Resilience of Biocontrol for Aflatoxin Minimization Strategies: Climate Change Abiotic Factors May Affect Control in Non-GM and GM-Maize Cultivars

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There has been significant interest in the development of formulations of non-toxigenic strains of Aspergillus flavus for control of toxigenic strains to reduce the aflatoxin B\textsubscript{1} (AFB\textsubscript{1}) contamination of maize. In the future, climate change (CC) abiotic conditions of temperature (+2–4°C), CO\textsubscript{2} (existing levels of 400 vs. 800–1,200 ppb), and drought stress will impact on the agronomy and control of pests and diseases. This study has examined (1) the effect of two-way interacting factors of water activity × temperature on colonization and AFB\textsubscript{1} contamination of maize cobs of different ripening ages; (2) the effect of non-toxigenic strains of A. flavus (50:50 inoculum ratio) on relative control of toxigenic A. flavus and AFB\textsubscript{1} contamination of ripening cobs; (3) post-harvest control of AFB\textsubscript{1} by non-toxigenic strains of A. flavus in non-GM and isogenic GM maize cultivars using the same inoculum ratio; and (4) the impact of three-way interacting CC factors on relative control of AFB\textsubscript{1} in maize cobs pre-harvest and in stored non-GM/GM cultivars. Pre-harvest colonization and AFB\textsubscript{1} production by a toxigenic A. flavus strain was conserved at 37°C when compared with 30°C, at the three ripening stages of cob development examined: milk ripe (R3), dough (R4), and dent (R5). However, pre-harvest biocontrol with a non-toxigenic strain was only effective at the R3 and R4 stages and not at the R5 stage. This was supported by relative expression of the aflR regulatory biosynthetic gene in the different treatments. When exposed to three-way interacting CC factors for control of AFB\textsubscript{1} pre-harvest, the non-toxigenic A. flavus strain was effective at the R3 and R4 stages and not at the R5 stage. This was supported by relative expression of the aflR regulatory biosynthetic gene in the different treatments. When exposed to three-way interacting CC factors for control of AFB\textsubscript{1} pre-harvest, the non-toxigenic A. flavus strain was effective at the R3 and R4 stages and not at the R5 stage. Post-harvest storage of non-GM and GM cultivars showed that control was achievable at 30°C, with slightly better control in GM-cultivars in terms of the overall inhibition of AFB\textsubscript{1} production. However, pre-harvest biocontrol with a non-toxigenic strain was only effective at the R3 and R4 stages and not at the R5 stage. This was supported by relative expression of the aflR gene in these treatments. This study suggests that the choice of the biocontrol strains, for pre- or post-harvest control, needs to take into account their resilience in CC-related abiotic conditions to ensure that control of AFB\textsubscript{1} contamination can be conserved.

Keywords: resilience, biocontrol, aflatoxins, climate change, non-toxigenic Aspergillus flavus, non-GM maize, GM maize
INTRODUCTION

There has been significant interest in developing biocontrol agents for aflatoxin B₁ (AFB₁) control in staple commodities, especially maize. Indeed, there are some commercial products based on individual non-aflatoxigenic A. flavus strains or mixtures of such strains for reducing AFB₁ contamination of maize and groundnuts in West and East Africa and in the USA for control of toxin contamination in cotton and groundnuts (Lyn et al., 2009; Abbas et al., 2011; Bandyopadhyay et al., 2016; Mauro et al., 2018; Kagot et al., 2019).

Abiotic factors, especially drought stress, can have a major impact on maize growth especially during the critical silking period, which can allow both pest damage and increase in the colonization by A. flavus resulting in AFB₁ contamination. In some cases, pest damage has been reduced by the use of GM cultivars, which have resistant genes for pesticides and/or herbicides. This can reduce the entry points for A. flavus and reduce contamination. However, under expected climate change (CC) conditions, which involves interactions between key abiotic factors such as the predicted increase in environmental CO₂ (400 vs. 1,000–1,200 ppm), elevated temperature (+2–4°C), and extreme changes in drought/wet regimes, there have been few studies to examine the resilience of non-toxigenic A. flavus biocontrol strains used for toxin reduction in maize and groundnuts. Many maize growing regions are considered hot spots for an impact of these environmental pressures. Indeed, work on A. flavus colonization of maize grain has shown that interacting conditions of +2–5°C, elevated CO₂ (650–1,000 ppm), and drought stress can result in an increase in both regulatory (aflR) and structural genes (aflD) involved in AFB₁ biosynthesis as well as other secondary metabolite genes in maize grain and lead to a significant stimulation in AFB₁ contamination (Battilani et al., 2016; Medina et al., 2017a; Gilbert et al., 2018). Bearing this in mind, it is thus surprising that while biocontrol of toxigenic A. flavus using microbial antagonists and non-toxigenic strains of A. flavus has been examined for many years, their resilience has never been examined under expected CC regimes (Cotty, 1994; Abbas et al., 2011; Bandyopadhyay et al., 2016; Weaver et al., 2016; Kagot et al., 2019). Some studies have examined the impact of non-toxigenic A. flavus strains and other microbial biocontrol agents on temporal control of AFB₁, in maize stored under different temperature and water availabilities (Mohale et al., 2013; Al-Saad et al., 2016; Medina et al., 2017a). However, the relative resilience of the biocontrol strains for control of mycotoxin production under CC scenarios has not previously received any attention (Medina et al., 2017b). It is very important to understand how the potential biocontrol strains targeting AFB₁ control in maize may behave under interacting CC abiotic factors and whether they have the necessary resilience to reduce biosynthesis of AFB₁ in situ.

