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Co-Inoculation of Aflatoxigenic and Non-Aflatoxigenic Strains of *Aspergillus flavus* to Assess the Efficacy of Non-Aflatoxigenic Strains in Growth Inhibition and Aflatoxin B_1 Reduction

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Abstract: The pre-harvest biocontrol approach currently used includes laboratory inoculations using non-aflatoxigenic strains of *Aspergillus flavus*. This strategy effectively suppresses the indigenous aflatoxigenic strains and reduces aflatoxin accumulation in sweetcorn. The current in vitro study's main objective is to determine the diametric growth rates of both Aflatoxin $(AF)^+$ and AF^- strains and improve the understanding of competitive relationships among these strains in sweetcorn (*Zea mays*). Sweetcorn kernels inoculated with AF^+ strains only, AF^- strains only, and co-inoculated with AF^+ + AF^- strains were investigated for aflatoxin concentrations. The diametric growth results revealed that growth rates of AF^- strains at 25 and 30 °C were much greater than AF^+ strains, which was in line with previous studies. The in vitro findings showed that the $AKR5^-$ and $AKL34^-$ biocontrol strains effectively inhibited the colony propagation and subsequent AFB_1 contamination (up to 79%) of AF^+ strains. On the other hand, the $AKR1^-$ and $AKL35^-$ were least effective in reducing AFB_1 contents only by 58% and 60%, respectively. There was a significant difference ($p < 0.05$) in the reduction of AFB_1 contents achieved by AF^- strains of *A. flavus*. The findings of the present study indicated the reduction in AFB_1 with population expressions of AF^+ strains by the AF^- strains and supports the notion of competitive exclusion through vigorous development and propagation of the non-aflatoxigenic fungi.

Keywords: sweetcorn; *Aspergillus flavus*; biocontrol; fungal competition; AFB_1

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1. Introduction

Aflatoxins (AFs) are predominantly produced by *Aspergillus flavus* and *A. parasiticus*. These fungi are typically saprophytic and occur in soil and plant material, causing food and stored grain decay. AFs are characterized by their carcinogenicity and other harmful health effects. AFs can enhance stress vulnerability and reduce growth competence. AFs also cause clinical symptoms of acute aflatoxicosis, including fatigue, increased anxiety, abdominal pain, diarrhea, and death [1]. AFs naturally exist in various cereal crops, including corn, nuts, wheat, and soybean, directly used by humans and animals. There are two important factors causing crop contamination by AFs at pre- and post-harvest stages, including high temperature and humidity [2]. As the food chain considered AFs to be inevitable contaminants, the US Food and Drug Administration (FDA) has developed standard levels for AFs of 20 ppb for all foods and animal feeds [3]. Following the Food Regulations, 1985 [4], Malaysia has defined the maximum permissible limits of total AFs in food as

5 ppb. Almost 450 million citizens are exposed to an undetectable amount of AFs in underdeveloped and developing nations of the world. While numerous AFs were presently identified, aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2) are the four most crucial AFs.

The toxicity pattern of AF is $B_1 > G_1 > B_2 > G_2$. The letters B and G represent their blue and green fluorescence under ultraviolet radiation, while the numbers 1 and 2 designate their small and large compounds [5]. The International Agency for Research on Cancer classifies AFB_1 as one of the most potent, mutagenic, Group 1 human carcinogens [6]. Ishadi and Mazlan [7] reported different concentrations of AFB_1 ranged between 5 and 400 ppb in Malaysian animal feeds. Moreover, a higher quantity of AFB_1 (106 ppb) was detected in Malaysian sweetcorn [8]. Later, Khan et al. [9] noticed the existence of aflatoxigenic *A. flavus* (AF^+) isolates isolated from sweetcorn samples based on UV fluorescence screening on coconut cream agar (CCA).

Consequently, various pre- and post-harvest methods were attempted to inhibit or minimize AFs contamination in food commodities. Biological control involves the application of different microbes such as bacteria, yeasts, and AF^- against AF^+ fungi [10]. Biocontrol is one of the most promising approaches, which involves field inoculation with AF^-, and has proven to be effective in reducing AFs contamination in the field [11,12]. The Environmental Protection Agency (EPA) has approved two bio-pesticides, including AF36 (NRRL 18543) and Afla-Guard®, for pre-harvest use to contain AF exposure. AF36 (NRRL 18543) and Afla-Guard® cannot synthesize AFs as they lack the polyketide synthase gene that is responsible for AF production [13–15]. The biocontrol method has been effectively tested in various commodities such as cotton [16], corn [17], and peanuts [18]. Although AF reduction has been accredited to the competitive exclusion of AF^+ by AF^- strains, the exact mechanism of exclusion has to be clarified.

