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Bio-competitive exclusion: efficacy of non-aflatoxigenic *Aspergillus* section Flavi-L morphotypes in control of aflatoxigenic *Aspergillus flavus* in groundnuts (*Arachis hypogaea* L.)

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Abstract

Background: The biological control mechanism of *Aspergillus flavus* (aflatoxigenic) strains in groundnuts with atoxigenic strains from the same species through competitive exclusion employed the use of endemic and well-adapted strains within the agro-ecological zones of Zimbabwe. The selected elite non-aflatoxigenic isolates of *A. flavus* native to Zimbabwe were evaluated for their capability to reduce aflatoxin contamination in groundnuts under laboratory conditions.

Results: Average reduction percentages in aflatoxin B concentration for the 2019 and 2020 set of experiments ranged from 91.6 ± 3.4 to 95.8 ± 3.1% and 90.29 ± 3.6% to 95.29 ± 4.1%, respectively. Levels of aflatoxin in the co-inoculation research experiments administered were significantly reduced in all the experimental units carried out. Treatment efficiencies of the tested isolates in this study at 4:1 and 2:1 ranged from 1.20 to 2.52 and from 1.02 to 1.21, respectively. The efficacy of the tested non-aflatoxigenic strains against the aflatoxigenic strain native to Zimbabwe (ZMW 0127) indicates that the non-aflatoxigenic isolates of *A. flavus.* have sound practical applications against vast communities of aflatoxin-producing fungi across all the agro-ecological zones in Zimbabwe.

Conclusion: The recognized non-aflatoxigenic isolates will be of an incentive as dynamic active ingredients in biocontrol formulations for the decrease in aflatoxins in groundnuts grown in Zimbabwe.

Keywords: Atoxigenic, Aflatoxin B, Biocontrol, Competition, Toxigenic

1 Background

Groundnut (*Arachis hypogaea* L.) is a major dietary and cash crop that is grown in Zimbabwe [1, 2]. The crop is primarily grown by small-scale farmers for household consumption [3]. However, groundnut production has for the recent years succumbed to pre- and post-harvest infection and contamination from *Aspergillus flavus* L morphotypes. The degree of the *Aspergillus flavus* infection leading to aflatoxin contamination in groundnuts has been entirely rampant in Zimbabwe. Regulations and strict limits for its containment have been plagued by an economic meltdown which have seen the nation failing to protect consumers from contaminated foods and feeds. The absence of mechanisms to enforce aflatoxin tolerance levels has resulted in chronic aflatoxin exposure to the general public [2].

Aflatoxin exposure to humans in Zimbabwe has been a matter of technical discourse and a public domain since there is a lack of data and awareness on the level of harm that these toxins actually cause. The recent

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strategies for aflatoxin management have been underway but their adoption by farmers has been so low owing to their inaccessibility and the prohibiting costs. Besides, no single mitigation measure has proven to be robust enough for wide-scale adoption in Zimbabwe owing to the immense shortfalls of each approach. However, the use of a single controlling approach in isolation may not prevent the initiation of aflatoxin contamination and be inadequate to reduce aflatoxin contamination to tolerable levels [4]. Therefore, aflatoxin management approaches must address the contamination progression throughout crop production until crops are consumed using all-inclusive interventions [4]. The most promising strategy to control aflatoxins therefore has been the use of atoxigenic *A. flavus* strains to competitively displace aflatoxin producers through Gause's Law of Competitive exclusion [5]. The competitive exclusion principle is a component of the biocontrol mechanism with atoxigenic isolates, locally adapted within the native agro-ecosystems against toxigenic strains of *A. flavus* [6]. Systematic studies on the use of native atoxigenic isolates of *A. flavus* in Zimbabwe to reduce aflatoxin contamination in groundnuts are needed in order to bring aflatoxin management with atoxigenic isolates closer to practical use in the nation. Identification of non-aflatoxigenic isolates of *A. flavus* which are endemic to Zimbabwe can be a resourceful base for mitigating aflatoxin contamination in groundnuts.

