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Non-Aflatoxigenic *Aspergillus flavus*: A Promising Biological Control Agent against Aflatoxin Contamination of Corn

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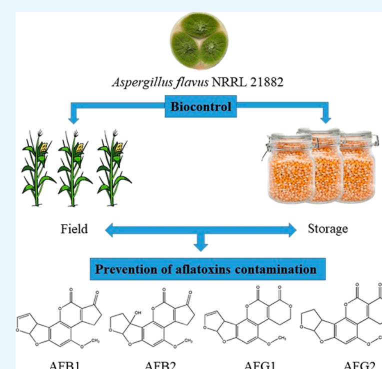
ABSTRACT: Aflatoxins (AFs) are a family of mycotoxins produced by molds in agricultural products. To deal with this problem, one of the control methods is the biological solution using a non-pathogenic strain *Aspergillus flavus* NRRL 21882 (Afla-Guard). This study was conducted to evaluate the potential of *A. flavus* NRRL 21882 to control the AF contamination of corn in the field and during storage in 2018 and 2019. The experimental design consists of treatment at different vegetative stages of infested corn in the field trial. After the field has been harvested, half the corn kernels from both treated and control plots were treated with biopesticide; the other half of the kernels from each group were not treated and used as the control of the storage. Consequently, storage applications consisted of kernels: (1) not treated at all; (2) treated prior to storage; (3) field-treated; and (4) treated both in the field and prior to storage. After field trials, the AF content was very low in the treated plots, ranging from 0.50 to 1.04 μg/kg and from 0.50 to 0.73 μg/kg in 2018 and 2019, respectively, while the AF content in the control was 98.3 and 73.9 μg/kg in 2018 and 2019, respectively. After storage, corn kernels from field plots that were treated with the biopesticide (treated/control) showed low levels of AFs, even after they have been stored under conditions conducive to AF contamination. The biopesticide effect ranged from 98 to 99% and from 69 to 99% in the field and during storage, respectively. This paper has provided the first indications on AF biocontrol based on a competitive exclusion in the corn-growing region of Turkey. The data showed that spraying during the storage period did not provide any further prevention of AF contamination, and only treatment in the field had a significant effect on AFs that occurred in storage.



1. INTRODUCTION

Corn (*Zea mays L.*) is a cereal grain and among the most important food and feed crops worldwide.[1] Over the past century, advances in corn genetics and agronomical technology have led to significant increases in corn yield.[2] Due to the high yield and economic importance of modern seed varieties, a rapid, uniform, and complete seedling establishment is a crucial element for successful corn crop cultivation.

The main mycotoxigenic fungi of corn are *Aspergillus* section *Flavi*, especially *Aspergillus flavus*.[3] *A. flavus* is a ubiquitous and soil-born fungal pathogen that can attack a wide range of crop species, mainly oilseed crops, including corn and peanuts, and many other commercially valuable cultures, damaging plants that are weakened by external stressors.[4,5] In addition, *A. flavus* causes considerable direct and indirect economic damages in numerous agricultural regions around the world.[6,7]

Populations of *A. flavus* consist of species exhibiting two distinguishable morphological variants of sclerotium size: large (*L*) variants with an average sclerotium size >400 μm in diameter and small (*S*) variants that produce high numbers of sclerotia <400 μm in diameter.[8] Both *L* and *S* strains are distinguished by certain characteristics including the production of mycotoxins.[9] It was reported that *A. flavus* S variants generally produce higher amounts of AFs than L variants.[10,11] *A. flavus* sclerotia are survival structures that can withstand hostile environmental circumstances. Besides, sclerotia germinate sporogenically in the soil by producing aerial conidiophores, which serve as an origin of the principal inoculum in the field and also have a role in sexual reproduction.[12,13]

Mycotoxins known as aflatoxins are made by the fungus *A. flavus* in the form of secondary metabolites. The four most common kinds of AFs are designated by the abbreviations AFB1, AFB2, AFG1, and AFG2. There is a potential that *A. flavus* isolates may produce AFB1 and AFB2, but neither AFG1 nor AFG2 can be produced.[14,15] AFs are known as the strongest mutagenic, teratogenic, and carcinogenic compounds among mycotoxins.[16,17] A lot of agricultural commodities are

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easily infested by pathogenic molds in the field, resulting in the contamination of most crops with AFs and/or other mycotoxins. Not only in the field but crops can also be heavily contaminated with AFs during improper storage.[18] Besides AFs, *A. flavus* can commonly produce cyclopiazonic acid (CPA), an indole tetramic acid known to be toxic to both animals and humans.[19]

AFs occur all over the world but are more prevalent in regions with warm and humid climate. Türkiye is defined by several geographical regions, and the climate varies according to each region and latitude. The Mediterranean climate is typically marked by warm, dry summers and mild, rainy winters. The oceanic climate is characterized by warm, wet summers and rainfall throughout the whole year. The continental climate is characterized by hotter summers and cold winters.[20] Climate change is also expected to cause serious consequences for the contamination of crops by AFs. Among all Rapid Alert System for Food and Feed notifications from 2002 to 2019, 10302 (20.9%) concerned AFs, the majority of them were from Iran (2473), followed by Turkey (2318).[21] A total of 1473 results on various food groups commercialized in Türkiye from 2002 to 2019 have been used in the evaluation of the risks. In the adult population, pistachios (44.4%) were the main source of chronic dietary exposure to AFB_1, which are followed by corn flour (16.2%).[22]

Due to their toxic effects, AFs are considered the most scientifically studied and extensively investigated mycotoxins. The LD_{50} values of AFs are from 0.5 to 10 mg/kg body weight, and AFs especially AFB_1 are classified as group 1 carcinogens. Thus, the presence of AFs in foods is strictly regulated by the United States Food and Drug Authority (US FDA) and the European Commission of European Countries (EC). Turkey's national legislation on mycotoxins is being aligned with EU legislation.[21,22] The maximum level (ML) in different nuts, dried fruits, spices, and grains is in the range of the ML of AFs in corn, wheat, rice, peanut, sorghum, pistachio, and almond set by FDA and EC ranging from 4 to 20 μg/kg.[23] Agricultural commodities that contain AFs above the MLs are not safe and are rejected at the border.[24] Therefore, it is of great importance to mitigate the contamination of AF-producing fungi and reduce the risk of AF contamination.