According to Wei et al. [19], the removal of AF^+ strains could be accomplished by the rapid growth of the competing AF^- fungal strain. On the other hand, contamination with AF could be reduced by the physical proximity of competitive strains that could send a signal inhibiting AF production in AF^+ strains by AF^- strains [20]. According to Bandyopadhyay et al. [21], the reduction in AF production is due to direct hyphal contact, known as "touch inhibition", and not by competitive exclusion. It recommends that AF biosynthesis be somehow affected when the strains of AF^+ and AF^- come close together. Thus, this study was aimed to assess (a) mycelial interactions of five AF^- and five AF^+ strains on malt extract agar (MEA) media and (b) the relative efficacy of the AF^- strains in inhibiting AF^+ growth and reducing AFB_1 production on sweetcorn kernels.

2. Materials and Methods

2.1. Fungal Strains

Ten *A. flavus* strains, isolated from sweetcorn kernels from four different farms in the Cameron Highlands, were used as competitors in this study. The AFB_1 producing strains ($AKR8^+$, $ARV17^+$, $ARV18^+$, $ARV20^+$, $ARV21^+$), and AF^- strains ($AKR1^-$, $AKR5^-$, $AKL34^-$, $AKL35^-$, and $AKL36^-$) were determined through morphological analysis, as described by Khan et al. [9]. Strain type, American Type Culture Collection (ATCC) 200,026 (synonym: NRRL3357; Northern Regional Research Laboratory), purchased from ATCC (University Blvd Manassas, VA, USA), was used as a positive control of AFB_1 synthesis while uninoculated plates were used as a negative control.

2.2. Preparation of Media and Inoculum

Malt extract agar (MEA) (Oxoid Ltd., Basingstoke, UK) was used to evaluate the hyphal expansion of AF^+ and AF^- strains and prepare a conidial suspension. Fifty grams of MEA was added to 1000 mL of dH_2O and boiled until dissolved. The media was autoclaved at 121 °C for 20 min and emptied into 9 cm Petri dishes. The inoculated dishes were incubated in the dark at 30 °C for 7 d. The conidia had been obtained through overflowing a single culture of individual strain employing distilled water with Tween 80 and scratching mycelia

by a sterilized scraper. The conidial suspensions were adjusted to 10^{-6}/mL with serial dilution and the haemo-cytometer. The spore suspensions were formulated 1 d earlier before being used and maintained at −4 °C. Spore suspensions for AF^+ and AF^- strains were conducted separately. Each inoculum of 50 mL, with a ratio of 1:1, was mixed for preparing the co-inoculation mixture.

2.3. Diametric Growth Rates of Aflatoxigenic and Non-aflatoxigenic Aspergillus flavus on Malt Extract Agar

The AF^+ and AF^- strains were inoculated on MEA and incubated in the dark for 7 d at various temperatures (25, 30, 35, and 40 °C). The diametric growth rates of the AF^+ and AF^- strains were recorded on days 3, 5, and 7 by assessing the colony diameter in two directions parallel to one another [22] using *ImageJ* software version 1.50 i. Diametric values were summed, and a curve of y = mx + c was constructed, with y being colony diameter (mm), x being incubation time (d), and m being diametric growth rate (mm/d).

2.4. In Vitro Inoculation

The trials were performed using sweetcorn kernels obtained from the local supermarket in 2019. Whole, uncontaminated kernels with a relatively unifying size were assigned randomly to four treatments and treated as per kernel screening assay [23]. The kernels were then sanitized with 75% ethanol and washed with distilled water. For each treatment, ten kernels were inoculated through soaking and then mixed thoroughly for 1 min. The treatments involved: (1) kernels inoculated by AF^- strains, (2) kernels inoculated by AF^+ strains, (3) kernels co-inoculated by AF^- + AF^- strains, (4) kernels inoculated by NRRL 3357 (positive control), and kernels inoculated by water (negative control). In the co-inoculated treatment, 50 mL of each AF^- and AF^+ strain, at a 1:1 ratio, was applied to the sweetcorn kernels. The individual compartments for each group of kernels were mounted in Petri plates. Kernels were incubated in the dark at 28 °C and observed at 3, 5, and 7 d of incubation. Three replicates for each treatment were made in this study. After incubation, the samples were ground to powder and preserved at −4 °C until HPLC analysis.

2.5. Aflatoxin Analysis

2.5.1. Chemical and Reagents

All the solvents and reagents utilized in this study were of HPLC grade unless otherwise stated. Methanol (MeOH) and acetonitrile (CAN) of HPLC grade were acquired from Sigma–Aldrich (St. Louis, MO, USA), and ultra-pure water (dH_2O) was attained from Elga LabWater (High Wycombe, UK). Likewise, a Mixed AF standard was purchased from Sigma–Aldrich (USA) with original concentrations of 1000 ng mL^{-1} (AFB_1, AFG_1) and 300 ng mL^{-1} (AFB_2, AFG_2). The standard was stored at −20 °C until further analysis. An HPLC C_{18} column, analytical, 12 nm, 5 µm, 150 × 4.6 mm was supplied by YMC (Kyoto, Japan).