2 Methods

2.1 Fungal isolation and fungal inoculum preparation

Isolates of *A. flavus.* were collected during previous studies [3]. Samples of groundnut seeds (CG 7, Seed-Co Zimbabwe) were collected in the representative natural agro-ecological regions of Zimbabwe in respective districts where the 240 isolates were obtained. The isolates of *A. flavus* used in this study were isolated from groundnuts collected from farmers in Natural Region V (NR V) (48 isolates), Natural Region IV (NR IV) (50 isolates), Natural Region III (NR III) (45 isolates), Natural Region I1a and IIb (NR IIa and IIb) (60 isolates), and Natural Region I (NR I) (37 isolates) as shown in Table 1, during the 2019 and 2020 growing seasons. The mean sample weight was 187 g (ranging between 97 and 320 g/sample). The groundnut sample seeds were ground finely with an Ultra Centrifugal Retsch Mill ZM200 (Retsch GmbH, Haan, NRW., Germany) to pass a 500-μm sieve. After grinding, they were homogenized to a powder-like consistency. Fungal isolates were isolated from finely ground groundnut seed through a dilution plate technique in a Rose Bengal Agar with 1.5 X Chloramphenicol (pH 7.2 ± 0.3 at 25 °C). Detection of the *Aspergillus flavus* strains from the finely ground groundnuts was done after the cultivation of the discovered aflatoxins in 5-2 agar (5% V-8 juice [Campbell Soup Company, Camden, NJ] and 2% Bacto-agar, pH 6 at 25 ± 2 °C [8]. The detected strains were detected and identified morphologically.

Conidial suspensions were put into Eppendorf tubes with 5 ml of deionized water. Conidial concentrations were estimated with a turbidity meter, determined with the nephelometric turbidity unit against the colony-forming units. For each isolate, the conidial concentration was adjusted to 1×10^6 conidia/ml.

2.2 Identification of *Aspergillus* section Flavi L morphotype isolates

Test isolates in the current study were evaluated for their capability to produce aflatoxin in a YES (Yeast Extract Sucrose + 0.3% Mβ-cyclodextrin and 0.6% sodium deoxycholate) medium. Strains were inoculated on 6-cm-diameter plates at a single central point and incubated at 25 ± 2 °C for 14 days, in the dark [9, 10]. Then,

Table 1 Origins of isolates of *Aspergillus flavus* utilized in this current study native to Zimbabwe

Isolate	Aflatoxin B (B_1 + B_2)[b]	Location	Coordinates	Agro-ecological zone[c]
CHv 105	–	Chivi	20° 05″ S, 31° 37′ 12″ E	NR V
CHr 1701	–	Chiredzi	18° 55" S, 29° 49' 18" E	NR V
GWe 2274	–	Gwenhoro	19° 46′ 2″ S, 29° 52′ 32″ E	NR IV
CHp 1019	–	Chipinge	20° 12′ 0″ S, 32° 37′ 12″ E	NR I
GKw 2471	–	Gokwe	18° 13′ 12″ S, 28° 56′ 24″ E	NR III
MRn 9932	–	Marondera	18° 15′ 0″ S, 31° 30′ 0″ E	NR IIa
MTd 0208	–	Mount Darwin	16° 45′ 54″ S, 31° 34′ 30″ E	NR IIb
NRRL 21882	–	Georgia (USA)	34° 7′ 36″ N, 83° 35′ 25″ W	–
ZMW 0127	+	Gweru (Zimbabwe)	19° 27′ 41″ S, 29° 48′ 08″ E	NR IV

[a]All the isolates in this section are native to Zimbabwe except NRRL 21882 which is native to the USA. Each isolate belonged to a distinct haplotype which corresponded to a unique African *Aspergillus flavus* vegetative compatibility group. Haplotype refers to multilocus haploid genotypes based on allele calls at each of 17 SSR loci [7]

