenic isolates on economically important food crops in the target regions (Callicott and Cotty, 2015). Atoxigenic *A. flavus* strains, especially with large deletions in the aflatoxin gene cluster, have been proposed for use in aflatoxin control as they compete for the niches occupied by toxigenic strains (Dorner, 2004; Pitt et al., 2015).

A potential candidate strain for biocontrol needs to have one or more deletions in key genes in the biosynthetic aflatoxin gene cluster responsible for both AFs and cyclopiazonic acid (CPA) (Mamo et al., 2018). The presence of CPA has been considered during selection of some atoxigenic biocontrol agents even though it has not been associated with actual outbreaks of mycotoxicosis (Chang et al. 2009). In addition Moore (2014) pointed out that if the biocontrol by an atoxigenic strain is lost the cause might be recombination between native toxigenic strains and the AF- strains

used for biocontrol. Their potential ability to evolve new phenotypes and genotypes via sexual recombination needs to be addressed.

Exploring the pathway of production of AFs requires expensive and time-consuming genetic and molecular techniques. However, screening techniques such as polymerase chain reaction (PCR) have been reported as rapid ways to detect deletions in the AFs cluster helping in the selection of potential biocontrol agents. Chang, Horn and Dorner (2005) describe a method able to detect deletions in the aflatoxin cluster requiring 32 separate PCR amplifications, whereas Donner et al. (2010) condensed the number of amplifications to 23. More recently, Callicott and Cotty (2015) designed a PCR able to provide detailed information about the both AF and CPA gene cluster. This approach can be used to evaluate atoxigenic strains for deletion of either structural or regulatory genes involved in aflatoxin biosynthesis. This is an important aspect to ensure that the atoxigenic strains can consistently be effective based on the knowledge that key genes have been deleted in the chosen strains (Bandyopadhyay, et al., 2016).

5.2 Objectives

a. To select atoxigenic *A. flavus* strains with potential biocontrol action and examine the deletion of key structural and regulatory genes involved in aflatoxin biosynthesis using Multiplex PCR
b. To examine the best atoxigenic/toxigenic strain ratio in stored maize kernels (*in situ* study) of two conventional (non-GM) and their respective isogenic genetically modified (GM) cultivars under different a_w conditions on relative control of AFB_1, and on relative expression of a structural (*alf*D) and regulatory (*afl*R) gene in the aflatoxin biosynthetic pathway

5.3 Materials and methods

5.3.1. Monitoring deletions in the aflatoxin and cyclopiazonic acid biosynthesis gene cluster using multiplex PCR

Fungal strains: Strains of *A. flavus* isolated in CHAPTER 2 (see Table 2.3) were selected for studies using a multiplex approach. Only strains from different cultivars were selected. Both types of strains (toxigenic and atoxigenic) were used and are described in Table 5.1.The type strain *A. flavus* AF70 was include as a positive control for its presence of genes biosynthetic genes in the aflatoxin and cyclopiazonic acid pathway (Callicott and Cotty, 2015).

The atoxigenic strains were studied with the aim of selecting potential BCAs, whereas the toxigenic strains were studied to verify the presence of the genes for AFs and CPA. The strains of *A. flavus* were sub-cultured on Yeast Extract Sucrose agar (YES - 2% yeast extract, 15% sucrose, 0.1% $MgSO_4$.7 H_2O and 2% agar) containing an overlay of sterile cellophane on the surface of the media. The plates were incubated at 30^oC for 3 days then the mycelia were removed from the cellophane, transferred to a sterile tube, snap frozen in liquid Nitrogen (N_2) and kept at -80^oC until genomic DNA extraction.

Table 5.1. Strains selected for monitoring deletions in the aflatoxin and cyclopiazonic acid biosynthesis gene cluster using multiplex PCR.

Strain	Code	AFB_1 production
*AF70**	AFL1	+
AfFRc	AFL2	-
AfPHx	AFL3	-
Af53H	AFL4	-
AfAS1c	AFL5	-
AfM20C	AFL6	-
AfBMC-b	AFL7	-
AfCDPW-b	AFL8	-
AfM20-a	AFL9	+
AfLRG-a	AFL10	+
AfASC-a	AFL11	+
AfP30C-a	AFL12	+
AfM20-c	AFL13	+

+ indicates the strain is toxigenic; - indicates no AFB_1 production; * type strain

Solutions for DNA extraction: The composition of CTBA buffer was 1% CTAB (cetyl trimethylammonium bromide, Sigma, USA), EDTA 100 mM (Ethylenediaminetetraacetic acid, Sigma, USA), Trizma® base 20 mM pH=8.0 (Tris-hydroxymethyl-aminomethane, Sigma, USA), NaCl 1.4 M (sodium chloride, Fisher Scientific, USA) and ultrapure water. CTBA is a cationic detergent that facilitates the separation of polysaccharides during purification, EDTA is responsible for chelation of divalent ions and stops the action of DNases, Trizma® is used to maintain the right pH for DNA isolation, and NaCl helps to remove the proteins bounded to DNA.

In addition to the buffer solutions prepared *in loco*, the DNA was extracted with the aid of buffer solutions commercially prepared and ready-to-use from the Qiagen DNeasy® Plant Mini kit (Qiagen, USA) named AP3, washing solutions AW1 and AW2, and elution buffer EA. For the clean-up and purification of DNA spin columns from the same kit were used: QIAshredder® Mini spin column (lilac) and DNeasy® Mini spin column (white).

Genomic DNA extraction process: Approximately 30 mg of the fresh mycelia was transferred to a sterile skirted 2 mL tube containing glass beads (0.3 mm) followed by the addition of 500 µL CTAB 1% buffer and immersion in liquid N_2. The cell lyses was performed using a tissue homogenizer Precellys®24 (Bertin, France). The tubes were placed in the homogenizer and lysed at 6500 RPM for 2 x 60 seconds with 15 seconds interval in between. After this, the tubes were immersed in liquid N_2 for 10 seconds and allowed to thaw completely at room temperature. Once the samples were thawed 130 µL buffer AP3 was added, mixed and incubated on ice for 5 min. This step precipitates detergents, proteins and polysaccharides. The following step was a centrifugation for 5 min at 20,000 x g and the lysate was then transferred into the QIAshredder® mini spin column and centrifuged for 2 min at 20,000 x g. Even though the QIAshredder® removes most precipitates and cell debris a small amount can pass through and form a pellet for this reason an additional separation was performed using chloroform for more efficient protein denaturation.

The lysate was transferred to a new tube and 500 µL chloroform added, mixed by inverting and centrifuged for 20 min at 20,000 x g. The aqueous layer was removed and transferred to a new tube and 1.5 volumes of buffer AW1 added and mixed by pipetting. The mixture was transferred to a DNeasy® mini spin column and centrifuged

for 1 min at 6000 x g. Afterwards, the DNeasy® mini spin column was transferred to a new collection tube and washed by adding 500 µL buffer AW2 and centrifuged for 1 min at 6000 x g. The flow-through was discarded and another 500 µL AW2 added followed by 2 min centrifugation at 20,000 x g. The spin column was placed in a new tube for elution with 60 µL EA buffer and incubated for 5 min at room temperature. The final genomic DNA was collected from the spin column by centrifugation for 1 min at 6000 x g. The concentration and quality of DNA was accessed using a Genova Nano spectrophotometer (JenWay, UK). The DNA was stored at -20^{o}C until use as template for polymerase chain reaction (PCR) amplification

Primers for multiplex PCR: The strains of *A. flavus* isolated from GM and non-GM maize were accessed for the presence/absence of biosynthetic genes for the aflatoxin and cyclopiazonic acid (CPA) cluster using a multiplex PCR approach developed by Callicot and Cotty (2015). Four multiplex PCRs were designed by Callicot and Cotty (2015) to amplify 32 markers (see Table 5.2). These markers are spaced approximately every 5 kb along the final 157 kb of the sub telomere region containing the sugar, aflatoxin and CPA clusters, as identified in the genome of NRRL 3357 (Payne et al., 2006).

PCR amplification: The multiplex PCR was performed using the genomic DNA from the isolated strains. The average quality for the DNA samples for ratio $A_{260/280}$ was ≥1.7 while for the ratio $A_{260}/_{230}$ was ≥2.5. A negative control (-C) reaction without the addition of DNA was included to confirm the absence of contamination, and a positive control (+C) sample was used to confirm the primes were working correctly. The +C was the type strain *A. flavus* AF70 that was used in the original work by Callicot and Cotty (2015) used as base for this experiment. Table 5.3 shows the amounts of reagent for 10 µL reaction mixture per individual PCR tube. All reagents stored at -20°C were brought to room temperature and vortexed gently after thawing. After the addition of all reagents, PCR tubes were vortexed gently, and placed in a Thermal cycler (Techne, UK). The PCR reaction conditions for M1 and M2 were set to 1 min at 94^{o}C, 35 cycles of 30s at 94^{o}C, 90s at 62^{o}C and 90s at 72^{o}C, followed by 10 min at 72^{o}C and held at 4^{o}C. The PCR M3 and M4 were set to 1 min at 94^{o}C, 35 cycles of 30s at 94^{o}C, 90s at 65^{o}C and 90s at 72^{o}C, followed by 10 min at 72^{o}C.

Table 5.2 Primers sequence used in multiplex screens of the aflatoxin gene cluster and neighbouring regions adapted from Callicot and Cotty (2015).

Panel[1]	Marker[2]	Forward	Reverse	Position[3]	Product size (bp)
M1	SC01	5'-ATACCTCATGATCTGGTGCACGG	5'-CTTCGCAGCGACAATGATACGTC	2181053–2181935	883
	IC01	50-GTCCCCAGGTACGATAGGTCTCT	50-GCTGGATATTCCAAGGAGTGGCT	2185681–2186422	742
	AC01	5'-GACTGCCACCCTATCACTCTTCC	5'-TGGCTCGACTGGGTATGAAATCC	2190536–2191148	613
	AC02	5'-GCATTGCCAGCATCGGTTTCATA	5'-AGGCAGACCGTACTAAGTGATGC	2195548–2196034	487
	AC03	5'-CATGATGGAGCATGACATTCGGC	5'-GCGCCACCATATCTTCTCAGTCT	2200655–2201041	387
	AC04	5'-TTTAACCCTTCAYGCCTCGAACT	5'-TGCGTARCTAATCTCATCGGGTT	2205695–2205991	297
	AC05	5'-TGCTGAGCGAGTAGGTAGTAGGT	5'-CCGGATCATCCCTCCAAATCTGT	2210926–2211119	194
	Iac	5'-GCTAGGGCGGGTCACGTTTTGCG	5'-GGCGTTGTTTAAGGGGAACCGACCC	N/A	115
M2	AC06	5'-CCTGTGAGGGACACAAAGACACT	5'-AAGAATAGCGGTGACATCCAGCA	2214679–2216105	1427
	AC07	5'-GAGGACAGGTTGTGTTGCTGTTG	5'-GTTCACGAGCTATCCTCAGCCAT	2219877–2220968	1092
	AC08	5'-GAACTGAGCCATTTCCATCAGCG	5'-GTCTTGTACAGGGAACGTGGTGA	2225164–2226060	897
	AC09	5'-AACGCTTCAACGTGGAGGACATA	5'-AATAGCGTTGGCGTTGAAGTCAC	2230697–2231432	736
	AC10	5'-CCCGCATTTTTCTCGATCCCTTG	5'-GCGACGACCAGTCATTATGAAGC	2235352–2235984	633
	AC11	5'-GTCAGACCACAGTGAGTGCTTCT	5'-AAGCTGACTGGGAGAATGTTGCT	2240596–2241131	536
	AC12	5'-CCCCTCAACTTCTGTCGTCCTAC	5'-GCTGGGTAGCGAACAATCCAATG	2245749–2246173	425
	AC13	5'-GCACACAGCAGAGGCATTTCTAC	5'-AATCTATCTAGCCATCGCCACCG	2250660–2250989	330
	IC02	5'-GCCTGCTAGGCTTGGAACTATGT	5'-CGCAATGCTAGTATGCCCTTGTC	2255869–2256077	209
	Iac	5'-GCTAGGGCGGGTCACGTTTTGCG	5'-GGCGTTGTTTAAGGGGAACCGACCC	N/A	115
M3	CC01	5'-GACACTCGTACCATCTATGCACC	5'-GATCCCTGATCCATTCCACCTTG	2260358–2261576	1219
	CC02	5'-ACGATACGAGCTTTAGTGCAAGG	5'-GATATAGACCTCAGGGTCGAGCA	2265671–2266595	925
	CC03	5'-AGAGCTGCGCACTCCATTT	5'-TGCCCAGGCAATAGGAAGTA	2269454–2270274	821
	CC04	5'-ACCTCAACAATTACACCGGATGG	5'-GTTGTAGCTCAACGTCACTAGCA	2275573–2276220	648
	ST01	5'-TATCTATCTGGGATACGGGCTGG	5'-TATGCCGTTGCTATCCAATGAGG	2280628–2281168	521
	ST02	5'-AAGTCAGATTCCGCGGTATGAAG	5'-TCATCGCATTAATCGAGGCAGTT	2285773–2286188	416
	ST03	5'-CCTCCTGCACAAAAATACTCCCA	5'-GATCAGATCTTTGAGCGTAGCGT	2290684–2291003	320
	ST04	5'-TCATGTTTCGGATCGGAGATTGG	5'-ACATTCCAAGTGAGAGATGTGGC	2295826–2296059	234
	Iac	5'-GCTAGGGCGGGTCACGTTTTGCG	5'-GGCGTTGTTTAAGGGGAACCGACCC	N/A	115
M4	ST05	5'-ACTGGTGTTGGATAGAGCTCAGA	5'-TGGAAGGTTCTCCGGATACTTGA	2300459–2301366	908
	ST06	5'-TACTCCGTTGCTGTCATTGGATG	5'-CGAATTCTTGGTTGAGCAGCTTG	2305524–2306305	782
	ST07	5'-TGCTGAATAACAACCTCGACCAG	5'-CAGGCTGGTATAGCACCAATGTT	2310806–2311489	684
	ST08	5'-GGTTTCGTCTTGCCTTCTTCTCA	5'-AGCAAAGTGATGCCGTTCAAATG	2315875–2316458	584
	ST09	5'-CGTACTTTGTTACGGCGTACATC	5'-GCTGTTTCGCGTTAGTTGGTAAC	2320567–2321078	512
	ST10	5'-GCCCGTAAATGAGGTGCAGATAA	5'-TTTGGGTGTGCTTCTTCATGCTA	2325663–2326066	404
	ST11	5'-GGGGACTTAGTCGCGAATGGTTA	5'-TATGAAGGCCACCAACTGAGGAC	2330725–2331009	285
	ST12	5'-AATGACGACACTTGAGGCACAG	5'-TCGGCTCCGTGACACCATATTA	2336181–2336365	185
	Iac	5'-GCTAGGGCGGGTCACGTTTTGCG	5'-GGCGTTGTTTAAGGGGAACCGACCC	N/A	115

[1] Panel contain primers used in a single multiplex PCR and are designed to be resolved in a single electrophoresis lane. [2]Marker name indicates the region in which it is found: SC - sugar cluster; IC- inter-cluster; AC- aflatoxin cluster; CC- cyclopiazonic acid cluster; ST- subtelomere; iac - internal amplification control; [3]Position Indicates the position in contig EQ963478.1 of the JCVI AFL1 v2.0 assembly of the Aspergillus flavus NRRL 3357 genome.

Table 5.3 Reagents (10 µL) used in reaction mixtures for each multiplex PCR.

Reagent	Amount per sample (µL)	Concentration
1 x PCR SuperMix *	5	1 x
Mix Primers Forward **	0.5	0.08 µmol. L^{-1}
Mix Primers Reverse **	0.5	0.08 µmol. L^{-1}
Sterile ultrapure water	3	-
Genomic DNA	1	8 ng. μL^{-1}

** AccuStart II PCR SuperMix (Quanta Biosciences, USA) containing dMTPs, Taq Polymerase and $MgCl_2$; ** Mix primers is the mixture of all the primers for each multiplex panel, the primers were produced by Sigma (USA).*

Gel electrophoresis: To access the PCR amplification, the amplicons were run on agarose gel electrophoresis. The gel was prepared with 1.5% agarose (Sigma, USA) in 1 x TAE buffer (40mM Tris, 20mM Acetate and 1mM EDTA pH=8.6). After complete dissolution of the agarose using heat, the solution was cooled down to 60-70^{o}C and added SafeView® (NBS Biologicals Ltd, UK) for nucleic acid staining as a safer alternative to ethidium bromide in a proportion of 5 µL per 100 mL of gel, gently mixed and casted. The gel was submerged in 1x TAE buffer and the 8 µL of PCR products were load with 2µL 6x DNA dye (Thermo Fisher Scientific, USA). In the first lane of each gel was loaded with 1kb DNA Ladder (Thermo Fisher Scientific, USA) as molecular size marker (250 to 10000 bp). Electrophoresis was run at 85 V for 90 min and the gel viewed under UV with the aid of G:Box imager and Genesnap version 7.09.02 image acquisition software (Syngene, Cambridge, UK). The deletion of genes by the strains of *A. flavus* was considered by the absence of bands for the markers.

5.3.2. Control of aflatoxin B_1 in maize kernels (*in situ*) using 50:50 ratios of pathogen:antagonist in different simulate storage conditions

Fungal strains: The atoxigenic strain of *A. flavus* (*Af*53H – AFL4^{-}) isolated from Brazilian maize was selected as a potential BCA based on the ability to reduce aflatoxin production in *in vitro* studies described in CHAPTER 4. Both the atoxigenic strain and the toxigenic strain used were native Brazilian strains from

Brazilian maize (*Af*LRG-a – AFLb^{+}). In addition, the toxigenic type strain NRRL3375 (AFLe^{+}) provided by Prof. D. Bhatnagar of the Southern Regional Research Centre, New Orleans, LA, USA was also used.

Spore suspension: The strains of *A. flavus* were sub-cultured on MEA (Malt Extract Agar, Oxoid, UK) and incubated for 7 days at 30^{o}C. Afterwards, the spores were removed from the Petri plate culture, transferred to a Tween®80 0.1% (v/v) solution and counted using a counting chamber (Neubauer improved, Paul Marienfield, GE). The final spore concentration obtained was 10^3 spores mL^{-1}. According to the experiments *in vitro* (Chapter 4) the ratio 50:50 (pathogen: antagonist) represent the most viable option for biocontrol. The spore suspensions were mixed in a 50:50 ratio prior to inoculation on the maize kernels. The controls consisted of 100:0 and 0:100 ratio antagonist: pathogen.

Maize: Two cultivars of non-GM maize and its isogenic GM lines were selected for the biocontrol *in situ* experiment (see Table 5.4). The *in situ* study (stored kernels) was chosen as an initial approach before applying the BCA direct to plants (*in vivo*). The kernels were gama-irratiated (12-15 kGy) in order to eliminate all the natural contamination but still keep the maize germinability. The a_w of the maize kernels was modified to 0.98 and 0.95 a_w by adding a solution of sterile water + *A. flavus* spores based on the moisture absorption curve for each cultivar (see Appendix E). The number of spores added to each cultivar was calculated as 10 spores per gram of maize from a solution of 10^3 spores. mL^{-1}. After the addition of the water, the maize kernels were kept at 4^{o}C for 24h for full absorption and equilibration. The water availability was checked by measuring the a_w using AquaLab® 4TE (Decagon, USA).

Incubation: 8 g of the maize grain were aseptically distributed into glass culture vessels (Figure 5.1, Magenta™, Sigma, USA) with vented lids (10 mm with a polypropylene membrane 0.22 µm pore size) to allow gases exchange but keeping the environment inside the vessel sterile. The jars were placed in sealed plastic boxes with a glycerol/water solution of the same a_w as the kernels to keep the equilibrium relative humidity the same as the target treatments. The

glycerol/water solution was renewed every 3 days. The boxes were incubated at 30ºC for 20 days. Samples were collected after 10 and 20 days for analysis of the aflatoxin quantification while for gene expression only samples on day 10 were used. The experiment was performed in three replicas per treatment. The samples for aflatoxin quantification were oven dried at 65ºC for 48h to remove the water and stop fungal growth. Afterwards, the samples were ground using a laboratory blender with stainless steel blade (Waring, Stamford, USA). The samples for gene expression (3 kernels per jar per treatment) were snap frozen in liquid N_2 and stored at -80ºC until RNA extraction.

Table 5.4. Maize cultivars (non-GM and GM) selected for biocontrol *in situ* as substrate for *A. flavus* development

Conventional cultivar (non-GM)	Isogenic line (GM)	Traits tolerance in the GM cvs
M20-A78 CON	M20-A78 PW®	HT- Glyphosate HT- Glufosinate ammonium IR- Lepidopteran
P30F53 CON	P30F53 H®	HT- Glufosinate ammonium Antibiotic resistance IR- Lepidopteran

IR- insect resistance; HT – herbicide tolerance.

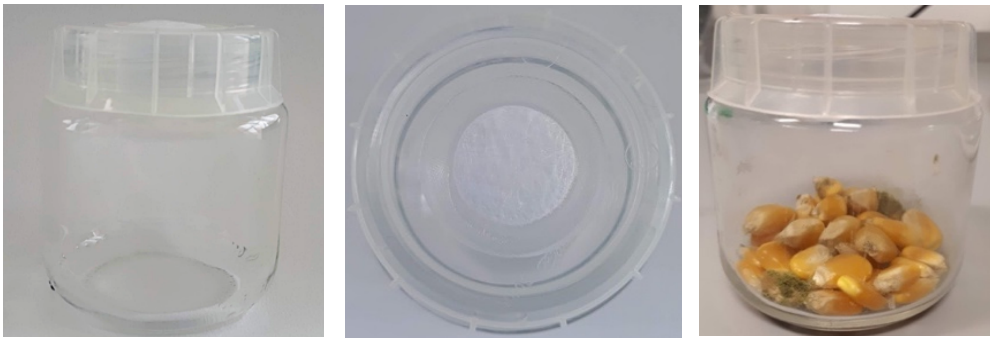

Figure 5.1. Glass culture vessels used in the experiments of biocontrol in situ with a 10 mm Ø vent in the lid and polypropylene membrane 0.22 µm pore size.

5.3.3. Aflatoxin quantification

AFs extraction: For the extraction of AFs 2 g of milled sample were transferred to a glass vial and 8 ml of extraction solution added (Methanol: water 80:20 v/v). The samples were agitated in a magnetic stirrer at high speed for 1h at room temperature and centrifuged afterwards to allow phase separation. The liquid phase was transferred to a new 15 mL polypropylene tube and 100 µL of the extract mixed with 900 µL of mobile phase (methanol: acetonitrile: water 30:15:60 v/v/v) in a 2 mL tube, vortex and filtered using nylon filters (13 mm× 0.22 µm) directly into an amber silanized vial. The samples were injected in the HPLC and those below the limit of detection (<1.0 ng. g^{-1}) were cleaned-up and concentrated using immunoaffinity columns (IAC) and then reinjected.

IAC represent an efficient way to clean samples prior to analysis but one of its limitations is the maximum capacity for binding the antibodies with aflatoxin (500 ng of total aflatoxin). Samples with a high concentration of aflatoxins needed to be diluted by several factors before passing into the IAC, for this reason, in this experiment a high amount of fungi biomass was inoculated resulting in elevated levels of aflatoxin dilutions of >50 times were required. Thus, the samples were injected directly into the HPLC only with a dilution in mobile phase. No interference peaks from the maize were detected in the chromatograms at the same retention time as AFB_1, indicating that high concentrations of aflatoxins can be injected in the HPLC without clean-up (see Validation of extraction of AF from maize in Appendix B-3).

Immunoaffinity column clean-up: The extract of the samples below the limit of detection in the first injection were diluted 2:20 in 1xPBS (phosphate buffered saline, Fisher Scientific, USA). The pH of the extracted was checked to ensure that this was not lower than 7.0 to ensure good performance of the IACs (AflaStar™ R, RomerLabs, Austria). The IACs were brought to room temperature prior to use and were attached to a SPE vacuum manifold (Phenomenex, California, USA). On top of each IAC a 25 mL reservoir was used to hold the sample extract. Following the manufacturers' instructions, the buffer in the IAC

was removed and the sample extract passed through the column at a speed of 1 to 3 mL.min^{-1}. Followed by 20 mL of 1xPBS for the clean-up (10 mL was added in the reservoir and 10 mL added directly into the IAC). The last step was the elution with 1.5 mL methanol. For best recovery the elution was performed by adding 3 x 500 µL of methanol. The eluted samples were evaporated to dryness using a vacuum evaporator (miVac Quattro Concentrator - Genevac, Leicestershire, UK) at 45^{o}C for 3h. The dried extract was resuspended in 500 µL mobile phase, transferred to an amber silanized vial and inject into the HPLC for AFs quantification.

HPLC analysis: The quantification of AFs in the biocontrol *in situ* study was done by reversed-phase HPLC. The HPLC system used was an Agilent 1200 series (Agilent, Santa Clara, USA) with a fluorescence detector (λ_{exc} 360 nm; λ_{em} 440 nm) and post column derivatization with a UVE photochemical reactor with UV-Light (LCTech GmbH, Germany). A C_{18} column (Agilent Zorbax® Eclipse Plus, 2.1 x 100 mm, 3.5 µm particle size) preceded by a Phenomenex® Gemini C18 guard column cartridge 3 mm x 3 µm (Phenomenex, California, USA) was used for separation. Followed by isocratic elution with methanol: water: acetonitrile (30:60:15, v/v/v) and a mobile phase flow rate of 1.0 mL min^{-1}. The injection volume was 5-50 µL according to each set of samples. A set of standards was injected (0.05–4 ng of AFs per injection) and standard curves were generated by plotting the peak areas against the amounts of aflatoxins. Only AFB_1 (R^2 = 0.999) was detected. The different sets of samples (highly contaminated - directly injected or lower contaminated - cleaned-up using IACs) were calculated based on the respective dilution factors. The recovery of the extraction method for AFB_1 in maize was > 80% (see validation method Appendix C section 0).

5.3.4. Gene expression studies

The gene expression studies were performed using samples from the *in situ* experiment after 10 days incubation only. This time frame was chosen based on previous studies with both *A. flavus* and *A. parasiticus* that suggested gene expression of several of the toxin biosynthetic genes had optimal peaks after 8-10 days of growth (Schmidt-Heydt, Magan and Geisen, 2008). The gene expression of the chosen genes was only performed for the interaction between toxigenic/atoxigenic strains AFLb^{+}: AFL4^{-}. The type strain AFLe^{+} (NRRL3357) treatments were excluded because the AFB_1 production was lower when compared to the native Brazilian toxigenic strain.

Sample grinding: The treatments from the *in situ* experiment were stored at -80^{o}C and transferred to reinforced 7 mL tubes designed for use in the Precellys 24® (Bertin, FR) homogenizer with 3 glass beads (6.5 mm). The tubes were kept in liquid N_2 until use. The breakage of kernels into a fine powder was performed by using the 6500 RPM cycle for 30 seconds (2 x 15 s) then the samples were immersed in liquid N_2 for 5 min and the cycle repeated. Approximately 50 mg of the powder was transferred to a 2 mL Eppendorf RNase/DNAse free to proceed with the extraction of the total RNA.

RNA extraction: Total RNA isolation was carried out using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich Co, USA) according to the manufacturer instructions. The observations for samples with high amounts of starch was taken into account. For this reason, samples were incubated at room temperature and 1 mL of lysis buffer added to the 50 mg of powder. RNA samples were treated with RNase-Free DNase set (Qiagen, Hilden, Germany). The purity and concentrations of RNA were examined by measuring the absorbance of 2 µL of sample Genova-Nano spectrophotometer (JenWay, Staffordshire, UK). Samples with good purity were consider when the ratio A_{260}/A_{280} was ≥2.0. The RNA integrity was verified using Experion™ RNA StdSens in an Experion™ automated electrophoresis system (Bio-Rad, California, USA). The quality of the samples

was considered as appropriated when RNA quality indicator (RQI) given by Experion™ was ≥7.0. The samples were kept at -80^{o}C until use.

Primers and probes: Nucleotide sequences of primers and probes used in this study follows the method described by Medina et al. (2015), and are shown in Table 5.5. The *nor*-Probe and *afl*R-Probe were labelled at the 5' end with the reporter molecule 6-carboxyfluorescein (FAM) and at the 3' end with the quencher Black Hole Quencher 2 (BHQ2). However, ben-Probe was labelled at the 5' end with the reporter cyanine-5 (CY5) and at the 3' end with the quencher BHQ2.

Relative gene expression using qPCR reactions: Real-time quantitative PCR (RT-qPCR) assays were used to amplify the structural *alfD* and the regulatory gene *afl*R of the aflatoxin biosynthetic pathway as target genes. The β-tubulin gene as used as the control gene. The *afl*D qPCR was previously optimised by Abdel-Hadi et al., 2010), whereas *afl*R was optimised by Medina et al. (2015) following the same method as that for the *afl*D gene.

Two RT-qPCR assays were carried out, one of them optimised to amplify the target *afl*D and the housekeeping β-tubulin genes, and the other one to quantify the *afl*R gene expression using as control the β-tubulin gene according to method described by Medina et al. (2015). The qPCR reactions were prepared in triplicate for each biological replicate (n=9). The TaqMan system with different primers and probes were used in all cases. Both reaction mixtures consisted of 6.25 µL Premix Ex Taq™ (Takara Bio Inc., Otsu, Japan), 830 nM of each primer, 330 nM of each probe, and 1.5 µl of cDNA template in a final volume of 12.5 µl. The optimal thermal cycling conditions included an initial step of 10 min at 95 °C and all 50 cycles at 95 °C for 15 s, 55 °C for 20 s and 72 °C for 30 s. The assays were carried out using a CFX96 Touch™ Real-Time PCR detection system (Bio-Rad, California, USA).

Table 5.5. Nucleotide sequences of primers for RT-qPCR assays designed on the basis of the *afl*D, *afl*R and β-tubulin genes.

Primer pairs	Gene	Nucleotide sequences (5'-3")	Position
*nor*Taq – 1		GTCCAAGCAACAGGCCAAGT	516[a]
*nor*Taq – 2	*afl*D	TCGTGCATGTTGGTGATGGT	562[a]
nor-Probe		[FAM]TGTCTTGATCGCGCCCG[BHQ2]	537[a]
*afl*RTaq – 1		TCGTCCTTATCGTTCTCAAGG	1,646[b]
*afl*RTaq – 2	*afl*R	ACTGTTGCTACAGCTGCCACT	1,735[b]
*afl*R-Probe		[FAM]AGCAGGCACCCAGTGTACCTCAAC[BHQ2]	1,689[b]
benTaq – 1		CTTGTTGACCAGGTTGTCGAT	65[c]
benTaq – 2	β - tubulin	GTCGCAGCCCTCAGCCT	99[c]
ben-Probe		[CY5]CGATGTTGTCCGTCGCGAGGCT[BHQ2]	82[c]

[a] Positions are in accordance with the published sequence of the *afl*D gene of *Aspergillus flavus* (GeneBank accession no. XM_002379908.1). [b] Positions are in accordance with the published sequences of *afl*R gene of *Aspergillus flavus* (GeneBank accession no. AF441435.2). [c] Positions are in accordance with the published sequences of β-tubulin (benA56) gene of *Aspergillus flavus* (GeneBank accession no. AF036803.1).