2.5.2. Preparation of Standard Solutions of Aflatoxin B_1

A series of standard solutions of 10 to 100 ppb for AFB_1 was prepared through dissolving stock standard solution with methanol. All the standard solutions were kept preserved at 4 °C until further analysis.

2.5.3. Aflatoxin Extraction and Cleanup

The extraction of AFB_1 was achieved, as formulated by Baquião et al. [24], with slight modifications. The ground corn sample (10 g) was mixed with 75 mL of a mixture of acetonitrile and deionized water at a ratio of 50:25 and was then shaken for 1 h. The extract was filtered through Advantec filter paper No.131. Two milliliters of the filtrate was then transferred into a test tube and pressed through sterile syringe filter cartridges (0.22 µM; HmbG Chemicals, Hamburg, Germany). One milliliter of the purified extract was removed and evaporated to dryness. The dried extract was then reconstituted into 1 mL of HPLC grade

methanol, vortexed for 30 s (LMS Co., Ltd., Tokyo, Japan) for 30 s, incubated in the dark at 37 °C for 30 min, and centrifuged (Sartorius, Germany) for 5 min at 10,000× *g*. The tresultant extracts were filtered into 2 mL HPLC vials (Thermo Scientific, Milford, MA, USA) using sterile syringe filter cartridges (0.22 µM; HmbG Chemicals, Hamburg, Germany).

2.5.4. High-Pressure Liquid Chromatography Procedure

A reversed-phase HPLC instrument (Waters 600, Milford, MA, USA), coupled with a fluorescence detector (Waters 2475, Milford, MA, USA) was employed in this study. A mobile phase of acetonitrile, methanol, and distilled water (10:35:55, *v*/*v*/*v*) with a 0.6 mL/min flow rate at 40 °C was used for separation through a C_{18} column (YMC-Triart, 5 µM, 12 nm, 150 × 4.6 mm; YMC, Tokyo, Japan). The injection volume was 20 µL, while the flow rate was 0.6 mL/min. The wavelengths of excitation and emission were 365 nm and 435 nm. The mobile phase (acetonitrile, methanol, and dH_2O) was filtered through a nylon filter membrane (0.45 µM; Merck, Darmstadt, Germany) and degassed in an ultrasonic bath (Power sonic 420, Seoul, Korea) for 30 min. A photochemical Reactor for Enhanced Detection (PHRED) (Aura Industries, New York, NY, USA) was employed to improve the fluorescence of AFB_1. The data software, Empower-2 Chromatography data software (Waters, Milford, MA, USA) was utilized for data acquisition and processing.

Before AF was analyzed for the sweetcorn samples, various performance parameters, including linearity, the limit of detection (LOD), the limit of quantification (LOQ), and recovery were assessed to validate the HPLC method. Linearity was determined by injecting mixed AF standards in methanol at six concentrations of 10, 20, 40, 60, 80, and 100 ppb for AFB_1. The calibration standards were assessed in triplicate before and during the analysis. The method was further validated for recovery by spiking AFB_1 into sweetcorn blank samples at a concentration of 20 and 100 ppb. The findings were expressed as relative standard deviation (RSD^r).

A calibration curve for AFB_1 (20, 40, 80, 100 ppb) was plotted to convert the luminescence unit (LU) regions attained by HPLC analysis into the actual amount of AFB_1 (ppb) of the sweetcorn samples. During sample analysis, four measurements (n = 4) have been noted for every calibrant solution ($\sum$ = 32) to test the reproducibility and the repeatability of the quantifications. For the calibration curve, the goodness of fit (R2) was 0.9960. The limit of detection (LOD) and limit of quantification (LOQ) were determined by the following formulae, as defined by the International Conference on Harmonization (ICH): LOD = 3.3 σ/s and LOQ = 10 σ/s. In the formulae, σ is the standard deviation of blank responses, while *s* is the slope of the calibration curve [25]. The separation and detection of AFB_1 was done as depicted by Kongetal [26], with slight changes.

2.6. Statistical Analysis

In this study, the experiment was performed in triplicate. The quantities were averaged and shown as a mean ± standard error. A one-way analysis of variance (one-way ANOVA) with a 95% confidence interval was performed with statistical software SPSS version 23. A p-value of ≤ 0.05 was accepted as a significant difference. Tukey's Honest Significant Difference (Tukey's HSD), with $\alpha = 0.05$, was performed to relate the significant difference between treatment means.