Many approaches to minimize AF contamination of agricultural products have been implemented, including adjustment of planting date, irrigation, improved fertility management, and fungicide application, but these have had limited effectiveness.[25] Biological approaches have been developed recently to minimize the risk of AF contamination by *A. flavus*, such as *Bacillus* isolates, puroindoline A protein, *A. flavus* volatile organic compounds, and non-pathogenic fungal strains.[26−29]

Alshanaaq et al. developed a biological approach using the *Aspergillus oryzae* M2040 strain, which was isolated from dry-fermented soybeans.[30] The developed method was successfully applied to inhibit AFB_1 production and spread of pathogenic *A. flavus* in vitro and in peanuts. Another study confirmed that the biopesticide strategy including non-aflatoxigenic *Aspergillus parasiticus* significantly reduced the contamination amount of AFs by 74 to 99%.[31]

Another natural biocontrol agent (Afla-Guard) was developed based on the competition between pathogenic and non-pathogenic *A. flavus* NRLL 21882. *A. flavus* is not an AF producer, nor does it produce CPA and other toxins. It naturally lacks the whole AF biosynthesis gene cluster.[32,33] It was seen that this biopesticide significantly reduces AF contamination levels and thus helps improve the marketability of corn and peanuts because of the effectiveness of *A. flavus* for controlling AF contamination of peanuts is well investigated.[34] It is also considered that the same biocontrol agent could be potentially applied to other crops such as corn.[35] Already, in one study, the researchers mentioned that *A. flavus* NRLL 21882 inoculated to corn plots greatly reduced AF contamination in harvested corn by 66 to 87%.[36]

Even after harvest, the same researchers found that the treatment with *A. flavus* NRLL 21882 in the field also has a carry-over impact of reducing AF contamination of peanuts during storage. Therefore, *A. flavus* NRLL 21882 is shown to be a very powerful and effective biocontrol agent in competing for the growth of aflatoxigenic *A. flavus* and reducing AF levels both in the field and during storage.

This study used *A. flavus* NRLL 21882 as a biopesticide to control AF contamination in corn. The study was conducted with 2 year field and depot trials, with the aim of reducing AF levels in corn with the biopesticide both in the field and during storage.

2. MATERIALS AND METHODS

2.1. Plant Material. *Z. mays L.* (corn) growing in the Adana Province (Southern Turkey) during 2018 and 2019 was used as vegetal material in this study.

2.2. Biocontrol Agent. In this study, we used Afla-Guard as a biopesticide (Syngenta, USA). The company received permission from the Republic of Turkiye Ministry of Agriculture and Forestry to bring the Afla-Guard from abroad as it would be used within the scope of the project. This biopesticide consists of non-aflatoxigenic *A. flavus* strain NRRL 21882; this fungicide comprises a 0.0094% active ingredient with a minimum of 1.2×10^8 CFU/lb of the product.[37]

2.3. Chemicals. The growth media used in this study were as follows: malt extract agar (MEA) and Czapek agar (CZ). The growth media were sterilized for 15 min at 121 °C and kept at 4 °C until needed. Both of the media were obtained from Biokar, France.

2.4. Sampling. The samples were collected during the 2018/2019 growing season. Corn cobs to be used as study samples were collected from 56 field plots in Adana province, located in Southern Turkey. The corn kernels were separated from the cobs by hand. The total of the sampled quantity was 5 kg which was divided into 5 subsamples. Then, the sub-samples were placed in sterilized paper bags and stored at 4 °C in the refrigerator until manipulation.

2.5. Fungal Isolation and Identification. To isolate the aflatoxigenic *A. flavus* strain, briefly, 100 corn kernels of each sample were immersed for 2 min in 70% ethanol, then in 0.4% sodium hypochlorite for 2 min, and washed with sterile distilled water (SDW) for 2 min. Disinfected grains were then plated on 9 cm Petri plates of MEA medium and incubated at 28 °C for 7 days.[38] After incubation, fungal colonies resembling *A. flavus* were subcultured for further identification. Briefly, a loop full of conidia was dissolved in 500 μL of 0.2% agar, and this suspension was used for three-point inoculations on Petri plates containing 20 mL of CZ. Petri dishes were incubated for 7 days at 28 °C in the presence of darkness and examined for the colony color, appearance, and morphology of conidia. Identification of isolates was carried out by using the available taxonomic keys and guidelines for the genera *Aspergillus*.[39] For the strain storage, All strains were cultured

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in MEA for 7 days at 28 °C. The strain agar plugs were kept at 4 °C in 4 mL vials of SDW, whereas for long-term storage, the strains were maintained as suspensions of conidia in 15% glycerol (w/v) and then kept at −80 °C.[40]

2.6. Sclerotium Production. Sclerotium production has been assessed by the Zanon method.[40] For this study, the strains were evaluated on Petri plates of CZ inoculated with a conidium suspension in soft agar (0.2%) prepared from a 7 day growth on MEA medium. Afterward, cultures were incubated in the presence of darkness for 21 days at 30 °C and visually observed for the detection of sclerotia. The strains were classified as S or L morphotypes as described by Cotty.[41]