The objectives of this study were to examine (1) the effect of ripening stage of maize cobs on rates of colonization and AFB₁ production by A. flavus in relation to interactions between two-way abiotic factors of temperature × water availability (water activity, a₃); (2) the effect of these two-way interacting factors on the control of AFB₁ contamination using 50:50 ratios of non-toxigenic and toxigenic strains in maize cobs of different ripening stages and in stored non-GM and isogenic GM maize cultivars; and (3) the effect of three-way interacting CC abiotic factors on resilience of non-toxigenic A. flavus strains in terms of reducing the expression of key biosynthetic genes involved in aflatoxin synthesis, and on phenotypic AFB₁ contamination, in these two types of maize cultivars.

MATERIALS AND METHODS

Fungal Strains

Two non-toxigenic strains of A. flavus isolated from Mexican and Brazilian maize were used in these experiments (AFL-Mex02; AFL4*). Both strains were examined molecularly to confirm that key genes in the biosynthetic cluster for aflatoxins were deleted using the multiplex PCR method developed by Callicott and Cotty (2015). Different toxigenic strains including one Mexican strain (ALF⁺-Mex01, isolated from Mexican maize), a type strain NRRL3375 (AFLe⁺), and one Brazilian toxigenic strain (AFLe⁺, isolated from Brazilian maize), all known AFB₁ producers were used. Table 1 summarizes the strains used in this study. The type strain was kindly provided by Prof. D. Bhatnagar, Southern Regional Research Centre, New Orleans, LA, USA.

Pre-harvest Studies With Maize Cobs of Different Ripening Ages for Resilience of Biocontrol of AFB₁

Maize cobs of different ripening stages (R3: Milk; R4: Dough; R5: Dent) were obtained from the NIAB farm (National Institute of Agriculture and Botany; Cambridge, UK). The type of maize was ES Regain (Euralis Semences; forage maize). Harvested maize cobs of different ripening ages were brought to the laboratory where the water activity (a₃) of sub-samples of detached kernels from the entire cob (5–10 maize kernels from the apical, middle, and distal parts of the cobs) was measured (AQUALAB® Series TE4; Decagon Devices Inc., Pullman, WA, USA). Maize cobs were then divided into batches and snap frozen in liquid nitrogen and stored at −20°C for later use in the experiments. The a₃ of the R3 (milk ripe), R4 (dough), and R5 (dent) stages was found to be 0.985, 0.976, and 0.958, respectively.

Table 1 | List of toxigenic and non-toxigenic strains used in this study.

| Strain code | Source | Aflatoxin B₁ producer +/− | Deleted genes |
|-------------|--------|--------------------------|--------------|
| AFL-MEX01⁻  | White maize/Mexico | Naturally very low producer* | None         |
| AFL-MEX02   | White maize/Mexico | Non-producer | aflN, aflE, aflS, aflB, aflA |
| AFL4*       | GM maize P30FS3 HB, Brazil | Producer |                       |
| AFB¹        | Landrace maize red grain, Brazil | Producer |                       |
| NRRL3357    | Peanuts (type strain, ARS, USDA) | Producer |                       |

*Less than the limit of quantification.
The fungal strains were all point inoculated on 3% Maize Meal Agar (MMA, 0.98 a,) and incubated at 25°C for 7 days, and the conidial spore suspensions made by using a loop and decanting spores into 9 ml of sterile water +0.5% Tween 8-0 solution in 25 ml Universal bottle. The conidial spore concentrations were measured with a hemocytometer, and then diluted with sterile water as required, to obtain a final concentration of 1 × 10⁶ spores/ml.

The flash-frozen cobs were thawed at 4°C for 24 h. The maize cobs were divided into three segments, and these were point inoculated. Each treatment consisted of 3–4 replicate maize cob segments taken at random from the batch of each ripening age. These were point inoculated by damaging a single kernel with a surface sterilized needle and then decanting a 10 μl droplet containing 10⁴ conidia/ml of the A. flavus type strain. These were incubation in separate environmental chambers at 30 and 37°C. The equilibrium relative humidity (ERH) of the atmosphere was maintained at the actual aₚ levels of the cobs by using glycerol/water solutions of the same aₚ as the maize cob ripening stages. The colonization rate was measured over a 10-day period, and the AFB₁ contamination quantified at the end of the experimental period only.

Subsequent studies examined the biocontrol of the toxigenic A. flavus strain and AFB₁ control in each of the ripening stages as described previously by Samsudin et al. (2017). The different ripening stages of the cobs were incubated in separate environmental chambers to stabilize for 3 hours at 25°C until inoculation. The treatment control used was point inoculated with the toxigenic A. flavus (MEX01⁴) or the type NRRL3375 strain alone. The biological control agent (BCA) treatments consisted of a 50:50 conidial inoculum ratio of pathogen:antagonist. This was used based on previous studies where different inoculum ratios were used to toxigenic:non-toxigenic strains of A. flavus, which showed that 50:50 or 25:75 ratios respectively, gave similar levels of AFB₁ control as described previously (Medina et al., 2017a). The cob sections were point inoculated with 100 μl of the treatments after damage to a single kernel using a surface sterilized needle and incubated at 30°C for 10 days (Samsudin et al., 2017). In all cases, 3–4 replicates per treatment were used. Each environmental chamber included 250 ml of a sterile solution of glycerol/water to maintain the ERH at the same level as the cob ripening stage aₚ. The colony diameter was measured at the end of the incubation period. For aflD and aflR gene expression, contaminated kernels from the colonized area were carefully removed at random with a pair of forceps and immediately frozen in liquid N₂ and kept at −80°C for subsequent RNA extraction and RT-qPCR as detailed previously (Al-Saad et al., 2016). The rest of the colonized cob was kept at −20°C for AFB₁ extraction; clean-up was done using an immune-affinity column (IAC) and quantified by HPLC-FLD. The limit of detection was <1.0 ng/g.