3. Results

3.1. Growth Rates of Aspergillus flavus on Malt Extract Agar

The diametric growth rates of AF^+ and AF^- strains on MEA after a 7 d period of incubation at various temperatures (25, 30, 35, and 40 °C) are shown in Figure 1. The growth rates have been attained by constructing a $y = mx + c$ curve in which y is the colony diameter (mm), x is the time of incubation (d), and m is the diametric growth rate (mm/d). At 1 d, both the AF^+ and AF^- strains displayed a very low growth rate at all temperatures. AT 5 d, the AF^- strains produced the highest growth rate at 25 °C and 30 °C compared to AF^+ growth. At 7 d, the overall growth rates of both AF^+ and AF^- strains at 40 °C were very small.

The growth rates of AF^- strains at 25 °C and 30 °C were very high compared to AF^+ strains, suggesting the efficacy of AF^- strains as biocontrol agents for the inhibition of AF^+ growth and AF reduction (Figure 2). In the negative control, no growth was observed.

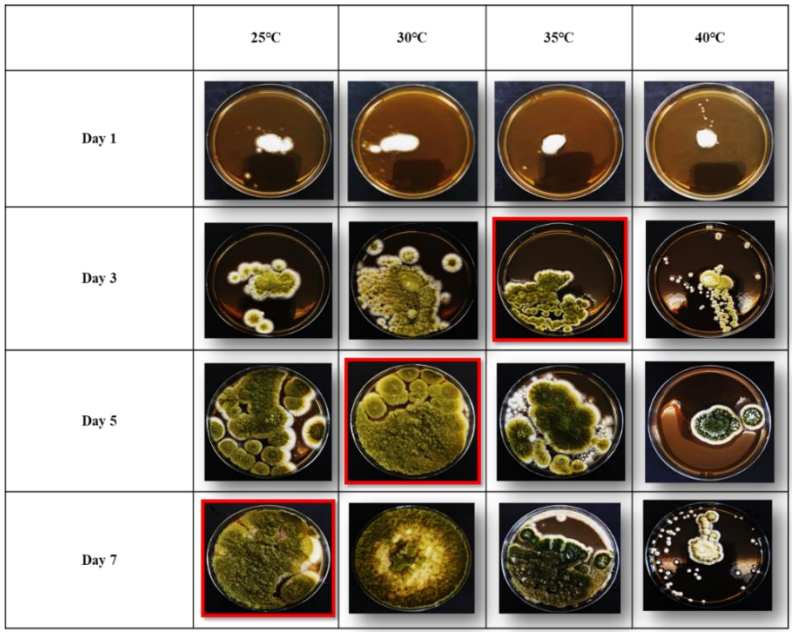

Figure 1. Diametric growth rates (mm/d) of $AKR5^-$ strain cultivated on malt extract agar (MEA) at four different temperatures (25, 30, 35, and 40 °C) for seven days.

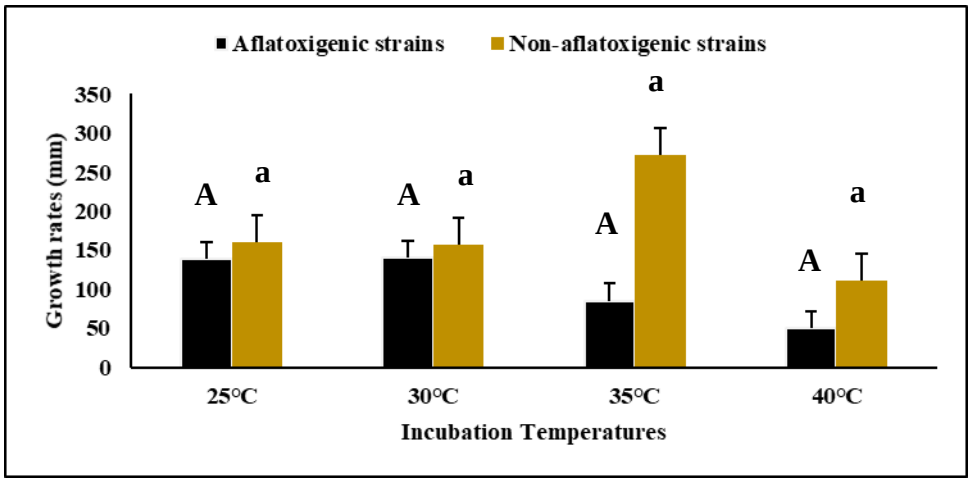

Figure 2. Diametric growth rates (mm/d) of AF^+ and AF^- strains cultivated on malt extract agar (MEA) incubated at four different temperatures (25, 30, 35, 40 °C) for 7 d. Data are means of triplicates ($n = 3$) with bars indicating a standard error (SE). Capital letters indicate a significant difference ($p < 0.05$) between AF^+ strains and small letters indicate a significant difference ($p < 0.05$) between AF^- using Tukey's Honest Significant Difference (Tukey's HSD).

3.2. Detection of Aflatoxin B_1 Using High-Performance Liquid Chromatography

On sweetcorn kernels, the perceived exterior growth of AF^+ strains in co-inoculated groups completely covered the pericarp (Figure 3). Even though the entry point was commonly at the pedicel, it is not strange to detect AFB_1 in other regions of a corn kernel. There has been a significantly complex fungal spread on the co-inoculated corn kernels as presented in Figure 3.