2.7. Aflatoxin Production. All isolated strains were assessed for AF production by growing them individually on MEA and incubating them at 28 °C in the presence of darkness for 7 days. Each strain was grown in a total of three separate Petri dishes. Using the tip of a pipette, we detached three discs with a diameter of 1 cm from the part of the Petri dish which was located between the center and the edge. Following the removal of the discs, each disc was placed in its own 20 mL vial, weighed, and then extracted with 10 mL of 70% methanol at a ratio of 1:10 (w/v) on the basis of its fresh weight. The vials were then placed in a shaker for 30 min at a slow speed. After this, the culture extract was filtered through filter paper.[42] After cleaning up the crude extract, an analysis for AFs was performed using an Agilent 1100 high-performance liquid chromatograph. The amount of the injection was 100 mL, and the flow rate was 1 mL per minute. The ex:360 nm and em:440 nm wavelengths were utilized for the detection. The mobile phase consisted of a mixture of water, acetonitrile, and methanol with a volumetric ratio of 6:2:3 (v/v/v), and 132 mg of KBr and 385 mL of HNO_3 were added to the mixture.[43]

2.8. Inoculum Preparation. The aflatoxigenic *A. flavus* AC 102 strain used in this work was originally isolated from corn kernels and presented the S morphotype (mean sclerotium diameter <400 μm). After AF analysis, the *A. flavus* AC 102 strain had a high aflatoxigenic potential producing AFB_1 and AFB_2 with a concentration between 50 and 312 μg/g. Inoculum preparation was performed using the method of Wilson and Bell (1984). Briefly, aflatoxigenic *A. flavus* was incubated at 28 °C for 5 days. Later, the conidium suspension was adjusted at 10^6 conidia/mL. One hundred grams of kernels was added to 50 mL of SDW and autoclaved for 30 min in an Erlenmeyer flask. Next, the flask was inoculated with 0.25 mL of the conidium suspension before being incubated at 30 °C for 10 days until overgrowth and sporulation. Then, the flask was washed off the corn seeds using 500 mL of SDW containing 50 μL of Tween 20 and filtered through a layer of sterile cheesecloth. The concentration of conidia was measured with a counting chamber and adjusted with SDW to 10^6 conidia/mL. Finally, 1400 corn kernels were coated with aflatoxigenic *A. flavus* conidia for planting in the field trial, to evaluate the capacity of the non-toxinogenic strain to control the AF contamination.

2.9. Field Trial Design. The field trials were conducted in a commercial field that had been previously used to grow corn and had never been used for biocontrol trials. The field was located in Seyhan, Adana Province, Southern Turkey. The experiments were carried out in separate plot designs. The field was divided into 7 (5 m × 70 cm) plots, and each plot contained 4 rows. Each plot was surrounded by a wide buffer area. The plants were planted in rows at a 15 cm distance. The planting dates were 03/07/2018 and 06/07/2019. All experimental treatments were organized in a completely randomized block design. The field was managed following conventional regional practices. During the plant growing seasons, the field trial was conducted by the following controls and treatments (Table 1): The treatment was carried out with

Table 1. Biopesticide Treatments in the Field[a]

field treatment	code treatment	Afla-Guard dose (g/da)
untreated plots	Control	
biopesticide application to the soil during seeding	Soil	2200
biopesticide application to foliar at the eight-leaf stage	V8	2200
biopesticide application to foliar at the 10-leaf stage	V10	2200
biopesticide application to foliar at 11-leaf and 13-leaf stages	V11 + V13	1100 + 1100
biopesticide application to foliar at the 12-leaf stage	V12	2200
biopesticide application to foliar at the silk stage	R1	2200

[a]V8: 8 leaves with the collar visible; V10: 10 leaves with the collar visible; V11: 11 leaves with the collar visible; V13: 13 leaves with the collar visible; V12: 12 leaves with the collar visible; and R1: onset of silking.

the *A. flavus* strain NRRL 21882 biocontrol agent at different vegetative stages (ground application: biopesticide had been applied in a band over crop rows by using a pump-back and aerial application: biopesticide had been applied in stage V8 to R1 to foliar by using a pump-back) as shown in Table 1, and in the control, we used corn kernels without spore coating. Biopesticide was applied five times from growth stage V8 to R1 (the V8 growth stage is when there are eight leaves with collar visible present, and the R1 growth stage is the onset of silking) as a spray from above the plants at a rate of 2200 g/da (Table 1). Soil application was made before the seeds were sowed at a rate of 2200 g/da (Table 1).

2.10. Harvesting. At the harvest stage, the corn cobs were harvested from each plot on 06/11/2018 and 05/11/2019. The corn cobs were dried for 5−10 days. Corn kernels coming from the control and treatment plots were shelled by hand. Then, a sample of 1 kg was taken from each plot and made into three sub-samples of about 333 g each. Subsequently, samples of 50 g for each sub-sample were stored at 4 °C until AF analyses as described below.

2.11. Storage Application. Field treatment was also followed by storage treatment. The treatments were performed according to the method described by J. W. Dorner and Cole (2002). For this purpose, corn kernels from the field plots were split in two to spray half the kernels in each group with the biocontrol agent prior to storage. Accordingly, 4 treatment regimens were included in the storage phase: (1) control−control, which was kernels never treated with the biopesticide; (2) control-treated, which was kernels from the untreated field plot that was sprayed with the biopesticide before storage; (3) treated−control, which was kernels from the treated field plot that was not sprayed prior to storage; and (4) treated−treated, which was kernels from the treated field plot that was also sprayed with the biopesticide prior to storage. Next, an aqueous conidial suspension of 2200 g/100 L of water of the biopesticide was sprayed for half samples, and the other half of the samples were used as control of the storage and remained