Post-harvest Studies With Non-GM and GM-Maize Cultivars

Two cultivars of non-GM maize and its isogenic GM lines were selected for the post-harvest biocontrol studies (Table 2). For these studies, batches of the maize grain were gamma-irradiated (12–15 kGy) in order to eliminate the natural contaminants but retain germinability of the kernels. The aₚ of the maize treatments was modified to 0.98 and 0.95 with the addition of sterile water based on the moisture absorption curve for each cultivar. These were mixed thoroughly to ensure that the spore inoculum was well distributed throughout the maize grain. The number of spores added to each cultivar was calculated as 10⁴ spores per gram of maize from a solution of 10⁴ spores/ml. After the addition of the water, the maize kernels were kept at 4°C for 24 h for full absorption and equilibration. The water availability was checked by measuring the aₚ using the AquaLab® 4TE (Decagon, USA).

The non-toxigenic and toxigenic strains of A. flavus were incubated on MMA for 7 days at 30°C and then used to prepare the conidial spore inoculum. A final concentration of 10⁴ spores/ml was obtained. A ratio of 50:50 (toxigenic:non-toxigenic strains) was used. Previous showed that this as an effective inoculum load for biocontrol of AFB₁ production (Mohale et al., 2013; Samsudin and Magan, 2016). The spore suspensions were mixed in a 50:50 ratio prior to inoculation of the maize kernels. The controls consisted of 100:0 and 0:100 ratios for the two control treatments.

A 10 g sample of maize grain were placed aseptically into glass culture vessels (Magenta®, Sigma, USA) with vented lids (10 mm with a polypropylene membrane 0.22 μm pore size) to allow gas exchange but keeping the environment inside the vessel sterile. These were inoculated with a 100 μl conidial mixture and shaken to distribute the conidia. The jars were placed in closed plastic environmental chambers that also contained a glycerol/water solution of the same aₚ as the maize kernels to keep the ERH the same as the target treatments. The glycerol/water solution was renewed every 3 days. The environmental chambers were incubated at 30°C for 20 days. Samples were destructively sampled after 10 and 20 days storage for quantification of AFB₁.

The samples for AFB₁ quantification were oven dried at 65°C for 48 h to remove the water and stop fungal growth. These were then ground using a laboratory blender with a
stainless steel blade (Waring, Stamford, USA). The effects on
aflD and aflR gene expression were only examined after 10 days
in all the treatments and replicates. The samples for gene
expression were snap frozen in liquid nitrogen and stored at
−80°C for subsequent RNA extraction. All studies were carried
out with two non-GM and their isogenic GM cultivars. All
experiments were carried out with at least three replicates per
treatments and repeated once.

Studies of Resilience of Non-toxigenic
Strains of A. flavus When Controlling
Aflatoxin B₁, Contamination of Maize
Cultivars Under Climate Change
Conditions

Fungal Strains

Studies were carried out with two non-aflatoxigenic strains of
A. flavus (AFL-Mex02; AFL4) isolated from Mexican and
Brazilian maize, respectively, based on their ability to reduce
AFB₁ in previous in vitro and in situ studies (Rodríguez-Sixtos,
2017; Marcon Gasperini, 2019). The native toxigenic strains
(AFL-MEX01; AFLb; and the AFLε type strain) with known
AFB₁ production capacity were used as the toxigenic pathogen
in these studies.

Pre-harvest studies involved the use of maize cobs at the
R4 (dough) and R5 (dent) stages only. Post-harvest studies
used the two non-GM and isogenic GM maize cultivars. The
same modification procedures were used as detailed previously.
A ratio of 50:50 conidia (toxigenic A. flavus: non-toxigenic
A. flavus) were used. The controls consisted again of only
the toxigenic or non-toxigenic conidial concentrations as
detailed previously.

The post-harvest maize grain treatments were modified to
0.98 and 0.95 aₚ with the addition of sterile water using the
moisture adsorption curve of each cultivar to obtain the target
 treatment regimes. The maize treatments and replicates were
inoculated with a conidial mixture of non-toxigenic and toxigenic
strains as described previously (10⁵ conidia/ml and addition
of the mixture resulting in approx. 10 conidia/g maize). The
only exception was that for the cultivars AS 1555 CON and
PRO*, and P2530 CON and Hx* were only conducted at 0.98
aₚ because of a limited amount of maize grain of these cultivars
available for the experiment.

For the CC study, the separate treatments were placed in
plastic environmental chambers (Lock & Lock HPL890 16 L)
containing a glycerol/water solution of the same aₚ as the
treatments as described previously. The environmental conditions
were set to flush the CO₂ treatments of 400 ppm (atmospheric
CO₂) and 1,000 ppm. The elevated CO₂ content was achieved
by using a gas cylinder containing a certified concentration of
1,000 ppm CO₂/synthetic air at 200 bar prepared by the
British Oxygen Company (Guildford, UK). The chambers were
vented, and for the 1,000 ppm treatment flushed with CO₂,
every 12 h for the 20-day experimental period. The concentration
of CO₂ was regulated to 3 L/min (LPM) with a gas flow meter
(Alicat Scientific, Arizona, USA) and flushed for 10–12 min,
which corresponded to 2× the volume of each chamber. After
flushing, the inlet and outlet valves of the chambers were
immediately closed, and they were incubated at 30 or 35°C.
The control chambers were flushed with air (400 ppm) and
similarly incubated. At the end of the experiment, samples
were destructively sampled for gene expression studies (10 days)
and for AFB₁ quantification (10, 20 days). The samples for
toxin quantification were oven dried at 65°C for 48 h to remove
the water and stop any fungal growth. The samples were ground
using the laboratory blender as described previously.