Relative quantification of the expression: Relative quantification of *afl*D and *afl*R genes was performed using the housekeeping gene β-tubulin (ben) as an endogenous control to normalise the quantification of the target in the relative quantification assays and used for all treatments. Quantification cycle (Cq) determinations were automatically performed by the instrument using default parameters, and the expression ratio was calculated using the $2^{-\Delta\Delta Ct}$ method proposed by Livak and Schmittgen (2001). The control of each condition corresponded to $AFLb^{+}$ without the presence of BCA.

5.3.5. Statistical analysis

The data from *in situ* experiments with an inoculum ratio 50:50 of pathogen: antagonist and its effects on AFB_1 production and relative gene expression were subjected to Shapiro-Wilk tests to determine normality and Levene's test to assess the equality of variance. However, the data violated the two assumptions

for ANOVA even after transformations and therefore non-parametric tests (Kruskal-Wallis/ Wilcoxon; p=0.05) were used for analyses (Chan and Walmsley, 1997). Where there was significance after the Kruskal-Wallis test, median comparisons for each pair were made using the Wilcoxon - Each Pair test (p=0.05). The correlation of relative gene expression × AFB_1 production was checked using non-parametric Spearman's (ρ) rank correlation coefficient for each water activity (a_w). The statistical package JMP®14 (SAS Institute Inc., 2018, Cary NC, USA) was used to perform the analyses.

5.4 Results

5.4.1. Monitoring deletions in the aflatoxin and cyclopiazonic acid biosynthesis gene cluster using multiplex PCR

Twelve strains of *A. flavus* isolated from the Brazilian maize cultivars were studied using a multiplex PCR approach developed by Callicot and Cotty (2015). Additionally, the type strain AF70 was used as a positive control in order to ensure the presence of amplicons. The multiplex was divided into 4 panels covering the subtelomere to the sugar cluster region. However, in this study, no amplification was achieved in the panel M4 corresponding to the end for the subtelomere (ST) for the markers ST05 to ST12. The results presented correspond to panels M1, M2 and M3. The amplification products are show in Figure 5.2 and a description of the markers and strains are shown in Table 5.6.

Eight out of 12 strains were demonstrated to be unable to produce AFB_1 when analysed by HPLC-FLD (see CHAPTER 2). According to lanes 2 to 9 in the electrophoresis gels (Figure 5.2), the atoxigenic strains showed intraspecific variance for presence or absence of the 32 markers used. The strains *Af*FRC and *Af*M20-a (lanes 2 and 9, respectively – Table 5.6.) had amplification for all the markers in the aflatoxin cluster, despite the strains being found to be non-producers.

In contrast, the strains *Af*AS1C and *Af*BMC-b (lanes 5 and 7 - Table 5.6.) had large deletion of genes in the aflatoxin cluster with 11 genes absent. The largest deletion for these strains corresponded to the region *afl*E (*nor*A) through *afl*U (*cyp*A) (Figure 5.2) in the aflatoxin cluster. Additionally, these strains did not have any of the amplification markers for cyclopiazonic acid (CPA). The strain *Af*53H (lane 4 – Table 5.6), used in the *in situ* biocontrol studies showed a deletion of 6 amplicons in the aflatoxin cluster in the region *afl*A (*fas*-2) through *afl*U. However, the strain also showed amplification for the genes in the CPA cluster.

Amongst the toxigenic ones, strain *Af*ASC-a (lane 11 – Table 5.6) showed deletions in the aflatoxin clusters. Whereas strains *Af*LRG-a and *Af*M20-c (lanes 10 and 13, respectively – Table 5.6) had amplification of all CPA markers in addition to all the aflatoxin markers.

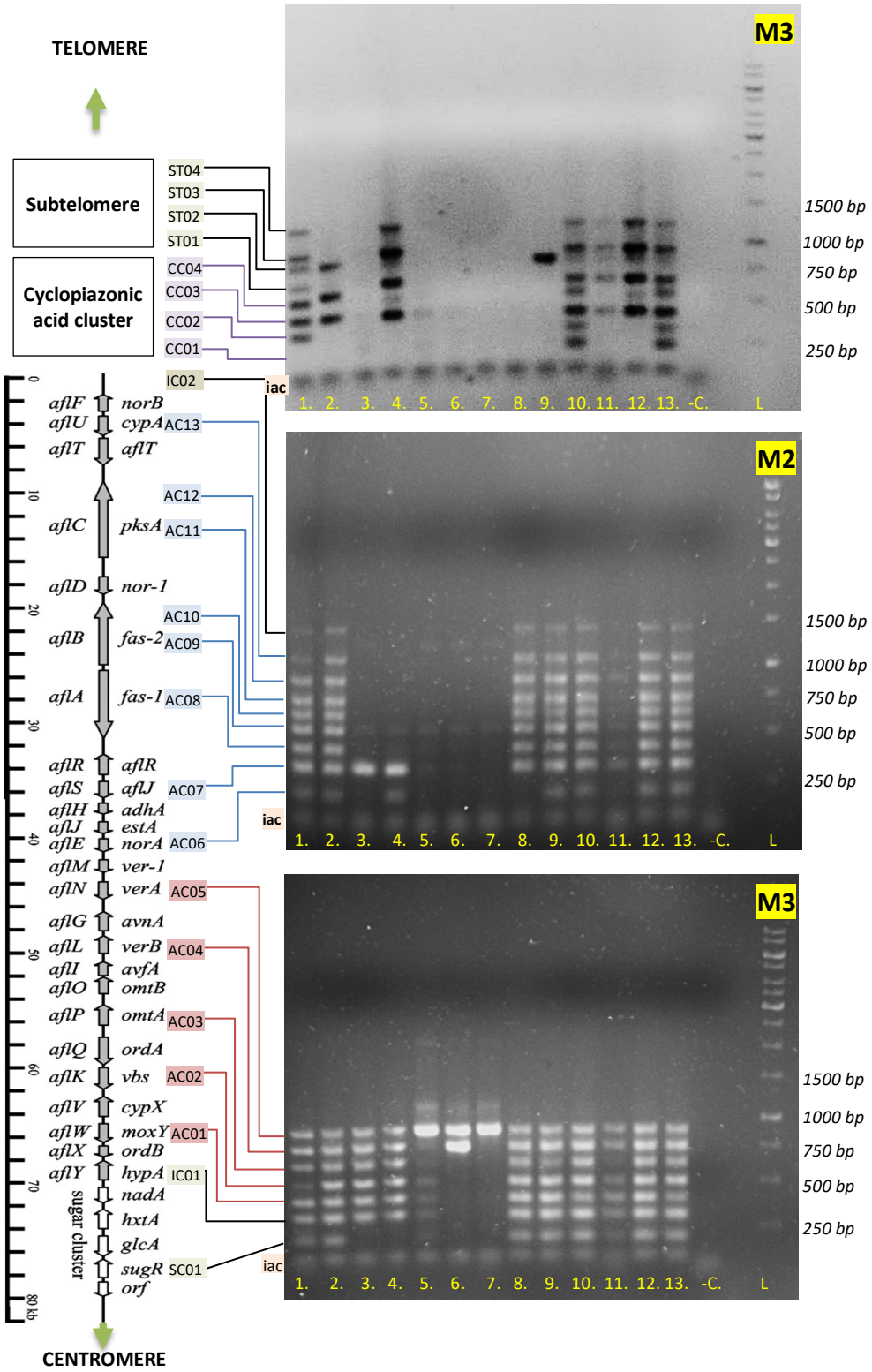

Figure 5.2. Imagens of multiplex amplifications for the panels M3, M2 and M1 aligned to a cartoon of the chromosome containing the aflatoxin, cyclopiazonic acid (CPA) and sugar cluster.

The pathway of genes for aflatoxin production are represented on the right side associated with the primers amplified starting in SC01 (sugR – AFLA_139110) from the bottom to the top. The 13 leftmost lanes contain 8 µL of multiplex amplification products, 14th lane is the negative control (-C) with no DNA template. The list with the corresponding strains from each lane is shown in **Table 5.5**. The rightmost lane in each image contains GeneRuler 1kB DNA ladder (Thermo Scientific), with visible bands from 250 to 10 000 bp. The smallest band in each panel is the internal amplification control (iac). Direction of migration during electrophoresis is from the top to bottom.

Table 5.6 Description of the electrophoresis gels from the multiplex amplifications for the panels M3, M2 and M1.

		A. flavus strains												
			TP	AFL⁻								AFL⁺		
		LANE	1	2	3	4	5	6	7	8	9	10	11	12	13
Panel	Marker	genes	AF70	*Af*FRC	*Af*PHx	*Af*53H*	*Af*AS1C	*Af*M20C	*Af*BMC-b	*Af*CDPW-b	*Af*M20-a	*Af*LRG-a	*Af*ASC-a	*Af*P30C-a	*Af*M20-c
M3	ST04	-	+	+	-	-	-	-	-	-	-	+	-	-	+
	ST03	-	+	+	-	-	-	-	-	-	-	+	+	+	+
	ST02	-	+	+	-	+	-	-	-	-	+	+	+	+	-
	ST01	-	+	+	-	+	-	-	-	-	-	+	+	+	+
	CC04	-	+	-	-	+	-	-	-	-	-	+	-	-	+
	CC03	-	+	+	-	+	-	-	-	-	-	+	+	+	+
	CC02	-	+	-	-	+	-	-	-	-	-	+	-	-	+
	CC01	-	-	-	-	+	-	-	-	-	-	+	-	-	+
M2	IC02	-	+	+	-	-	-	-	-	+	+	+	-	+	+
	AC13	*aflU*	+	+	-	-	-	-	-	+	+	+	-	+	+
	AC12	*aflC*	+	+	-	-	-	-	-	+	+	+	-	+	+
	AC11	*aflC*	+	+	-	-	-	-	-	+	+	+	-	+	+
	AC10	*aflA*	+	+	-	-	-	-	-	+	+	+	-	+	+
	AC09	*aflA*	+	+	-	-	-	-	-	+	+	+	-	+	+
	AC08	*aflB*	+	+	-	-	-	-	-	+	+	+	-	+	+
	AC07	*aflS*	+	+	+	+	-	-	-	+	+	+	+	+	+
	AC06	*aflE*	+	+	-	+	-	-	-	-	+	+	-	+	+
M1	AC05	*aflN*	+	+	+	+	+	+	+	+	+	+	+	+	+
	AC04	*aflL*	+	+	+	+	-	+	-	+	+	+	+	+	+
	AC03	*aflP*	+	+	+	+	-	-	-	+	+	+	-	+	+
	AC02	*aflK*	+	+	+	+	-	-	-	+	+	+	+	+	+
	AC01	*aflW*	+	+	+	+	-	-	-	+	+	+	+	+	+
	IC01	*aflX*	+	+	+	+	-	-	-	+	+	+	+	+	+
	SC01	*glcA*	+	+	-	-	-	-	-	+	+	+	+	+	+

TP: type strain *A. flavus* AF70; AFL⁻ : atoxigenic strains of *A. flavus* isolated; AFL⁺ : toxigenic strains of *A. flavus* isolated.(+) indicates the presence of bands and (-) indicates the absence of it. Markers represent the primers amplified. ST: subtelomere cluster; IC: inter cluster; SC: sugar cluster; AC: aflatoxin cluster; CC: cyclopiazonic acid cluster.

5.4.2. Control of aflatoxin B_1 in stored maize using 50:50 ratios of pathogen:antagonist in different simulate storage conditions

The effect of atoxigenic strains on AFB_1 production in stored maize grain is shown in Figure 5.3. The use of 50:50 mixture of the two toxigenic *A. flavus* with the atoxigenic $AFL4^-$ (*Af*53H) demonstrated the ability to reduce toxin content significantly in all the maize cultivars used as substrate for fungal development at both a_w (0.98 and 0.95) levels after both 10- and 20-days storage. The relative control of AFB_1 ranged from 54 to 100%. However, the toxin production pattern was affected by the type of cultivar used.

Interestingly the AFB_1 production by the toxigenic strains was different in the stored maize grain study than found previously in the *in vitro* assays (see Chapter 4), the type strain NRRL3357 ($AFLe^+$) was able to produce more toxin, however *in situ* conditions the production was affected. The native toxigenic strain $AFLb^+$ on the other side was able to produce almost 10-fold more toxin after 10 and 2-fold more after 20 days. However, the relative control was not affected, despite the higher production by the target toxigenic strain.

The overall control of the toxigenic type strain NRRL3357 ($AFLe^+$) was affected when using maize grain of the cultivar M20-A78 CON. The relative control was lower when compared with the other cultivars in all the conditions tested. The same was observed for the strain $AFLb^+$, but only at 0.95 a_w after 10 days. While control using M20-A78 CON was 58%, and with the other types of maize cultivars there was total control of toxin production. The different a_w levels did not appear to individually have an effect on biocontrol when compared to the controls.

The most effective control of AFB_1 production occurred when the cultivar P30F53 CON was used. Even though the native antagonist $AFLb^+$ produced more toxin with this cultivar, the relative control was 100% at both a_w treatments after 10 and 20 days. In contrast, the overall control of toxin by $AFL4^-$ as a biocontrol strain appeared to be affected by the use of conventional maize (Figure 5.4). The ability to reduce toxin content was significantly lower when *A. flavus* was inoculated in the non-GM maize. However, the biocontrol effect of AFL4- against the 2 toxigenic strains was similar ($p=0.05$) despite the production pattern by the antagonist strains were different.

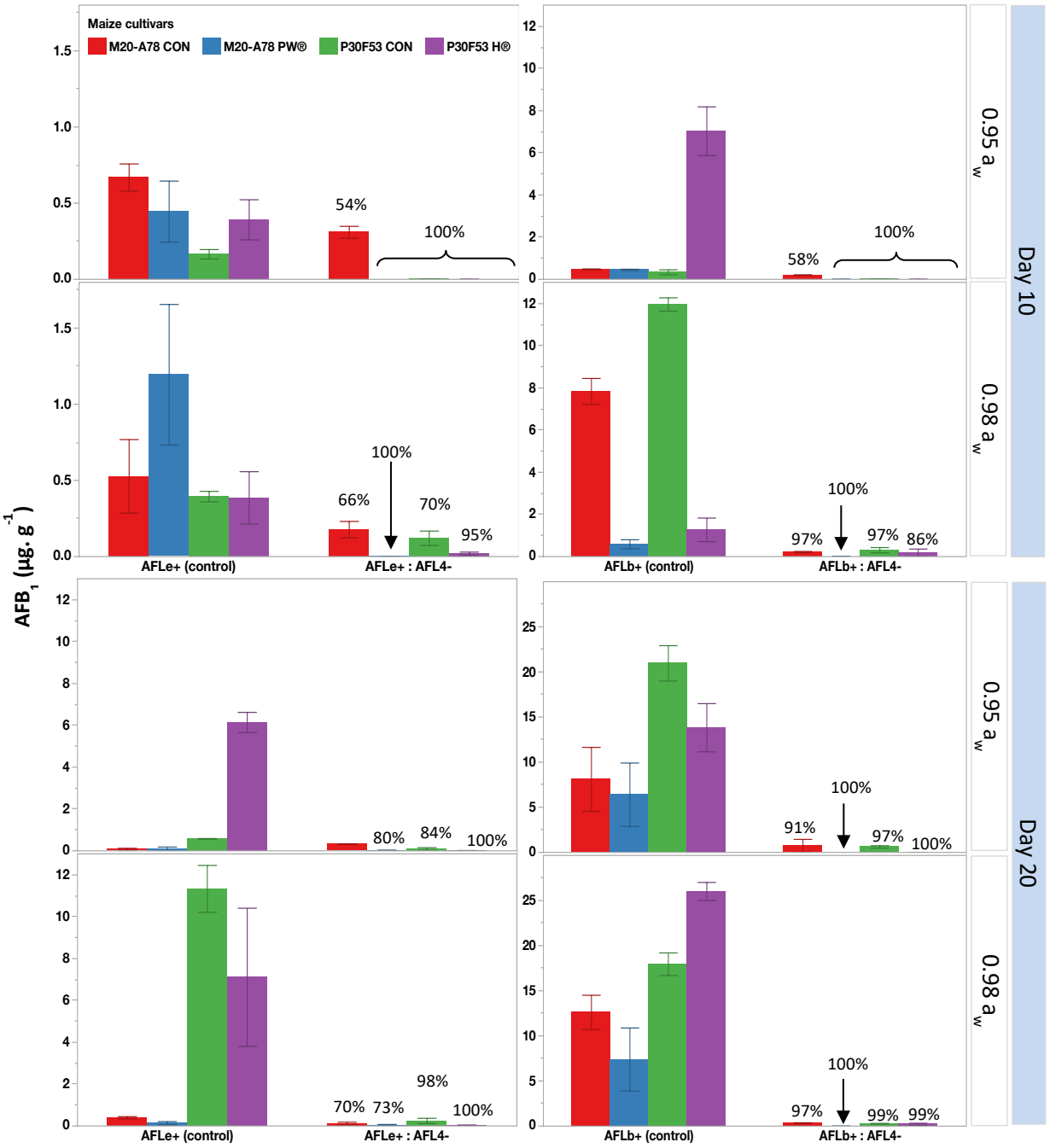

Figure 5.3. Effect of antagonist atoxigenic strains in controlling toxigenic strains in stored maize grain on AFB_1 production using maize from conventional cultivars (M20-A78 CON and P30F53 CON) and their respective isogenic GM lines (M20-A78 PW® and P30F53 H®) at 0.98 and 0.95 a_w analysed after 10- and 20-days storage at 30°C. The Pathogen (AFL^+) and antagonist (AFL^-) were mixed in a 50:50 spore ratio. Antagonist: atoxigenic *A. flavus* strain AfP53H ($AFL4^-$); Pathogen: native *A. flavus* strain $AFLb^+$ and type strain NRRL3357 ($AFLe^+$). Control corresponded to 100:0 inoculum ratio AFL^+: AFL^-. Bars represent AV±SE (n=3). The values above the bars represent the relative % control.

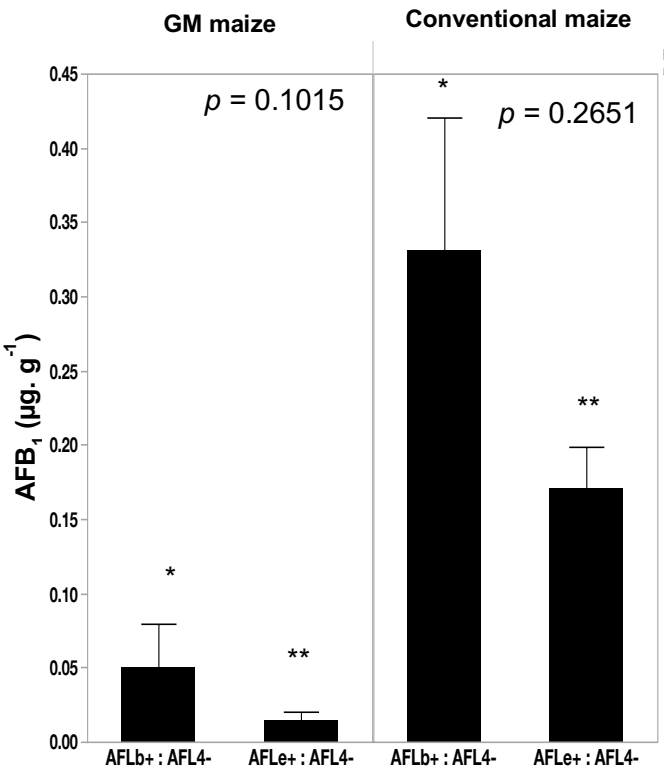

Figure 5.4. Overall content of AFB_1 in stored maize by the atoxigenic strains in GM and conventional maize cultivars taking into account the combined parameters (a_w, days and maize cultivars) for the interaction AFL^+: AFL^- 50:50 inoculum ratio. Asterisks (*) indicates significant differences ($p<0.05$) for the same strain in different maize cultivars. P-value (≥0.05) represents no difference between $AFLe^+$: $AFL4^-$ and $AFLb^+$: $AFL4^-$ in the same type of maize. Bars represent AV±SE.

5.4.3. Gene expression studies

The gene expression of the target structural gene *alfD (nor*-1) and the regulatory gene *afl*R in the aflatoxin biosynthetic pathway were only investigated in the treatments with the native Brazilian toxigenic strain $AFLb^+$ since this strain was able to produce higher amounts of toxin and the BCA strain $AFL4^-$ was able to significantly control the AFB_1 content. The results for the gene expression are presented as relative gene expression and shown in Figure 5.5. The relative gene expression indicates that there was a significant reduction for both *alfD* and *afl*R genes when the strain $AFL4^-$ was used as the BCA. The most significant reduction of relative expression occurred in the cultivar M20-A78 PW® at both a_w levels. The isogenic line (M20-A78 CON), on the other hand, indicated lesser effect on gene expression under biocontrol conditions. This cultivar had the lower relative control of AFB_1 in both a_w levels tested. Interestingly there was no overall effect of the maize cultivars in the expression in the control conditions ($AFLb^+$). However, the types of maize (GM or non-GM) had an effect on AFB_1 contamination levels (Figure 5.6).

The correlation of relative gene expression × AFB_1 was tested using non-parametric Spearman's coefficient. There was a positive correlation (Table 5.7). When the AFB_1 content was higher it resulted in the higher expression of both target genes. The coefficient for *aflD* at both a_w levels was higher (0.83 and 0.85 for 0.95 and 0.98 a_w, respectively). For *afl*R the coefficients of correlation where lower but still positive.

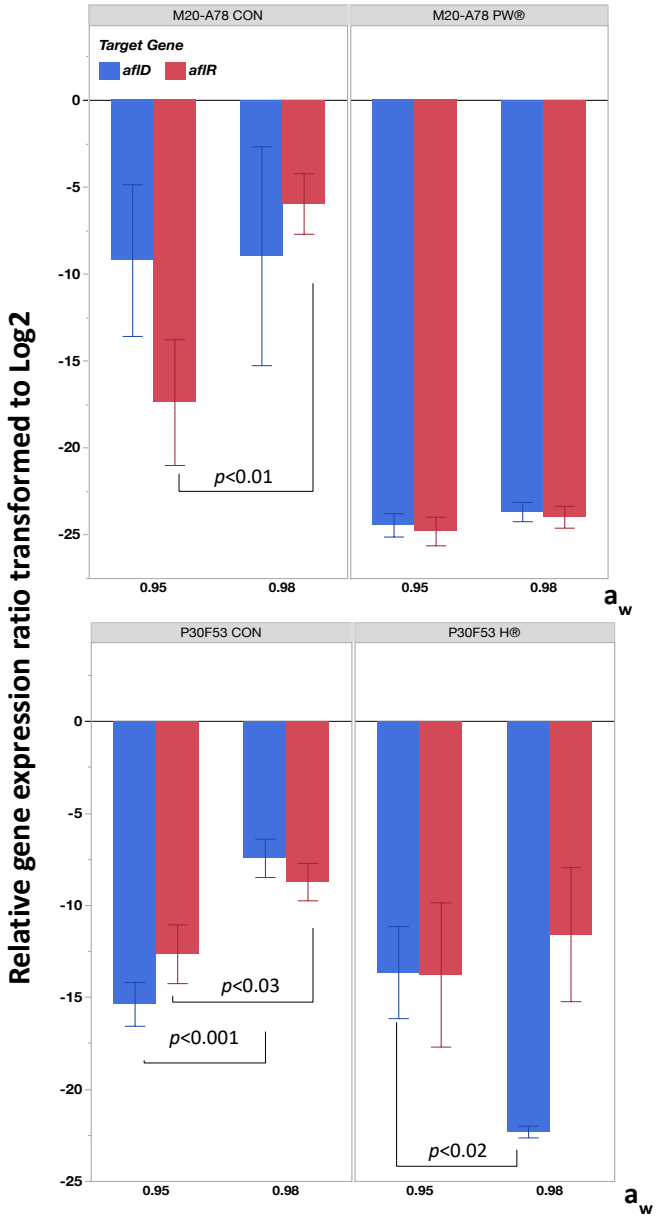

Figure 5.5. Effect of antagonist strain AFL4$^-$ on relative gene expression of target genes (aflD and aflR) at 30°C after 10 days using conventional (M20-A78 CON and P30F53 CON) and GM (M20-A78 PW® and P30F53 H®) cultivars modified to 0.98 and 0.95 a_w. AFLb$^+$: AFL4$^-$ biocontrol treatment with 50:50 spore's ratio. The values were normalized for de control AFLb$^+$ toxigenic strain (expression = 1.0 – log2 = 0); Bars represents AV±SD

Table 5.7. Spearman's correlation coefficient (ρ) of Log_2 relative gene expression × AFB_1 production for the different water activities (a_w) in the *in situ* assays after 10 days storage at 30°C.

a_w	gene	Coefficient (ρ)	Probability
0.95	*afl*D	0.83	<.0001
	*afl*R	0.71	<.0001
0.98	*afl*D	0.84	<.0001
	*afl*R	0.67	0.0003

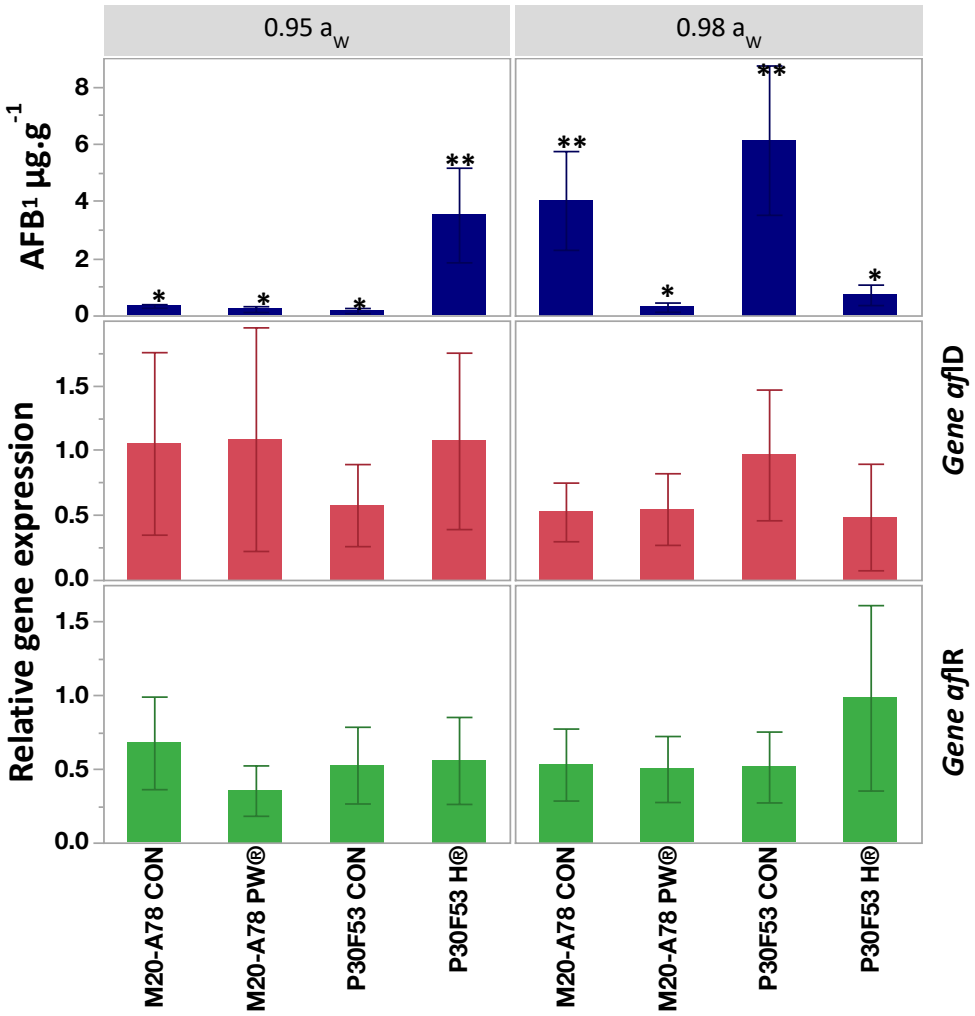

Figure 5.6. Effect of maize cultivars on AFB_1 content and relative gene expression of aflD and aflR by the toxigenic *A. flavus* strain ($AFLb^+$) after 10 days at 30°C with water activity levels of 0.95 and 0.98 a_w. Cultivars M20-A78 CON and P30F53 CON correspond to non-GM maize; Cultivars M20-A78 PW® and P30F53 H® correspond to GM maize. Bars with same symbol (*) are statistically different (p<0.05). Bars represent AV±SE.

5.5 Discussion

The present study aimed to monitor deletions in the AFs and CPA biosynthesis gene clusters using multiplex PCR for 12 *A. flavus* strains isolated from Brazilian maize cultivars. Nine of these strains lacked the ability to produce AFs when analysed by both the visual fluorescence CAM (Coconut Cream Agar) medium and by HPLC-FLD. Monitoring deletions of genes involved in the AFs and CPA biosynthetic pathways can represent a quick and cheaper approach to select potential BCAs. The method used in this study was developed by Callicot and Cotty (2015) and it covers markers from the AFs and CPA mycotoxin cluster.

A substantial number of *A. flavus* isolates have been found to contain several deletions in the aflatoxin gene cluster resulting in non-AF producers strains (Chang, Horn and Dorner, 2005). In this study, amongst the 9 non-aflatoxigenic strains tested, 5 (*Af*PHx, *Af*53H, *Af*AS1C, *Af*M20C and *Af*BMC-b) showed large deletions of the genes *afl*U (*cyp*A) through *afl*B (*fas*-1), including deletion of *afl*C (*pks*A) and *afl*A (*fas*-2), and *afl*L (*ver*B) through *afl*W (*mox*Y). Additionally, the strains *Af*AS1C, *Af*M20C and *Af*BMC-b showed absence of the markers *afl*E (*nor*A) and *afl*S (*afl*J). The non-aflatoxigenic isolates *Af*FRC, *Af*M20-a and *Af*CDPW-b, in contrast, had the majority of AF markers present, with the exception of the *afl*E which was absent in the last strain mentioned.

Aflatoxin biosynthesis has been proposed to involve at least 23 enzymatic reactions (Yu and Cleveland, 2007) and at least 34 genes (Yu et al., 2004). Thus far, at least 15 structurally well-defined aflatoxin intermediates have been identified in the aflatoxin biosynthetic pathway (Yu et al., 2004; Bhatnagar et al., 2018). The genes involved in toxin biosynthesis are in a 70 kb gene cluster. They encode a DNA-binding protein functioning in the AF pathway gene regulation, and other enzymes such as cytochrome P450-type monooxygenases, dehydrogenases, methyltransferases, and polyketide and fatty acid synthases (Bhatnagar, Ehrlich and Cleveland, 2003).

The gene *afl*U (cytochrome P450 monooxygenase) is reported to encode a polypeptide of 498 amino acids (Yu et al., 2004). Expression studies using RT-PCR showed that the transcript of *afl*U was detected only under aflatoxin-conducive conditions (Payne, 1998). However, Ehrlich et al. (2004) reported that a sequence comparison of the aflatoxin biosynthesis pathway gene cluster upstream from the *aflC*, revealed that *A. flavus* isolates are missing portions of the genes *afl*U and *afl*L (*nor*-B) at the end of the AFs biosynthetic cluster causing the lack of aflatoxin G production.