[b]Aflatoxin B (B_1 + B_2): +, toxigenic, aflatoxin production; –, atoxigenic, no aflatoxin production

[c]*NR* natural agro-ecological regions in Zimbabwe: *NRI* Natural Region I, *NR IIa* Natural Region IIa, *NR IIb* Natural Region IIb, *NR III* Natural Region III, *NR IV* Natural Region IV, *NR V* Natural Region V

3 agar plugs from each Petri dish were removed from a distinct colony, and they were transferred into Eppendorf tubes with the addition of 1 ml methanol. After 60 min, the extract was filtered by Millipore filters of 0.22 mm with an aliquot of 200μl derivatized in a 70:20:10, v/v/v, water:700μl of trifluoroacetic acid:acetic acid, and the solution was analyzed by a reverse phase HPLC [11]. The HPLC equipment was used for sample analysis and it was equipped with a fluorescence detector (Jasco-FP 920). The relative emission and excitation wavelengths (nm) were determined with a photochemical post-column derivatization reactor at 435 nm and 365 nm, respectively. Waters Spherisorb Colums ODS2, 4.6 mm × 25 cm, 5 μm particle size, C18 column was used for separation chromatography in a reverse phase system. The isocratic program consisted of a (3:1:1, v/v/v) ratio of water to methanol to acetonitrile respectively purchased from Fisher Scientific [10, 11]. The flow rates for the reagent and mobile optimum phase were 0.6 ml^{-1} and 1 ml^{-1}, respectively. The injection volume was 80 μl from Sigma-Aldrich. A mix of aflatoxins, containing 2 μg/ml each of AFB_1 ($C_{17}H_{12}0_6$) and AFB_2 ($C_{17}H_{14}0_6$)-difurocoumarocyclopentenones, was used from Romer Labs. Samples were taken as positive (aflatoxigenic) when yielding a peak at a retention time similar to each standard, with a height five times higher than the reference point, whereas the negative (non-aflatoxigenic) strains were identified as those which were not in compliance with the standard reference point. Isolates that were invariably negative for aflatoxins were considered non-aflatoxigenic for the purpose of this study.

2.3 Cyclopiazonic acid detection

The collected isolates of *A. flavus.* were tested for cyclopiazonic acid production in the Czapek Yeast Autolysate medium according to the method by Ostrý and Polster [12]. The identified strains were inoculated on 6-cm-diameter plates and incubated at 25 °C for 14 days, in the dark [13]. The spherical high-performance HPLC method connected to a Varian UV Detector running at a wavelength of 285 nm was used for cyclopiazonic analysis in this study and a EuroSpher 100-5 NH_2, Column 250 × 4.6 mm was also used for chromatographic separation. The eluent used was pumped at 1.0 ml^{-1} and consisted of an isocratic program as follows: acetonitrile:50 mM ammonium acetate (3:1, v/v), pH 5. The injection volume was 100 μl.