Gene Expression Studies

The gene expression studies were performed using the samples
from the different maize ripening stages (pre-harvest studies)
and those from the stored maize grain experiments after 10 days
incubation. 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 of expression after 8–10 days growth (Schmidt-
Heydt et al., 2008). The gene expression of the chosen genes
was only performed for the interaction between toxigenic/
non-toxigenic strains MEX01*:MEX02* and AFLb*:AFL4*,
respectively. The type strain AFLε* (NRRL3357) treatments
were not included because the AFB₁ production was lower
than the native Mexican or Brazilian toxigenic strains.

The treatments/replicates were stored at ~80°C and transferred
to reinforced 7 ml tubes designed for use in the Precellys 24®
(Bertin, FR) homogenizer with three glass beads (6.5 mm).
The tubes were kept in liquid N₂ until use. The kernels were
homogenized into a fine powder using a 6,500 rpm cycle for
30 s (2 × 15 s) and then immersing them in liquid N₂ 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 were 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 were
considered to be of good purity when the ratio A₂₆₀/A₃₄₀ was
≥2.0. The RNA integrity was verified using Experion™ RNA
StdSens in an Experion™ automated electrophoresis system
(Bio-Rad, California, USA) or by gel electrophoresis, and an
RQI >6 was used as a threshold for integrity. The samples
were kept at ~80°C until use.

Relative Gene Expression Using Quantitative
Polymerase Chain Reactions

Real-time quantitative PCR (RT-qPCR) assays were used to
amplify the structural aflD and the regulatory gene aflR of
the aflatoxin biosynthetic pathway as target genes (Table 3). The β-tubulin gene was used as the control gene. The afdID qPCR was previously optimized by Abdel-Hadi et al. (2010), whereas afdIR was optimized by Medina et al. (2015) following the same method as that for the afdID gene.

Two RT-qPCR assays were carried out, one optimized to amplify the target afdID and the housekeeping β-tubulin genes, and the other one to quantify the afdIR gene expression and for the β-tubulin gene. The qPCR reactions were prepared in triplicate for each biological replicate (n = 9). The TaqMan system with different primers and probes was 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, CA, USA).

Relative Quantification of the Expression
Relative quantification of afdID and afdIR genes was performed using the housekeeping gene β-tubulin (ben) as an endogenous control to normalize 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 the default parameters, and the expression ratio was calculated using the 2−∆∆Ct method as proposed by Livak and Schmittgen (2001). The control of each condition corresponded to AFLb1 without the presence of the BCA.

Mycotoxin Quantification
For the extraction of AFB1, a 2 g sample was ground and transferred to a glass vial and mixed with 8 ml of extraction solution (methanol:water; 80:20 v/v). The samples were agitated with a magnetic stirrer for 1 h at room temperature and then centrifuged 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 Eppendorf tube and vortexed and filtered using a nylon filter (13 mm × 0.22 μm) directly into an amber silanized vial. The samples were injected into the HPLC system, and samples below the limit of detection (<1.0 ng/g) were cleaned-up and concentrated using an IAC as described previously and then reinjected.

IAC Analysis
The extract of the samples was diluted 2:20 in 1× PBS (phosphate buffered saline, Fisher Scientific, USA). The pH of the extract was checked to ensure that this was not lower than 7.0 to ensure good performance of the IACs (AlfaStar™ R, RomerLabs, Austria). The IACs were brought to room temperature prior to use and were attached to a SPE vacuum manifold (Phenomenex, CA, USA). Above 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. This was followed by 20 ml of 1× PBS 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 of methanol. For best recovery, the elution was performed by adding 3 × 500 μl of methanol. The eluted samples were evaporated to dryness using a vacuum evaporator (miVac Quattro Concentrator – Genevac, Leicestershire, UK) at 45°C for 3 h. The dried extract was re-suspended in 500 μl mobile phase, transferred to an amber silanized vial, and injected into the HPLC for AFB1 quantification.

HPLC Analysis
The quantification of AFB1 in the maize grain was done by reverse-phase HPLC. The HPLC system used was an Agilent 1,200 series (Agilent, Santa Clara, USA) with a fluorescence detector (λ_em 360 nm; λ_exc 440 nm) and post-column derivatization with a UVE photochemical reactor with UV-Light (LCTech GmbH, Germany). A C18 column (Agilent Zorbax® Eclipse Plus, 2.1 × 100 mm, 3.5 μm particle size) preceded by a Phenomenex® Gemini C18 guard column cartridge 3 mm × 3 mm (Phenomenex, CA, 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. The injection volume was 5–50 μl according to each set of samples. A set