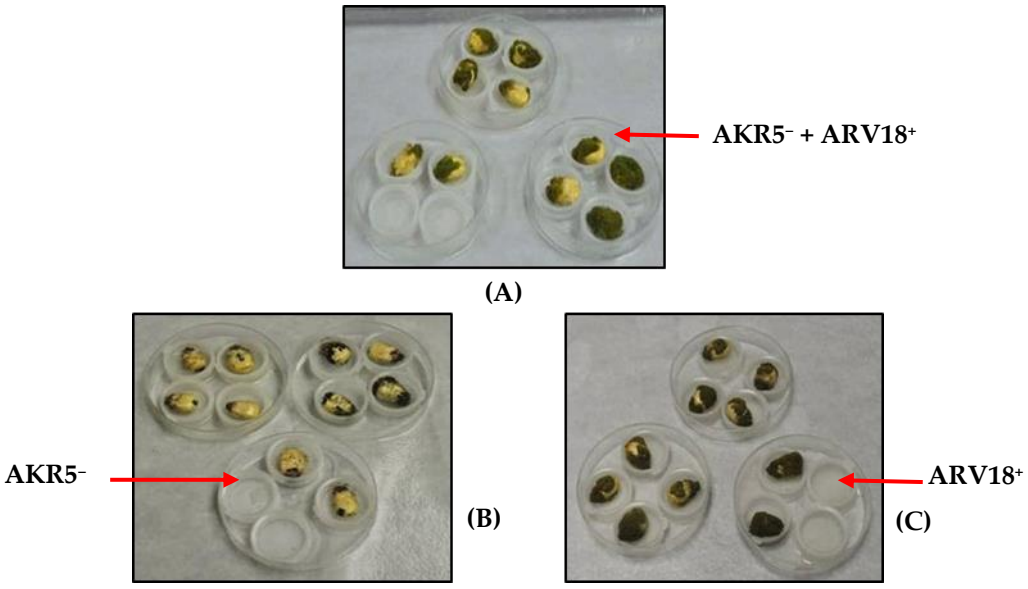

Figure 3. Images of the AKR5^{-} and ARV 18^{+} strains; (**A**) AKR5^{-} and ARV 18^{+} strains were grown on corn kernels in mixtures; (**B**,**C**) AKR5^{-} and ARV 18^{+} strains were grown on corn kernels separately following on 7 d of incubation.

The growth of AF^{+} strains (AKR8^{+}, ARV17^{+}, ARV18^{+}, ARV20^{+}, ARV21^{+}) was restricted to the margins of the sweetcorn kernels, and is most likely outcompeted by the AF^{-} strains (AKR1^{-}, AKR5^{-}, AKL34^{-}, AKL35^{-}, AKL36^{-}). The corn kernel samples inoculated with AF^{-} strains alone or with negative control (dH2O) did not produce AFB_1 when examined using the HPLC technique. On the other hand, the co-inoculated corn kernel samples exhibited a small amount of AFB_1 production, indicating that AF^{-} strains dominate AF^{+} strains. Data were assessed in a one-way ANOVA through the mean of AFB_1 produced by AF^{+} and co-inoculated (AF^{+}-AF^{-}) strains of *A. flavus*.

3.3. LOD and LOQ for Aflatoxin B_1

The LOD and LOQ for AFB_1 were used to validate the HPLC system performance. A standard solution of 20–100 ppb of mixed AF standard was used to construct a four-point calibration curve. The LOD for AFB_1 was 0.072 ppb, according to the fluorescence detector FLD. Likewise, the LOQ for AFB_1 was 0.220 ppb (Table 1).

Table 1. Validation of AF determination by HPLC analysis.

AF	LOD (ppb) [a]	LOQ (ppb) [b]	Calibration Curve [c]	R^2
AFB_1	0.072	0.220	y = 55,006,523.62	0.9960

[a] Limit of detection (LOD); [b] Limit of quantification (LOQ); [c] x = Concentrations of AF (ppb), y = intensity.

3.4. Linearity

The linearity of the method was determined by four-point calibration curves over a range of 20 ppb to 100 ppb for AFB_1, using the correlation coefficient (R^2) and the slope to demonstrate a relative association of responses versus AFB_1 concentrations. Calibration curves were constructed by plotting the peak area (y) versus AFB_1 concentrations (x) (Figure 4). For the FLD detector, calibration curves with linear regression (R^2) demonstrated excellent linearity for AFB_1 (0.9960). The average recoveries for AFB_1 at 20–100 ppb spiking levels ranged from 70–94%.