In an earlier study, Chang et al. (1995a) proposed that *afl*C gene was linked to the *afl*D (*nor*-1) gene, which is required for the conversion of norsolorinic acid to averantin at the beginning of AFs pathway. Consequently, a disruption in *afl*C could prevent the synthesis of norsolorinic acid resulting in interruption of AFs biosynthesis. Trail et al. (1995) using knockout experiments demonstrated that *afl*C is important for aflatoxin biosynthesis. Atoxigenic isolates were found to be genetically different from toxigenic isolates because of polymorphism in *aflC,* which resulted in the production of a defective polyketide synthase (PKS) (Chang and Hua, 2007). Moreover, Abdel-Hadi, Carter and Magan (2011) showed that the *afl*D gene expression was a reliable marker to discriminate between aflatoxin and non-aflatoxin producers.

Similarly, the genes *afl*B (*fas-1*) and *afl*A (*fas*-2*)* located side by side in the AFs cluster (Yu et al., 2004) have being reported as enzymatic synthase genes (Amaike, Affeldt and Keller, 2013) involved in AF biosynthesis. The *afl*B and *aflA* encode alpha and beta subunits of fatty acid synthetase (FAS), respectively. Together with PKS that is encoded by *afl*C (*pks*A), the FAS are involved in the conversion of acetate to norsolorinic acid that is the first stable intermediate of AFs biosynthesis. Thus, disruption on *afl*C and *afl*B can lead to loss of AFs production (Watanabe and Townsend, 2002).

Interestingly, for the strains *Af*FRC and AfM20-a, using HPLC-FLD and a conducive medium toxin production was not detected. However, the strain showed all the markers for AF biosynthesis using the multiplex PCR. When all

AFs genes are present in a strain, but a single nucleotide polymorphism causes an early termination in the *aflC* gene it can result in lack of toxin production (Ehrlich and Cotty, 2004). Callicot and Cotty (2015) highlighted that the multiplex PCR method will not detect isolates with mechanisms of atoxigenicity lacking an indel. Further investigation using more complex molecular techniques should be used to find the region where the polymorphism is causing the absence of AF production by such strains.

Non-aflatoxigenic *A. flavus* strains with large deletions in the aflatoxin gene cluster, have been proposed for use in aflatoxin control as they compete for the niches occupied by toxigenic strains (Dorner, 2004; Pitt et al., 2015). Competition for nutrients and space necessary for growth and toxin production is important in deciding which isolate grow fastest (Chang and Hua, 2007). Identification of genetic diversity within *A. flavus* populations through vegetative compatibility groups (VCGs) may help to understand the population dynamics since the genetic exchange would likely be greater among isolates from same VGC group (Pildain, Vaamonde and Cabral, 2004). Moore (2014) pointed out that if the biocontrol by atoxigenic strains is lost, the cause might be recombination between native toxigenic strains and the atoxigenic ones used for biocontrol. Their ability to evolve new phenotypes and genotypes via sexual recombination needs to be considered.

Although the present work did not investigate the genetic variation within the atoxigenic *A. flavus* strains, Chang, Horn and Dorner (2005) hypothesized that isolates in the same vegetative compatibility group (VCG) have identical deletion patterns but isolates with identical deletion patterns also belong to different VCGs. Multiple strains studied by McAlpin, Wicklow and Horn (2002) produced identical DNA fingerprints when belonged to the same VCG, with a few exceptions. Within all the strains isolated from different maize cultivars from Brazil and studied using the multiplex PCR method, no two strains showed the same pattern of deletions. The differences were detected in AF, CPA or sugar cluster, and in the subtelomeric region of the multiplex M3.

In addition to aflatoxins, the main toxin produced by *A. flavus*, CPA, became notably important. This toxin is a indole-tetramic acid neurotoxin (Chang, Ehrlich and Fujii, 2009) also produced by other *Aspergillus* species, such as *A. tamarii* (Dorner, 1983) *A. oryzae*, *A. versicolor*, *A. clavatus , A. fumigatus* and *A. phoenicis* (Vinokurova et al., 2007). In *A. flavus*, the gene cluster that codes for CPA biosynthesis is adjacent to the aflatoxin gene cluster (Chang, Horn and Dorner, 2009). In this study 4 markers from the cluster of CPA were used to verify the ability to produce this toxin. The non-aflatoxigenic strain *Af*53H demonstrated ability to amplify all the markers in this cluster, and *Af*FRC presented only amplification for the marker CC03. Regarding the toxigenic strains, *Af*LRG-a and *Af*M20-c showed the presence of all the CPA genes, whereas both *Af*ASC-a and *Af*P30C-a had deletions of the markers CC01, CC02 and CC04. The production of CPA by the isolated strains used in this work was not confirmed using analytical approaches, however it has being reported that strains missing genes in CPA cluster are unable to produce this toxin (Chang, Horn and Dorner, 2009).

Based on the data obtained using the multiplex PCR and associated with previous tests for biocontrol *in vitro*, the strain *Af*53H (AFL4-) was selected for the *in situ* assays. This strain was able to significantly reduce the AFB_1 content *in situ* when paired with 2 toxigenic strains: a native Brazilian strain $AFLb^+$ and the type strain NRRL3357 ($AFLe^+$) in a 50:50 inoculum ratio with the atoxigenic strain. The relative AFB_1 control ranged from 58 to 100% under different a_w (0.98 and 0.95) condition for up to 20 days storage. According to the multiplex PCR, the strain $AFLb^+$ (AfLRG-a) showed the presence of all the markers for AFs and CPA.

The *Af*53H strain showed the presence of all the CPA markers, and possibly was able to produce this metabolite. Regarding the AF markers, this strain had a large deletion supporting this strain had potential for biocontrol. *Af*53H showed deletion of *afl*C, the same gene which is absent in the commercial strain already being used in the field for biocontrol: AF36. A defect in the early aflatoxin biosynthesis gene (*afl*C) in AF36 resulted in its inability to produce the polyketide precursor necessary for aflatoxin formation (Ehrlich and Cotty, 2004). Although AF36 is capable of competitively displacing toxigenic strains, the use of this strain in

biocontrol is questioned, since maize inoculated with AF36 accumulated significant quantities of the mycotoxin CPA (Abbas et al., 2011b). AF36 does have a fully functional CPA cluster, and although it is effective at excluding toxigenic strains and reducing AF levels, it is reported to significantly increase CPA accumulation in food and feed commodities (Chang, Horn and Dorner, 2005).

Furthermore, the present study reports, for the first time, an attempt to investigate the impacts triggered by the use of conventional (non-GM) and GM maize when atoxigenic biocontrol strains are used. The results showed that *Af*53H was successful in controlling AFs in both GM and non-GM cultivars. However, some alterations in the phenotypic AFB_1 production were observed. The overall AFB_1 reduction indicated that when biocontrol was applied in maize kernels of the non-GM maize, the final control was lower than that in GM cultivars.

As previously detailed in CHAPTER 3, the way cultivars of GM or non-GM maize affect *A. flavus* development is complex and not completely elucidated. Based on the patterns of AFB_1 production using the different maize cultivars either GM or non-GM, in the ecophysiological studies on maize-based matrices suggested that interacting environmental conditions affected the development of *A. flavus* (see CHAPTER 3). The biochemical composition has been demonstrated as very similar in GM and the isogenic non-GM cultivars (Reuter et al., 2002). The fatty acids in the maize could play a major role in pathogen susceptibility and seed colonisation (Dall'Asta et al., 2012). As free FAs, linoleic acid levels partly regulate development, colonisation and mycotoxin production by *Aspergillus* spp (Calvo et al., 1999; Scarpari et al., 2014). Changes in nutritional or environmental factors can also influence secondary metabolite production (Magan and Aldred, 2007a).

In addition to the alterations in the AFB_1 production patterns, the gene expression of target structural gene *afl*D and the regulatory gene *afl*R were investigated. Quantification of these were based on the relative expression using a reference gene (ß-tubulin gene). ß-tubulin is reported to be constitutively expressed and

constant during the growth phases of *A. flavus* (Mayer et al., 2003). The results showed that *A. flavus* AfP53H (AFL4$^-$) as a biocontrol agent in an inoculum ratio 50:50 inhibited both the target structural and regulatory genes (*alf*D and *afl*R). The correlation with phenotypic aflatoxin production shows that gene inhibition resulted in less toxin after 10 days storage.

Al-Saad et al. (2016) studied action of bacterial antagonists as BCAs for the control of *A. flavus* and although there was relative inhibition of gene expression of *afl*D and *afl*R there was sometimes a stimulation of AFB_1 production. Thus, the inoculum ratio × nutritional parameters may be important factors which need to be controlled to avoid the potential for interacting stress factors stimulating toxin production.

The use of different maize cultivars (non-GM and respective isogenic GM line) did not affect the gene expression of *afl*D and *afl*R at 30^{o}C after 10 days storage in the control (AFLb$^+$). However, the phenotypic AFs production was affected by the type of maize. For interactions between AFLb$^+$: AFL4$^-$, when the conventional cultivar M20-A78 CON was used, the relative reduction of the toxins was lower and gene inhibition was less pronounced. The a_w level also had an effect on the relative gene expression when the atoxigenic antagonistic strain was applied, under the same storage conditions. Abdel-Hadi, Carter and Magan (2010) reported that at lower a_w levels (e.g., 0.90) *afl*D expression can increased, and it is optimally expressed at 0.98 a_w on a conducive medium.

The regulatory gene *afl*R encodes a positive regulator (AFLR) which activates the pathway gene transcription (Chang et al., 1995b), whereas the *afl*S (*afl*J) gene encodes for a protein factor (AFLS) which was found to be involved in the regulation of transcription. The gene aflS, is adjacent to the *afl*R gene and has been shown to be associated with the expression of *afl*C, *afl*D (*nor*-1), *afl*M (*ver*-1), and *afl*P (*omt*A) (Chang and Hua, 2007). Disruption of *afl*S and *afl*R results in a failure to produce AFs (Meyers et al., 1998; Yu et al., 2011). Additionally, *afl*D gene encoding an enzyme (norsolorinic acid ketoreductase) that catalyses the conversion of the first stable AF biosynthesis intermediate, norsolorinic acid to

averantin is a key early structural gene in the biosynthetic pathway (Zhou and Linz, 1999).

In summary, *Af*53H (AFL4$^-$) was able to effectively control AFB_1 production against both the toxigenic strains when a spore ratio of 50:50 was used in the stored maize studies at 30^{o}C for up to 20 days. The effect of *Af*53H as a BCA was also verified with the gene expression: *afl*D and *alf*R were significantly suppressed. The use GM and non-GM maize grain resulted in differences in phenotypic aflatoxin production patterns for the atoxigenic strain AFL4$^-$. However, the same was not observed on relative gene expression of *afl*D and *alf*R. These effects require more in-depth investigation as this was the first attempt to verify the impact of conventional versus GM maize in biocontrol approaches using atoxigenic strains.

5.6 Conclusions

- 5 strains of nonaflatoxigenic *A. flavus* demonstrated large deletion of genes in the AFs cluster
- non-aflatoxigenic strains amplified most of the markers in the AF cluster, indicating the inability to produce AF might occur in a different position in the biosynthetic toxin cluster
- The strain selected for biocontrol *in situ* (*Af*53H – AFL4$^-$) had a large deletion of AF markers but had all the CPA markers
- The native Brazilian toxigenic strain *Af*LRG-a (AFLb$^+$) used in the biocontrol study as a pathogen had all the markers for both AF and CPA clusters and seems to be genetically different from the chosen BCA strain
- *Af*53H was able to significantly reduce AFB_1 production when paired with both toxigenic strains in a 50:50 spore ratio. The relative reduction varied from 58 to 100%
- The relative gene expression of *afl*D and *afl*R in the toxigenic strain AFLb$^+$ was significantly inhibited by the BCA strain
- The correlation gene expression $\times$ AFB_1 was positive. Suppression in the gene expression resulted in lower toxin levels

- The overall biocontrol action seems to have been most effective when used in stored GM maize cultivars

CHAPTER 6

BIOCONTROL RESILIENCE UNDER SIMULATED PEST DAMAGE AND CLIMATE CHANGE SCENARIOS IN CONVENTIONAL AND GM BRAZILIAN MAIZE

CHAPTER 6. BIOCONTROL RESILIENCE UNDER SIMULATED PEST DAMAGE AND CLIMATE CHANGE SCENARIOS IN CONVENTIONAL AND GM BRAZILIAN MAIZE

6.1 Introduction

The potential effect of climate change (CC) scenarios on food production and food security is now receiving significant interest world-wide. Available evidence and predictions suggest overall negative effects on agricultural production as a result of CC, especially when more food is required by an ever increasing population (Dwivedi et al., 2013). According to United Nations' projections (USDA/DESA, 2011) the world population would reach 9,3 billion by 2050 and the climate will be warmer by 2°C due to the increase in the CO_2 and ozone concentrations (Jaggard, Qi and Ober, 2010). The concentration of CO_2 in the atmosphere has continuously increased from pre-industrial levels at 280 ppm to 412 ppm at present (NOAA and Climate.gov, 2018), and is expected to reach above 800 ppm by 2050 (IPCC, 2014). By the year 2100 temperatures could increase by 4ºC, and CO_2 levels are anticipated to reach approximately 1000 ppm (Gilbert et al., 2016).

Magan et al. (2003) cited climate as a crucial factor for fungal colonization and mycotoxin production. The effect of CC on mycotoxin production is very complex and difficult to predict completely because many of the factors will interact with each other (Dwivedi et al., 2013). Crops subjected to drought and high temperature, especially during the reproductive phase, are under greater risk of aflatoxin contamination by *A. flavus* and *A. parasiticus*, with perhaps also a greater risk of increasing aflatoxin contamination during the storage phase (Paterson and Lima, 2011).

Furthermore, higher temperatures due to climate warming could create new challenges related to more rapid insect population growth rates, increasing insect overwintering and voltinism (number of generations of an organism in a year), altering crop-pest synchrony, changing geographical ranges of important pest

species (DeLucia et al., 2012; Wu et al., 2011) and abundance in agricultural systems (Miraglia et al., 2009). All these factors could lead to increasing the damage to ripening maize kernels and allow more rapid entry for mycotoxigenic fungal pathogens, resulting in perhaps increase mycotoxin contamination levels. Thus, the use of GM *Bt* crops could be a strong ally under CC conditions where problems associated with insects may increase. However, there is little knowledge about this correlation under climatic variations in maize.

Maize can be infected by several pathogens during the growing season, harvesting, drying and storage causing significant losses. Pre-harvest pests represent an average of 35% of potential yield losses worldwide (Oerke, 2006), whereas post-harvest losses range between 14 and 36% (López-Castillo et al., 2018). Post-harvest maize insect pests include many species from the orders Coleoptera and Lepidoptera, which are distributed worldwide, trigger nutritional losses which impacts on the economics of downstream processing (García-Lara, Chuck-Hernández and Serna-Saldivar, 2013). Species such as *Sitophilus zeamais*, *Prostephanus truncatus*, *Sitotroga cerealella*, *Rhyzopertha dominica* and *Tribolium castaneum* are considered major post-harvest pests of maize (López-Castillo et al., 2018). Indeed, *S. zeamais* is a major stored grain pest of maize in Brazil where insecticides are heavily used for its control (Santos et al., 2006).

Damage caused by pest invasion is variable but often starts in the field before harvest, when the eggs are laid, and continues through to storage. Larvae and adults bore into the grains through neat round holes and feed on the grain resulting in average losses of 30% in stored maize (Taruvinga, Mejia and Sanz Alvarez, 2014). Other pests attack ripening grain standing in the field, especially during silking, and the insects are then usually transported inside the grain to the storage facilities. Infestations produce abundant heat and moisture that may encourage mould spoilage to be initiated and well as secondary pests (Taruvinga, Mejia and Sanz Alvarez, 2014), feeding on the germ and endosperm which leaves behind a fine powder, shells of bran and particles of faeces (Agri-Facts, 2014).

The correlation of pest invasion and fungal infection have been extensively reported (Blandino et al., 2014; Battilani et al., 2013; Tirado et al., 2010; Dowd, 2000). Insect damage is one factor that predisposes maize to mycotoxin contamination, because herbivory insects, mainly Lepidopterans, produces wounds that encourage fungal colonization and vectors of fungal spores (Munkvold and Hellmich, 1999; Alma et al., 2005). The most effective way to manage Lepidopteran insects and reduce the associated mycotoxin risk is with GM insect resistance genes with Crystal (Cry) toxin from *Bacillus thuringiensis* (Bt) (Munkvold et al., 1999; Wu, 2006). The *Bt* toxin attaches itself to the cell membrane in the stomach and intestinal canal of the insects causing cell rupture and leaking, having a lethal effect (Schnepf et al., 1998), reducing the populations pre-harvest.

The GM maize with *Bt* toxin was developed against herbivorous lepidopteran pests attacking leaves, but the toxin also affects Lepidoptera attacking the kernels. However, coleopteran maize weevils (*S. zeamais*) were not affected by transgenic *Bt* maize (*Cry1*Ab toxin) (Hansen, Lövei and Székács, 2013). On the other hand, *Bt* toxins were efficient at controlling *S. cerealella*. Furthermore, the spectrum and efficacy of *Bt* protoxins against stored grain insects may also have some effect on storage pests (Bushra and Aslam, 2014).

In Brazil today most of the maize cultivated is genetically modified (GM). The GM trait, which is most commonly cultivated in both winter and summer crops, is a combination of insect-resistance (*Bt*) and herbicide-tolerance (HT). The use of GM crops with HT traits may represent a significant decrease in the use of pesticides and a reduction in the farming costs. While the GM crops with *Bt* traits are essentially to control insects, mainly in the order Lepidoptera, that are common problems in tropical and sub-tropical areas such as Brazil. The environmental variations caused by CC may have impacts on the number of insects contaminating maize crops in Brazil and pest damage of *Bt* crops. In Brazil, where GM maize is commonly the main crop grown there is little information on whether CC might predispose maize to more or less AFs. This may be important in understanding the future risks of aflatoxin and might have an

impact on control strategies, including biocontrol resilience (Medina et al., 2017a).

6.2 Objectives

a. To simulate three different levels of pest damage (0, 5 and 15%) in gamma-irradiated maize kernels of conventional (non-GM) and genetically modified (GM) cultivars at 0.98 and 0.95 a_w and 30°C to examine effects on the temporal aflatoxin B_1 (AFB_1) production (10 and 20 days), and on the relative expression of the *aflD* and *aflR* biosynthetic genes
b. To examine the effects of interacting CC conditions using the optimal temperature and +5°C at 400 vs 1000 ppm CO_2 on *A. flavus* colonisation, aflatoxin B_1 production and on the relative expression of the *aflD* and *aflR* biosynthetic genes using one GM maize cultivar and its respective isogenic non-GM line
c. To examine the relative effectiveness of the best BCA under CC scenarios and with simulated pest damage on AFs control

6.3 Materials and Methods

6.3.1. *A. flavus* strains and maize cultivars

Fungal strains: The non-aflatoxigenic strain of *A. flavus (Af*53H – $AFL4^-$) isolated from Brazilian maize was selected as the biocontrol agent (BCA) based on the ability to reduce AFs in previous *in vitro* and *in situ* studies described in CHAPTER 4 and 5. The native toxigenic strain (*Af*LRG-a – $AFLb^+$) also isolated from the Brazilian maize with a high AFB_1 production capacity was used as the pathogen.

Fungal strain and spore suspension: The strains were sub-cultured on MEA (Malt Extract Agar, Oxoid, UK) and incubated for 7 days at 30°C. Afterwards, the spores were removed from the colony using a sterile loop, transferred to a water/Tween®80 0.1% (v/v) solution and spores counted using a counting

chamber (Neubauer improved, Paul Marienfield, GE). A final spore concentration at 10^3 spores mL^{-1} was obtained and considered as the stock solution. The spore stock solution was used to modify the a_w of the kernels as described in the section '*Moisture adsorption curve'.* According to the experiments *in vitro* (Chapter 4) the ratio 50:50 (pathogen: antagonist) represent the most viable option for biocontrol. The spore suspension was mixed in a 50:50 ratio with the pathogen and the maize kernels inoculated. The controls consisted of the 100:0 and 0:100 ratio for pathogen: antagonist.

Maize cultivars: Simulated insect damage, for 6 cultivars of GM maize with insect resistance (IR) and their respective isogenic conventional (non-GM) were selected. All the cultivars had different traits with between one and 5 genes inserted for this purpose. The complete information about the cultivars and their characteristics are shown in Table 6.1.

For the CC study P30F53 CON and P30F53 H® were selected. The kernels were irradiated (12-15 kGys) to eliminate all the microbiota but still conserve germinative capacity. The a_w of the kernels was modified to 0.98 and 0.95 a_w by adding a solution of sterile water + *A. flavus* spores according to the absorption curve of each cultivar to obtain the target treatment regimes.

Moisture adsorption curve: The water activity (a_w) of the kernels was modified prior the experiment based on the moisture adsorption curves of each cultivar. The initial a_w of the maize kernels from the cultivars selected was $\leq$0.7 (see CHAPTER 2 - Figure 2.2). For the moisture adsorption curves (Appendix E) sub-samples of 5 g of maize grain were placed in 25ml Universal glass bottles and different amounts of water were added to the maize grain. These were allowed to equilibrate at 4°C for 24h then brought to room T °C (20-25°C) and the a_w was measured using an AquaLab® 4TE DUO (Decagon Devices, USA). The moisture content was determined by oven drying at 110°C for 24 h. The adsorption curve was plotted in relation to the amount of added water (mL) $\times$ a_w and this was used to determine the exact amount required to modify the actual a_w of the maize grain for each cultivar.

The a_w of the kernels was modified to 0.98 and 0.95 a_w by adding a solution of sterile water + *A. flavus* spores according with the absorption curve of each cultivar. The exception were the cultivars AS 1555 CON and PRO®, and P2530 CON and Hx® were only 0.98 a_w was used because of the limited amount of these cultivars available for the experiment. The number of spores added was calculated as 10 spores per gram of maize from the stock solution (10^3 spores. mL^{-1}). After the addition of water + spore solution, the treatments were thoroughly mixed, and kept at 4°C for 24h for complete equilibration. The final a_w was confirmed using the AquaLab®.

Table 6.1. Characteristics of the GM and non-GM cultivars of maize grain selected for the insect damage study.

Conventional cultivar (non-GM)	Isogenic line (GM)	Traits tolerance
AS 1555 CON*	AS 1555 PRO®	IR - Lepidopteran
P30F53 CON	P30F53 H®	HT - Glufosinate ammonium Antibiotic resistance IR - Lepidopteran
P2530 CON*	P2530 Hx®	IR - Lepidopteran
BM-709 CON	BM-709 PRO_2®	HT - Glyphosate IR - Lepidopteran
CD-384 CON	CD-384 PW®	HT - Glyphosate HT - Glufosinate ammonium IR - Lepidopteran

IR- insect resistance; HT – herbicide tolerance; * cultivars used only in the condition 0.98 a_w

6.3.2. Effect of simulated pest damage

Insect damage simulation: The insect damage was simulated by making a 2 mm diameter hole through to the endosperm tissue with an electrical drill using a surface sterilised drill bit (Figure 6.1). The kernels used were selected based on similar sizes and the integrity of pericarp. Kernels with visual defects were

discarded. The size of the holes was based on the parameters for grain quality described by Agbizgrain (http://www.agbizgrain.co.za). The characteristic of damage was similar to that caused by the primary storage pests that are able to invade the kernel initially in the field and bore into the grain during storage creating characteristics damage holes.

Figure 6.1. Representation of damage caused by insects and the simulated damage.

Incubation: The intact and damaged kernels with respective a_w modified to 0.98 and 0.95 were distribute into glass culture vessels (Magenta™, Sigma, USA - Chapter 5 - Figure 5.1) with the lid vented (10 mm with a polypropylene membrane 0.22 µm pore size) to allow gases exchange but keeping the environment inside the vessel sterile. The kernels were mixed to have a portion of 0% (all kernels intact), 5% and 15% damage in a total of 8 g per jar (see Table 6.2). The jars were placed in sealed plastic boxes with a glycerol/water solution of the same a_w as the kernels to keep the relative humidity (RH) of the atmosphere at the same treatment a_w level. The glycerol/water solution was renewed every 3 days. The boxes were incubated at 30°C for 20 days. Samples were collected after 10 and 20 days for AFs quantification. For gene expression, only samples of the day 10 were used. The samples for AFs quantification were oven dried at 65°C for 48h to remove the water and stop the fungal growth.

Afterwards, the samples were ground using a laboratory blender with a stainless-steel blade (Waring, Stamford, USA). The samples for gene expression (3 kernels per treatment) were snap frozen in liquid nitrogen (N_2) and stored at -80^{o}C until processing.

Table 6.2. Proportion of intact and damaged kernels present in each jar.

Damage	Intact kernels	Damaged kernels
0%	8 g	0 g
5%	7.6 g	0.4 g*
15%	6.8 g	1.2 g**

* equivalent to 1 kernel; ** equivalent to 3 kernels

6.3.3. Impact of climate change on atoxigenic strain resilience and aflatoxin control

For the CC study, 8 g maize kernels with the a_w modified to 0.98 and 0.95 as described previously, were aseptically distributed into the sterile glass culture vessels (See CHAPTER 5 - Figure 5.1, Magenta™, Sigma, USA). A 5% simulated damage treatment was included in these experiments (Table 6.2). The jars were placed in sealed plastic boxes (Lock & Lock HPL890 16 L) containing a glycerol/water solution of the same a_w as the treatments as described previously. The environmental conditions were set to have CO_2 flushing of 400 ppm (atmospheric) and 1000 ppm. The atmospheric concentration of CO_2 was 408 ppm in May 2018 when the experiment was carried out according to NOAA (National, Oceanic & Atmospheric Administration – U.S Department of Commerce).

The elevated CO_2 content was achieved by using a gas cylinder at required 1000 ppm CO_2/synthetic air 200 bar prepared by BOC (Guildford, UK). The boxes were vented, and for the 1000 ppm treatment flushed with CO_2, every 12h as shown in Figure 6.2 during the 10 days experimental period. The concentration of CO_2 was regulated to 3 liter.min^{-1} (LPM) with a gas flow meter (Alicat Scientific, Arizona, USA) and flushed for 11 minutes corresponding to 2x the volume of each box. After flushing, the boxes were immediately sealed and incubated at 30 and

35ºC along with the control boxes at 400 ppm. At the end of the experiment samples were collected for AFs quantification and gene expression studies. The samples for toxin quantification were oven dried at 65ºC for 48h to remove the water and stop any fungal growth. The samples were ground using the laboratory blender as described previously. Mycotoxin extraction, quantification, clean-up and injection was performed according to described in the CHAPTER 5 - Section 5.3.3.

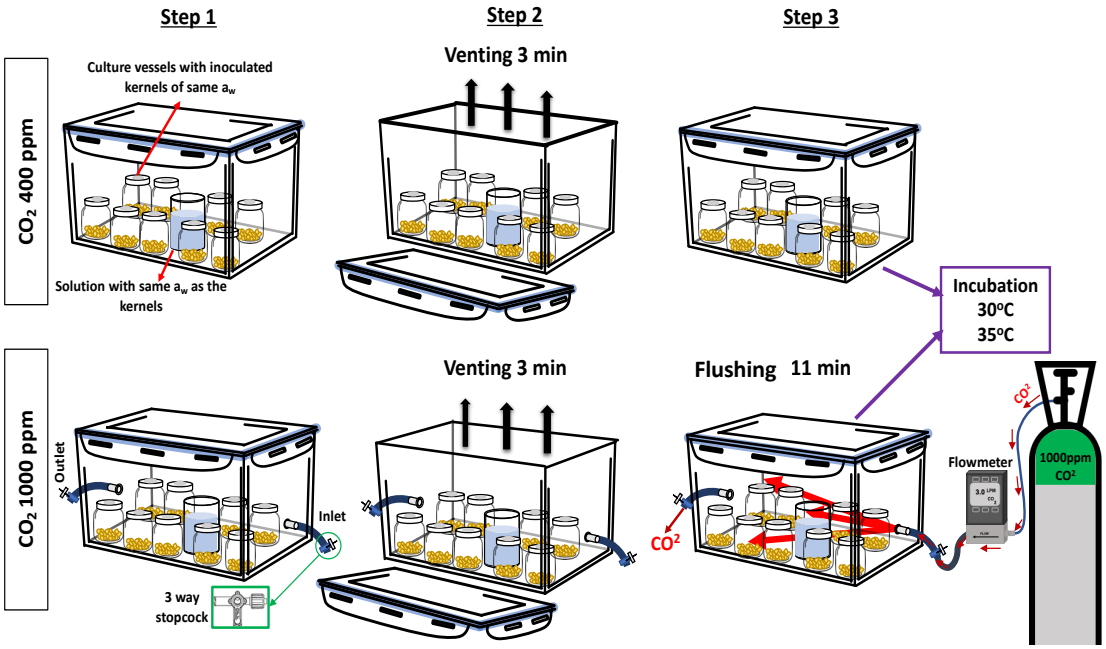

Figure 6.2. Schematic representation of the climate change experiment using plastic boxes with CO_2 at 400 ppm (atmospheric) and 1000 ppm.

6.3.4. Gene expression studies

The gene expression was performed using samples simulated pest damage and climate change collected on day 10. This time frame was chosen based on previous studies with both *A. flavus* and *A. parasiticus* that suggested gene expression of several of the biosynthetic genes had optimal peak after 8-10 days of growth (Schmidt-Heydt et al., 2008). For the simulated pest damage, 2 cultivars of GM (P30F53 H® and CD-384 PW®) and the respective isogenic non-GM maize

(P30F53 CON and CD-384 CON) were select for gene expression study. In the case of climate change effect, the gene expression was analysed for the condition of 35ºC x 0.98 a_w and CO_2 (400 and 1000 ppm).

The samples grinding, total RNA extraction, information about primers and probes used, cDNA and RT-pPCR method flowed the described in the CHAPTER 5 - Section 5.3.4

Relative quantification of the expression: Relative quantification of *afl*D and *afl*R genes was performed using the housekeeping gene β-tubulin (ben) as an endogenous control to normalise the quantification of the target in the relative quantification assays and used for all treatments. Quantification cycle (Cq) determinations were automatically performed by the instrument using default parameters, and the expression ratio was calculated using the $2^{-\Delta\Delta Ct}$ method proposed by Livak and Schmittgen (2001) and transformed to fold change Log_2. The control sample for simulated pest damage corresponded to 0% damage for each cultivar and a_w level. The control sample in CC study was 30ºC-400 ppm for each cultivar, whereas for the biocontrol the calibrant corresponded to pathogen strain $AFLb^+$: $AFL4^-$ 100:0 spore's ratio for each condition.

6.3.5. Statistical analysis

The data from simulated insect damage and climate change study on aflatoxin B_1 production and relative gene expression were subjected to Shapiro-Wilk tests to determine normality and Levene's test to assess the equality of variance. However, the simulated insect damaged effect on AFB_1 production and gene expression data violated the two assumptions for ANOVA even after transformations and therefore non-parametric tests (Kruskal-Wallis/ Wilcoxon; p=0.05) were used for analyses (Chan and Walmsley, 1997). Where there was significance after the Kruskal-Wallis test, median comparisons for each pair were made using the Wilcoxon - Each Pair test (p=0.05).