2.4 Co-inoculation of viable groundnut pods with aflatoxigenic and non-aflatoxigenic isolates of *A. flavus*

Ninety-six non-aflatoxigenic strains were tested for their efficacy on the aflatoxigenic strain (ZMW O127) of *A. flavus.* which was done through a co-inoculated process under the same ambient conditions. ZMW 0127 aflatoxigenic strain in the current study was obtained from highly contaminated regions in Zimbabwe which were hardly hit by the 2008 acute aflatoxicosis outbreak [12–14]. Prior to inoculation, groundnut seeds of CG 7 variety (Seed-Co Zimbabwe) were surface-sterilized according to the modified method by Craufurd et al. [15]. Surface sterilization efficiency was then tested on selective media for 14 days in a dark environment and germination of groundnuts was tested as well. More than 95% of the seeds germinated and there were no fungal contaminations or opportunistic organisms observed after the incubation period. Equal inoculum (10^6 conidia/isolate/flask) of both the aflatoxigenic and non-aflatoxigenic isolates was mixed into the flasks (0.70 ml/flask), and they were gently shaken to enable coating of the groundnut seeds with the aflatoxin inoculum. The ambient moisture for the test experimental units was maintained at 25 ± 2 °C. The inoculated groundnut seed was incubated at 30 ± 2 °C for a week in a non-illuminated room. For termination of the co-inoculation treatment unit, 50 ml of methanol (70%) was added to the flasks, and the quantity of aflatoxins obtained was observed. Aflatoxin inhibition in these experimental units was computed as a percentage of the aflatoxin content in groundnut seed inoculated with only the aflatoxin-producing isolate (ZMW 0127). Seven *A. flavus* L strain isolates (CHv 1051, CHr 1701, CHp 1019, GKw 2471, MRn 9932, GWe 2274, and MTd 0208) were most effective at reducing aflatoxin contamination of groundnut seed and they were tested further for consistency and stability in efficacy trials against the toxigenic strain (ZMW 0127). In these experiments, comparisons were made with NRRL 21882, the active ingredient of the Afla-Guard biopesticide [16, 17], the non-aflatoxigenic isolate that is the active ingredient in AflaGuard (Syngenta, Wilmington, DE), a biocontrol product currently registered for the management of aflatoxins in grain crops (Table 2).

2.5 Co-inoculation treatment efficiency

The efficacy of a treatment effect administered for biological control of infective *A. flavus* aflatoxigenic strains (TE), which entails the capability of a certain amount of non-aflatoxigenic inoculum to reduce infection, was evaluated comprising of distinct proportions of aflatoxigenic strain 100:50% (2:1) and 80:40% (4:1). An efficiency of 1 means that 50% of the non-aflatoxigenic strain caused a 50% reduction in aflatoxin [18]. TE > 1.0 indicated that aflatoxin was reduced by a non-aflatoxigenic strain to a greater extent than that explained by its proportionate ratio. Increased TE indicates an improvement in the amount of aflatoxin reduction each unit of

Table 2 Competitive exclusion and efficacy of atoxigenic *Aspergillus* section Flavi against toxigenic L morphotypes

	Aflatoxin B (B_1 + B_2) ($\mu g g^{-1}$)[b]			Aflatoxin B (B_1 + B_2) ($\mu g g^{-1}$)		
	2019-(ZMW 0127)[b]			2020-(ZMW 0127)		
Atoxigenic isolate[a]	1st Exp	2nd Exp	Av (R %)	1st Exp	2nd Exp	Av (R %)
CHv 1051	13.3^{a}	19^{a}	91.9 ± 4.3	9.9^{a}	11.2^{a}	94.39 ± 6.4
CHr 1701	12.3^{a}	8.7^{b}	94.7 ± 3.8	19.9^{b}	12^{a}	91.49 ± 4.5
GWe 2274	15.9^{b}	9^{b}	93.7 ± 4.1	14.8^{bc}	20.5^{b}	90.49 ± 5.2
CHp 1019	11.8^{a}	12.9^{c}	93.8 ± 3.8	12.6^{c}	12^{a}	93.39 ± 5.8
GKw 2471	18.6^{c}	8.8^{b}	93.1 ± 3.8	12.9^{c}	23.1^{b}	90.29 ± 3.6
MRn 9932	19.3^{bc}	14^{c}	91.6± 3.4	7.4^{a}	20.2^{b}	92.49 ± 4.4
MTd 0208	8.9^{a}	7.9^{b}	95.8 ± 3.1	8.9^{a}	18.9^{b}	92.59 ± 4.0
NRRL 21882	10.1^{a}	6.8^{b}	95.7 ± 3.8	7.2^{a}	10.4^{c}	95.29 ± 4.1
None	207.3^{d}	189.7^{d}	. . .	175.9^{d}	192.9^{d}	. . .