| Primer pairs | Gene   | Nucleotide sequences (5’–3’*)  | Position  |
|--------------|--------|--------------------------------|-----------|
| norTaq – 1   | aflD   | GTCCAAGCAGCACGGCCAGAT         | 516°      |
| norTaq – 2   | aflD   | TCCTGCTATGTGTGTTGAGGT         | 562°      |
| nor-Probe    |        | [FAM]TGCTTGGATGCGGCGCCT[GEO][BHQ2] | 537°      |
| aflRTaq – 1  | aflD   | TCGTCCTATGTGTCTCAAGG          | 1,646°    |
| aflRTaq – 2  | aflD   | ACGTTGCTACAGCTGCCACT         | 1,735°    |
| aflR-Probe   |        | [FAM]AGCAGAGCAGCCAGTGGACACAC[GEO][BHQ2] | 1,689°    |
| benTaq – 1   | β-tubulin | GTCACGGCCCGCTACAGCT          | 99°       |
| benTaq – 2   | β-tubulin | [CY5]CGATGCTGGCTGGCTGGCGCTGCT[BHQ2] | 82°       |

aPositions are in accordance with the published sequence of the afdID gene of Aspergillus flavus (GenBank accession no. XM_002379908.1); bPositions are in accordance with the published sequences of aflD gene of Aspergillus flavus (GenBank accession no. AF441435.2); cPositions are in accordance with the published sequences of β-tubulin (benVA56) gene of Aspergillus flavus (GenBank accession no. AF036803.1).
of standards was injected (0.05–4 ng of a mixture of aflatoxins per injection), and standard curves were generated by plotting the peak areas against the amounts of each aflatoxin. The recovery of the extraction method for AFB<sub>1</sub> in maize was 80%.

Statistical Analyses
The data from pre-harvest cobs of different ripening stages and the in situ post-harvest experiments (gene expression data, AFB<sub>1</sub>) were analyzed using the Shapiro-Wilk tests to determine normality and Levene’s test to assess variance homogeneity. 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<sub>1</sub> production was checked using non-parametric Spearman’s (ρ) rank correlation coefficient for each a<sub>w</sub> level. The statistical package JMP®14 (SAS Institute Inc., 2018, Cary, NC, USA) was used to perform the analyses.

The data sets for the effect of CC scenarios on AFB<sub>1</sub> production satisfied the requirements for ANOVA after transformation to the cube root. Tests were thus performed comparing the interactions of temperature × CO<sub>2</sub> × a<sub>w</sub> 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 toxigenic pathogen strain (AFib<sup>+</sup>) in the same conditions as the non-toxigenic strain was applied. For the effects of CC, the control sample refers to normal environmental conditions (30°C, 400 ppm CO<sub>2</sub>) for each cultivar. The statistical package JMP®14 (SAS Institute Inc., 2018, Cary, NC, USA) was used to perform the analyses.

RESULTS
Effect of Maize Cob Ripening Stage and Temperature on Colonization and Aflatoxin B<sub>1</sub> Contamination
Figure 1 shows that a toxigenic strain of *A. flavus* is able to colonize ripening maize cobs of different ages at both

![Figure 1](image1.png)

**FIGURE 1** | Relative colonization rates of maize cobs of different ripening ages (R3, R4, R5) by a toxigenic strains of *A. flavus* (NRRL 3375) when point inoculated and incubated at the water activity levels of the different growth stages at 30 and 37°C. Statistical test performed after lesion diameter (mm) data was transformed to Log(x + 1) to achieve normality fit (Shapiro Wilk p > 0.05). The values of p indicate no difference between temperatures. Treatments with the same letters indicate no differences in the ripening stages based on the Tukey’s HSD test (p > 0.05).

![Figure 2](image2.png)

**FIGURE 2** | Relative contamination of maize cobs of different ripening ages (R3, R4, R5) with aflatoxin B<sub>1</sub> when incubated at the actual water activity levels of the maize kernels at the different growth stages at 30 and 37°C. The toxigenic strain NRRL 3375 was used. The values of p indicate no evidence of difference between temperatures. Ripening stage treatments with the same letters were not significantly different using the Wilcoxon for each pair test (p ≥ 0.05). Overall analyses was done using the Non-Parametric data – Wilcoxon Test. Ripening stages: R3 × R4 × R5, p = 0.0009; Temperatures: 30 × 37°C, p = 0.7728.

![Figure 3](image3.png)

**FIGURE 3** | Effect of a non-toxigenic strain (AFib-MEX02) of *A. flavus* when co-inoculated with a wild type toxigenic strain (AFib-MEX01) on aflatoxin B<sub>1</sub> production when applied as a mixed inoculum (50:50) to maize cobs of different ripening ages (R3, R4, R5) after 10 days at 30°C. The percentage values above the bars show relative reduction of AFB<sub>1</sub>, and the values of p (<0.05) indicate evidence of difference from the control (toxigenic strain). Treatments with the same letters show no differences in the ripening stages using the Tukey’s HSD test at 5% significance. n.d. – AFB<sub>1</sub> reduction not detected.
30 and 37°C in a relatively similar manner with no significant difference between cob ripening stage and temperature. AFB₁ production was significantly lower initially at the R3 (milk) stage and 37°C and then significantly higher at the R4 (dough) stage and the same temperature. There was no difference at the R5 (dent) stage (Figure 2). Statistically, there was a significant overall effect of ripening stage but not of temperature.

**Relative Control of AFB₁ Contamination in Ripening Ages of Maize Cobs Using a Non-toxigenic A. flavus Strain**

Figure 3 shows the effect of the non-toxigenic strain (AFL⁻:MEX02) on control of AFB₁ production by the toxigenic strain (AFL⁺:MEX01) on maize cobs of different ripening stages. This shows that the level of control of AFB₁ was maximum at the R3 (milk) and R4 (dough) stages (p = 0.05). At the R5 (dent) stage, there was no control of toxin production. Plate 1 shows an example of the colonization of maize cobs segments at the R5 stage. This was confirmed by measurement of the relative expression of the regulatory aflR gene, which was downregulated at the R3 and R4 stages in the maize cobs, while in the R5 stage, there was no difference from the toxigenic control gene expression levels (Figure 4).