The effect of climate change on AFB_1 production, after transformation to cube root ($\sqrt[3]{}$), satisfied the requirements for ANOVA and effect test was performed

comparing the interactions of T°C × CO_2 × a_w × simulated pest damage for each cultivar of maize. The relative gene expression for this study violated the two assumptions for ANOVA and the differences were compared using non-parametric tests (Kruskal-Wallis/ Wilcoxon; p=0.05)**.** The calibrant (control sample) for the biocontrol experiment was the pathogen strain ($AFLb^+$) in the same conditions as the antagonist was applied. For the effects of climate change, the control sample refers to normal environmental conditions (30°C and 400 ppm CO_2) for each cultivar**.** The statistical package JMP®14 (SAS Institute Inc., 2018, Cary NC, USA) was used to perform the analyses.

6.4 Results

6.4.1. Effect of simulated pest damage on AFB_1 production

The simulated pest damage results are present in the Figure 6.3 and Figure 6.4.Three cultivars of conventional maize (CD-384 CON, BM 709 CON and P30F53 CON) and their respective isogenic GM lines (CD-384 PW®, BM 709 PRO® and P30F53 H®) were examined at 3 levels of simulated damaged (0% - control, 5% and 15%) at 0.98 and 0.95 a_w. For cultivars AS 1555 CON and P2530 CON and the respective GM lines, AS 1555 PRO® and P2530® were only studied at 0.98 a_w.

The differences in AFB_1 content were not consistently higher as the damage level was increased. However, overall the *A. flavus* infection seemed to be increased with the levels of damage (Figure 6.5). Differences were observed for some of the cultivars. The clearest differences were detected between the 0 and 15%, damage with the latter level of simulated damage resulting in lower toxin content when the cultivars GM lines P2530 Hx®, CD-384 PW®, BM 709 PRO® and P30F53 H® were inoculated with *A. flavus.* Whereas, for the GM cultivar AS 1555 PRO® at 0.98 a_w 5% damage resulted in higher AFB_1 contamination, but only after 10 days storage. The most significant difference in terms of different levels of damage was observed with the Non-GM cultivars P30F53 CON and P30F53 H® after 20 days storage where a higher level of AFB1 was found in the

undamaged treatments, but only at 0.95 aw. However, at 0.98 a_w there were significant differences between these cultivars when comparing them with GM isogenic lines.

The use of the isogenic GM line resulted in more AFB_1 at all levels of simulated damage. Interestingly, for the cultivar P2530, the GM line (P2530 Hx®) there was a 2-fold reduction of AFB_1 contamination when compared with its conventional isogenic line at 15% damage level at 0.98 a_w after 20 days storage (Figure 6.3). The other cultivars did not show any consistent differences between the conventional and related isogenic GM lines. Differences were found but varied with the treatment a_w and level of damage. The overall AFB_1 production combining data for all the cultivars (Figure 6.6) showed that toxin production increased at 0.98 a_w after 20 days storage but there was no significant overall trend for the levels of simulated damage or any overall distinction between using GM over non-GM cultivars. The effect of a_w was more pronounced after 10 days storage with 15% damage ($p=0.001$), whereas after 20 days there was no significant effect ($P=0.05$) of a_w.

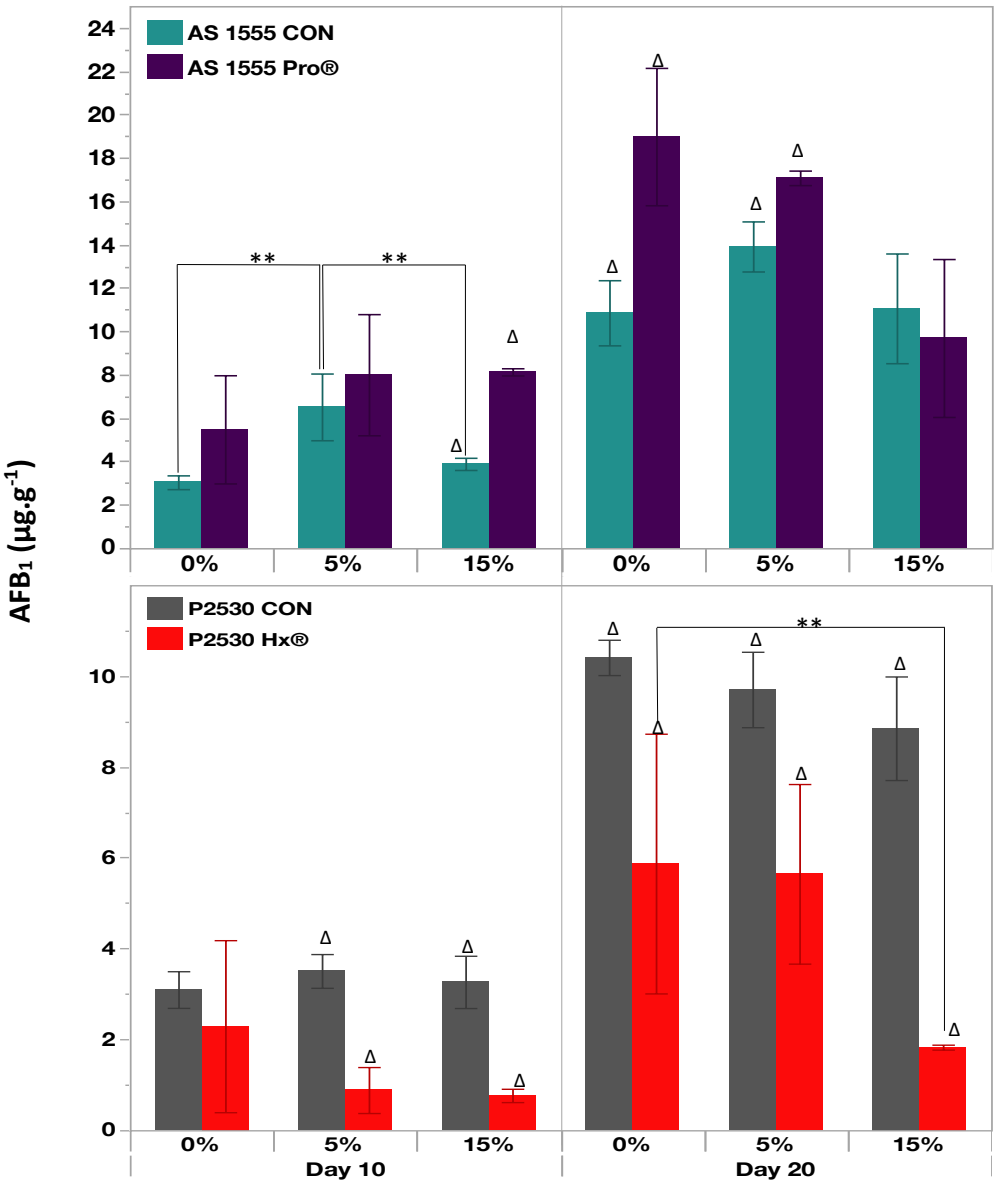

Figure 6.3. Effect of different levels of simulated pest damage (0, 5 and 15%) on AFB_1 production by *A. flavus* strain AfLRG-1 in stored maize kernels of conventional (AS 1555 CON and P2530 CON) and GM maize (AS 1555 PRO® and P2530 Hx®) at 0.98 water activity, incubated at 30°C for 20 days. Asterisks represents differences between the levels of damage: ** $p<0.05$; Δ represents differences between GM and non-GM cultivars for each level of damage on each condition ($p<0.05$). Bars represent AV±SE.

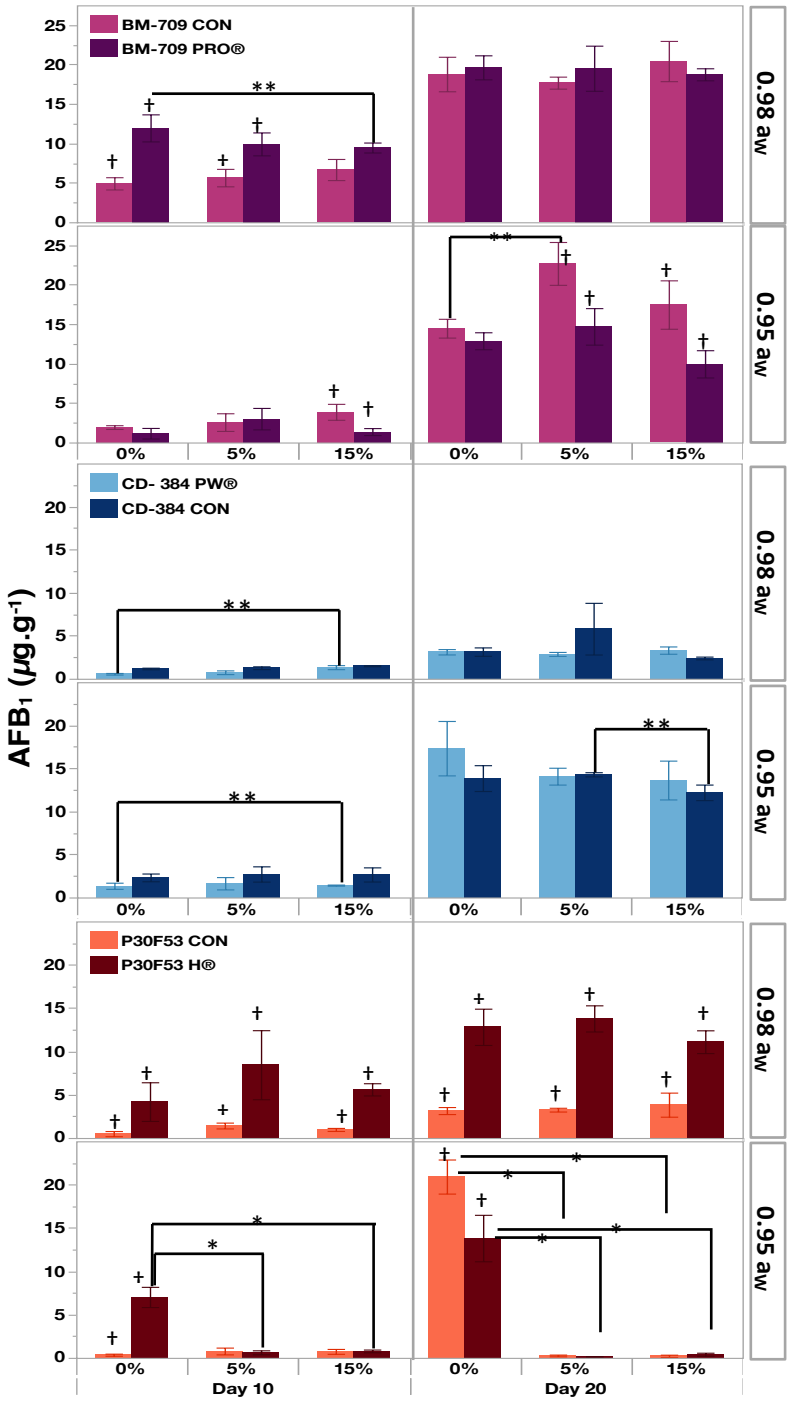

Figure 6.4. Effect of different levels of simulated pest damage (0, 5 and 15%) on AFB_1 production *by A. flavus* strain *Af*LRG-1 ($AFLb^+$) in sored maize kernels of conventional (BM 709 CON, CD-384 CON and P30F53 CON) and GM maize (BM 709 PW®, CD-384 PRO® and P30F53 H®) at both 0.98 and 0.95 a_w incubated at 30°C for 20 days. Asterisks represents differences between the levels of damage: ** p<0.05, * p<0.01; † represents differences between GM and non-GM cultivars for each level of damage on each condition (p<0.05). Bars represent AV±SE.

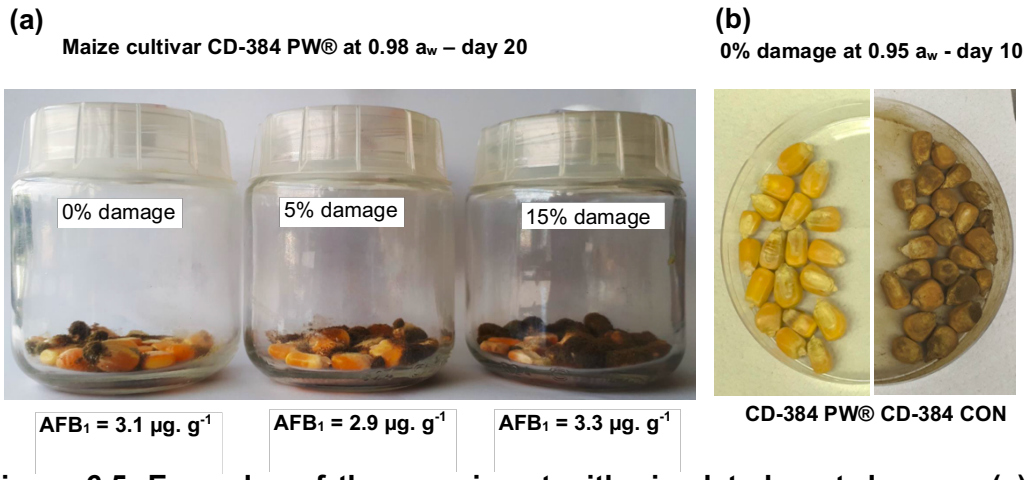

Figure 6.5. Examples of the experiment with simulated pest damage: (a) shows the 3 levels of damage for the GM maize CD-384 PW® and the AFB_1 detected; (b) shows visual comparation of *A. flavus* invasion between GM (CD-384 PW®) and its isogenic non-GM (CD-384 CON) cultivars under the same conditions.

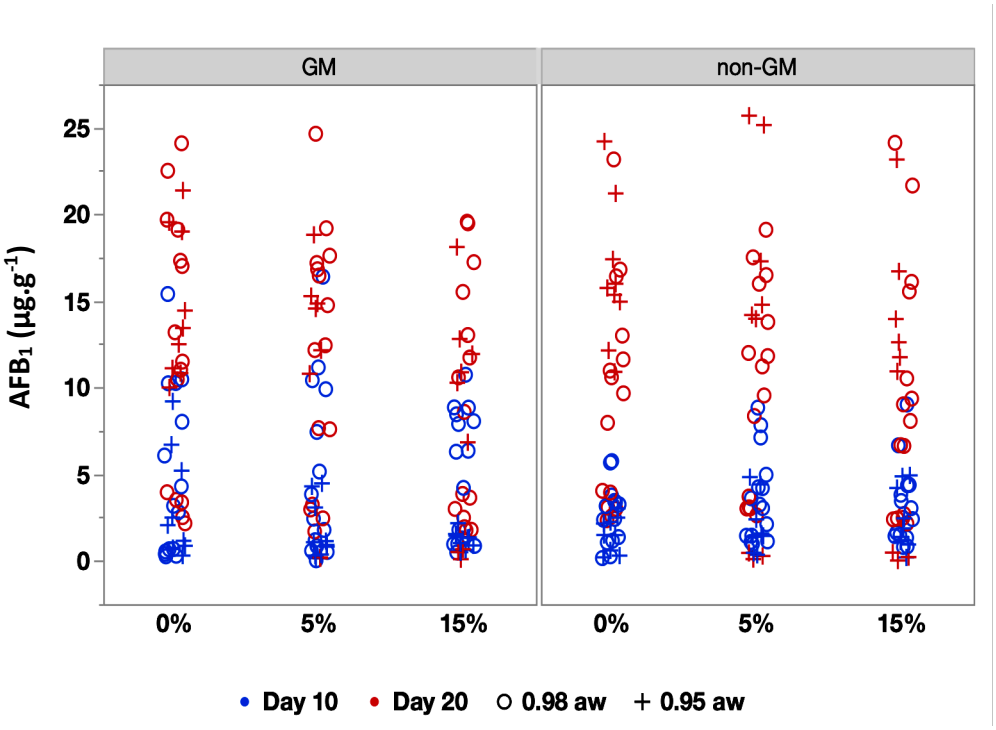

Figure 6.6. Overall AFB_1 production for all the maize cultivars by type (GM and non-GM) in relation to different levels of simulated pest damage: 0, 5 and 15% and 0.98 and 0.95 a_w.

6.4.2. Effect of simulated pest damage on *afl*D and *afl*R genes expression

The relative gene expression for the structural gene (*afl*D) and regulatory gene (*afl*R) were tested for the cultivars CD-384 PW® and CD-384 CON, and P30F53 H® and P30F53 CON using kernels after 10 days storage at 0.98 and 0.95 a_w at 30°C. The relative gene expression is shown in Figure 6.7**.** The expression of *afl*D and *afl*R showed differences in each maize cultivar used. The expression was more consistent in the 5% damage and 0.98 a_w treatment. The lower a_w (0.95) and higher damage (15%) showed lower expression indicating that day 10 for the study of gene expression might have been too late based on the kinetics of gene expression.

The gene *afl*R was upregulated for the cultivars CD-384 CON, P30F53 CON and P30F53 H® with 5% damage at 0.98 a_w, with a significant increase compared to the control (0% damage). Interestingly, for the cultivar CD-384 PW® this same gene had expression reduced. In the case of *afl*D, using the CD-384 PW® at 5%/0.98 a_w, the expression was significantly higher ($p<0.03$) (Figure 6.7**).** For the non-GM cultivar P30F53 CON the opposite occurred in the same conditions: *afl*R expression increase, whereas *afl*D was reduced. The isogenic GM line (P30F53 H®) had consistent upregulation of both *alf*R and *alf*D with 5% damaged kernels at 0.98 a_w. However, this was not statistically significant ($p=0.05$) when compared to the non-damaged treatment (control, 0%).

With maize kernels modified to 0.95 a_w, there was a clearer pattern of the *afl*D gene expression, with a significant increase in the non-GM CD-384 CON and its isogenic GM line CD-384 PW® cultivars. However, for the non-GM cultivar P30F53 CON, *afl*D expression was reduced at 5% damage. However, there was no difference between the control (0% damage; $p=0.05$) and 15% kernel damage treatments at 0.95 a_w. Its isogenic GM line P30F53 H® showed a significant downregulation at 5% damage, but not at other damage treatment levels. The correlation coefficient (Spearman, ρ**)** between gene expression and AFB_1 production was weak and negative at 0.95 a_w and 15% damage for both of these

genes (-0.1 and -0.5 for *afl*R and *afl*D, respectively). The coefficients for 5% of damage were positive: 0.47 and 0.31 for *afl*R and *afl*D, respectively.

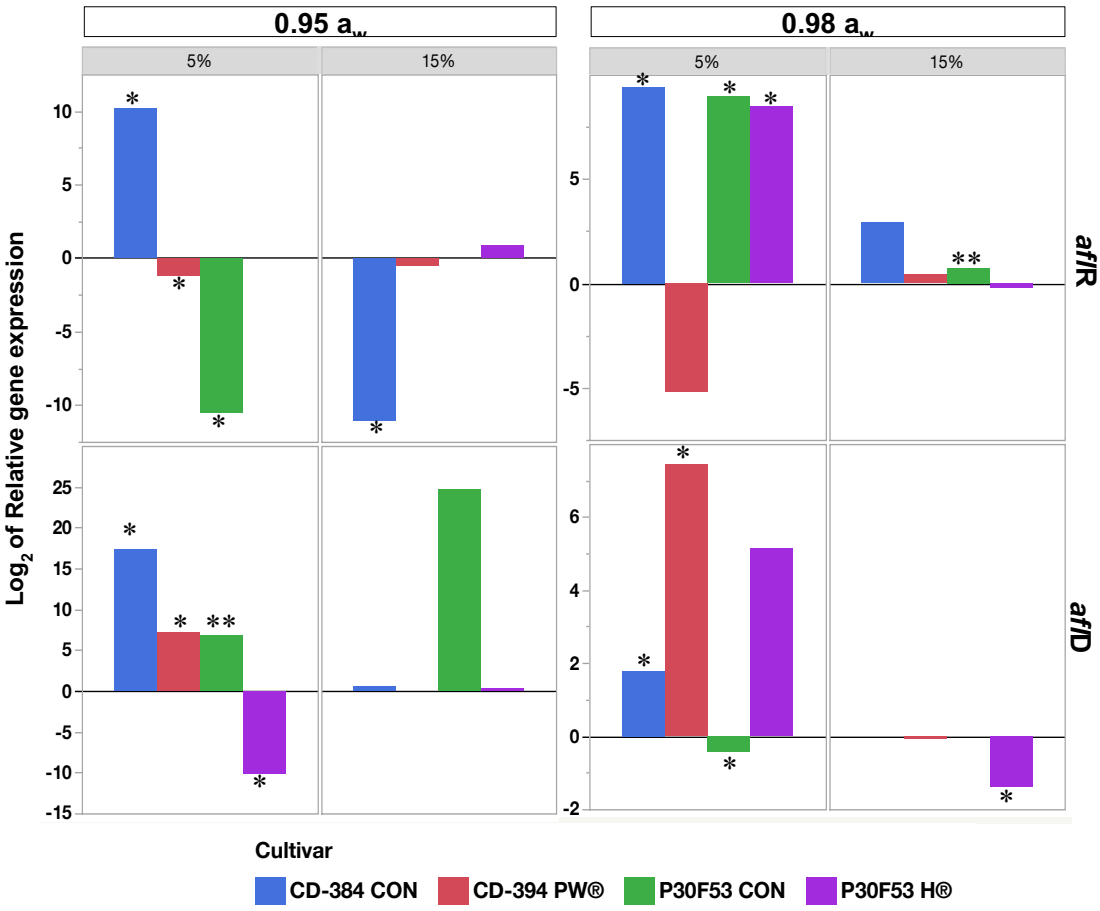

Figure 6.7. Effects of simulated damage (5% and 15%) on relative gene expression (log2 transformed) of the structural (aflD) and regulatory (aflR) genes for the GM cultivars (CD-384 PW® and P30F53 H®) and their respective isogenic non-GM lines (CD-384 CON and P30F53 CON) after 10 days storage at 30°C. Asterisks (*) represents significant difference for up or down regulation of genes from the control (p<0.03). ** reduction on gene expression. The control sample consisted of non-damaged kernels (0%) for each condition.

6.4.3. Effect of climate change scenarios on AFB_1 production

In the overall study of the effect of increased temperature and CO_2 levels using conventional and GM maize with undamaged kernels and 5% of simulated damaged, the *A. flavus* strain produced more AFB_1 when inoculated onto GM kernels (Figure 6.8). The AFB_1 production was significantly lower with 5% kernel damage at 30°C/400 ppm CO_2/0.98 a_w for both types of maize. At 0.95 a_w there was no significant difference between undamaged and damaged kernels when the GM cultivar was used. However, for conventional maize, 5% damage increased AFB_1 production significantly ($p<0.05$). When the temperature was increased to 35°C, but the CO_2 level kept at existing levels (400 ppm), the AFB_1 was reduced in both treatment damage levels at 0.98 a_w for all the cultivars. The effect of +5°C at 0.95 a_w showed some differences in behaviour between the GM and non-GM maize. While GM maize had reduction of AFB_1, the conventional maize had higher toxin contamination in the undamaged maize kernel treatments.

The effect of 30°C x 1000 ppm CO_2 resulted in lower AFB_1 contamination in undamaged kernels of GM maize at 0.98 and 0.95 a_w. On the other side, using non-GM maize resulted in more toxin when 5% of kernels were damaged for both a_w levels. The effect of CC (35°C x 1000 ppm CO_2) at 0.98 a_w was more evident in conventional maize, where there was a significant increase of AFB_1 compared with 35°C/400 ppm. Under water stress (0.95 a_w) conditions there was a significant effect of elevated CO_2. In the GM cultivar, a CC scenario also incurred more toxin at 0.98 a_w for both levels of damage. However, when abiotic stress and damage where combined this resulted in a 4-fold increase in AFB_1 contamination.

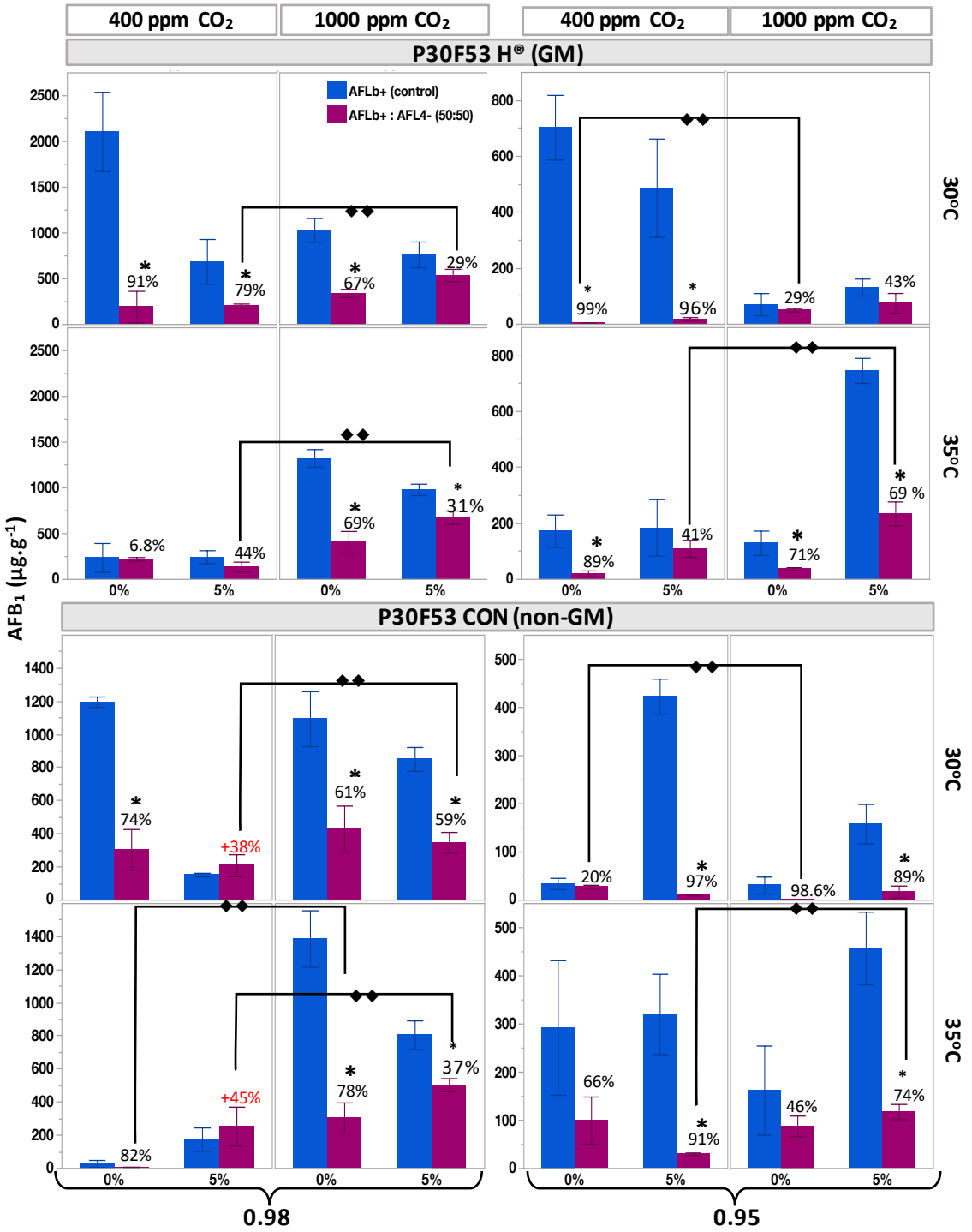

Figure 6.8. Effect of temperature (30 and 35°), CO_2 (400 and 1000 ppm), simulated pest damage (0 and 5%) and a_w (0.98 and 0.95) on AFB_1 contamination by toxigenic strain $AFLb^+$ (control) and on biocontrol action with $AFLb^+$: $AFL4^-$ spore ratio 50:50 pathogen: antagonist in conventional (P30F53 CON) and GM (P30F53 H®) maize kernels. Values above bars represent relative control (%) of AFB_1; *represents significant reduction ($p<0.05$) on AFB_1 from the control ($AFLb^+$); ◆◆ represents significant differences of biocontrol action between CO_2 levels.

6.4.4 Relationship between climate change scenarios and biocontrol resilience

Furthermore, there were a few differences in the biocontrol action when CO_2 was increased. At 30°C the relative reduction of AFB_1 was affected at 1000 ppm CO_2 in the 0.98 and 0.95 a_w treatments using GM maize. For this cultivar, at 35°C x 1000 ppm, the biocontrol efficacy was enhanced in undamaged kernels at 0.98 a_w but reduced where 5% damage maize kernels were present. Using non-GM maize at 0.98 a_w + 5% damaged kernels, at both 30 and 35°C, the biocontrol efficacy was affected. AFB_1 contamination increased from 38% at 30°C to 45% (control) at 35°C in atmospheric CO_2 levels.

Under climate change conditions (35°C/1000 ppm CO_2) and 0.98 a_w the AFB_1 contamination was increased in both 0 and 5% damage. However, the efficacy of biocontrol was significantly affected when compared to the control. The AFB_1 relative control ranged from 37 to 78%. Similar patterns were found using GM maize in these environmental conditions. The relative AFB_1 control at 0% damage/35°C/0.98 a_w and 1000 ppm CO_2 was 69% and 78% using GM and non-GM maize, respectively. When CO_2 was increased to 1000 ppm, but the temperature kept at 30°C, the AFB_1 was lower in GM maize in both 0.98 and 0.95 a_w.

The effect test (Table 6.3) shows that the interaction of T°C × CO_2 × a_w × simulated pest damage caused a significant effect only in the $AFLb^+$ control in conventional maize. For the relative biocontrol, and effects on GM maize, the interaction of all the factors was not significant. The insect damage level individually did not have a significant effect on biocontrol in both types of maize, however the CO_2 effect was evident.

Table 6.3. Effect Tests (ANOVA) on AFB_1 production for the interaction of Temperature (30 and 35°C) × CO_2 (400 and 1000 ppm) × a_w (0.98 and 0.95) × simulated pest damage (0 and 5%) in conventional and GM maize for the *A. flavus* control (toxigenic strain $AFLb^+$) and for the biocontrol ($AFLb^+$: $AFL4^-$) in a 50:50 spore ratio pathogen: antagonist.

Maize		Effects	DF	SQ	F Ratio	*p*-value
P30F53 H®	$AFLb^+$ (control)	CO_2	1	2.4	0.9	0.3395
		Insect damage	1	11.8	4.6	0.0376
		a_W	1	15.4	6.0	0.0185
		T°C	1	93.8	36.7	<.0001
		CO_2 × Insect damage	1	15.3	6.0	0.0188
		CO_2 × a_W	1	24.3	9.5	0.0037
		CO_2 × T°C	1	95.8	37.5	<.0001
		Pest damage × a_W	1	16.2	6.4	0.0158
		Pest damage × T°C	1	16.2	6.3	0.0159
		a_W × T°C	1	10.8	4.2	0.0461
		CO_2 × pest damage × a_w × T°C	1	8.47	3.47	0.0705
	$AFLb^+$: $AFL4^-$ (50:50)	CO_2	1	48.2	34.6	<.0001
		Insect damage	1	5.2	3.7	0.0620
		a_W	1	37.7	27.0	<.0001
		T°C	1	4.7	3.4	0.0734
		CO_2 × Insect damage	1	1.1	0.8	0.3823
		CO_2 × a_W	1	2.3	1.6	0.2102
		CO_2 × T°C	1	0.3	0.2	0.6664
		Pest damage × a_W	1	3.0	2.2	0.1509
		Pest damage × T°C	1	0.3	0.2	0.6700
		a_W × T°C	1	1.5	1.1	0.3030
		CO_2 × pest damage × a_w × T°C	1	0.63	0.5	0.5785
P30F53 CON	$AFLb^+$ (control)	CO_2	1	31.8	9.5	0.0039
		Insect damage	1	2.6	0.8	0.3816
		a_W	1	0.0	0.0	0.9441
		T°C	1	13.7	4.1	0.0511
		CO_2 × Insect damage	1	0.1	0.0	0.8730
		CO_2 × a_W	1	74.1	22.0	<.0001
		CO_2 × T°C	1	22.0	6.5	0.0149
		Pest damage × a_W	1	48.0	14.2	0.0006
		Pest damage × T°C	1	3.1	0.9	0.3436
		a_W × T°C	1	35.7	10.6	0.0024
		CO_2 × pest damage × a_w × T°C	1	34.20	19.36	0.0001
	$AFLb^+$: $AFL4^-$ (50:50)	CO_2	1	15.0	9.2	0.0043
		Insect damage	1	1.6	1.0	0.3307
		a_W	1	20.5	12.6	0.0011
		T°C	1	2.8	1.7	0.1952
		CO_2 × Insect damage	1	0.1	0.1	0.7853
		CO_2 × a_W	1	16.4	10.1	0.0030
		CO_2 × T°C	1	16.2	9.9	0.0032
		Pest damage × a_W	1	4.1	2.5	0.1212
		Pest damage × T°C	1	6.6	4.0	0.0516
		a_W × T°C	1	31.3	19.2	<.0001
		CO_2 × pest damage × a_w × T°C	1	1.49	1.39	0.2460

$p < 0.05$ represents significant effect on AFB_1 production; P30F53 CON – conventional maize; P30F53 H® GM maize cultivar.