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Table 2. Biopesticide Treatments during Storage[a]

storage treatments	code of corn kernels in storage	treatment dose (g/100 L of water)
untreated biopesticide control plot corn kernels	control-control	
biopesticide application to the control plot corn kernels	control-treated	2200
corn kernels from the soil-treated plot	soil treated-control	
biopesticide application to the corn kernels from the treated soil plot	soil treated−treated	2200
corn samples from the V8-treated plot	V8 treated-control	
biopesticide application to the corn kernels from the V8-treated plot	V8 treated−treated	2200
corn samples from the V10-treated plot	V10 treated-Control	
biopesticide application to the corn kernels from the V10-treated plot	V10 treated−treated	2200
corn kernels from the V11 + V13-treated plot	V11 + V13 treated-control	
biopesticide application to the corn kernels from the V11 + V13-treated plot	V11 + V13 treated−treated	2200
corn samples from the V12-treated plot	V12 treated-control	
biopesticide application to the corn kernels from the V12-treated plot	V12 treated-treated	2200
corn samples from the R1-treated plot	R1 treated-control	
biopesticide application to the corn kernels from the R1-treated plot	R1 treated-treated	2200

[a]V8: 8 leaves with the collar visible; V10: 10 leaves with the collar visible; V11: 11 leaves with the collar visible; V13: 13 leaves with the collar visible; V12: 12 leaves with the collar visible; and R1: onset of silking.

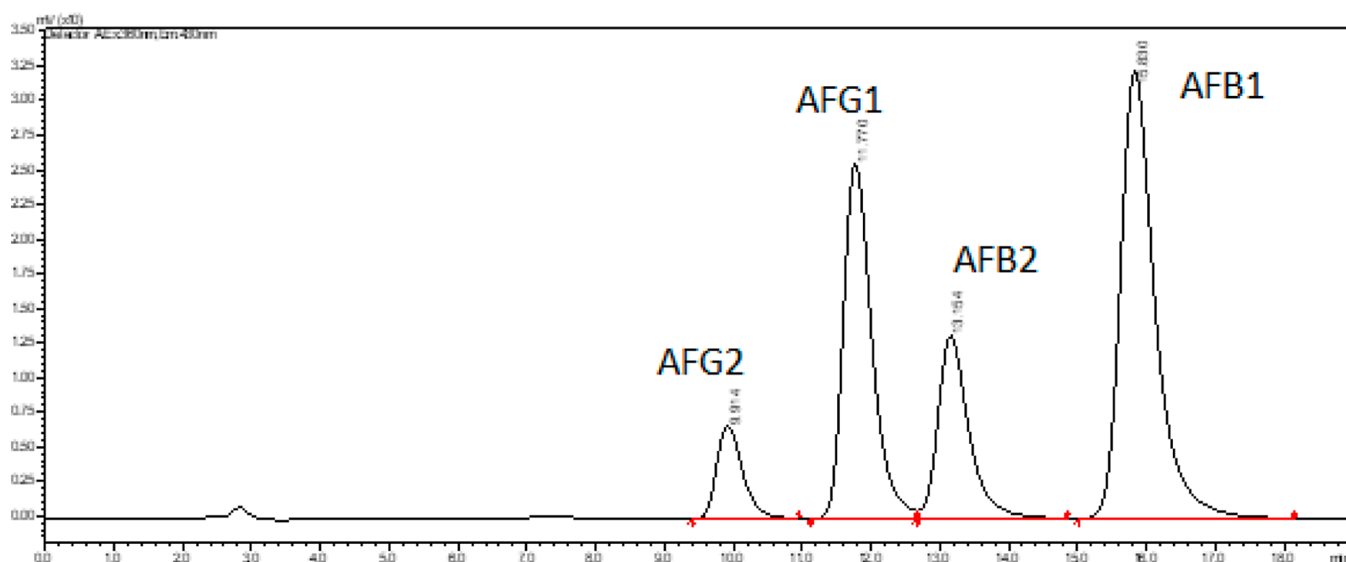

Figure 1. HPLC-FD chromatogram of AFG_2, AFG_1, AFB_2, and AFB_1 (AF concentrations: 0.3 μg/L for AFG_2,1 μg/L AFG_1, 0.3 μg/L for AFB_2, and 1 μg/L for AFB_1. Retention times were 9914 min for AFG_2, 11,740 for AFG_1, 13,154 for AFB_2, and 15,830 for AFB_1. Excitation wavelength:360 nm. Emission wavelength: 430 nm).

without any treatment. All the kernels were stored in barrels at room temperature for 3 months. All the experimental procedures presented are in Table 2. At the end of storage, samples of 1 kg were collected and refrigerated at 4 °C until AF analysis as described below.

2.12. Aflatoxin Analysis. AFs were analyzed by high-performance liquid chromatography with fluorescence detection (HPLC/FD). AF analysis was performed as described by Lavlor, 2019,[46] with some modifications.

2.12.1. Sample Preparation. Using a Waring blender (Waring Products Co., Connecticut, USA) at high speed for 1 min, 50 g of corn samples was extracted with methanol: water (6:2, v/v) and 5 g of NaCl. AFs are soluble in methanol and most organic solvents but are poorly soluble in water. NaCl, when added to the sample, has the ability to break the hydrogen bonds of AFs, thus improving the distribution coefficient between methanol and water for extraction, although other inorganic salts have the same effect as NaCl. Then, the sample extract was filtered through Whatman no. 4 filter paper. The mixture was then filtered by using Whatman 4 filter paper, and 5 mL of the filtered extract was diluted with 15 mL of phosphate buffered saline (PBS).

2.12.2. Clean-Up with an Immunoaffinity Column (IAC). An immunoaffinity column was loaded with 10 mL of PBS, and 5 mL/min PBS was passed through the column. Then, 20 mL of the diluted filtrate was run through the column at a rate of 3 mL/min. The column was then rinsed with 22 mL of water and dried under a gentle vacuum for 10 s 1 mL of methanol and 1 mL of water were then eluted from the column and filtered through a 0.45 μm filter prior to HPLC injection using 1 mL/min solvent flow column rates.