**Post-harvest Control of Aflatoxin Under Different Water Availability Conditions in Non-GM and GM Maize Grain**

Figure 5 summarizes the effect of a non-toxigenic strain (AFL⁻) on AFB₁ control in maize grain contaminated with two different toxigenic A. flavus (AFL⁺; AFl⁺⁺⁺⁺) strains on AFB₁ control in GM and non-GM cultivars (mean of two cvs of each). This showed that the toxigenic AFLb⁺ strain produced significantly more AFB₁ than the AFl⁺⁺⁺⁺ (NRRL3375) strain. Overall, there was better control of AFB₁ in the GM-maize cultivars than the non-GM ones. Although in both GM and non-GM cultivars, >90% control of AFB₁ contamination of the maize grain was achieved using the initial inoculum ratio of 50:50 of non-toxigenic:toxigenic conidial inoculum. These studies were carried out at 30°C and showed that efficacy was consistent across non-GM and GM cultivars. Figure 6 compares the relative expression of both the aflD and aflR genes in one of the non-GM and GM cultivars at 0.98 and 0.95 a_w. The efficacy of the non-toxigenic strain on the toxigenic strains was supported by the effects on the structural and regulatory genes examined. These were significantly downregulated in both non-GM and GM cultivars, although this was more pronounced in the latter one.

**Resilience of Non-toxigenic Strains of A. flavus for Control of Aflatoxin B₁ Under Three-Way Interacting Climate Change Abiotic Conditions in Non-GM and GM Strains**

Two sets of studies were carried out. Pre-harvest studies with a 50:50 mixed conidial inoculum of the non-toxigenic/toxigenic strains were used in maize cobs at the R4 and R5 stages and incubated at 30 or 35°C and exposed to elevated CO₂ for 10 days. This showed that there were no statistically significant differences in AFB₁ contamination between the control and the elevated CO₂ treatments (p = 0.05; means of R4: 545 vs. 390; R5 = 1,125 vs. 780 ng/g AFB₁ in maize, respectively). The relative expression of the aflD and aflR genes showed that there was an inhibition of the former structural gene expression but not of the latter regulatory gene (data not shown).

Exposure to interacting climate change abiotic factors in post-harvest storage studies was carried out with the two non-GM and isogenic GM cultivars. Figure 7 compares the relative effect of the biocontrol strain AFL4⁻ when co-inoculated with the toxigenic strain AFLb⁺ in one of the non-GM and GM cultivars when carried out under existing CO₂ conditions (400 ppm) at two a_w stress levels and comparisons with exposure...
to 1,000 ppm CO₂. There was higher AFB₁ production at 0.98 aₚ than at 0.95 aₚ. In addition, in the non-GM cultivar, more AFB₁ was produced by the toxigenic strain. Overall AFB₁ control was more effective at 0.98 aₚ than at 0.95 aₚ in both existing and future CO₂ scenarios. For both GM and non-GM maize cultivars, the control of AFB₁ was similar, suggesting relative resilience of this non-toxigenic strain.

Figure 8 compares the relative expression of the regulatory aflR gene at 35°C and 0.98 aₚ in the different CO₂ treatments (400 and 1,000 ppm CO₂) with the control at 30°C/400 ppm CO₂ in both non-GM and GM cultivars. This shows that the aflR gene expression was significantly affected at 35°C when comparisons were made between existing (400 ppm) and future (1,000 ppm) CO₂ treatments. The relative expression values were relative to those at 400 ppm CO₂ and 30°C (existing conditions).

**DISCUSSION**

This study has examined the pre-harvest and post-harvest resilience of non-toxigenic strains of *A. flavus* for control of AFB₁ contamination of maize cultivars, including non-GM and isogenic non-GM with herbicide/pesticide resistant traits for the first time. The ability of the toxigenic strains of *A. flavus* to colonize ripening maize cobs at both 30 and 37°C suggests that it is important to screen non-toxigenic biocontrol strains for resilience to fluxes in temperature and the ability to tolerate a range of water availability conditions to ensure that competitiveness both pre- and post-harvest can be maintained. The fact that AFB₁ control was more effective at the milky ripe (R3) and dough stages (R4) of maize cobs pre-harvest suggests that the window for control based on the aₚ range at these stages may be an important consideration for application of the biocontrol strain. Recently, Giorni et al. (2019) showed that relative infection of ripening maize cobs by *A. flavus, Fusarium verticillioides* and *F. graminearum* influenced the colonization and the mixture of mycotoxins contaminating the maize at harvest. Thus, the presence of *A. flavus* impacted on the contamination of the maize grain with other mycotoxins such as fumonisin B₁ (FB₁) and deoxynivalenol, perhaps because of the its wider range of temperature and water availability during silking.