6.4.4. Effect of climate change scenarios on gene expression

The non-aflatoxigenic *A. flavus* strain AFL4- previously tested in normal climatic conditions (30ºC atmospheric CO_2; see CHAPTER 5), was tested under increased temperature (+5ºC), CO_2 (1000 ppm) and simulated damage in the kernels (0 and 5%). The study for gene expression was done with samples from 35ºC using GM and conventional maize (cultivar P30F53 H® and P30F53 CON, respectively) after 10 days storage. The control sample used for normalizing the relative expression was the antagonist AFLb+ for each condition. The RT-pPCR was performed from both genes (*afl*R and *afl*D). However, the structural gene *afl*D was not detected in any of the samples in the time frame selected (Figure 6.9).

There was a reduction in *afl*R expression when the biocontrol strain was applied in a spore ratio of 50:50 of pathogen: antagonist. However, when the CO_2 level was increased to 1000 ppm + 5% kernel damage in the GM cultivar, the inhibition of *afl*R was not significantly different from the control ($p=0.05$) even though the AFB_1 production was reduced in this condition by 35%. Interestingly, in the same scenario with the conventional cultivar, the same gene was strongly inhibited but the AFB_1 relative reduction was 37%. Overall, CO_2 had an effect on the biocontrol action for the expression of *afl*R with conventional maize ($p<0.01$). The used of GM maize showed that increasing CO_2 + 5% damaged kernels did not have an overall effect on the on the expression of *afl*R when the biocontrol agent was used.

The gene expression of *A. flavus* $AFLb^+$ under climate change was compared to the normal scenario. Expression at 35ºC and 1000 ppm was normalized with control sample at 30ºC/400 ppm with no damaged kernels. The results are shown in Figure 6.10. The expression of *afl*R at 400 ppm/35ºC was similar to the control with no damage, but it was inhibited in the 5% damage treatment. With 1000 ppm CO_2 and both levels of damage (0 and 5%) using conventional maize, *afl*R expression was reduced when compared to the control. The opposite occurred with GM maize at 400 ppm/35ºC: the gene expression increased. When CO_2 was

increased to 1000 ppm, the expression decreased when compared to existing climatic conditions (30ºC/400 ppm). The overall effect of climate change was more evident on GM maize, where the expression was significantly reduced at 1000 ppm (p=0.0039). The increase of CO_2 on conventional maize did not have an effect on the *afl*R relative expression (p-0.62)

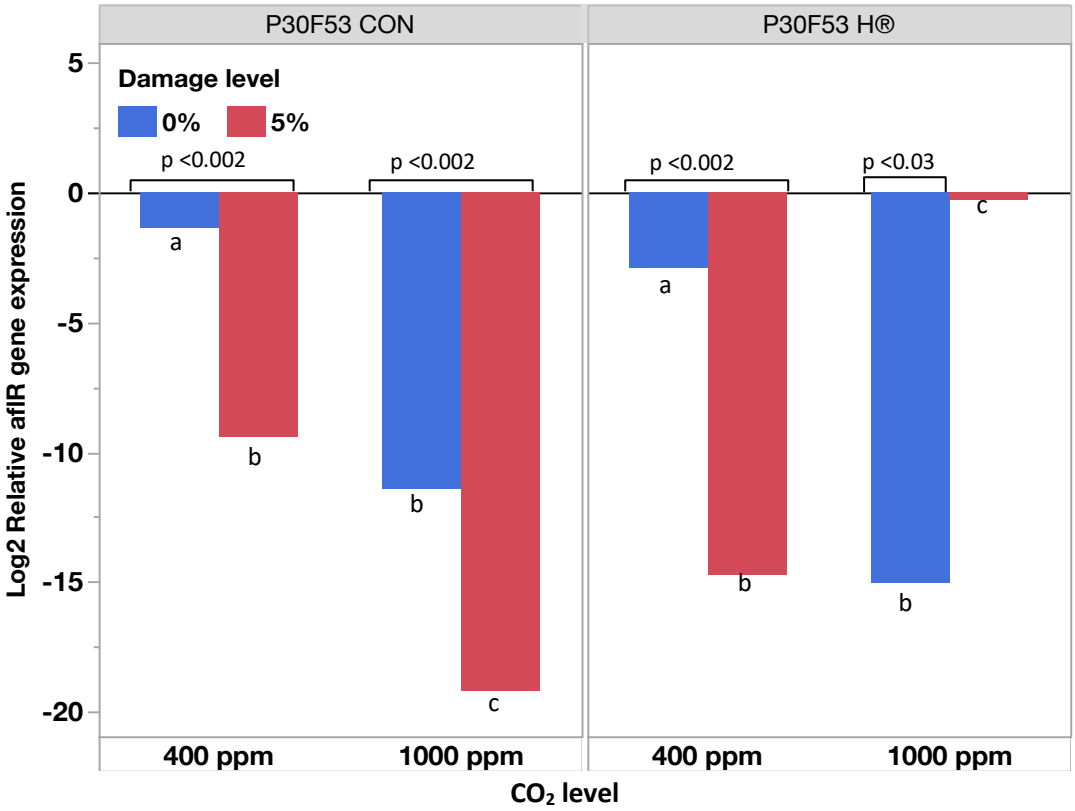

Figure 6.9. Effect of antagonist strain AFL4$^-$ as a biocontrol agent (BCA) in a spore ratio of 50:50 pathogen: antagonist (AFLb$^+$: AFL4$^-$) on relative expression of the regulatory gene aflR using conventional (P30F53 CON) and GM (P30F53 H®) maize with 0.98 a_w after 10 days at 35ºC in different CO_2 levels (400 ppm and 1000 ppm) and simulated pest damage (0% and 5%). The expression was normalised for the control sample (AFLb+) in each condition. p-values represent significant differences from the control; different letters indicate difference in the damage and CO_2 levels (p<0.05).

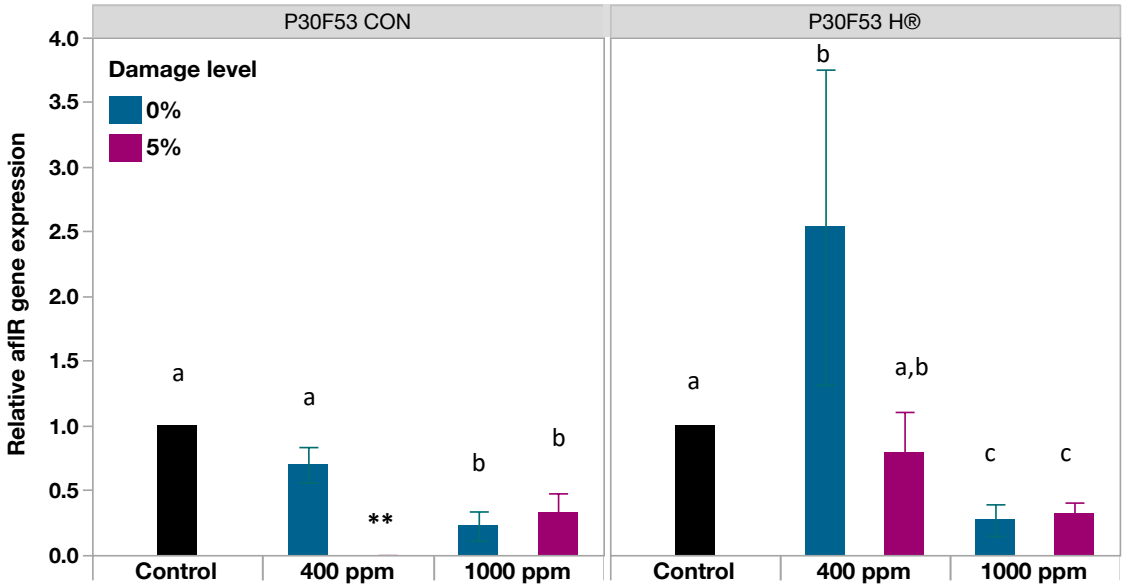

Figure 6.10. Relative gene expression of regulatory gene (aflR) at 35°C in different levels of CO_2 (400 and 1000 ppm) using conventional (P30F53 CON) and GM (P30F53 H®) maize with 0.98 a_w and simulated pest damage (0 and 5%). Control sample refers to gene expression at 30°C- 400 ppm CO_2. Same letter indicates significant differences ($p<0.05$). ** inhibition of expression.

6.5 Discussion

This study explored the effects of elevated CO_2 on AFB_1 production and the subsequent effects caused when non-aflatoxigenic biocontrol strains were used under these scenarios for reducing AFB_1 contamination in relation to non-GM and GM maize cultivars for the first time. By the year 2100 temperatures could increase by 4°C, and CO_2 levels are anticipated to reach approximately 1000 ppm (Gilbert et al., 2016). Thus, we used optimal conditions for *A. flavus* growth (30°C) and +5°C under atmospheric CO_2 (400 ppm) and predicted climate change scenarios (1000 ppm).

The effect of CC on AFB_1 production in feed maize has been examined previously. Medina et al. (2015), conducted the first study which examined the effect of three-way interacting CC factors (water stress × temperature × elevated

CO_2) on *A. flavus* and AFB_1 production *in vitro*. They increased temperature (34 and 37°C), a_w (0.97 to 0.95 and 0.91) and CO_2 was increased from 350 to 650 and 1000 ppm. The growth of *A. flavus* was relatively unaffected but the expression of key genes such as the *aflR* and *aflD* were significantly increased. In addition, the production of AFB_1. Similarly, Vaughan et al. (2014) demonstrated that elevated CO_2 (800 µmol CO_2 mol^{-1} air) also enhanced maize susceptibility to *Fusarium verticillioides* infection, but the increase in fungal biomass did not result in higher fumonisin levels. Although, subsequent studies suggested that there was an interaction between drought stress and elevated CO_2 which increased fumonisin production (Vaughan et al., 2016).

More recently, Gilbert et al. (2018) using RNA-Sequencing demonstrated that AFB_1 production on stored maize grain was altered by a_w × temperature × elevated CO_2. Also, several genes involved in the biosynthesis of secondary metabolites, exhibit different responses to a_w or temperature stress depending on the atmospheric CO_2 content. At 37ºC and 1000 ppm CO_2 the transcription factor *afl*R was decreased. After 10 days incubation the expression of biosynthetic genes in maize stored at 30ºC generally decreased. However, the effects of high CO_2 (1000 ppm) and water stress (0.91 a_w) showed decreased values, possibly in response to elevated AFB_1 levels (Gilbert et al., 2018).

In the present study, the effect of increasing CO_2 at 30 and 35ºC varied depending on the type of maize used (GM or conventional). This was the first attempt to analyse whether using GM or conventional maize under CC scenarios can cause differences in AF production by *A. flavus*. Overall, more AFB_1 was produced when *A. flavus* was inoculated in GM maize using the cultivars P30F53 in atmospheric CO_2 levels. However, in CC conditions, the use of GM or conventional maize did not show significant differences in AFB_1 content.

Additionally, higher temperatures due to climate warming could create new challenges related to alterations in insect populations (Wu et al., 2011). Insects are ectotherms, thus an increase in ambient temperature directly influences their metabolic rates, developmental rates and activity patterns (Altermatt, 2010). This

could lead to increased numbers of insects, increased injury to crops, higher occurrence of fungal contamination, and by extension, increased levels of mycotoxins (Medina et al., 2017a).

Considering the importance of pest damage in maize production and its alterations due to CC, this study also attempted to analyse the effect increasing damage might have in stored maize. The response in terms of AFB_1 contamination by adding different levels of simulated damage to the kernels when several cultivars where used in current climate conditions (30^{o}C and atmospheric CO_2 – 400 ppm) showed that an increase in damage in stored kernels did not result in a direct increase on toxin content.

However, it was unclear whether the lack of increase in AF production when more damage was imposed to the kernels was due to the simulation without the actual insect damage dynamics and introduction simultaneously of spores of *A. flavus* allowing more rapid invasion and expanded surface area which can result in increased toxin contamination. Alternatively, the nutritional composition of the maize used could also have affected the AF contamination. All the GM cultivars used had different insect resistance (IR) traits and were compared with the respective isogenic non-GM cultivars. The effect of the presence of IR was not consistent for all the cultivars. Only the GM P30F53 H® and its conventional isogenic line (P30F53 CON) showed consistent differences between them, with the latter type resulting in less AFB_1 but only at 0.98 a_w.

The full interaction of T^{o}C × CO_2 × a_w × simulated pest damage had significant effects only in conventional maize. However, this effect did not occur when biocontrol was applied. The action of the atoxigenic Brazilian strain $AFL4^-$ as a biocontrol agent was significant with increased CO_2, but the overall efficacy was lower than in non-CC conditions. The use of GM cultivar (P30F53 H®) showed better results for biocontrol under abiotic stress (0.95 a_w) and increased CO_2 when the kernels were undamaged. Interestingly, when there were damaged kernels, the biocontrol efficacy was affected, and control in conventional maize was improved.

The expression of biosynthetic genes (*afl*R and *afl*D) under simulated insect damage varied consistently depending on which maize cultivar was used. The expression of both genes with 15% of kernels damage and at 0.95 a_w was not strongly detected. The correlation with the AFB_1 production was also not significant, either positive or negative. Additionally, the structural gene (*afl*D) was not detected in the CC study at the temperature chosen for the analysis (35^{o}C) using RT-qPCR. It has been suggested that optimum a_w for *aflD* expression was at 0.90 a_w, which is different from that for growth and the levels used in this study (0.98 and 0.95 a_w) (Abdel-Hadi, Carter and Magan, 2010). The expression of *afl*R, on the other hand was detected.

Interestingly, for the expression of *afl*R gene in the biocontrol study, the relative expression in the GM maize (P30F53 H®) + 5% simulated damaged at 35^{o}C/1000 ppm CO_2 resulted in no inhibition of gene expression. However, AFB_1 contamination was reduced by 37%. In the same scenario but using conventional maize (P30F53 CON), the relative AFB_1 control was 35% and *afl*R was significantly inhibited. It has been described that interacting a_w × temperature × elevated CO_2 have a significant impact on aflatoxin biosynthetic gene expression (*afl*D and *afl*R) and can significantly stimulate the production of AFB_1 (Medina et al., 2014).This study, additionally shows that intrinsic factors present in the maize cultivars could trigger different responses in terms of gene expression. The regulatory mechanism underlying the biosynthesis of aflatoxin is not completely understood, perhaps due to its complexity through different levels of regulation (Gallo et al., 2016). While the experimental procedure facilitated observations of changes in gene expression and AF production after 10 days, a kinetic study may provide more insights into the change which may occur in relation to toxin control and relating this to the biosynthetic gene expression observed.

The increase in CO_2 and ozone concentrations and resulting changes in temperature and precipitation patterns will affect plant physiology. Significant effects on the sensitivity of plants to environmental stresses such as drought, waterlogging or heat, and susceptibility to pathogenic microorganisms might

appear (Mikkelsen, Jørgensen and Lyngkjaer, 2015). Thus, agricultural practices may need to change to address these CC related challenges.

The predictions of CC should be considered for the development of BCAs since it may affect their resilience and thus efficacy. Magan, Medina and Aldred (2011) pointed out that changes in temperature and water availability can modulate the response of different mycotoxin producing species to control agents, both pre- and post-harvest. Therefore, it is imperative to seek ways to control AF and explore the potential impacts of CC on the pathogenicity of *A. flavus* (Moore, 2014). Moreover, the expected increase in pest damage caused by CC could have impacts on the efficacy of the BCA since this might affect the invasion and competitiveness of target pathogen and the BCA. Perhaps formulations of the biocontrol agents may need to be modified to conserve antagonistic activity/competitiveness to maintain efficacy under CC scenarios. In addition, CC might also cause accelerated resistance of pests to *Bt* hybrids currently in use creating the need for evolutionary biotechnology strategies (Venugopal and Dively, 2017).

In summary, this study has shown that AFB_1 production by *A. flavus* can be affected by a number of interacting variables: type of cultivar, T^{o}C, CO_2 levels, water availability and presence of damaged kernels in the maize storage system. These interactions may also significantly affect BCA resilience and relative action. However, these interactions are complex and require more detailed study to identify the key parameters which determine relative toxin contamination levels and the potential for effective biocontrol under the expected CC conditions. This may also require evolution of cultivars, whether they are non-GM or GM to ensure that the production of maize would not be severely affect by CC scenarios resulting in losses in quality and enhance contamination with AFs due to *A. flavus* infection.

6.6 Conclusion

- Different levels of simulated pest damage (0, 5 and 15%) showed that AFB_1 production did not increased with a higher level of damage
- The toxin production with 15% of damage was lower or equal to that at 0 or 5% damage
- There was not a strong distinction of using GM cultivars with IR compared to conventional cultivars when simulated pest damage was introduced
- The gene expression of *afl*R and *afl*D genes involved in AF biosynthesis showed distinctions between the maize cultivars
- The correlation of gene expression × AFB_1 for the pest damage was not significantly positive
- The interaction $T^oC \times CO_2 \times a_w \times$ simulated pest damage had significant effects only for conventional maize, but not when biocontrol was used
- Using GM maize (P30F53 H®) with 5% simulated damage at 35°C/1000 ppm CO_2 did not caused inhibition of gene expression but the AFB_1 production was reduced by 35%.
- Using Conventional (P30F53 CON) cultivar the relative AFB_1 control was 37% and *afl*R was significantly inhibited with damaged kernels at $0.98 \times 35^oC \times 1000$ ppm CO_2
- The action of the atoxigenic Brazilian strain $AFL4^-$ as a biocontrol agent in a 50:50 spore ratio of pathogen: antagonist was significant with increased CO_2, but the overall efficiency was lower than in non-CC conditions
- The used of GM cultivar (P30F53 H®) showed better results for biocontrol under abiotic stress (0.95 a_w) and increased CO_2 at 35°C when the kernels were undamaged
- Biocontrol in conventional maize was better when there were damaged kernels at $0.95\ a_w \times 35^oC \times 1000$ ppm CO_2. This suggests implications of CC factor for maize production because of the vulnerability of the cvs used today, and the importance of using resilience of BCAs as part of control strategy.

CHAPTER 7

OVERALL DISCUSSION, FINAL CONCLUSIONS AND FUTURE RECOMMENDATIONS

CHAPTER 7. OVERALL DISCUSSION, FINAL CONCLUSIONS AND FUTURE RECOMMENDATIONS

7.1 Overall discussion

In Brazil, despite the large GM production, few surveys have investigated the similarities and differences between the mycobiota in these two groups of maize cvs, especially related to dominant species and the mycotoxin contamination profiles. Furthermore, as the projections for future decades suggest significant changes in the climate, this will affect the way *A. flavus* invades maize both pre- and post-harvest. This study explored the effects of using different non-GM and GM maize cvs on potential control strategies such as biocontrol using native atoxigenic *A. flavus* strains, and their resilience under increased pest damage and climate change (CC) scenarios.

Projections (USDA/DESA, 2011) show that by 2050 the climate will be warmer by +2°C due to the increase in the CO_2 and ozone concentrations (Jaggard, Qi and Ober, 2010). The concentration of CO_2 in the atmosphere is expected to reach above 800 ppm by 2050 (IPCC, 2014), and is anticipated to reach approximately 1000 ppm with a +4°C in temperature by 2100 (Gilbert et al., 2016). Changes in rainfall patterns with extreme wet and drought periods are expected to occur (Magan et al., 2011). Thus, the three-way interacting factors (temperature × elevated CO_2 × drought stress) could significantly impact crop yield and quality. Magan et al. (2011) and Medina et al. (2014; 2015) suggested that significant impacts would occur due to these three-way interacting factors on growth and mycotoxin production by key mycotoxigenic fungal species and also on nutritional quality of staple grains.

Maize is particularly prone to drought stress (Lobell et al., 2011), which can predispose it to increased disease susceptibility and pest damage impacting on yield and quality (Pandey et al., 2017). This could influence levels of contamination with aflatoxins (AFs) and may also influence the production of

other mycotoxins produced by *A. flavus* and related species, such as cyclopiazonic acid (CPA). Immunocompromised groups, especially children could thus be exposed to toxin contaminated maize which may have serious health implications (Bennett, 2010).

Differences in mycotoxin contamination profile between GM cvs (representing pesticide, herbicide or pesticide + herbicide resistance) and their isogenic non-GM maize were found in this study. Conversely, frequency of fungal isolation was similar across the cultivars. Indeed, there was a strong trend indicating that the GM maize cvs could be a factor in reducing the contamination with different mycotoxins (e.g. fumonisins). However, because the samples of maize cvs, both GM- and non-GM, obtained were harvested and efficiently dried and stored there was no AFs present. Normally, in Brazil, AFs contamination is the major safety risk in maize production by farmers because of relatively slow or poor drying conditions and storage (Calvo et al., 2002; Sabino et al., 1989).

Understanding the potential implications of genetic traits inserted in the maize cvs in relation to *A. flavus* colonization and AF production requires further investigation. Some studies have suggested that there are lower mycotoxins in GM maize when compared with non-GM cvs with pesticide resistant genes due the reduction in insect damage which can usually provide entry points for mycotoxigenic fungal pathogens and can also act as vectors for spore inoculum. Mainly Lepidopteran species act as vectors for fungal spores as well as damaging the ripening maize kernels, allowing entry of *A. flavus* and other pathogenic spoilage moulds to infect the cobs (Alma et al., 2005). Pellegrino et al. (2018) observed mycotoxin contamination in relation to plants expressing resistance to Lepidoptera (GM *Bt*) and this suggested that all stacked *Cry*1Ab maize hybrids contained significantly less fumonisins and trichothecenes. It has been reported that when plants were infested with Southwestern corn borers, a GM (*Bt*11) hybrid had >75% reduction in aflatoxin compared with its non-*Bt* counterpart (Windham et al., 1999).

Furthermore, higher temperatures due to climate warming could create new challenges related to pest problems. More rapid insect population growth rates, increasing insect overwintering and voltinism (number of generations of an organism in a year), altered crop-pest synchrony, changing geographical ranges of important pest species (DeLucia et al., 2012; Wu et al., 2011) and abundance in agricultural systems (Miraglia et al., 2009) could influence the levels of damage in these crops. This could lead to increased damage to ripening maize during silking and allow more rapid entry for mycotoxigenic fungal pathogens, perhaps resulting in an increase in mycotoxin contamination levels. Thus, the use of GM *Bt* crops could be a strong ally under CC conditions where problems associated with insects may increase. However, there was little knowledge previously about the impact of such climatic changes on GM- vs non-GM cvs and mycotoxin contamination, especially post-harvest.

This study showed that different native toxigenic *A. flavus* strains were able to colonise and contaminate both the GM and non-GM nutritionally-based matrices with AFB_1. The environmental factors, a_w and temperature, and their interactions had a significant effect on the ecology of the strains examined. The concentration of AFB_1 was not significantly influenced by the GM or isogenic non-GM cv maize-based nutritional source. The nutritional content of each hybrid could be responsible for the distinct toxin production patterns as this may affect the metabolism of *A. flavus* strains although there were no significant changes in the colonisation patterns.

The biochemical composition of GM maize has been hypothesized as different from conventional hybrids (Bakan et al., 2002). However, from the point of view of a macro-nutritional assessment, GM maize can be considered as substantially equivalent to the parental maize line (Reuter et al., 2002). A second hypothesis links the distinct AFB_1 production by the strains perhaps being related to the fatty acid (FAs) content in maize, and this influencing pathogen susceptibility and seed colonisation of the cultivar (Dall'Asta et al., 2012) as weel as mycotoxin production (Scarpari et al., 2014). Exploring the metabolomic fingerprint, amongst

40 water-soluble metabolites, those responsible for discrimination between GM and non-GM, included ethanol, citric acid, trehalose and glycine-betaine, which were found in higher levels in the GM maize samples (Piccioni et al., 2009). Additionally, oxylipins have been linked as responsible for defence roles or promoting virulence. They may be related to specific host and fungal genes and govern the interactions between maize and *F. verticillioides* (Battilani et al., 2018). In *A. flavus*, the oxylipins play a crucial role as signals for regulating the biosynthesis of aflatoxins, conidiogenesis and the formation of sclerotia (Scarpari et al., 2014). Furthermore, no studies have reported comparisons of oxylipins in GM and non-GM cultivars and their effect on *A. flavus* development. Although this study does not explore the individual macro and micro composition of the maize hybrids it reveals the importance of understanding how the different types of maize can impact on AF content, especially subsequently during post-harvest storage.

Subsequently, native Brazilian strains from the mycobiota were screened for potential as BCAs. The colony interaction approach showed that *A. flavus* was very competitive with dominance against many other species *in vitro*. However, when paired with atoxigenic *A. flavus* strains, significant control of AFB_1 was achieved. Although the interaction scores between toxigenic (AFL^+) and atoxigenic (AFL^-) strains were mutual intermingling, with no effect on growth, the production of AFB_1 can still be influenced. The production of mycotoxins depends on a wide range of environmental, epidemiological and genetic factors, and thus perhaps growth inhibition alone may not be a good indicator of whether a reduction in mycotoxin production will be achieved (Pfliegler, Pusztahelyi and Pócsi, 2015; Medina et al., 2017).

This study considered that using a 50:50 ratio of AFL^-: AFL^+ would be the most effective approach for trying to achieve effective biocontrol. However, other studies suggested that increasing the spore inoculum of the atoxigenic strains relative to toxigenic ones would give better control of AFs production (Degola, Berni and Restivo, 2011; Pitt and Hocking, 2006) although it may not be

economically cost effective. It has also been suggested that the use of indigenous strains may also be very important to obtain effective control of AFB_1 contamination (Probst at al., 2011). The advantage of using different inoculum ratios under different environmental regimes is to obtain information on what level of inoculum of the BCA you may need for effective control. This is important when considering practical use and the economics of their use (Medina et al., 2017).

Biocontrol has been considered among the most promising technologies for sustainable agriculture (Tracy, 2014). The use of native atoxigenic *A. flavus* isolates may reduce some concerns about safety and environmental impacts (Atehnkeng et al., 2008). The effectiveness of biocontrol using atoxigenic strains is based on the fact that these are predominantly asexual, genetically stable and aggressive as competitors coupled with their inability to recombine with native toxigenic strains (Abbas et al., 2011a; Ehrlich and Cotty, 2004). Although the mechanism by which atoxigenic strains interfere with aflatoxin biosynthesis has not been definitively elucidated (Huang et al., 2011).

Furthermore, the stability of BCA over the range of environmental factors necessary for effective control is also a source of concern (Medina et al., 2017a). In the case of *A. flavus*, these concerns are typically addressed by restricting the biocontrol agents to native atoxigenic isolates on economically important food crops in the target regions (Callicott and Cotty, 2015). Atoxigenic *A. flavus* strains, especially with large deletions in the aflatoxin gene cluster, have been proposed for use in aflatoxin control as they compete for the niches occupied by toxigenic strains (Dorner, 2004; Pitt et al., 2015). A substantial number of *A. flavus* isolates have been found to contain several deletions in the aflatoxin gene cluster resulting in non-AF producers strains (Chang, Horn and Dorner, 2005). In this study, amongst the 9 atoxigenic strains tested, 5 (*Af*PHx, *Af*53H, *Af*AS1C, *Af*M20C and *Af*BMC-b) showed large deletions of the genes in the AF cluster. Moreover, based on previous studies the strain *Af*53H (AFL4-) was select to use as the candidate BCA *in situ*.

The Brazilian strain AFL4$^-$ was able to effectively control AFB_1 production when a spore ratio of 50:50 was used in stored maize at 30^{o}C for up to 20 days. The effect of AFL4$^-$ as a BCA was also verified with the gene expression studies: *afl*D and *alf*R were significantly suppressed. The use GM and non-GM maize grain resulted in differences in phenotypic aflatoxin production patterns in the presence of colonisation by the atoxigenic strain AFL4$^-$. However, the same was not observed when measuring relative gene expression of *afl*D and *alf*R. These effects require more in-depth investigation as this was the first attempt to verify the impact of conventional versus GM maize in biocontrol approaches using atoxigenic strains. Perhaps a more detailed kinetic study would be useful to address these questions.

This study also explored the effects of elevated CO_2 on AFB_1 production and the subsequent effects caused when atoxigenic biocontrol strains were used under these scenarios for reducing AFB_1 contamination in relation to non-GM and GM maize cvs for the first time. We used optimal conditions for *A. flavus* growth (30^{o}C) and +5^{o}C under atmospheric CO_2 (400 ppm) and predicted climate change scenarios (1000 ppm). The effect of increasing CO_2 at 30 and 35^{o}C varied depending on the type of maize used (GM or conventional). This was the first attempt to analyse whether using GM or conventional maize under CC scenarios can cause differences in AF production by *A. flavus*. Overall, more AFB_1 was produced when a GM cultivar was inoculated with *A. flavus* in atmospheric CO_2 levels. However, in CC conditions, the use of GM or conventional maize did not show any significant differences in AFB_1 content.

AFB_1 production by *A. flavus* can be affected by a number of interacting variables: type of cultivar, T^{o}C, CO_2 levels, water availability and presence of damaged kernels in the maize storage system. These interactions may also significantly affect BCA resilience and relative action. However, these interactions are complex and require more detailed study to identify the key parameters which determine relative toxin contamination levels and the potential for effective biocontrol under the expected CC conditions. This may also require evolution of the maize cultivars, whether they are non-GM or GM.

7.2 Final conclusions

Overall, the present project has largely been able to address the original questions posed at the begin of the research. The fungal biodiversity of 10 pairs of GM- and isogenic non-GM maize cultivars from Brazil was studied, followed by their relative mycotoxin contamination profiles for the first time. Several native atoxigenic strains of *A. flavus* were isolated and used for biocontrol of toxigenic *A. flavus* strains and AFs control. The ecology of strains of *A. flavus* in terms of growth and toxin production were shown to be similar, regardless of whether the nutritional medium was based on GM or non-GM maize cvs. The efficacy of one chosen native atoxigenic BCA candidate ($AFL4^-$ - AfP53H) was shown to significantly inhibit AFB_1 production *in vitro* and *in situ* in stored maize. In addition, the resilience of the BCA under climate change scenarios and high incidence of pest damage was largely conserved, regardless of storage conditions or levels of simulated pest damage. The use of GM- cvs based on pesticide or herbicide or pesticide + herbicide and isogenic non-GM cvs was shown to be an important factor to consider in relation to control strategies for the reduction of *A. flavus* and more importantly AF contamination of maize during post-harvest storage.