2.12.3. HPLC Analysis. HPLC with a Kobra cell was used to achieve this goal. FD was at an excitation wavelength of 365 nm and an emission wavelength of 435 nm. The HPLC system consisted of a C18 column with a mobile phase of water/methanol/acetonitrile (62:22:16, v/v/v), and 120 g of potassium bromide (KBr) and 4 M 350 μL of nitric were added to each liter. The flow rate was 1 mL/min; the injection volume was 100 μL. The HPLC column was kept at 40 °C temperature. Calibrant solutions were obtained by diluting

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stock solution of the total AF standard (Aflastandard, 1000 ng/L, R-Biopharm, Glasgow, Scotland) with the mobile phase at concentrations of 0.30, 0.50, 1.5, 3.0, 5.0, 7. 5, and 10 ng/mL.[47] Concentrations of the total AFs, AFB_1, AFB_2, AFG_1, and AFG_2 in corn samples were determined as μg/kg.

In Figure 1, HPLC-FD chromatograms of AFG_2 (1.2 μg/L), AFG_1 (4 μg/L), AFB_2 (1.2 μg/L), and AFB_1 (4 μg/L) standards injected into the HPLC device to create a calibration curve are shown. The retention times have been determined of AFG_2, AFG_1, AFB_2, and AFB_1 as 9.79 , 11.73 , 13.12 , and 15.96 min, respectively.

2.12.4. Validation Method. In-house validation was performed on the analytical method for the determination of AFs in maize samples. The method's linearity, the limit of detection (LOD), the limit of quantification (LOQ), recovery, and precision were all examined. Using a set of six standard solutions with concentrations between 0.4 and 20 g/L for AFB1 and AFG1 and between 0.12 and 6 g/L for AFB2 and AFG2, calibration curves were constructed to assess linearity. A coefficient of the determination (R^2) value greater than 0.99 was acceptable (Table 3).

Table 3. Linearity Data for AFs by the HPLC-FD Method[a]

analyte	range (μg/L)	linear regression equation	R^2
AFB_1	0.4−20	y = 418424x + 76061	0.99
AFB_2	0.12−6	y = 669453x + 35497	0.99
AFG_1	0.4−20	y = 237238x + 42391	0.99
AFG_2	0.12−6	y = 256585x + 10046	0.99

[a]R2: coefficient of determination; AF: aflatoxin.

On the same day, the experiment was carried out at a single concentration of analytes in the sample (0.5 g/kg for AFB1 and AFG1 and 0.15 g/kg for AFB2 and AFG2). The LODs were determined by multiplying the standard deviation (SD) of the 10 repeat analyses of strengthened blank materials by 3. The LOQs were determined by multiplying the matching SD by 10 (Table 4). These recovery results met the standards of the Commission Regulation guideline, which calls for a recovery rate of 70−110% for a mass fraction of 1−10 lg kg^{-1}.[48]

Table 4. Aflatoxins LOD, LOQ, Recovery, and Repeatability Values[a]

	LOD (μg/kg)	LOQ (μg/kg)	recovery (%)	repeatability (%)
AFG_1	0.05	0.17	92.9	4.51
AFG_2	0.04	0.16	94.2	1.42
AFB_1	0.06	0.21	90.4	4.49
AFB_2	0.05	0.18	91.3	0.64

[a]LOD: limit of detection; LOQ: limit of quantification; and AF: aflatoxin.

The relative SD (RSD) between replicates has also been calculated to a certain degree of linearity. The accuracy (relative error of the mean,%) was determined by the examination of calibration samples. It is claimed that the RSD value must be less than 0.66xCV of the value derived by the Horwitz equation under repeatable conditions (European Commission, 2006b). The formula is

$$\% \text{ CV: } 2^{(1-0.5\log C)}$$

This means that for mass fractions of 1.3 g/kg, the RSD level under repeatability parameters must be less than 24%. Nonetheless, RSD levels should be kept as low as feasible.[49]

2.13. Statistical Analysis. A complete analysis of the data was performed using analysis of variance. The test referred to as the least significant difference (LSD) was used to do the comparison between the treatment means. The software MSTAT-C was utilized in order to carry out the statistical analysis.[50] For each field trial, a complete randomized block design was utilized, and each replicate consisted of four replicates. To calculate the efficiency of the biopesticide in the AF biocontrol in order to differentiate the effects of biopesticide application from the effects induced by natural variables, we utilized Abbott's formula, which is a method that is frequently utilized for analyzing phytopathological field data. The average biopesticide efficiency was calculated by the Abbott formula, and all the values were corrected concerning the control using Abbott's formula: % effect of pesticide = [AF content in the control plots (x) − average AF content in the treatment plots(y)/AF content in the control plots(x)] × 100.

3. RESULTS AND DISCUSSION

In this study, *A. flavus* NRRL 21882 was used as a biopesticide to control AF contamination in corn, and the study was conducted with 2 year field and depot trials, with the aim of reducing AF levels of the biopesticide both in the field and during storage.

The results of LOD, LOQ, recovery, and reproducibility studies carried out for method validation are summarized in Table 3.

When Table 4 is examined, the LOD values of AFG2, AFG1, AFB2, and AFB1 in maize are found to be 0.04, 0.05, 0.05, and 0.06 μg/kg, respectively, with the analysis method used. The LOQ values obtained by this method were 0.160 μg/kg for AFG2, 0.17 μg/kg for AFG1, 0.18 μg/kg, and 0.21 μg/kg for AFB2. The recovery values of AFG_2, AFG_1, AFB_2, and AFB_1 from maize were 94.2, 92.9, 91.3, and 90.4%, respectively. The recovery values of AFs from maize were found to be in accordance with the analysis method parameters determined by the EU (between 70 and 110% for the 1−10 μg/kg toxin concentration).