The biocontrol of AFB₁ contamination at different ripening stages is important. They represent different aₚ levels as well as different nutritional compositions during the silking process. However, since ripening stage did not affect the ability of the toxigenic strain to colonize the cobs, this suggests that toxigenic *A. flavus* is adapted to the ripening stages of maize, expressing specific gene in maize. Reese et al., 2011; Dolezal et al., 2014. The non-toxigenic strain was effective in controlling AFB₁ production at the milky ripe and dough stages. However, at the dent stage, it was less effective. This was supported by the effects on the gene expression of one of the key regulatory genes (aflR) involved in secondary metabolite production. Previously, Verheecke et al. (2015) studied the efficacy of *Streptomyces* strains against toxigenic *A. flavus* strains on synthetic media. They examined five different...
biosynthetic genes involved in aflatoxin production. In their study, the aflR expression was decreased by a *Streptomyces* strain, but aflD expression was unaffected by all the biocontrol strains examined. However, these studies did not include the impact of water availability, which may have affected the relative control achieved. In the study by Al-Saad et al. (2016), the efficacy of bacterial biocontrol strains showed significant efficacy with a decrease in the aflD and aflR relative expression, although this was not always translated into effective phenotypic toxin control.
The non-toxigenic strain AFL4− was able to significantly reduce the AFB1 content in situ when paired with two different toxigenic strains, AFLb+ and the type strain NRRL3357 (AFLe+) in a 50:50 inoculum ratio. The relative AFB1 control ranged from 58 to 100% under different a (0.98 and 0.95) conditions for up to 20 days storage. Furthermore, the present study has shown for the first time the comparison between impacts of such biocontrol in the reduction of AFB1 in conventional (non-GM) and isogenic GM maize cultivars. The overall AFB1 control indicated that the non-toxigenic strains applied were less resilient in non-GM stored maize cultivars, with the final control achieved being lower than that in the isogenic GM cultivars.

The question is whether this is due to differences in the biochemical composition of non-GM and GM cultivars due to the manipulation of the genes, e.g., the presence of the Cry1Ab gene. There are few studies, which have examined this. There is a study, which has suggested that Cry1Ab protein in maize residues had no direct effect on F. graminearum and Trichoderma atroviride. However, some corresponding BT/non-BT maize hybrids differed more in composition than that due to the Cry gene alone. However, this may affect the saprophytic growth of such fungi on crop residues (Naef et al., 2006). Changes in free fatty acids (FFA) in maize are an indicator of fungal infections and a deterioration in quality. Thus, fatty acids (FAs) in the maize could play a role in pathogen susceptibility and seed colonization (Dall’Asta et al., 2012). FFAs, especially of linoleic acid levels, partly regulate development, colonization, and mycotoxin production by Aspergillus spp. (Scarpari et al., 2014). Thus, changes in nutritional or environmental factors or both may also influence secondary metabolite production (Magan and Aldred, 2007).

The efficacy of the non-toxigenic strains in controlling AFB1 production was supported by the gene expression of the target structural and regulatory genes (afID, aflR). The results showed that competition in the maize grain niche inhibited the relative expression of both these genes. The correlation with phenotypic AFB1 production showed that the lowered expression of these genes resulted in less toxin after 10 days storage. Previously, Al-Saad et al. (2016) when screening bacterial antagonists as biocontrol agents of toxigenic A. flavus strains sometimes found that while relative inhibition of afID and aflR expression was evident, sometimes there was concomitant stimulation of AFB1 production. Thus, the resilience of such potential biocontrol strains may well be influenced by water and temperature stress limitations, as well as inoculum ratio × nutritional parameters to avoid such effects. Recently, Venkatesh and Keller (2019) suggested that there are complex interactions between bacteria and mycotoxigenic fungi and focused on key ecological factors being light, nutrients, and pH. Two probably more important abiotic factors are water availability and temperature. Bacteria require almost freely available water (>0.98–0.99 a) for growth and have significantly less resilience to water stress than many mycotoxigenic fungi, which are either xerotolerant or xerophilic, the only exceptions being some of the toxigenic Fusaria. However, stored cereals are seldom harvested or stored under wet conditions, and thus, the interactions with bacteria may in fact be relatively limited, although pre-harvest interactions certainly must occur. Thus, the competitive exclusion of toxigenic strains of A. flavus is more likely to be successful with non-toxigenic strains or relatively xerotolerant species that may have the temperature and water stress tolerance to compete effectively. Indeed, Kohl et al. (2019) suggested that biocontrol agents use a cascade of mixed mechanisms of action to control plant pathogens, and this needs to be borne in mind when developing biocontrol strategies, including for toxigenic fungal pathogens.

It was noted that the relative gene expression of biosynthetic genes of the toxigenic A. flavus strains in non-GM and isogenic GM-maize was similar for the afID and aflR at 30°C after 10 days storage. However, the phenotypic AFB1 production was different in the two types of cultivars. For example, in the conventional M20-A78 CON cultivar, the relative reduction in AFB1 was lower, and this was supported by the less pronounced reduction in the gene expression of the two genes examined. Furthermore, the a level also had an effect on the relative gene expression when the non-toxigenic strain was applied, under the same storage conditions. Previously, Abdel-Hadi et al. (2010) reported that under water stress levels (e.g., 0.90 a), the afID expression can...
be increased, although it is optimally expressed at 0.98 a_{w} on a conducive medium.

It has been shown previously that the ratio of the two regulatory genes aflR and aflS changes with a_{w} \times \text{temperature conditions} (Abdel-Hadi et al., 2012). The aflR gene encodes for a positive regulator (AFLR), which activates the pathway gene transcription (Chang et al., 1995), whereas the aflS (=-aflJ) gene encodes for a protein factor (AFLS), which is involved in the regulation of transcription. These key genes are adjacent to and associated with the expression of a number of structural genes, e.g., aflC, aflD (nor-1), aflM (ver-1), and aflP (omtA; Chang and Hua, 2007). The capacity for disrupting the functioning of either or both of these two genes (aflS, aflR) perhaps by competition from non-toxigenic strains can reduce or completely inhibit aflatoxin production (Meyers et al., 1998; Yu et al., 2004).