7.3 Future recommendations

Based on the findings of this work future research should focus on:

a) Exploration of the intrinsic differences in the macro- and micro- nutrients caused by insertion of genetic traits to provide insect resistance and/or herbicide tolerance using advanced metabolomic approaches
b) Sequencing the DNA of the Brazilian strain AFL4^{-} to further confirm deletions in the AF and CPA clusters to guarantee the profile of the atoxigenic strain for future larger scale trials and its use in a sustainable system in Brazil.
c) Testing other native strains with potential inclusion as a mixture with this atoxigenic strain AFL4^{-} to improve the ability to overcome the diverse range of toxigenic strains in the maize agroecosystem
d) Investigate in more depth the kinetics of impacts on the key structural and regulatory genes involved in biosynthesis of AFs and CPA.
e) Increase the pool of variations in the cvs of maize from different geographic regions in Brazil
f) Apply the BCA *in planta* using formulations, either as a spray or seed coating to control *A. flavus* in Brazilian maize agroecosystems
g) Potential relationship between CC scenarios in different maize growing regions and potential for increased contamination with AFs and ways to minimise the impact on toxin contamination

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APPENDICES

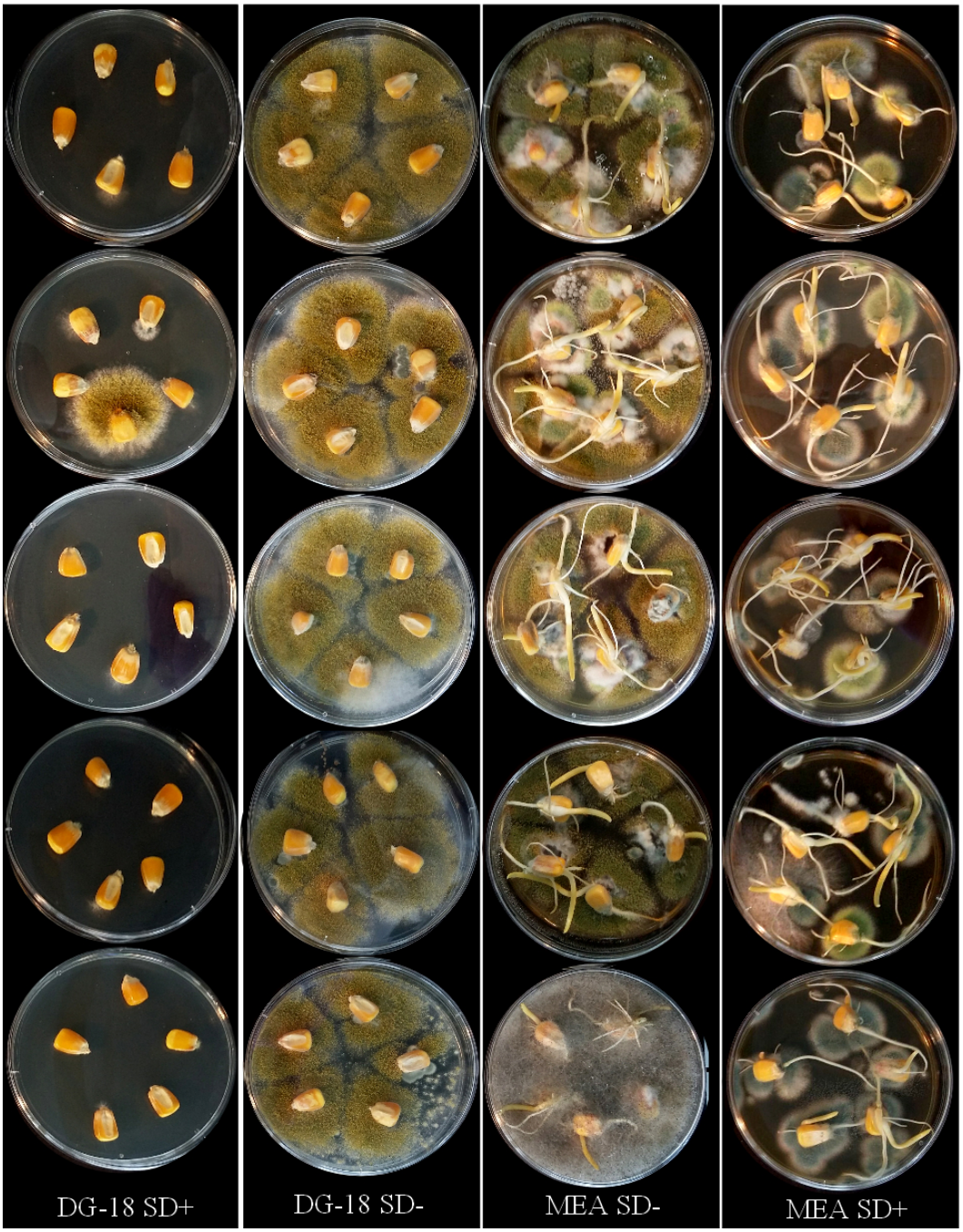
Appendix A

Appendix A-1 Characteristics of the GM and non-GM cultivars of maize grain used in this study.

Conventional cultivars	Isogenic GM line	Event name[1]	Inserted gene in the GM cvs	Traits tolerance in the GM cvs
AS 1555 CON	AS 1555 PRO®	MON89034	Cry2Ab2 Cry1A.105	IR - Lepidopteran
P30F53 CON	P30F53 H®	DAS1507×T25	PAT BLA Cry1F	HT - Glufosinate ammonium Antibiotic resistance IR - Lepidopteran
P2530 CON	P2530 Hx®	MON810	Cry1F	IR - Lepidopteran
BM-709 CON	BM-709 PRO_2®	MON89034×NK603	CP4 EPSPS Cry2Ab2 Cry1A.105	HT - Glyphosate IR - Lepidopteran
M20-A78 CON	M20-A78 PW®	NK603×TC1507×MON89034	CP4 EPSPS PAT Cry1F Cry1A.105 Cry2Ab2	HT - Glyphosate HT - Glufosinate ammonium IR - Lepidopteran
CD-384 CON	CD-384 PW®	NK603×TC1507×MON89034	CP4 EPSPS PAT Cry1F Cry1A.105 Cry2Ab2	HT - Glyphosate HT - Glufosinate ammonium IR - Lepidopteran
AS 1556 CON	AS 1556 PRO_2®	NK603×MON89034	CP4 EPSPS Cry2Ab2 Cry1A.105	HT - Glyphosate IR - Lepidopteran
none	2B587 Hx®	NK603×TC1507×MON89034	CP4 EPSPS PAT Cry1F Cry1A.105 Cry2Ab2	Glyphosate HT - Glufosinate ammonium IR - Lepidopteran
none	Hybrid YH®	MON810×TC1507	Cry1F Cry1Ab	IR - Lepidopteran
none	AG 9030 PRO_3®	NK603×MON88017×MON89034	CP4 EPSPS Cry1A.105 Cry2Ab2 Cry3Bb1	HT - Glyphosate IR - Lepidopteran
Landrace - Yellow kernel	none	-	-	-
Landrace - Round white kernel	none	-	-	-
Landrace -Red kernel	none	-	-	-

IR- insect resistance; HT – herbicide tolerance; [1] event name refers to the unique code to access the information about the trait at http://www.isaaa.org/gmapprovaldatabase/eventslist/default.asp.

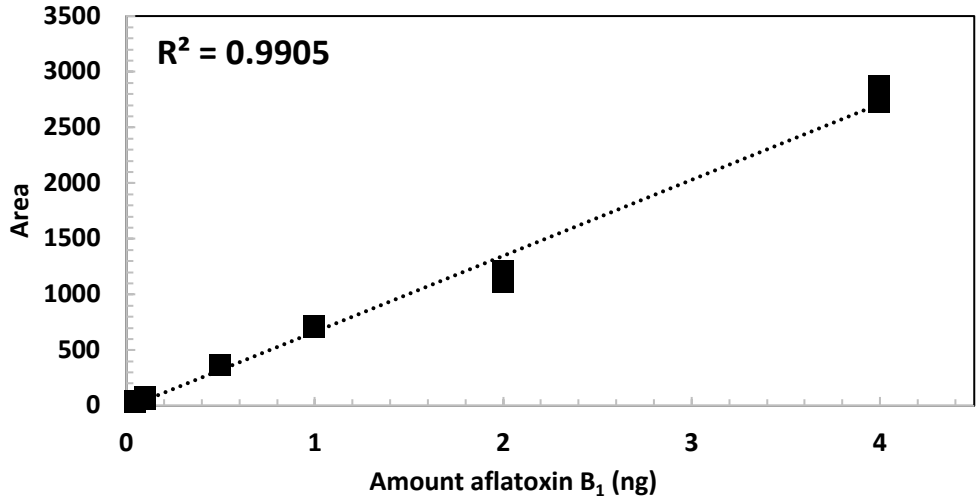
Appendix B



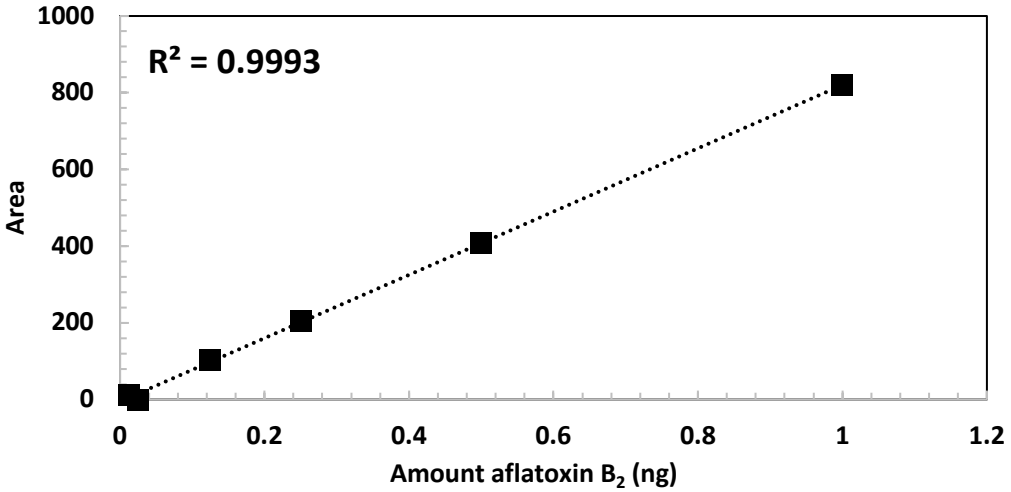
Appendix B-1 Example of Plates from direct plating on Dycloran-18%-glycerol (DG18) and malt extract agar (MEA) media with and without surface disinfection (SD+; SD-).

Appendix C

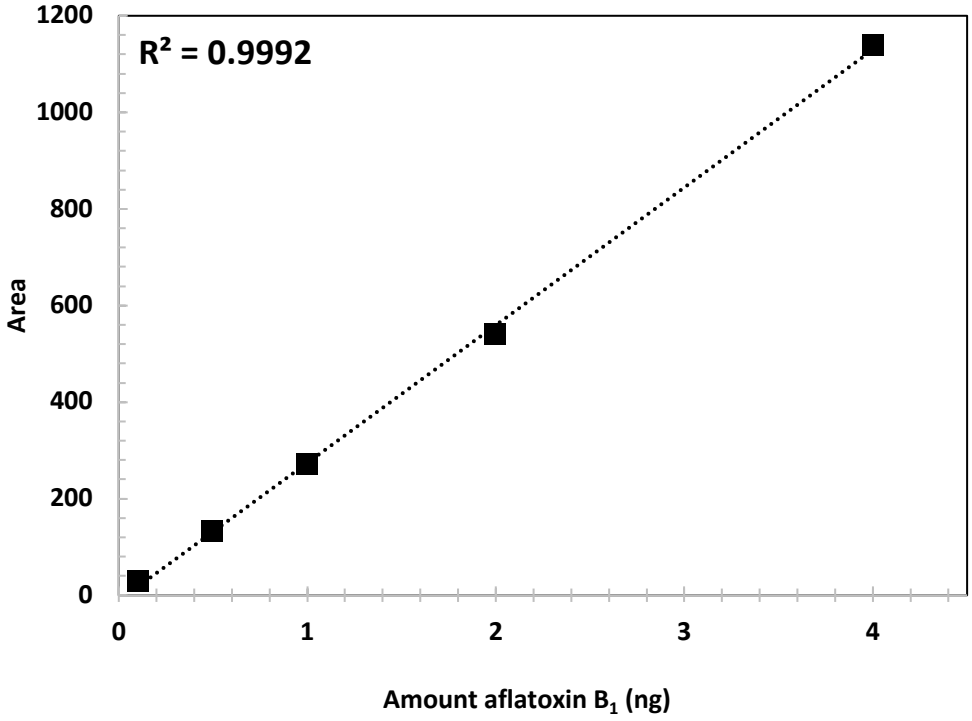
Calibration curves for aflatoxin quantification



Appendix C-1 Example of calibration curve for aflatoxin B_1 using the Agilent Poroshell® 120 EC-18, 4.6 x 100 mm, 2.7 µm particle size preceded by a Phenomenex® Gemini C18 column, 3mm, 3µm guard cartridge with manual derivatization using trifluoracetic acid.

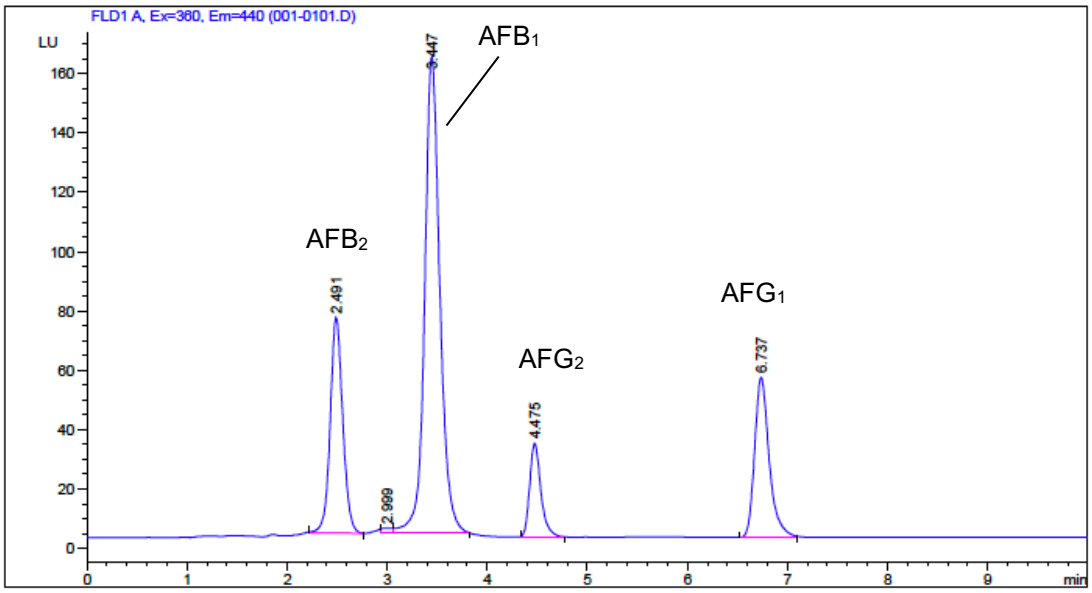


Appendix C-2 Example of calibration curve for aflatoxin B_2 using the Agilent Poroshell® 120 EC-18, 4.6 x 100 mm, 2.7 µm particle size column preceded by a Phenomenex® Gemini C18 column, 3mm, 3µm guard cartridge with manual derivatization using trifluoracetic acid.

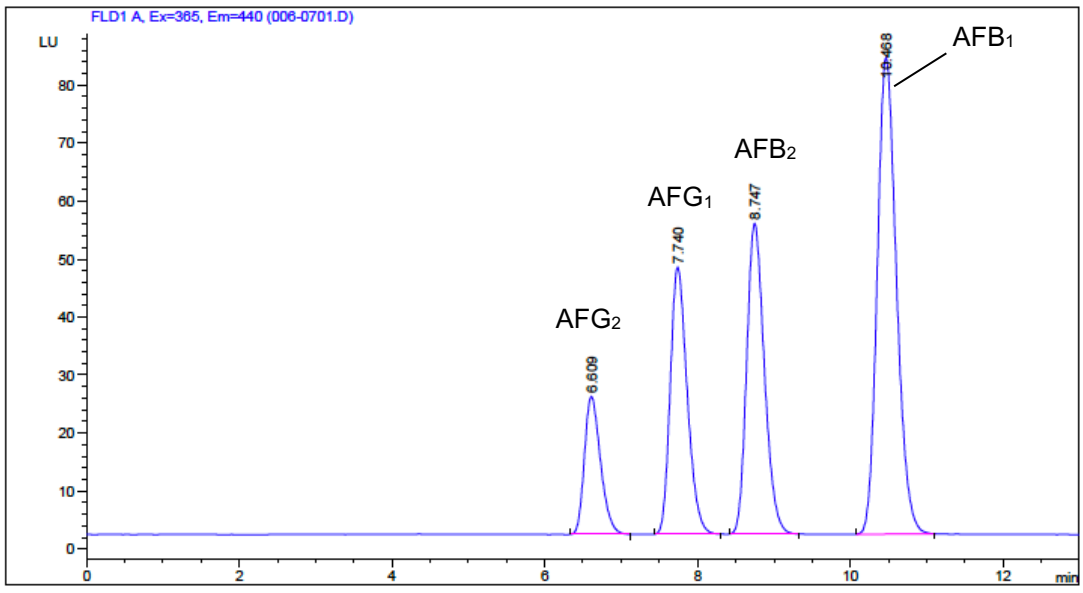


Appendix C-3 Example of calibration curve for aflatoxin B_1 using the Agilent Zorbax® Eclipse Plus, 2.1 x 100 mm, 3.5 µm particle size) column preceded by a Phenomenex® Gemini C18 guard column cartridge 3 mm x 3 µm post column derivatization with a UVE photochemical reactor with UV-Light.

HPLC chromatograms with fluorescence detector (λ_{exc} 360 nm; λ_{em} 440 nm)



Appendix C-4 Chromatogram of standard mix of aflatoxins using the Agilent Poroshell® 120 EC-18 (4.6 x 100 mm, 2.7 µm particle size0 column preceded by a Phenomenex® Gemini C18 column (3mm, 3µm particle size) guard cartridge with manual derivatization using trifluoracetic acid. Isocratic elution with methanol:water:acetonitrile (30:60:10 v/v/v) at flow rate of 1.0 mL min^{-1}.



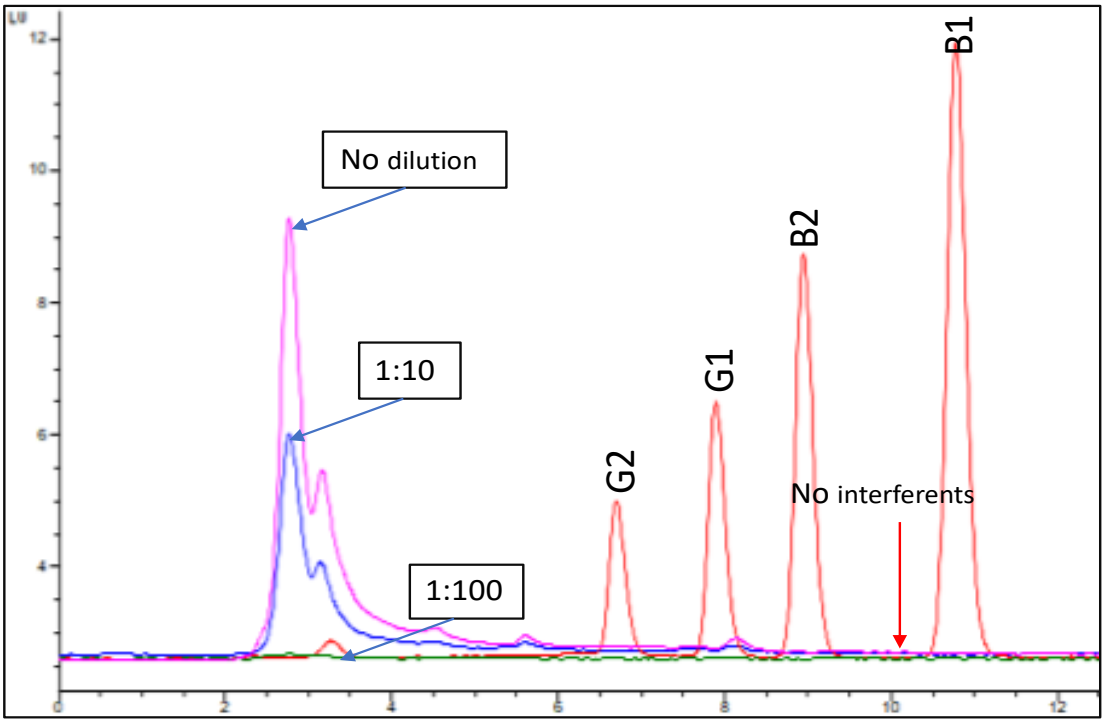
Appendix C-5 Chromatogram of standard mix of aflatoxin using the Agilent Zorbax® Eclipse Plus (2.1 x 100 mm, 3.5 µm particle size) column preceded by a Phenomenex® Gemini C18 guard column cartridge (3 mm x 3 µm particle size) with post column derivatization using UVE photochemical reactor with UV-Light. Isocratic elution with methanol: water: acetonitrile (30:60:15, v/v/v) at flow rate of 1.0 mL min^{-1}.

Validation of extraction of AF from maize

Appendix C-6 Recovery values for the validation of the method for extraction of aflatoxin B_1 (AFB_1) from maize for samples injected directly ("dilute and shoot") and samples that were cleaned-up using the immunoaffinity columns (IAC).

Solvent (v/v)	Extraction process (1h)	Sample	Spiking level – AFB_1	Recovery (%) [AV ± SD]
MeOH: H_2O (60:40)	shaker	5 g	50 ng. g^{-1}	58 ± 1.3
MeOH: H_2O (80:20) + 2 g NaCl	shaker	5 g	50 ng. g^{-1}	73.5 ± 6.0
MeOH: H_2O (80:20)	shaker	5 g	50 ng. g^{-1}	65.5 ± 9.0
Validation for "dilute and shoot" injections				
MeOH: H_2O (80:20)	Mag. Stirrer	5 g	50 ng. g^{-1}	86.6 ± 2.4
MeOH: H_2O (80:20)	Mag. Stirrer	2g	50 ng. g^{-1}	83.3 ± 1.1
Validation with clean-up using IAC				
MeOH: H_2O (80:20)	Mag. Stirrer	5 g	50 ng. g^{-1}	81.7 ± 1.3
MeOH: H_2O (80:20)	Mag. Stirrer	2g	50 ng. g^{-1}	84.6 ± 3.2

MeOH – methanol; H_2O – water; Mag. Stirrer – magnetic stirrer; NaCl – sodium chloride. AV of n=5 each condition.



Appendix C-7 Chromatograms of injection of standard of mix of aflatoxin B_1 + B_2 + G_1 + G_2 (red line, concentration 50 $ng.g^{-1}$) paired with injection of blank extracts for the "dilute and shoot" method with no dilution of the extract, 1:10 and 1:100 dilution in mobile phase (methanol: water: acetonitrile (30:60:15, v/v/v)).

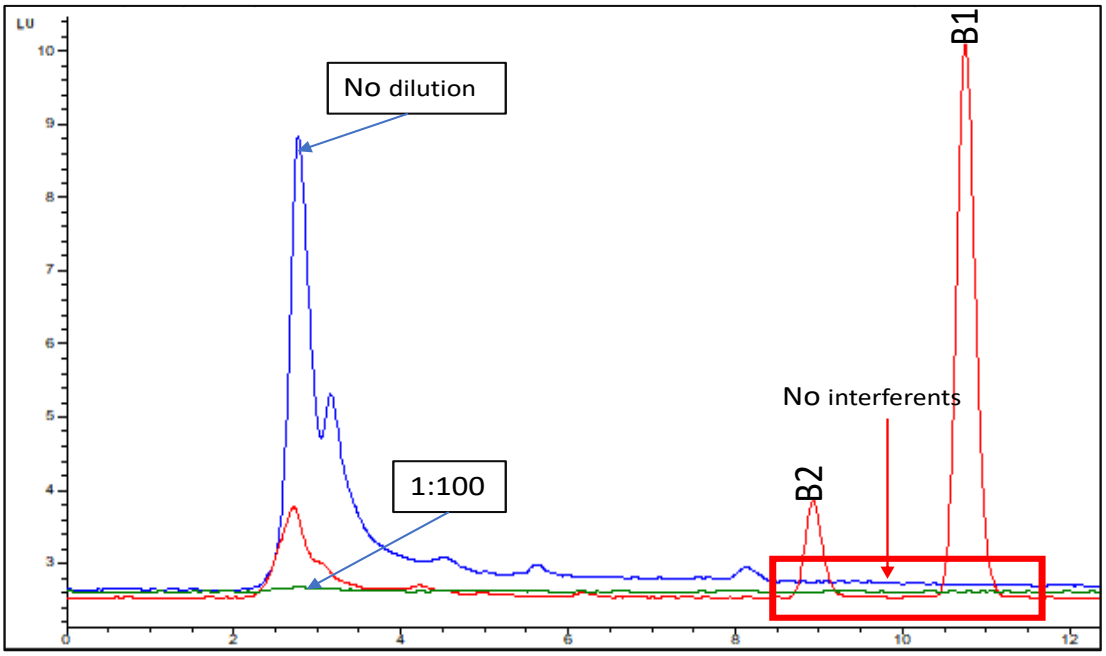
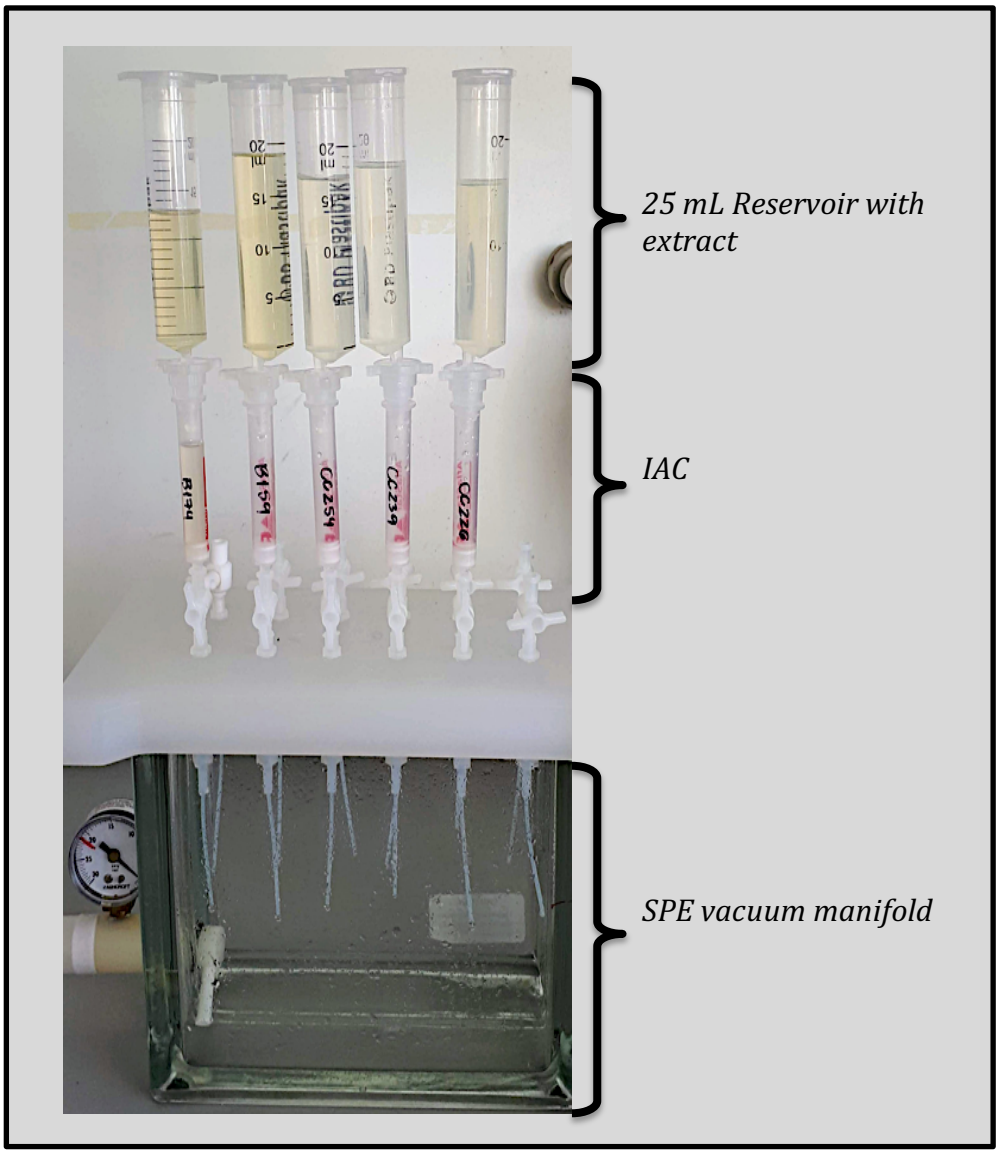
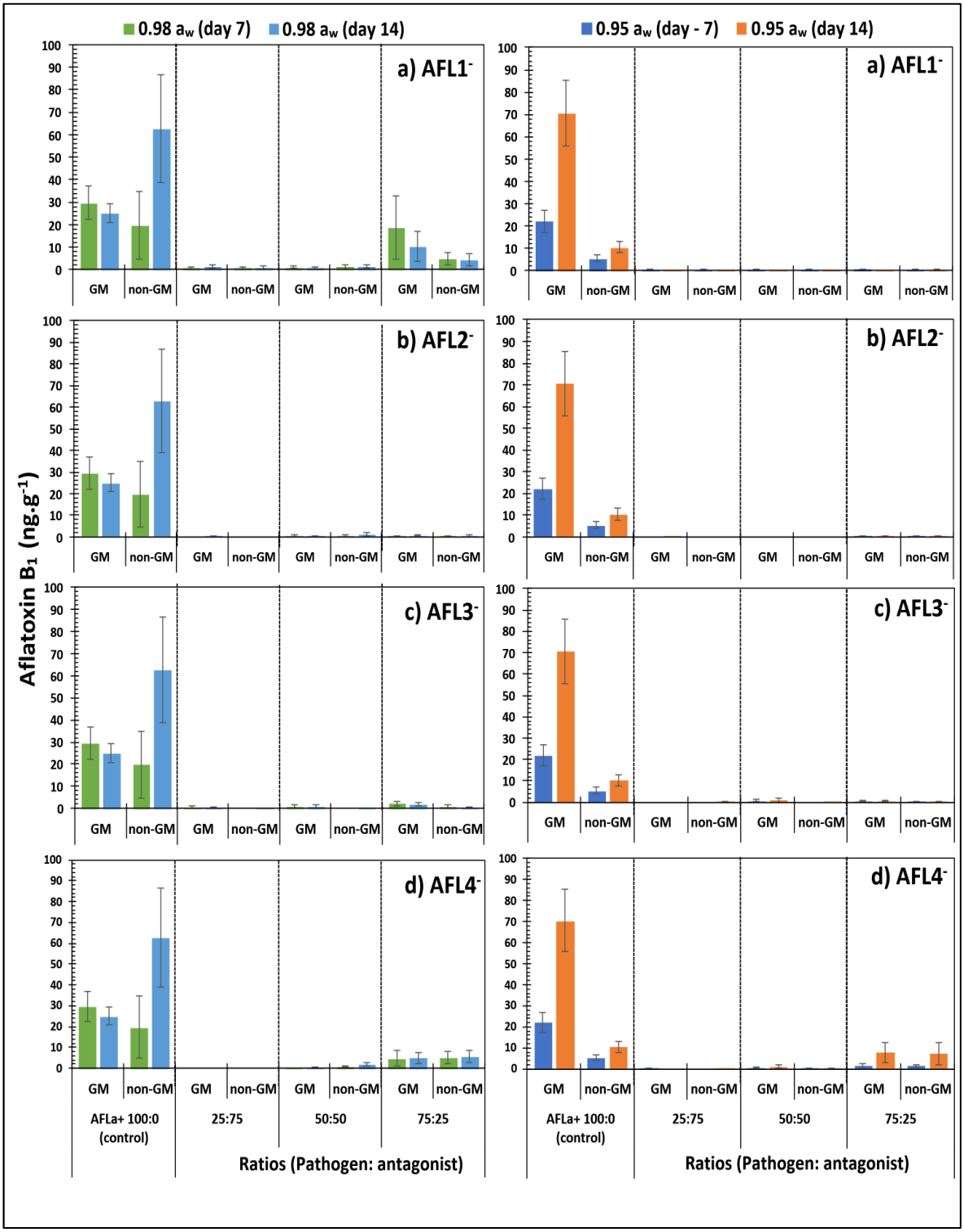


Figure C-8 Chromatograms of injection of a control sample (highly contaminated diluted at 1:100 - red) paired with injection of a blank extract for "dilute and shoot" method. Blue line: blank with no dilution; green line: blank diluted 1:100 in mobile phase (methanol: water: acetonitrile (30:60:15, v/v/v)).