First, the results of the field experiment, which contained the AF content after treatment in the field during 2018 and 2019, were given and are shown in Table 5.

As seen in Table 5, after field plot treatment, we found that the untreated plots had a significantly higher AF concentration than treated corn in both years. In 2018, the control plots possessed a very high concentration of AFs of 98 μg/kg. For this year, AF concentrations were very low in the treated groups, ranging from 0.50 μg/kg to 1.04 μg/kg. Moreover, the percentage of reduction ranged from 98 to 99% in the plots treated with biopesticide (Figure 2). In 2019, the AF concentration in control plots was 73.9 μg/kg, whereas corns from the six treated plots contained AFs doses between 0.50 and 0.73 μg/kg (Table 5). The obtained results showed no significant difference ($P \leq 0.05$) between the treated groups with biopesticide, while a significant difference was noted between the treatments and the control ($P \leq 0.05$). According to the results, the field treatment controlled AF production, regardless of the method used, soil treatment, or foliar treatment. Regardless of the environmental and growth conditions in 2 years, the biopesticide treatment significantly reduced AF contamination in corn. When compared to the

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Table 5. AF Content after Treatment in the Field during 2018 and 2019[a]

after field treatments	2018		2019	
	AFB_1 (μg/kg)	total AFs (μg/kg)	AFB_1 (μg/kg)	total AFs (μg/kg)
control	93.5	98.0[a]	72.7	73.9[a]
soil	1.00	1.04[b]	0.51	0.73[b]
V8	0.50	0.52[b]	0.48	0.52[b]
V10	0.49	0.52[b]	0.50	0.52[b]
V11 + V13	0.45	0.50[b]	0.49	0.50[b]
V12	0.49	0.50[b]	0.50	0.50[b]
R1	0.50	0.50[b]	0.50	0.50[b]
standard deviation	15.9	8.8		

[a]V8: 8 leaves with the collar visible; V10: 10 leaves with the collar visible; V11: 11 leaves with the collar visible; V13: 13 leaves with the collar visible; V12: 12 leaves with the collar visible; and R1: onset of silking. The data shown are the result of four replicates. AF levels are expressed in μg/kg. Within the column, values not sharing a common letter are significantly different ($p < 0.05$).

negative controls, it was seen that the AFs were found to be reduced by 98−99% in 2018 and by 99% in 2019 (Figure 2). In both years, the highest AFB_1 levels were observed at 93.5 and 72.5 μg/kg, respectively. When the biopesticide was applied in 2018−2019, AB_1 levels were reduced by up to 1 μg/kg (Table 5).

The results obtained from this study using Afla-Guard in corn showed parallelism with the results of studies conducted in different countries on peanuts and corn using Afla-Guard, and it was seen that it could successfully solve the AF problem on a field basis.[35,34,42,51] In a study conducted in Turkey for the biological control of AF contamination in peanuts in 2015, Afla-Guard was shown to give effective results.[52] In this study, the researchers reported that AF concentrations were generally quite low in treated samples (between 0.04 and 0.71 μg/kg), and AFs were reduced by 97 to 99% compared with the controls. Besides, in some studies, the researchers used locally non-aflatoxigenic *A. flavus* isolates to control AF contamination. However, the results of these studies showed that the AF reduction was not strong in comparison with Afla-Guard. In the study of Alaniz Zanon et al.,[38] the competitive ability of 9 non-aflatoxigenic strains was assessed in co-inoculations of corn kernels with an aflatoxigenic *A. flavus*. All evaluated strains reduced AF contamination in corn kernels. The AFB_1 reduction ranged from 6 to 60%. The same study was conducted on peanuts by the same researcher, where the efficacy of single and mixed inocula (three strains of non-aflatoxigenic *A. flavus*) as potential biocontrol agents was evaluated. According to the findings, most treatments reduced the occurrence of aflatoxigenic *A. flavus* strains in soil and peanut kernel samples, and no AF was detected in kernels. Reductions in AF contamination of 78 and 89% were found in treated plots compared to the uninoculated control plots.[40] In another interesting study, the biocontrol of AF contamination in corn was evaluated by using non-aflatoxigenic *A. flavus* delivered as a bioplastic-based seed coating. The biocontrol efficiency reached 85.2%.[53]

On the other hand, regarding the postharvest practices, the study conducted by Kinyungu et al.[54] has shown that preharvest biomonitoring applies will not substitute for the need for optimal post-harvest measures to minimize the drying period between harvest and storage. During the incubation period, aflatoxigenic and non-aflatoxigenic *A. flavus* species developed and dispersed in the grain, and AF levels increased; this was the case even in samples taken from biocontrolled fields.

Biopesticide application timing in corn cultivation under plot conditions is important for successful treatment. In particular, it was seen that the application of biopesticide should be done from the air until the tassel, which is the most open time of the field corn. Therefore, the timing of application of *A. flavus* NRRL 21882, which fights pathogenic *A flavus* in treated plots, is an important factor for good results.[51]

At this stage of the study, *A. flavus* NRRL 21882 was applied by spraying it to only one of the divided corn kernels as an aqueous conidial suspension, while the remaining half was left as a negative control. The experimental design of the storage