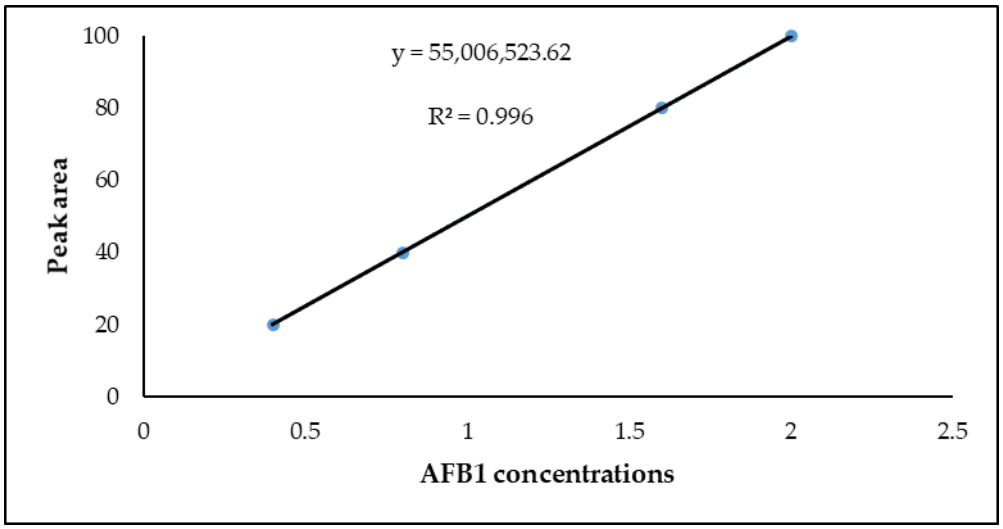

Figure 4. Standard curve for AFB_1 standard solution.

3.5. Quantification and Reduction of Aflatoxin B_1

Figure 5 shows the amount of AFB1 produced by the AF^+ strains of *Aspergillus flavus*, including $AKR8^-$, $ARV17^+$, $ARV18^+$, $ARV20^+$, and $ARV21^+$. These AF^+ produced AFB_1 ranging from 0.91 to 3.96 ppb; however, they did not exceed the permissible limit of 5 ppb. The $AKR8^+$ and $ARV17^+$ strains were found to be the highest AFB_1 producers (2.61, 3.96 ppb), while the $ARV21^+$ strain was found to be the lowest producer of AFB_1 (0.91 ppb) (Figure 5).

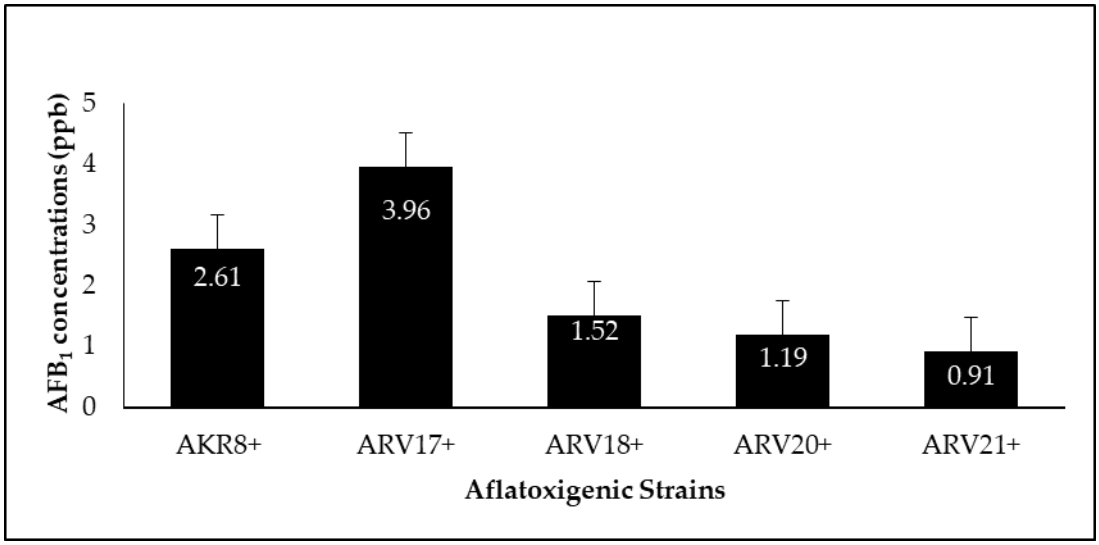

Figure 5. AFB_1 production by AF^+ strains following seven days of incubation.