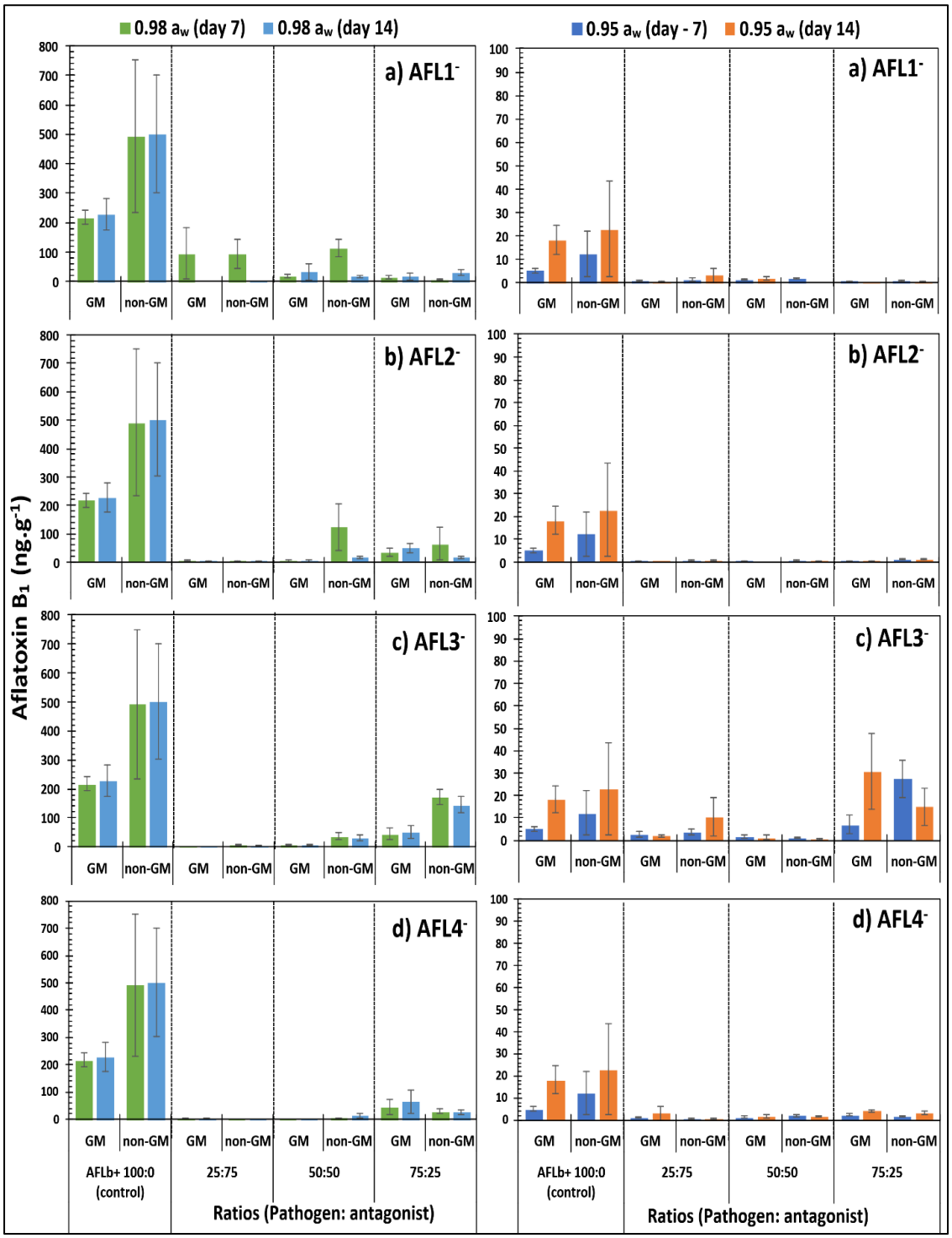


Appendix C-9 Assembly of the IAC attached to the 25 mL reservoir in a SPE vacuum manifold for clean-up of samples for AFB_1 quantification.

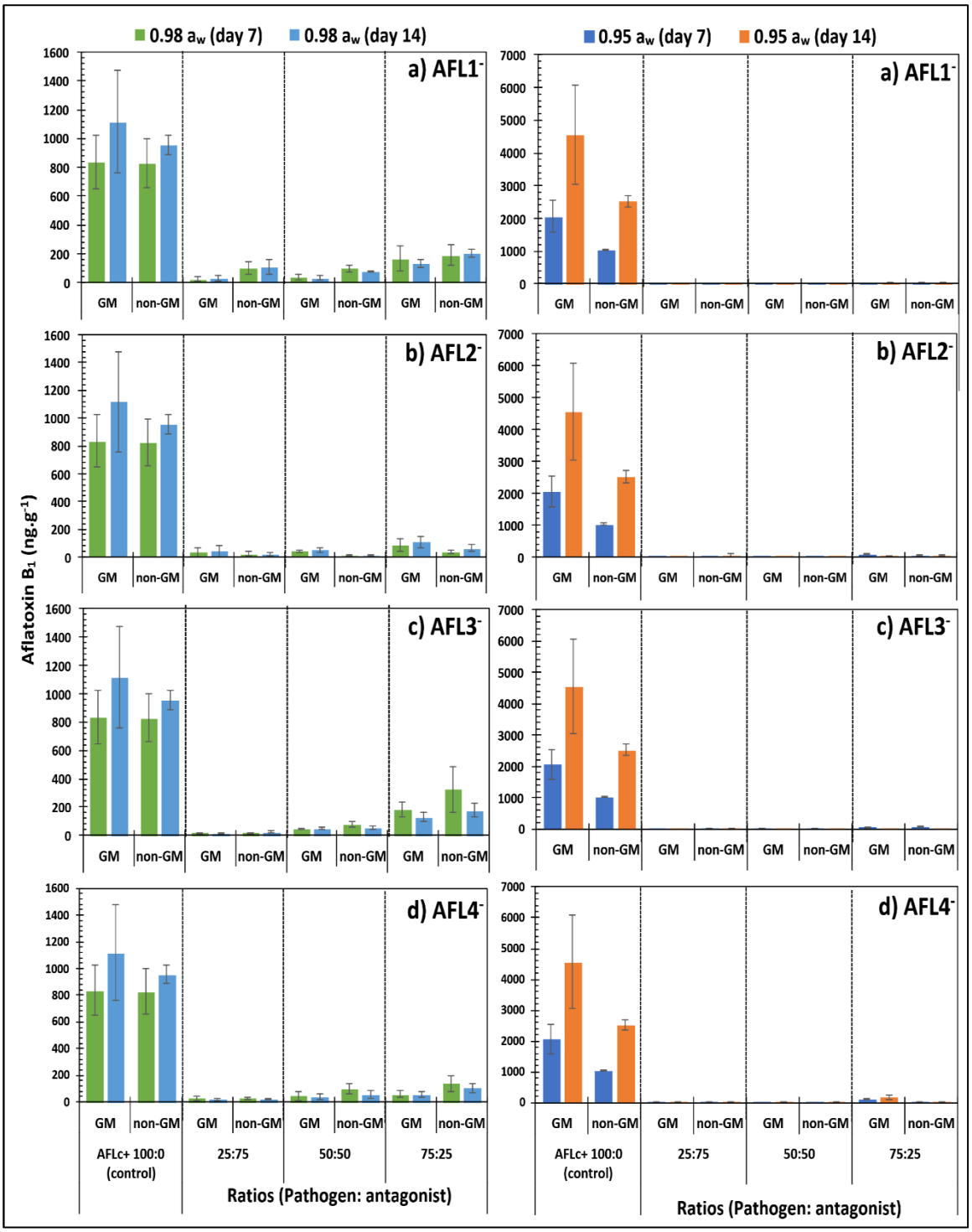
Appendix D



Appendix D-1 Aflatoxin B_1 (AFB_1) production for the interaction pathogen: antagonist in different ratios of inoculum using GM and non-GM maize as media substrates and modified water availability (0.98 and 0.95 aw) after 7 and 14 days incubation at 30°C. AFLa+ (pathogen) - *A. flavus* strain producer of AFB1; AFL1-, AFL2-, AFL3- and AFL4- - A. flavus atoxigenic strains (antagonist). The ratio 100:0 indicates the control of the pathogen. No AFB_1 production was detected (<LOD=0.1 ng. g^{-1}) for the ratio 0:100 – control of the antagonist.

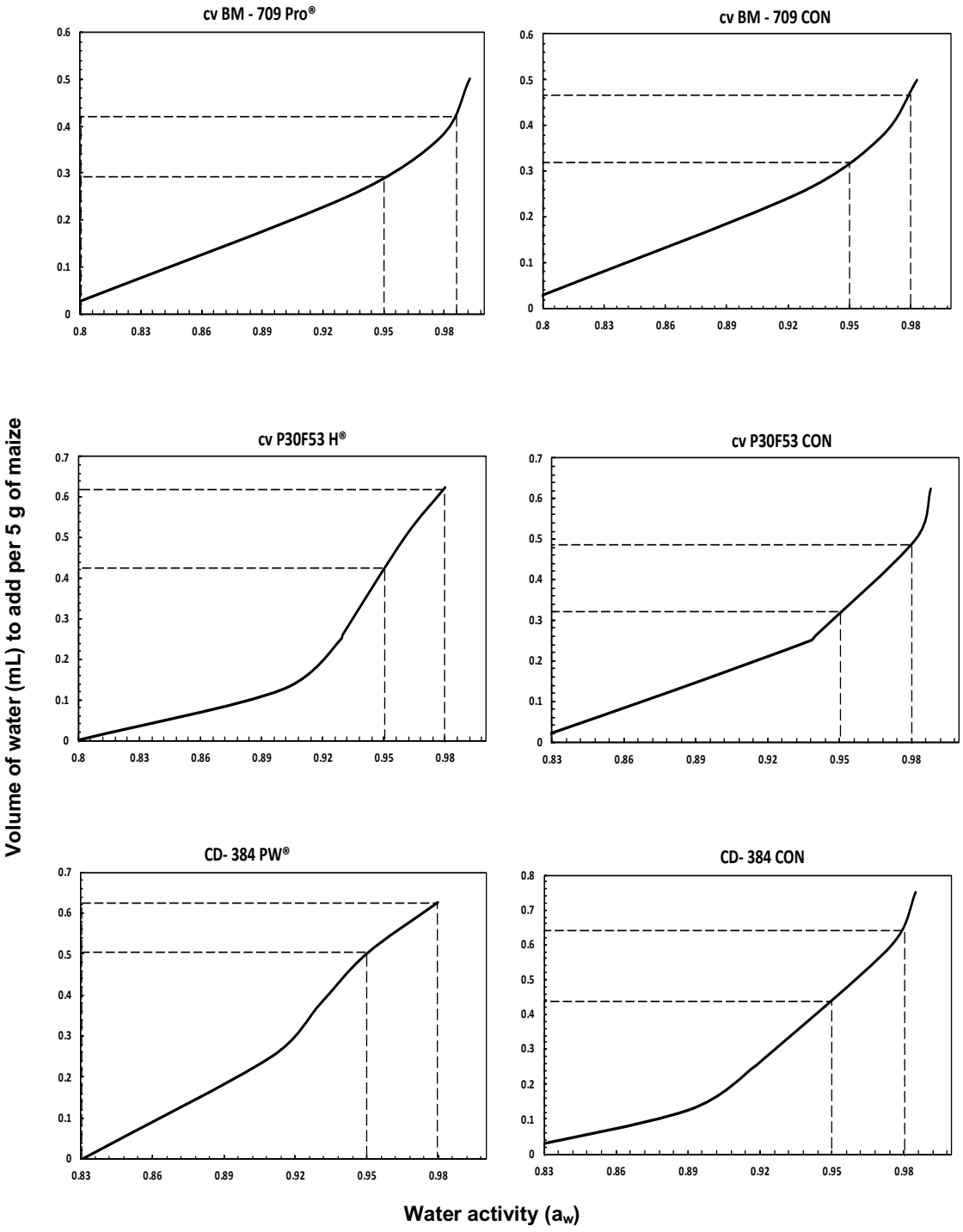


Appendix D-2 Aflatoxin B_1 (AFB_1) production for the interaction pathogen: antagonist in different ratios of inoculum using GM and non-GM maize as media substrates and modified water availability (0.98 and 0.95 aw) after 7 and 14 days incubation at 30°C. AFLb+ (pathogen) - *A. flavus* strain producer of AFB1; AFL1-, AFL2-, AFL3- and AFL4- *A. flavus* atoxigenic strains (antagonist). The ratio 100:0 indicates the control of the pathogen. No AFB_1 production was detected (<LOD=0.1 ng. g^{-1}) for the ratio 0:100 – control of the antagonist.



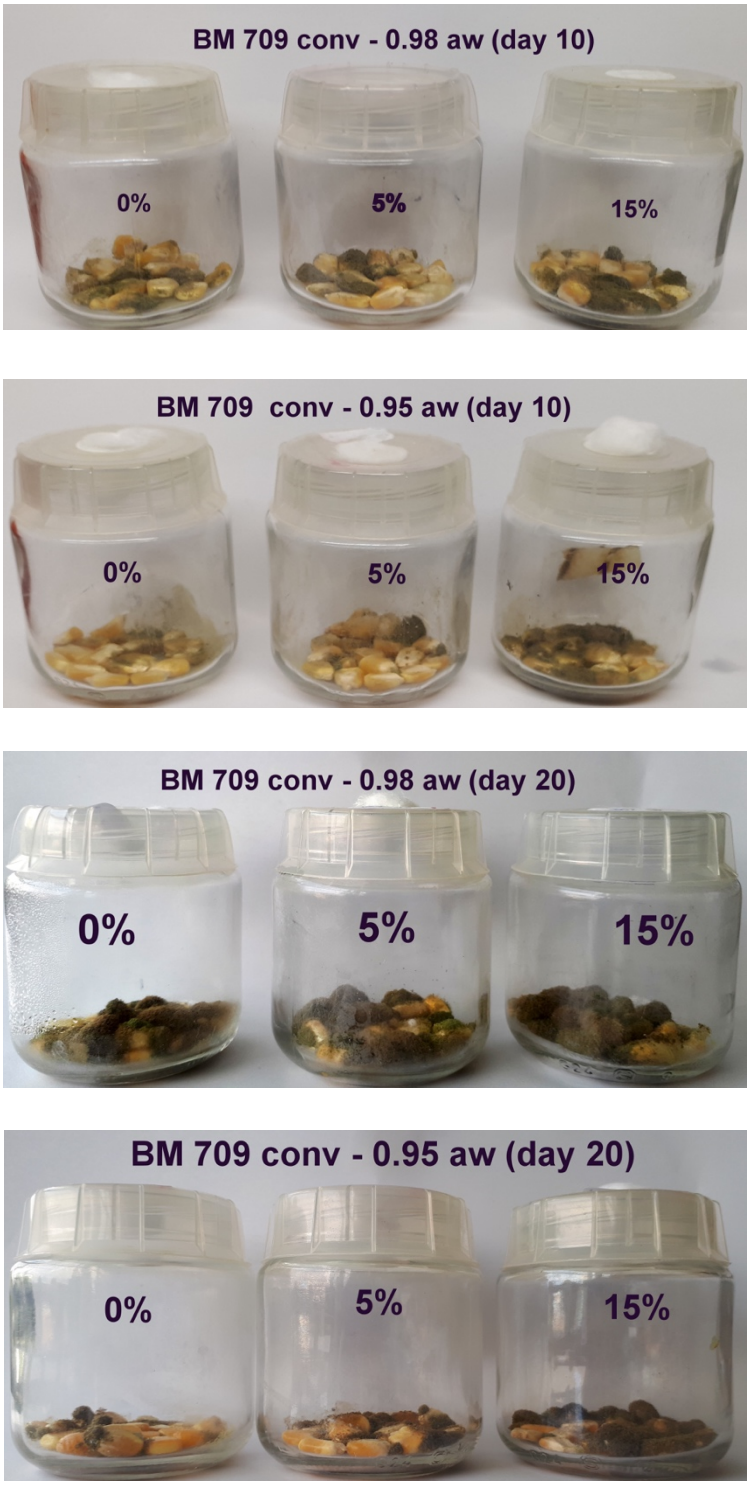
Appendix D-3 Aflatoxin B_1 (AFB_1) production for the interaction pathogen: antagonist in different ratios of inoculum using GM and non-GM maize as media substrates and modified water availability (0.98 and 0.95 aw) after 7 and 14 days incubation at 30°C. AFLc+ (pathogen) - *A. flavus* strain producer of AFB1; AFL1-, AFL2-, AFL3- and AFL4- *A. flavus* atoxigenic strains (antagonist). The ratio 100:0 indicates the control of the pathogen. No AFB_1 production was detected (<LOD=0.1 ng. g^{-1}) for the ratio 0:100 – control of the antagonist.

Appendix E



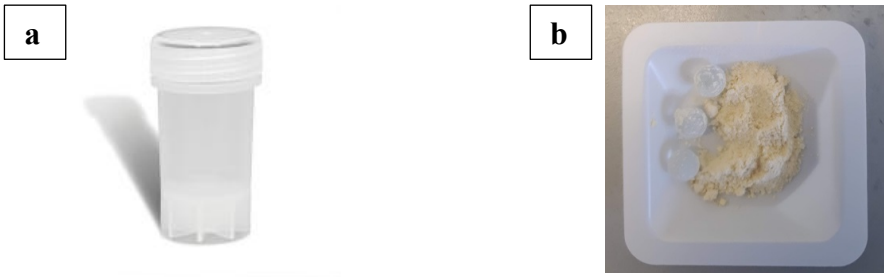
Appendix E-1 Absorption curves of main cv of maize used in this work.

Appendix F

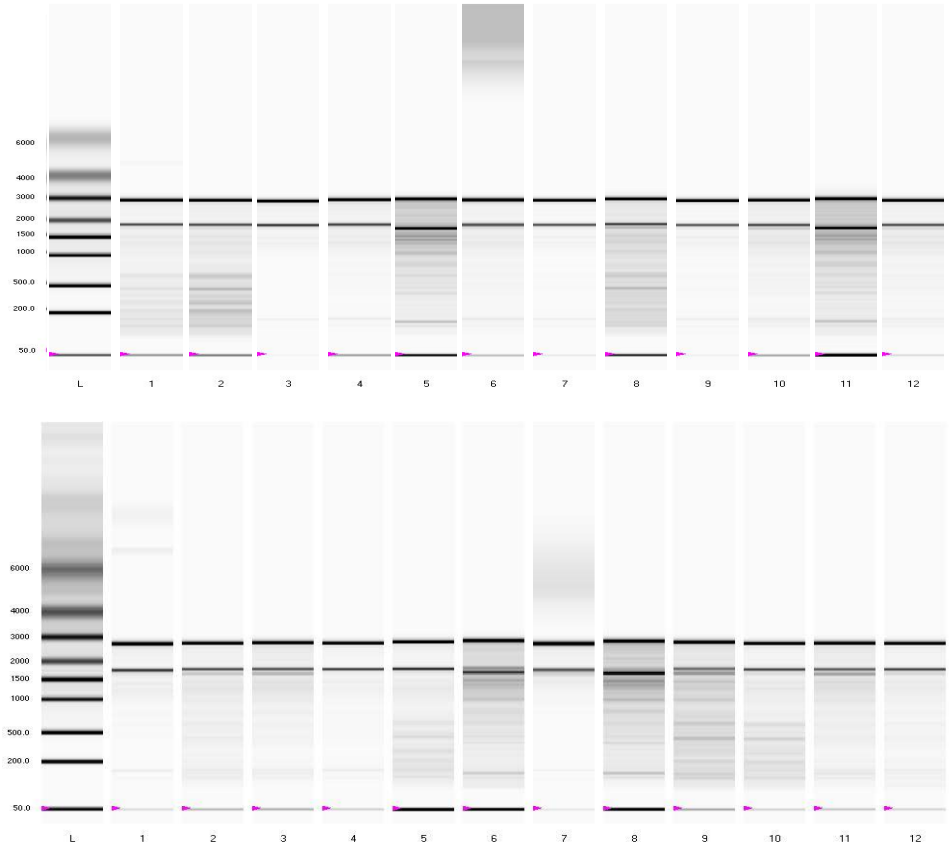


Appendix F-1 Pest damage simulation (0, 5 and 15%) in kernels of the non-GM cultivar BM-709 CON at 0.98 and 0.95 a_w after 10 and 20 days incubated at 30°C

Appendix G



Appendix G-1 (a) Reinforced tube used for gridding the kernels to a (b) fine powered prior RNA extraction.



Appendix G-2 Examples virtual gel of RNA quality extract from maize kernels used in the RT-qPCR obtained using Experion™ RNA StdSens Chips in the automated electrophoresis Experion™ system (Bio-Rad, California, USA). The first lane (L) is the ladder. Lanes 1 to 12 refer to samples. Samples show good quality (RQI ≥7.0) and no degradation of bands.

Appendix H

Appendix H-1 Recovery (%) for the Identification of mycotoxins and related compounds in the maize cultivars by LC-MS/MS performed by Dr. Michael Sulyok in BOKU, Tulln, Austria using aQTrap 5500 LC-MS/MS System (Applied Biosystems, Foster City, CA).

	Mycotoxins	% recovery
Fusarium metabolites	Fumonisin B1	75.0
	Fumonisin B2	75.0
	Fumonisin B3	79.0
	Fumonisin B4	75.0
	hydrolysed Fumonisin B1	104.0
	Fusarin C	83.0
	Bikaverin	70.3
	Beauvericin	100.1
	Fusaric acid	77.7
	Fusarinolic acid	100.0
	Equisetin	180.0
Alternaria metabolites	Alternariol	80.0
	Alternariolmethylether	106.0
Penicillium metabolites	Berkedrimane B	79.0
	Chrodrimanin	95.5
	Demethylsulochrin	264.0
	Penicillide	71.3
	Pinselin	81.0
	Purpactin A	93.5
	Questiomycin A	84.9
	Rugulovasine A	38.0
other species	Dehydroaustinol	70.0
unspecific metabolites	Asperglaucide	91.0
	Asperphenamate	131.0
	cyclo(L-Pro-L-Tyr)	90.7
	Emodin	90.1
	Iso-Rhodoptilometrin	114.0
	N-Benzoyl-Phenylalanine	80.5
	Tryptophol	76.2

Appendix I

Appendix I-1 Poster presented at International Commission on Food Mycology Workshop 2016, Freising, Germany – June 2016.

Fungal biodiversity of Brazilian GM and non-GM maize

Alessandra Marcon Gasperini, Angel Medina and Naresh Magan

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

Cranfield UNIVERSITY

ICFM International Commission of Food Mycology

Introduction

Maize is at the centre of global food security as one of the most important cereal crops in diets worldwide.

GMOs? Genetically modified (GM) organisms are plants or animals that have been modified by the addition of a small amount of genetic traits from other organisms through molecular techniques. The traits are able to provide different characteristics.

Maize in Brazil represents an important economic and social product in both family farming and agribusiness. Brazil is the 2nd largest GM maize producer with an adoption of 82%.

Maize is prone to infection by *Aspergillus flavus* and contamination with **AFLATOXINS** during ripening and poor post-harvest storage.

There is little information on the correlation between GM crops, fungal contamination and levels of AFLATOXIN contamination.

The objectives of the present work was to study (a) the fungal diversity in GM/non-GM maize cultivars from Brazil; (b) the ecology of growth and aflatoxin B_1 production by *A.flavus* strains.

Methods & Materials

- **Samples**: 7 GM and non-GM maize cultivars from Brazil were used in this study
- **Water activity (a_w) & Moisture content (MC w/w %):** Subsamples were checked for a_w using a Aqualab 3TE and the MC by oven drying at 120ºC overnight.
- **Fungal biodiversity:** direct plating and serial dilution techniques were used and incubation at 25ºC for 5-7 days.
  - *Direct plating:* 100 grains of each sample were direct plated in malt extract agar (MEA) and dichloran (18%) glycerol agar (DG18) – ½ of the grains were surface disinfected in sodium hypochlorite 4%. The frequency of isolation of fungal species was determined. Strains of *A. flavus* were isolated and the AFB_1 production evaluated.
  - *Serial dilution:* 10 g of maize were soaked in 90 ml sterile water for 3h. The sample was then homogenised in Stomacher 400 for 5 min. After serial dilution (10^{-2} to 10^{-4}) then 0.1 mL were spread plated on MEA and DG18 for fungal populations and on nutrient agar (NA) for bacteria.
- **Ecology of *A. flavus* strains:** 2 strains of *A.flavus* were select and grown on milled maize media from 3 GM and 3 non-GM related maize cultivars) where the aw was modified with glycerol to 0.99 and 0.95 a_w. The plates were incubated at 30 & 25ºC. The diameter of growing colonies were measure over 10 days. Agar plugs (5 x 4 mm) of each replicate and treatment were collect for AFB1 analysis.
- **Aflatoxin B_1 analysis: samples were** extracted with chloroform, followed by derivatization and quantification using HPLC-FLD.

Results

Table 1. Samples of maize used in this work and its characteristics.

	Sample	Cultivar	Traits
Genetically modified	GM -1	Hybrid YH	Bt
	GM -2	AS 1555 PRO[a]	Bt
	GM -3	P2530 Hx[b]	Bt
	GM -4	2B587 Hx	Bt & HT
	GM -5	AS 1556 PRO2	Bt & HT
	GM -6	P30F53 H[c]	Bt & HT
	GM -7	AG 9030 PRO3	Bt & HT
Non-Genetically modified	non GM -1	AS1555[a]	Convencional Hybrid
	non GM -2	P30F53[c]	Convencional Hybrid
	non GM -3	P2530[b]	Convencional Hybrid
	non GM -4	AS1656	Convencional Hybrid
	non GM -5	Yellow grain	Landrace
	non GM -6	White grain	Landrace
	non GM -9	Red grain	Landrace

Bt: cultivar with trait for insect resistance/ HT: cultivar with trait for herbicide tolerance. Same letter indicate the same cultivar with and without the GM trait.

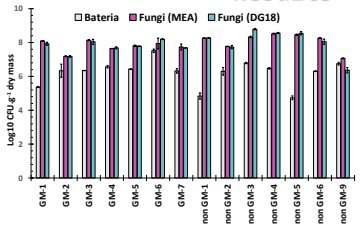


Figure 1: Colony unit forming (Log10(2×CFU÷1).g^{-1}) values of the maize; bars indicate SEs.

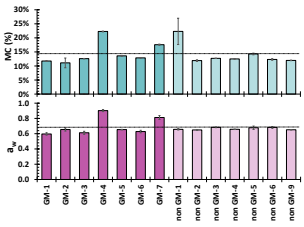


Figure 2: Values of water activity (a_w) and moisture content (MC; %) of the maize samples; bars+ S.E.s

Table 2. Aflatoxin B_1 (AFB_1) production by the *A. flavus* strains isolated from the maize samples.

Strain	AFB_1
*Af*GM-1a	+
*Af*GM-1b	+
*Af*GM-3	-
*Afn*GM-1a	-
*Afn*GM-1b	-
*Afn*GM-2a	+
*Afn*GM-2b	+
*Afn*GM-4	+
*Afn*GM-9a	+
*Afn*GM-9b	+

+ AFB_1 producer/ - AFB_1 non producer

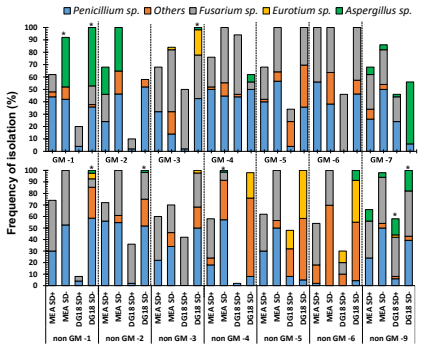


Figure 3. Fungal infection (%) in maize and the identified species. In the samples marked (*) *A. flavus* was present and isolated. "Others" sp. include: *Rhyzopus, Epicocum, Cladosporium, Mucor, Alternaria, Wallemia* and *Trhichoderma.* SD* surface disinfected/ SD- non surface disinfected.

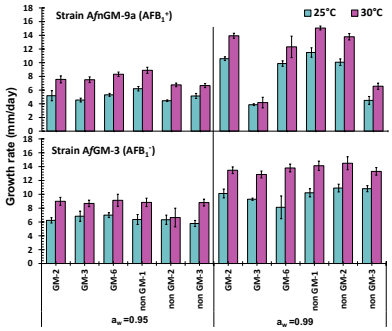


Figure 4. Comparison of the relative growth of the toxigenic and atoxigenic strain of *A. flavus* at different temperatures and a_w levels on three different GM and non-GM milled maize media. Bars=S.Es.