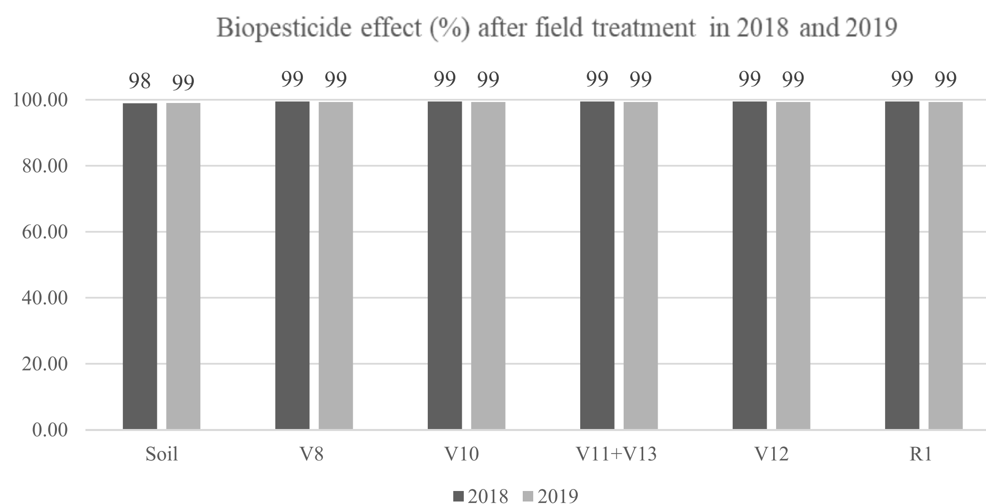

Figure 2. Biopesticide effect on the AF amount after field treatment in 2018 and 2019. V8: 8 leaves with the collar visible; V10: 10 leaves with the collar visible; V11: 11 leaves with the collar visible; V13: 13 leaves with the collar visible; V12: 12 leaves with the collar visible; and R1: onset of silking.

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period and the results of the AF content of the samples in 2018 and 2019 are given in Table 6.

Table 6. AF Content after Treatment during Storage in 2018 and 2019[a]

	2018		2019	
	AFB_1	total AFs	AFB_1	total AFs
after storage treatment	(μg/kg)			
control−control	626	627[a]	462	466[a]
control-treated	179	193[b]	87.9	89.6[b]
soil treated-control	8.70	8.80[c]	5.00	5.19[c]
soil treated−treated	0.90	1.00[c]	1.63	1.65[c]
V8 treated-control	6.10	7.45[c]	6.10	6.38[c]
V8 treated−treated	0.50	0.50[c]	1.38	1.50[c]
V10 treated-control	6.71	7.28[c]	4.42	4.50[c]
V10 treated−treated	0.50	0.50[c]	1.00	1.25[c]
V11 + V13 treated-control	6.80	7.10[c]	4.90	5.27[c]
V11 + V13 treated−treated	0.50	0.50[c]	1.50	1.75[c]
V12 treated-control	5.80	6.30[c]	6.21	6.31[c]
V12 treated- treated	0.50	0.50[c]	1.70	2.00[c]
R1 treated-control	6.80	7.58[c]	4.05	4.08[c]
R1 treated −treated	0.50	0.50[c]	2.23	2.25[c]
standard deviation	32.2	5.7		

[a]V8: 8 leaves with the collar visible; V10: 10 leaves with the collar visible; V11: 11 leaves with the collar visible; V13: 13 leaves with the collar visible; V12: 12 leaves with the collar visible; and R1: onset of silking. The data shown are the result of four replicates. AF levels are expressed in μg/kg. Within the column, values not sharing a common letter are significantly different ($p < 0.05$).

According to Table 6, significant AFB_1 and AF contamination was seen in corn kernels that were not treated with biopesticides under any condition during the study (control−control) at the end of the storage and experiment (Table 6). However, corn kernels that came from field plots treated with biopesticide contained very few AFs, even after they have been stored under circumstances that are conducive to AF production. In fact, this meant that only the application in the field was sufficient. It was shown in this study too that there was no significant difference in the AF concentration in corn kernels treated with biopesticide in the field alone (treated-control) when compared to the AF concentration in both fields and prestorage [treated−treated biopesticide-treated corn kernels (Figures 3 and 4]. Therefore, it was seen that additional treatment before storage did not have an increased beneficial impact when we look at the data. Also, as a result, it can be said that the best control of AF contamination during storage occurred when corn kernels were treated with biopesticide in the field (Figures 3 and 4). When looking at the data, the levels in the treated samples before storage are lower than those in the untreated samples. The standard deviation is in Table 5. Although there does not seem to be a statistically significant difference in the levels in the treated samples before storage, considering the carcinogenic potential of AFs, the experiment may be worth it.[44]

Regarding the storage application, these results showed similarity with a previous study performed to assess the biological control potential of AF contamination of peanuts by applying competitive, non-toxigenic strains of *A. flavus* and *A. parasiticus*.[45] In this similar study, Dorner (2009) emphasized that after 3 months of storage, peanut AFs (control−control) were very high. Besides, Dorner noticed that also the peanuts that were treated in storage only (control−treated) had a significantly lower level of AFs than those without treatment (control−control).

In our study, as seen in Table 6, corn from field groups treated with the biopesticide had very few AFs, even after it