This study has explored the effects of elevated CO_{2} and temperature on potential for control by non-toxigenic fungi, both pre- and post-harvest with both non-GM and GM cultivars. Pre-harvest results suggest that the non-toxigenic strain used (MEX02) was not as resilient under elevated climate change interacting factors with no effective control of AFB_{1} production in maize cobs of the dent stage. The post-harvest studies were able to examine the relative efficacy of the non-toxigenic strain (AFL04^+) on control of AFB_{1} in related non-GM and isogenic GM cultivars for the first time. The effect of increasing CO_{2} at 30 and 35°C varied depending on the type of maize used (conventional or GM). This was the first attempt to analyze whether using these two types of cultivars under climate-related abiotic factors can cause differences in AFB_{1} production and the level of control achieved when using non-toxigenic biocontrol strains.

The action of the non-toxigenic A. flavus strain (AFL^+4) as a biocontrol agent was significant in the elevated CO_{2} treatments, although the overall efficacy was lower than in non-climate change abiotic conditions. The use of the GM cultivar (P30F53 H^+) showed better results in terms of relative biocontrol under abiotic stress (0.95 a_{w}) and increased CO_{2}.

There are limited previous studies to examine the effect of three-way interacting abiotic factors of elevated temperature \times \text{elevated CO}_{2} \times \text{drought stress effects on biocontrol of mycotoxin production} (Verheecne-Vaessen et al., 2019). Previously, three-way interacting climate change abiotic factors were shown to stimulated AFB_{1} production by the type strain of A. flavus (NRRL strain) both in vitro and in stored maize grain under increased temperature (30 vs. 34–37°C), 350 vs. 650/1,000 ppm CO_{2}, and different a_{w} stress levels (Medina et al., 2015, 2017b). While growth of A. flavus was relatively unaffected, expression of key genes such as the aflR and aflD was significantly increased and translated into a stimulation of AFB_{1} production. Indeed, Vaughan et al. (2014) demonstrated that elevated CO_{2} (800 \mu mol CO_{2} \text{ mol}^{-1} \text{ air}) enhanced maize susceptibility to F. verticillioides infection, but the increase in fungal biomass did not result in higher FB_{1} toxin levels. Although subsequent studies suggested that there was an interaction between drought stress and elevated CO_{2}, which increased FB_{1} production (Vaughan et al., 2016).

More recently, Gilbert et al. (2018) using RNA-sequencing demonstrated that AFB_{1} production in stored maize grain was altered by a_{w} \times \text{temperature} \times \text{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 1,000 ppm CO_{2}, the transcription factor aflR 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} (1,000 ppm) and water stress (0.91 a_{w}) showed decreased values, possibly in response to elevated AFB_{1} levels (Gilbert et al., 2018).

Other imposed chemical stresses may also result in physiological impacts on toxigenic fungi such as A. flavus. Recent studies by Hanano et al. (2019) have shown that the most toxic congener of dioxin, the 2,3,7,8-tetrachlorinated dibenzo-p-dioxin, reduced growth but stimulated both conidial sporulation and AFB_{1} production supported by levels of biosynthetic gene expression. Indeed, such exposure to chemical stress was shown to increase the production of superoxide dismutase and catalase. Of particular interest was the activity of caleosin/peroxygenase enzyme, which was activated in the presence of such recalcitrant compounds. This suggests that exposure to elevated levels of atmospheric particulates and gaseous stresses such as CO_{2} and interaction with elevated temperatures may impact on physiological functioning of A. flavus and influence the toxin contamination levels.

**CONCLUSIONS**

This study has shown that the relationship between pre-harvest ripening stage of maize cobs and their inherent water availability will influence both colonization and AFB_{1} production by toxigenic A. flavus strains and also influence the potential for effective control of toxin contamination. Thus, by using a 50:50 ratio of BCA:pathogen composition, the efficacy of the non-toxigenic strain was more effective at the R3 and R4 ripening stages, supported by the downregulation of the two toxin genes (aflD, aflR) relative expression, accompanied by a significant reduction in AFB_{1} contamination. At the dent stage, perhaps a higher inoculum of the non-toxigenic strain is necessary for effective control.

Post-harvest, biocontrol of AFB_{1} production in non-GM and GM cultivars was affected by interacting variables: type of cultivar, T °C, CO_{2} levels, and water availability conditions. These interactions may also significantly affect BCA resilience and relative action. This study suggests that in GM cultivars, the relative control was slightly more effective than in the equivalent isogenic non-GM maize cultivars post-harvest. The resilience of the non-toxigenic strains appeared to vary between these. Overall, it is very important to include resilience to climate-related abiotic factors to ensure that
the identified strains of non-toxigenic strains and indeed other biocontrol candidates have the necessary ecological competence to compete effectively and reduce toxin contamination, whether the approach is for controlling pre- or post-harvest. Formulation approaches for such biocontrol agents may also play an important role in conserving resilience under a range of interacting abiotic conditions in the maize agroecosystem.

DATA AVAILABILITY STATEMENT
All the data sets are deposited with Cranfield University via the senior author and can be openly accessed via him.

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AUTHOR CONTRIBUTIONS
AG and AR-S carried out the research work. EG-C and CV-V assisted with molecular work and quantification of toxins. AM and NM supervised the research and drafted the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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