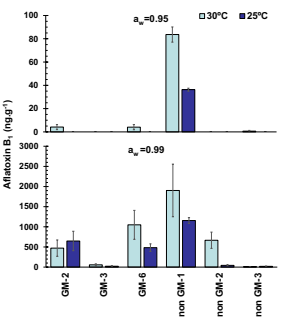


Figure 5. AFB_1 production by the strain AfnGM-9a in MMEA from different maize samples at different temperatures and a_w levels. Bars=SEs

Conclusions

- The GM/non-GM maize samples had a high contamination with populations of fungi supported by both the serial dilution and the frequency of isolation from the samples. With the exception of two maize samples the a_w/MC of the samples were close to the level for safe storage (=0.70 a_w). Overall, the isolation of *A. flavus strains* was higher in the GM maize samples..
- Using GM and non-GM maize as a nutritional substrate for colonisation by strains of *A. flavus showed that both a toxigenic and non-toxigenic strain colonised both types of maize-based matrices.* The production of AFB_1 by a toxigenic strain on the different GM/non-GM maize matrices suggests that for this strain more AFB_1 is produced on non-GM substrates than on GM substrates at both 0.95 and 0.99 a_w.
- Studies are in progress on the ecology of *A. flavus* strains on GM and non-GM cultivars, on the interactions between toxigenic *A. flavus* strains and other fungal colonists to identify potential antagonists and on the impact of climate change parameters on AFB_1 contamination of GM and non-GM maize cultivars.

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bms British Mycological Society promoting fungal science

65 anos CAPES

This research was supported by CAPES Foundation, Ministry of Education of Brazil – Project BEX 12937/13-4.

Appendix I-2 Poster presented at Focused Meeting 2016: The Dynamic Fungus – Microbiology Society, Exeter, UK- September 2016

Ecology of *Aspergillus flavus strains* on GM and non-GM maize-based media

Alessandra M. Gasperini, Angel Medina and Naresh Magan

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Introduction

MAIZE is an important commodity worldwide and can be infected by fungi during the growing season, harvest and storage.

As adoption of genetically modified (GM) crops continues to increase on a global scale, no studies have compared the influence of GM maize with either herbicide-tolerance or herbicide-tolerance + insect-resistance colonisation by *Aspergillus flavus* and aflatoxin contamination, in relation to non-GM maize cultivars.

OBJECTIVE: to study the ecology of *A. flavus* strains on GM- and non-GM based maize media to examine the impact on growth and aflatoxin B_1 (AFB_1) production.

Materials and Methods

- Strains: *A. flavus* strains were from GM- and non-GM maize: strains *fn*GM-9a, *Afn*GM-1a and *Afn*GM-2a were toxigenic. The *Af*GM-3 was atoxigenic.
- Ecology of *A. flavus*: The strains of *A. flavus* were grown on a 3% milled maize medium made from 3 GM and 3 non-GM related maize cultivars. The water activity (a_w) was modified with glycerol to 0.90, 0.95 and 0.99 and treatments incubated at 25, 30 and 35°C. The temporal mycelial extension rates of colonies were measured over 10 days and the growth rates determined. Agar plugs (5 x 4 mm) of each replicate and treatment were then collected for AFB_1 analysis.
- Aflatoxin B_1 analysis: samples were extracted with chloroform, followed by derivatization and quantification using HPLC – FLD.

Results

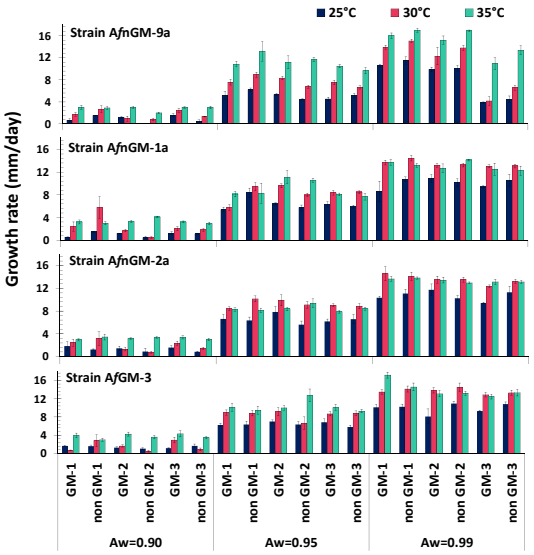

Figure 1: Comparison of the relative growth of the toxigenic and atoxigenic strains of *A. flavus* at different temperatures and a_w levels on three different GM and non-GM milled maize media. Bars=S.Es.

Table 1. Samples of maize used in this work and its characteristics.

Sample	Cultivar	Traits
GM -1	AS 1555 PRO[a]	Bt
GM -2	P30F53 H[c]	Bt & HT
GM -3	P2530 Hx[b]	Bt
non GM -1	AS1555[a]	Convencional Hybrid
non GM -2	P30F53[c]	Convencional Hybrid
non GM -3	P2530[b]	Convencional Hybrid

Bt: cultivar with trait for insect resistance/ HT: cultivar with trait for herbicide tolerance. GM – Genetically modified/ non-GM – non genetically modified

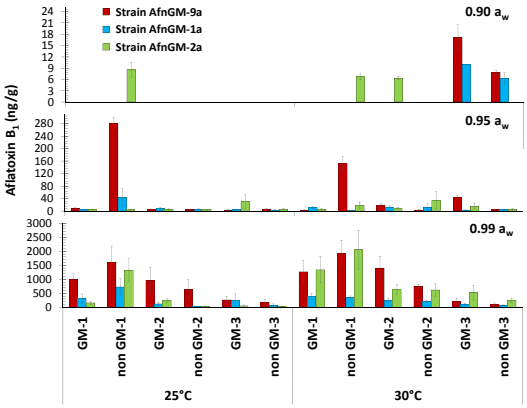

Figure 2: AFB_1 production by strains of *A. flavus* at different temperatures and a_w levels on three different GM and non-GM in MMEA. Bars=S.Es.

Conclusions

- Using GM and non-GM maize as nutritional substrate for colonisation by strains of *A. flavus* showed that both a toxigenic and non-toxigenic strain colonised both types of maize-based matrices at all levels of temperature and a_w.
- The production of AFB_1 on the different GM/non-GM maize matrices varieties in relation to strains and substrate. For strains *Afn*GM-9a and *Afn*GM-1a more AFB_1 was produced on non-GM substrates than on GM substrates at both 0.95 and 0.99 a_w. While the strain *Afn*GM-2a showed less AFB_1 production in all substrates.
- Studies are in progress on the interactions between toxigenic *A. flavus* strains and other fungal colonists to identify potential antagonists and on the impact of climate change parameters on AFB_1 contamination of GM and non-GM maize cultivars.

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This research was supported by CAPES Foundation, Ministry of Education of Brazil – Project BEX 12937/13-4.

Appendix I-3 Poster presented at SfAM- ECS Research Symposium, University of Westminster (Marylebone Campus), London, UK - 19th April 2017

Fungal biodiversity and ecology of *Aspergillus flavus* in Brazilian GM and non-GM maize

Alessandra M. Gasperini, Angel V. Medina and Naresh Magan

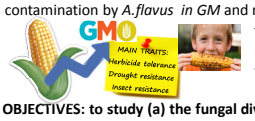
Applied Mycology Group, Environmental and AgriFood Theme, Cranfield University, Bedford, U.K

INTRODUCTION

Maize is prone to infection by *Aspergillus flavus* and contamination with the mycotoxins AFLATOXINS, both pre- and post-harvest.

Aflatoxins are class 1a carcinogens and of greatest concern worldwide.

However, while the adoption of genetically modified (GM) crops continues to increase on a global scale, few studies have compared colonisation and toxin contamination by *A.flavus in GM* and non-GM maize.

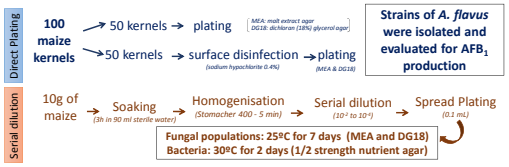


This will have implications for the relative levels of contamination on GM maize used for human consumption.

OBJECTIVES: to study (a) the fungal diversity in different cultivars of GM and non-GM maize from Brazil, and (b) examine the ecology of isolated strains of *A.flavus* on GM- and non-GM based maize matrices on growth and aflatoxin B_1 (AFB_1) production.

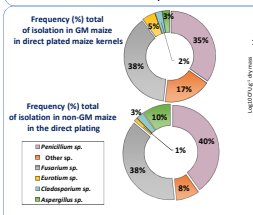
METHODOLOGY

- **Fungal biodiversity:** direct plating and serial dilution techniques were used and incubation at 25ºC for 5-7 days.



- **Ecology of *A. flavus*:** 4 strains of *A.flavus* isolated from GM/non-GM maize were select and grown on milled maize media from 3 GM and 3 non-GM related maize cultivars where the a_w was modified with glycerol to 0.90, 0.95 and 0.99 and incubated at 35, 30 and 25°C. The diameter of growing colonies were measure over 10 days. Agar plugs of each replicate and treatment were used to quantify AFB_1 production.
- **Strains:** The isolated strains *Af*nGM-7a, *Af*nGM-1a and *Af*nGM-2a were toxigenic. The *Af*GM-3 was atoxigenic.
- **Aflatoxin B_1 analysis:** samples were extracted with chloroform, followed by derivatization and quantification using HPLC – FLD.

RESULTS



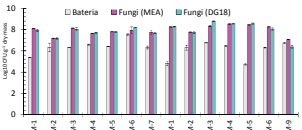


Figure 1: Colony unit forming ($Log10_{(2\times CFU\cdot L)}\cdot g^{-1}$) values of the maize samples used in this study; bars indicate SEs.

Table 1. Aflatoxin B_1 (AFB_1) production by the *A.flavus* strains isolated from the maize samples.

Strain	AFB_1
*Af*GM-1a	+
*Af*GM-1b	+
*Af*GM-3	-
*Af*nGM-1a	-
*Af*nGM-1b	-
*Af*nGM-2a	+
*Af*nGM-2b	+
*Af*nGM-4	-
*Af*nGM-7a	+
*Af*nGM-7b	+

+ AFB_1 producer/ - AFB_1 non producer

Table 2. Samples of maize used in the ecology study and its characteristics.

Sample	Cultivar	Traits
GM -1	AS 1555 PRO®	Bt
GM -2	P30F53 H©	Bt & HT
GM -3	P2530 Hx®	Bt
non GM -1	AS1555®	Convencional Hybrid
non GM -2	P30F53®	Convencional Hybrid
non GM -3	P2530®	Convencional Hybrid

Bt: cultivar with trait for insect resistance/ HT: cultivar with trait for herbicide tolerance. GM – Genetically modified/ non-GM – non genetically modified

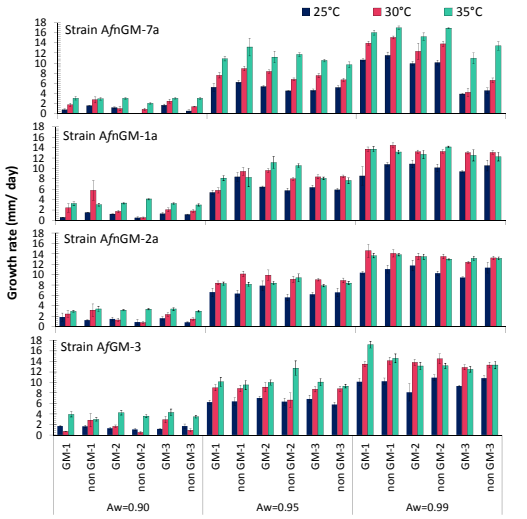


Figure 2: Comparison of the relative growth of the toxigenic and atoxigenic strains of *A. flavus* at different temperatures and a_w levels on three different GM and non-GM milled maize media. Bars=S.Es.

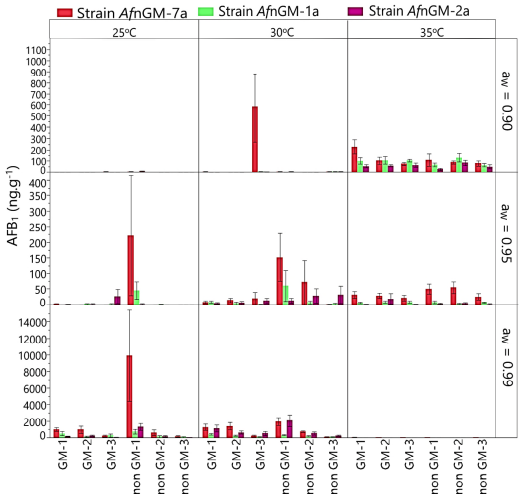


Figure 3: Comparison of the relative production of AFB_1 by toxigenic strains of *A. flavus* at different temperatures and a_w levels on three different GM and non-GM milled maize media. Bars=S.Es.

Conclusions

- Biodiversity on GM- and non-GM cultivars: frequency of isolation (%) for all samples showed high incidence of *Fusarium* and *Penicillium* sp. A total of 11 strains of *A. flavus* were isolated: 6 are AFB_1 producers, and 5 strains atoxigenic (non-producers).
- There was little difference in the ability of strains of *A.flavus* examined to colonise GM- and non-GM as nutritional substrates.
- Ecological studies showed that the growth patterns of strains tested on both types of maize-based media was similar. Optimum growth was at 0.99 a_w and 30ºC. The strain *Af*nGM-7a was a higher AFB_1 producer than the others. The production of AFB_1 for all the strains was similar at 0.90 a_w and 35ºC and 0.99 a_w and 30ºC.

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This research was supported by CAPES Foundation, Ministry of Education of Brazil – Project BEX 12937/13-4.

Appendix I-4 Poster presented at 1st MycoKey "International Conference on Global Mycotoxin Reduction in the Food and Feed Chain", Ghent, Belgium - 11-14 September 2017. WINNER OF BEST POSTER PRIZE

Potential biological control agents from strains isolated from GM and non-GM Brazilian maize for control of *Aspergillus flavus* and aflatoxin B_1 production

Alessandra M. Gasperini, Angel V. Medina and Naresh Magan

Applied Mycology Group, Environmental and AgriFood Theme, Cranfield University, Bedford, U.K

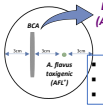
Introduction

Contamination of maize by *Aspergillus flavus* and the production of aflatoxins imposes an extensive socio-economic cost. Among the measures studied to reduce the risk of mycotoxins, **biocontrol has been considered a promising technology for sustainable agriculture**. A potential option for *A. flavus* management in the field has largely been focused on the use of atoxigenic isolates of *A. flavus* which may be able to compete with the toxigenic strains by displacing them and reducing aflatoxin B_1 (AFB_1) contamination.

OBJECTIVES: to identify potential biological control agents isolated from distinct Brazilian GM/non-GM maize cultivars for control of AFB_1 by studying (a) the interactions, competitiveness and Index of Dominance (ID) and (b) co-cultivation of different inoculum ratios of pathogen: antagonist and effects on aflatoxin B_1 production.

Methodology

➢ **Interactions, competitiveness and Index of Dominance (I_D)**



BCA (biocontrol agent) = atoxigenic A. flavus (AFL-); yeasts and other fungal species isolated from GM and non GM Brazilian maize

- Media: Malt extract agar and maize meal agar
- Water availability: 0.95 and 0.98 a_w
- Incubation: 10 days at 30°C

The Petri plates were examined periodically and each interacting species given a score according to Magan and Lacey (1984):

- *mutual intermingling (1/1)*
- *mutual antagonism on contact (2/2)*
- *mutual antagonism at a distance (3/3)*
- *dominance of one species on contact (4/0)*
- *dominance at a distance (5/0)*

➢ **Co-cultivation of different inoculum ratios of pathogen: antagonist and effects on aflatoxin B_1 production**

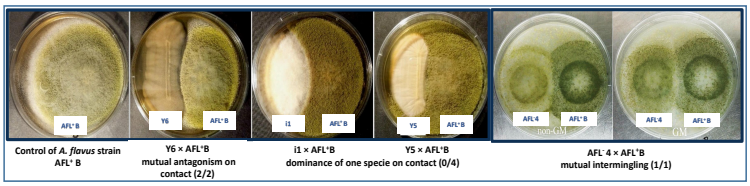
SPECIES	Volume of spore suspension [number of spores. mL^{-1}]										TOTAL (mL)
A. flavus	6ml	[1.0×10^6]	4.5ml	[7.5×10^5]	1.5ml	[5.0×10^5]	3ml	[2.5×10^5]	0ml	[0]	15
BCA	0ml	[0]	1.5ml	[2.5×10^5]	4.5ml	[5.0×10^5]	3ml	[7.5×10^5]	6ml	[1.0×10^6]	15
Final ratio	100:0		75:25		50:50		25:75		0:100		

100:0 – control *A. flavus* toxigenic / 0:100 – control of Biocontrol agent (BCA)

- Media: Maize meal agar (cultivar GM (P30F53H) and the respective isogenic non-GM (P30F53)
- Water availability: 0.95 and 0.98 a_w
- Incubation: 14 days at 30°C

AFB_1 analysis by HPLC-FLD= 5 agar plugs collect on day 7 and 14

Results



Control of *A. flavus* strain AFL+ B | Y6 × AFL+B mutual antagonism on contact (2/2) | i1 × AFL+B dominance of one specie on contact (4/0) | Y5 × AFL+B dominance of one specie on contact (4/0) | AFL-4 × AFL+B mutual intermingling (1/1)

The interaction of toxigenic and atoxigenic *A. flavus* strains and other candidate species isolated from maize on relative competitiveness under different water availabilities (0.98 and 0.95 a_w) at 30°C showed that *A. flavus* was largely dominant against other species tested and there was no inhibition of growth when paired with atoxigenic *A. flavus* strains.

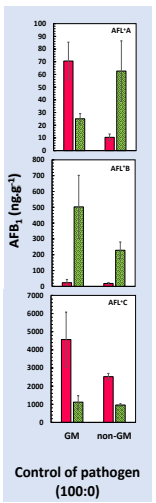


Figure 1: AFB_1 production of the toxigenic *A. flavus strains* A, B and C using GM and non-GM maize as media substrate and modified water availability (0.98 and 0.95 aw) on 14th day of incubation at 30°C.

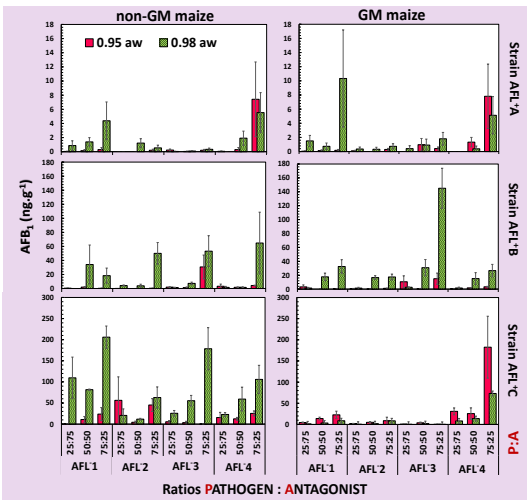


Figure 2: AFB_1 production for the interaction pathogen: antagonist in different ratios of inoculum using GM and non-GM maize as media substrate and modified water availability (0.98 and 0.95 aw) on 14th day of incubation at 30°C. Toxigenic *A. flavus* (pathogen) *strains* A, B and C; *A. flavus* atoxigenic (antagonist) strains AFL- 1, AFL- 2, AFL- 3 and AFL- 4 . For the ratio 100:0 (control of the pathogen) no AFB_1 production was detected (<LOD=0.01 ng. g^{-1})

Table 4. Relative control of AFB1 production for the interaction pathogen: antagonist in different ratios of inoculum using GM and non-GM maize as media substrate and modified water availability (0.98 and 0.95 a_w) in the 7th and 14th day of incubation at 30°C for the strain AFL+C - higher producer of toxin in the interaction study (>4×10^3 ng.g^{-1})

Relative % of AFB_1 control for the strain AFL+C

		non GM maize				GM maize			
a_w		0.98		0.95		0.98		0.95	
Pathogen:Antagonist	day	7	14	7	14	7	14	7	14
AFL-1	25:75	88	89	100	100	97	98	100	100
	50:50	88	91	100	100	95	97	100	100
	75:25	77	78	98	99	80	88	99	100
AFL-2	25:75	97	98	100	98	96	96	100	100
	50:50	98	99	100	100	95	95	100	100
	75:25	95	93	95	98	90	90	96	100
AFL-3	25:75	97	97	99	100	98	99	100	100
	50:50	90	94	98	100	95	96	100	100
	75:25	61	81	93	100	78	88	97	100
AFL-4	25:75	97	98	98	99	96	98	99	99
	50:50	88	94	100	100	95	96	100	99
	75:25	83	89	99	99	93	95	94	96

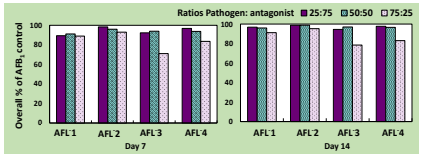


Figure 3: Overall % of AFB_1 control by ratios for the interaction pathogen: antagonist on 7th and 14th day of incubation at 30°C. *A. flavus* atoxigenic (antagonist) strains AFL- 1, AFL- 2, AFL- 3 and AFL- 4 . Bars indicate the average of relative reduction considering the overall production at different a_w and GM/non-GM maize and toxigenic strains.

Conclusions

- All the AFL- strains were able to reduce the AFB_1 production in both 0.98 and 0.95 aw treatments after 7 and 14 days at 30°C;
- The ratios of 50:50 and 25:75 of AFL+: AFL- were not statistically different at 0.95 aw;
- the overall relative reduction of AFB_1 at the end of the incubation period was 46%-100%;
- There was no difference in the results obtained using GM and non-GM maize as a nutritional matrix in terms of AFB_1 production or the relative amounts of control achieved;
- These results are a good method for effective screening of candidates for choosing the best strains for in vivo assays.

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Deciphering mycological complexity

This research was supported by CAPES Foundation, Ministry of Education of Brazil – Project BEX 12937/13-4.

Appendix I-5 Poster presented at World Mycotoxin Forum, Amsterdam - 12-14 March 2018.

Potential biocontrol agents for control of *Aspergillus flavus* and aflatoxin B_1 production

Alessandra Marcon Gasperini, Carol Verheecke-Vaessen, Angel Medina and Naresh Magan

Applied Mycology Group, Environmental and AgriFood Theme, Cranfield University, Bedford, U.K

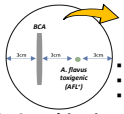
Introduction

A potential option for *A.flavus* management in the field has largely been focused on the use of atoxigenic isolates of *A.flavus* which may be able to compete with the toxigenic strains by displacing them and reducing aflatoxin B_1 (AFB_1) contamination.

OBJECTIVES: The aim of this study was to (a) isolate atoxigenic *A.flavus* strains from GM and non-GM maize hybrids for insect resistance and/or herbicide tolerance from Brazil and (b) compare competitiveness and control of AFB_1 production *in vitro* GM- and non-GM maize hybrids

Methodology

➢ **Interactions, competitiveness and Index of Dominance (I_D)**



BCA (biocontrol agent) = atoxigenic A. flavus (AFL-); yeasts and other fungal species isolated from GM and non GM Brazilian maize

- Media: Malt extract agar and maize meal agar
- Water availability: 0.95 and 0.98 a_w
- Incubation: 10 days at 30ºC

➢ **Co-cultivation of different inoculum ratios of pathogen:antagonist and effects on aflatoxin B_1 production**

SPECIES	Volume of spore suspension [number of spores. mL^{-1}]										TOTAL (mL)
A.flavus	6ml	[1.0×10⁶]	4.5ml	[7.5×10⁵]	1.5ml	[5.0×10⁵]	3ml	[2.5×10⁵]	0ml	[0]	15
BCA	0ml	[0]	1.5ml	[2.5×10⁵]	4.5ml	[5.0×10⁵]	3ml	[7.5×10⁵]	6ml	[1.0×10⁶]	15
Final ratio	100:0		75:25		50:50		25:75		0:100		

100:0 – control *A. flavus* toxigenic / 0:100 – control of Biocontrol agent (BCA)

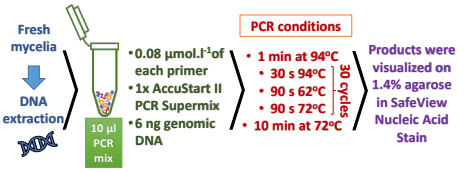
Water availability: 0.95 and 0.98 a_w / Incubation: 14 days at 30ºC

AFB_1 analysis by HPLC-FLD= 5 agar plugs collect on day 7 and 14

Different conidial ratios of toxigenic and atoxigenic strains were used and spread plated on maize-based media using maize grain from a **GM *Bt* hybrid P30F53H (gene Cry1.F, Herculex®I) and the isogenic *non*-GM hybrid (P30F53).**

➢ **Multiplex PCR analysis for deletions in the aflatoxin biosynthesis gene cluster**

The AFL^- strains were studied for the presence/absence of key genes involved in the biosynthesis to produce aflatoxin according to method described by Callicott & Cotty (2015)[1]. This method uses PCR primers developed for markers spaced approximately every 5 kb from aflatoxin biosynthesis gene cluster.



Results

The interaction of toxigenic and atoxigenic *A.flavus* strains and other candidate species isolated from maize on relative competitiveness under different water availabilities (0.98 and 0.95 a_w) at 30ºC showed that *A.flavus* was largely dominant against other species tested and there was no inhibition of growth when paired with atoxigenic *A. flavus* strains (Figure 1).

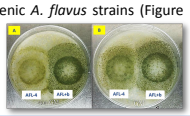


Figure 1: Interaction AFL^-:AFL^+ in maize-based media using non-GM (A) and GM hybrid (B).

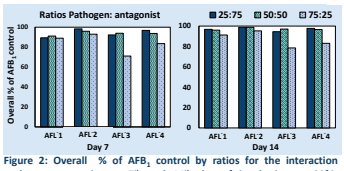


Figure 2: Overall % of AFB_1 control by ratios for the interaction pathogen:antagonist on 7th and 14th day of incubation at 30ºC. *A.flavus* atoxigenic (antagonist) strains AFL^-1, AFL^-2, AFL^-3 and AFL^-4 . Histograms indicate mean relative reduction in AFB_1 production at different a_w levels on GM/non-GM maize.

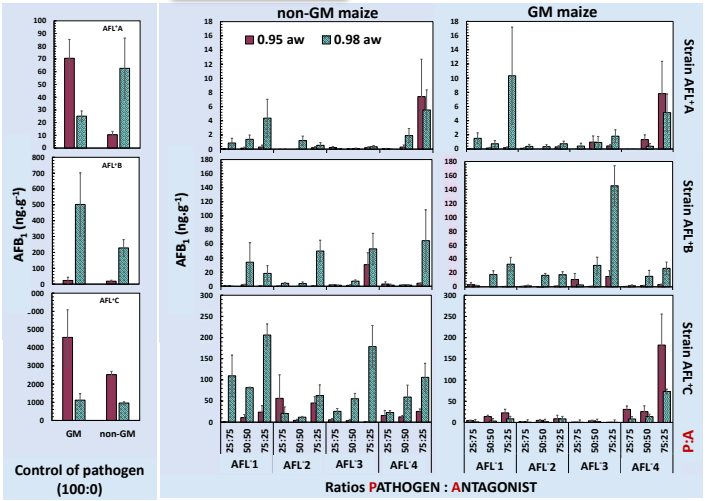


Figure 3: AFB_1 production of the toxigenic *A. flavus strains* A, B and C using GM and non-GM maize as media substrate and modified water availability (0.98 and 0.95 aw) after 14 days incubation at 30ºC. Bars = S.E.s

Figure 4: AFB_1 production after interaction between pathogen:antagonist at different inoculum ratios using GM and non-GM maize as media substrates and modified water availability (0.98; 0.95 a_w) after 14 days at 30ºC. Toxigenic *A.flavus* (pathogen) *strains* A, B and C; *A. flavus* atoxigenic (antagonist) strains AFL^-1, AFL^-2, AFL^-3 and AFL^-4 . For the ratio 100:0 (control of the pathogen) no AFB_1 production was detected (<LOD=0.01 ng. g^{-1}).

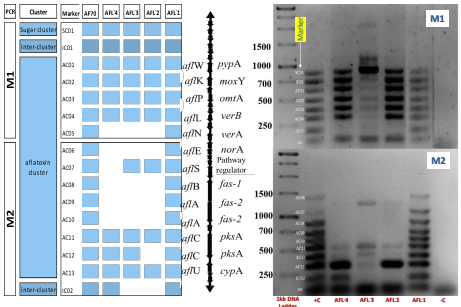


Figure 5: Schematic representation of the multiplex PCR divided into M1 and M2. The column "markers" indicates the primers used in this study designed by Callicott and Cotty (2015). The absence of colour in the columns represents the deletion of genes in the aflatoxin biosynthesis cluster. The columns represent: AF70- *A.flavus* as positive control for the PCR; AFL^-4 to AFL^-1 - strains of atoxigenic *A.flavus* studied as BCAs.

Figure 6: Images of multiplex PCR (M1 and M2) amplifications in electrophoresis gel. The extreme right lane contains GeneRuller 1kb DNA ladder (Thermo Scientific) with bands from 250 to 1500 bp visible. The lanes represent: **+C - *A.flavus*** type strain AF70 as a positive control for the PCR due to the fact this strain has all the genes for aflatoxin biosynthesis; **AFL^-4 to AFL^-1** - strains of atoxigenic *A.flavus* studied as BCAs; -C as negative control for the PCR. The smallest band in lanes of each panel is the iac (internal amplification control).

Conclusions

- All the AFL^- strains were able to reduce the AFB_1 production in both 0.98 and 0.95 a_w treatments after 7 and 14 days at 30ºC
- The ratios of 50:50 and 25:75 of AFL^+: AFL^- were not statistically ($p<0.05$) different at 0.95 a_w
- The overall relative reduction of AFB_1 at the end of the incubation period was 46%-100%
- There was no difference in the results obtained using GM and non-GM maize as a nutritional matrix in terms of AFB_1 production or the relative amounts of control achieved
- AFL^-4 had deletion of 6 genes in the aflatoxin cluster. Screening for such deletions is a rapid method for identification of atoxigenic isolates for potential biocontrol
- The AFL- strains with the larger number of biosynthetic genes deleted are being examined for *in situ* biocontrol of AFB_1, and the resilience under potential future climate change environmental conditions

[1] Callicott, K.A. and Cotty, P.J. (2015) 'Method for monitoring deletions in the aflatoxin biosynthesis gene cluster of *Aspergillus flavus* with multiplex PCR', *Letters in Applied Microbiology*, 60(1), pp. 60–65.

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This research was supported by CAPES Foundation, Ministry of Education of Brazil – Project BEX 12937/13-4.

Appendix J

Appendix J-1 List of possible papers to be published in scientific journals

- FUNGAL DIVERSITY AND MYCOTOXIN PROFILES IN CONVENTIONAL AND GM ISOGENIC MAIZE LINES FROM BRAZIL

- ECOLOGICAL STUDIES ON GROWTH AND AFLATOXIN B1 PRODUCTION BY *ASPERGILLUS FLAVUS* IN CONVENTIONAL AND GM MAIZE-BASED MATRICES

- SCREENING FOR POTENTIAL BIOCONTROL AGENTS (BCAS) FOR CONTROL OF *ASPERGILLUS FLAVUS* AND AFLATOXIN B_1 PRODUCTION USING DIFFERENT INOCULUM RATIOS AND WATER REGIMES *IN VITRO*

- MONITORING DELETIONS IN THE AFLATOXIN GENE CLUSTER OF *ASPERGILLUS FLAVUS* STRAINS WITH POTENTIAL BIOCONTROL ACTION AND EFFECTS ON BIOSYNTHETIC TOXIN GENE EXPRESSION AND AFLATOXIN B_1 PRODUCTION

- BIOCONTROL RESILIENCE UNDER SIMULATED PEST DAMAGE AND CLIMATE CHANGE SCENARIOS IN CONVENTIONAL AND GM BRAZILIAN MAIZE