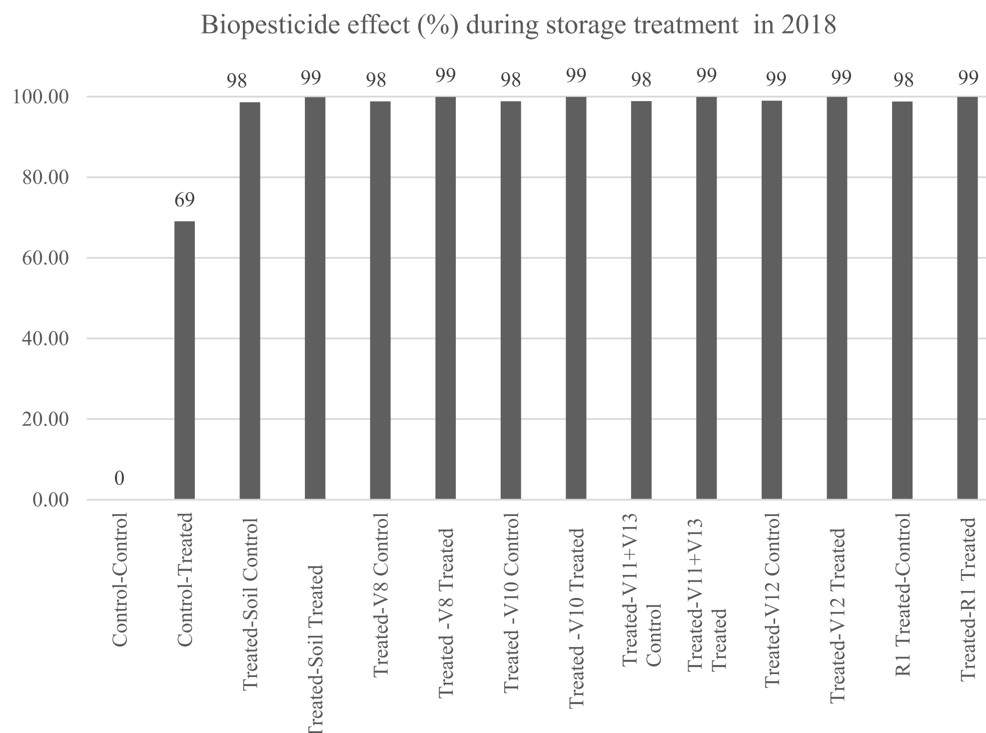

Figure 3. Biopesticide effect after treatment during storage in 2018. -V8: 8 leaves with the collar visible; V10: 10 leaves with the collar visible; V11: 11 leaves with the collar visible; V13: 13 leaves with the collar visible; V12: 12 leaves with the collar visible; and R1: onset of silking.

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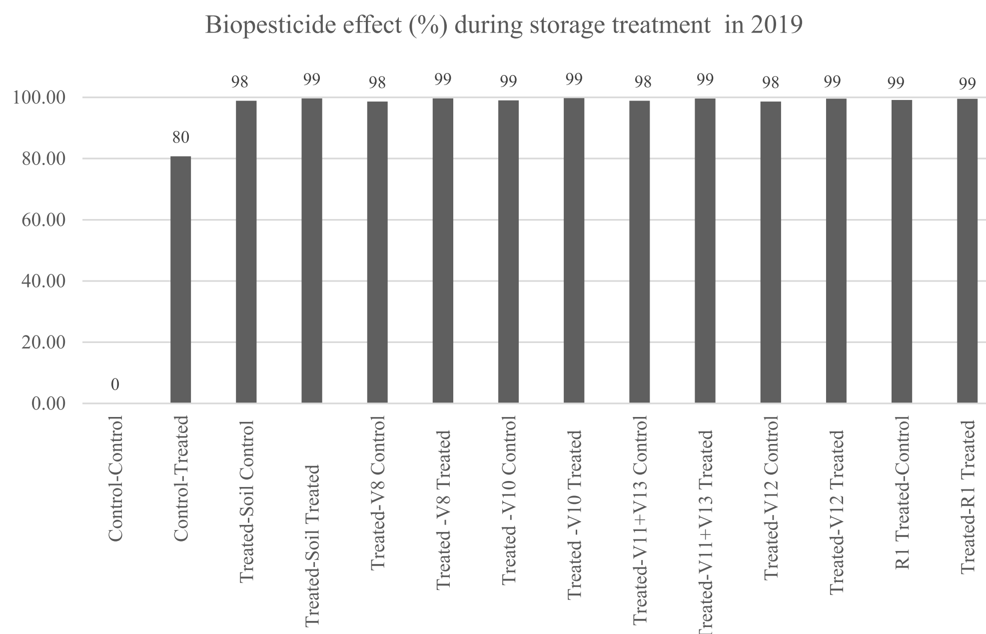

Figure 4. Biopesticide effect after storage treatment in 2019. V8: 8 leaves with the collar visible; V10: 10 leaves with the collar visible; V11: 11 leaves with the collar visible; V13: 13 leaves with the collar visible; V12: 12 leaves with the collar visible; and R1: onset of silking.

had been stored under conditions conducive to AF production. Also, in this study, it was seen also that corn kernels from the treated−control group were not significantly different in the AF level compared to corn kernels from the treated−treated group (Table 6).

On the other hand, it was noticed that the growth of *A. flavus* started in corn seeds after 6 months of storage. In fact, *A. flavus* is an obligate aerobic organism, and it is generally known that it cannot grow under anaerobic conditions[55,56] and especially when the storage period is kept short. In our study, the storage was done in barrels, which helps *A. flavus* to get little free oxygen, so as to conduct its growth in micro-aerophilic condition, and the growth starting from 6 months can be accepted as an indication of the correctness of this situation.

As a result, although the corn kernels are inoculated with *A. flavus* conidia, mold growth may not be possible when the storage is done under anaerobic conditions, and the storage time is kept short. In addition, corn and other grains that will be processed as raw materials are purified from mold spores by pre-treatment including cleaning. Thus, also the grains will not pose a risk in terms of food safety while preserving their commercial value.

4. CONCLUSIONS

A. flavus NRRL 21882 was first used as a biopesticide for peanuts in Turkey, and following this study, a similar subject was set up for corn, where both field and in-storage studies were conducted. It was seen that the *A. flavus* NRLL 21882 strain effectively limited AF production in the field and during storage for corns throughout this study. As a result, once again in this study, it was shown that the field treatment with *A. flavus* NRLL 21882 which can be used as a sustainable phytoprotective agent has great potential in order to reduce the amount of preharvest AF contamination and the amount of contamination that develops during storage.

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Notes

The authors declare no competing financial interest.

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