Figure 6 shows a significant ($p < 0.05$) decrease in AFB_1 concentration in sweetcorn kernels by the AF^- strains including $AKR1^-$, $AKR5^-$, $AKL34^-$, $AKL35^-$, and $AKL36^-$ when co-inoculated with AF^+ strains ($AKR8^+$, $ARV17^+$, $ARV18^+$, $ARV20^+$, $ARV21^+$). In co-inoculated treatments, the AFB_1 concentrations ranged between 0.321 and 1.093 ppb, while the reduction in AFB_1 concentrations in corn kernels ranged from 23% for $AKL36^-$ to 79% for the $AKR5^-$ strain. The highest reductions in AFB_1 concentrations were reported for $AKR5^-$ and $AKL34^-$, decreasing AFB_1 by 79% and 75%, respectively. On the contrary, $AKL36^-$ reduced the AFB_1 synthesis to a very small degree and would therefore not be a suitable

candidate for a potential biocontrol agent. In the positive control (inoculation with NRRL 3357), the mean concentration of AFB_1 was 1.142 ppb (Table 2).

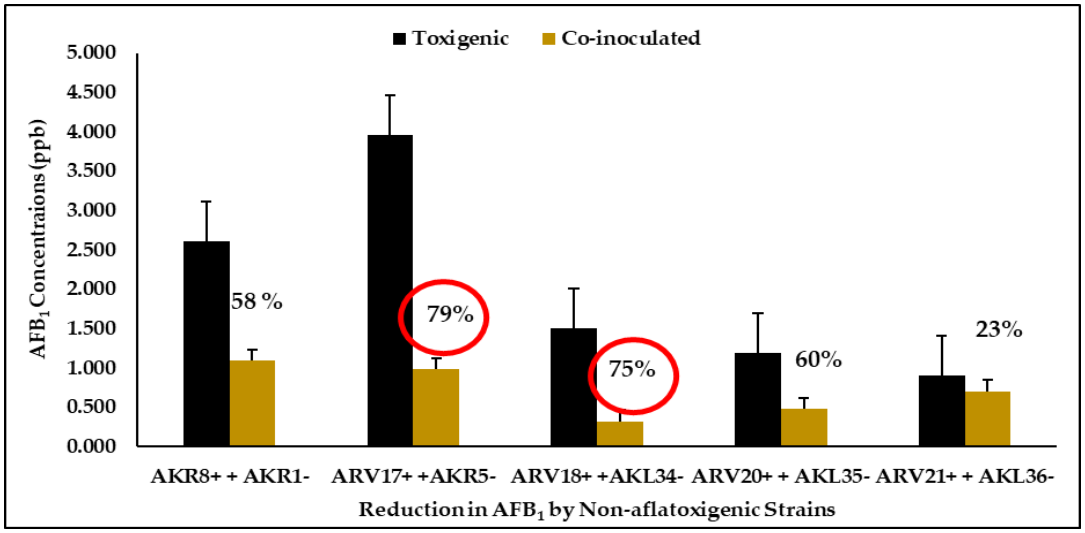

Figure 6. The percentages of reductions in AFB_1 by the AF^- strains following seven days of incubation.

Table 2. Detection of AFB_1 in AF^+ and co-inoculated ($AF^+ - AF^-$) strains following seven days of incubation.

	Strains	Concentrations (ppb)
		AFB_1
Non-aflatoxigenic strains	$AKR1^-$	ND
	$AKR5^-$	ND
	$AKL34^-$	ND
	$AKL35^-$	ND
	$AKL36^-$	ND
Aflatoxigenic strains	$AKR8^+$	2.615 ± 0.12
	$ARV17^+$	3.962 ± 0.32
	$ARV18^+$	1.509 ± 0.39
	$ARV20^+$	1.195 ± 0.51
	$ARV21^+$	0.907 ± 0.11
Co-inoculated strains	$AKR1^- + AKR8^+$	1.093 ± 073
	$AKR5^- + ARV17^+$	0.983 ± 0.10
	$AKL34^- + ARV18^+$	0.321 ± 0.30
	$AKL35^- + ARV20^+$	0.479 ± 0.11
	$AKL36^- + ARV21^+$	0.702 ± 0.21
Positive Control	(NRRL 3357)	1.142 ± 0.42
Negative Control	Water	ND

ND = not detected.

4. Discussion

In this study, AF^- strains were used as potential biocontrol agents, and their efficacy in inhibiting AF^+ growth and the reduction of AFB_1 in sweetcorn was investigated. While all the co-inoculated kernels demonstrated fungal growth at 3 d, external fungal growth was primarily attributed to AF^- strains across all incubation periods. The AF^- appears to have a competitive advantage compared to AF^+ strains due to its ability to grow rapidly and vigorously colonize the host (Figure 2). AF production needs energy in the form of ATPs [27], possibly affecting the capability of a toxigenic strain to colonize rapidly because it may divert energy to a secondary metabolism pathway. Because this does not seem to be a tactical approach aimed at AF^- strains, it seems that AF^+ and AF^- strains do not compete in normal settings and coexist until their habitats are disrupted [28].

The AF^+ strains' growth was significantly reduced in the co-inoculated kernels and was confined to the edges of the kernel, probably due to the rapid growth of the AF^- biocontrol strains. A significant difference in population observed between $AKR5^-$ + $ARV17^+$ and $AKL34^-$ + $AKR8^+$ treatments are in line with previous studies, while the AFB_1 synthesis was reduced by AF^- strains between 75 and 79%. In the current study, the average reduction in AFB_1 production was 59% compared to previous studies' results on cotton [29,30]. The difference could be due to the short time and small sample size of the lab testing compared to the field tests. Inoculation time could also be attributed to the limited reduction of AFB_1 synthesis found in the present research. Maximum decline (80–90%) in AF production has been observed in corn [26] and cotton [30] when the AF^- strains were introduced 1 d before the AF^+ strains. It does not, however, represents events in the field.

The field environments would be better demonstrated by studies that investigate the pre-inoculation of AF^+ strains. The observed discrepancies in the population among co-inoculated and the individual treatments back the hypothesis that the growth inhibition of AF^+ strains is caused by competitive removal by AF^- strains [26,30]. This exclusion may be owing to the preliminary attainment of resources and tissue volume by an AF^- strain, or by some mysterious explanation as to why fungus needs to live in a particular condition [29]. Moreover, knowing the relative inoculum level is a crucial element in the transport of biocontrol agents. By employing different quantities of AF^- and AF^+ strains in the matrix protocol, useful data can be obtained on the impact of larger inoculum of either strain on the production of AFB_1. This suggestion showed that AF^+ and AF^- inoculum treatment in the ratio of 50:50 or 25:75 delivered viable results for the reduction of AFB_1 production. It presents essential data on inoculum quantity that could be critical in inhibiting AFB_1 production in sweetcorn in a wide range of environments. According to Pittetal et al. [31] and Degola et al. [32], the increase in AF^- spore inoculum against AF^+ would provide a higher reduction in AFB_1 production. Meanwhile, inocula spore quantities were approximately the same in our competitive study; rapid development and a vigorous expansion of AF^- strains successfully removed the AF^+ strains and thus reduced AFB_1 concentrations.

There are some other explanations for the reduction in AF production, including the presence of different volatile compounds in both the AF^+ and AF^- strains. Some terpenes, such as alpha-pinen, have antifungal properties [33] that are found in volatile compounds synthesized by AF^- strains [34]. Furans are other types of antimicrobial compounds formed by *A. flavus*; nonetheless, it is released by the AF^- strains several days before the AF^+ strains [34]. The synthesis of specific volatiles from AF^- strains could be involved in preventing AF^+ strains from growing, thus reducing the quantity of AFs produced. As the AF^- strains are not affected by the proximity of the AF^+ strains, AF^- strains seem unlikely to target AF production directly. The reduction of AFs may, therefore, be an incidental suppression of the population density of the AF^+ strains. It would help to clarify if the relative success of the used AF^- strain effectively manages AFs contamination in field conditions. Regretfully, this research has not investigated its long-term consequence.

Our study showed that, even though AF^- strains reduced AFB_1 synthesis, the kernels occupied by the AF^- strains have been severely spoiled by 7 d of inoculation. The AF^- did not produce AF; however, the majority of kernels lost their integrity by the end of the study and were inundated by the fungus, which would not be beneficial to the crop if the biocontrol remained after harvesting. Another possible precaution against using AF^- stains as biocontrol agents is the production of cyclopiazonic acid (CPA), a toxin that allegedly specifically targets inner major organs and skeletal muscles [35–37].

While the harmful impressions of CPA are not as well described as those of AFs [35], misuse of AF^- strains, leading to the production of CPA, could lead to unintended human and animal health effects. It is also essential to take account of the possible path of inhibited AF^+ strains in instances of inundation and containment by competitive AF^- strains when using them as biocontrol agents. For instance, its latent capacity to replicate in laboratory conditions may result in consistently inhibited strains, leading to generations of AF^+

descendants becoming more contagious than anticipated [37]. These issues concerning possible impacts on crop quality and unforeseen health threats have to be thoroughly assessed while selecting particular AF^- strains for AF biocontrol uses.

5. Conclusions

The current study demonstrated, under in vitro conditions, that the $AKR5^-$ and $AKL34^-$ biocontrol strains have effectively inhibited colony propagation and subsequent AFB_1 contamination (79%, 75%) by the AF^+ strains. In comparison, the $AKR1^-$ and $AKL35^-$ were the least effective, reducing AFB_1 contents by only 58% and 60%, respectively. Thus, there was a significant difference ($p < 0.05$) in the reduction of AFB_1 contents achieved by AF^- strains of *A. flavus*. Using AF^+ strains, we have been able to monitor invasion and colonization easily and understand the competition of the two opposing strains in sweetcorn kernels. Based on its vigorous development and propagation, our results accept the competitive exclusion theory in favor of biocontrol strains. This study also points out that there are important issues concerning the long-term use of AF^- strains for the elimination of native AF^+ strains in biocontrol methods.

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