[a]Co-inoculated isolates (non-aflatoxigenic and aflatoxigenic isolates simultaneously co-inoculated at a conidial concentration of 1×10^6 conidia ml^{-1}). For None, two aflatoxigenic isolates of *A. flavus* were used

[b]Mean values with common superscript letters within a column are not significantly different according to Fischer's unprotected significant difference test @ 5% significance level

atoxigenic achieves. Treatment efficiency (TE) was calculated as follows: TE = *r/[a/(a + t)]*, where "*r*" is % aflatoxin reduction, "*a*" is non-aflatoxigenic conidia quantity, and "*t*" denotes aflatoxigenic conidia quantity applied.

2.6 Statistical analysis

The results obtained from the study were analyzed using the randomized complete block design (RCBD) in all experiments with GenStat software 19th version. Fischer's unprotected test was used for mean separation.

3 Results

3.1 Co-inoculation of viable groundnut pods with aflatoxigenic and non-aflatoxigenic isolates of *A. flavus*

Co-inoculation of atoxigenic isolates with the toxigenic isolate ZMW 0127 had significant ($p < 0.05$) effects on aflatoxin content on groundnut seed. In the first and second experiments in 2019, groundnut seed co-inoculated with a toxigenic native strain (ZMW 0127) and atoxigenic strains (CHv 1051, CHr 1701, CHp 1019, GKw 2471, MRn 9932, GWe 2274, and MTd 0208) had aflatoxin B content ranging from 8.9 to 19.3 $\mu g g^{-1}$ and 6.8 to 19.0 $\mu g g^{-1}$, respectively, and these contamination levels were significantly different ($p < 0.05$) across all the different strains with comparable average differences. MTd 0208 strain (atoxigenic) had the greatest average efficacy with 8.9 $\mu g g^{-1}$ and 7.9 $\mu g g^{-1}$ of conidial spores for the first and second set of experiments, respectively, and it was comparable to NRRL 21882 (10.1 $\mu g g^{-1}$ and 6.8 $\mu g g^{-1}$ of conidial spores for the first and second set of experiments, respectively). Average reduction percentages in aflatoxin B concentration for the 2019 set of experiments ranged from 91.6 ± 3.4 to 95.8 ± 3.1% which were noted in co-inoculated groundnut seed compared with groundnut seed inoculated with two strains of the toxigenic (ZMW 0127) strains. Aflatoxin B levels in co-inoculation experiments conducted with the selected isolates were significantly lower in both experiments. For the 2020 season, the seven isolates endemic to Zimbabwe performed similarly during the additional evaluations as in the preliminary experiment of 2019, causing an average of 90.29 ± 3.6% to 95.29 ± 4.1% aflatoxin reduction. All atoxigenic isolates were statistically comparable in their capability to obstruct aflatoxin production in groundnut seed.

3.2 Co-inoculation treatment efficiency

Groundnut seed which was co-inoculated with two different quantity concentrations of 2:1 ratio of conidia from aflatoxigenic and non-aflatoxigenic isolates, respectively, was contaminated with less aflatoxin spores compared to groundnut seed which was co-inoculated with a 4:1 ratio which is four times more from the aflatoxigenic strain than from non-aflatoxigenic isolates (Table 3). With the 2:1 ratio, the concentration of aflatoxin B (B_1 + B_2) ranged between 40 and 49 $\mu g g^{-1}$, while with the 4:1 ratio, aflatoxin B (B_1 + B_2) ranged from 55 to 80 $\mu g g^{-1}$. Reduction percentage in terms of inhibition efficiency of the atoxigenic native strains ranged from 51.0 to 60.4% for the 2:1 ratio and from 24.5 to 49.1% for the 4:1 ratio. Therefore, on average, aflatoxigenic isolates exhibited considerable effectiveness in terms of percentage reduction for the proportion of the inoculum composed by the aflatoxigenic strains, when composing 100% of the aflatoxigenic inoculum (as in the 2:1 disease pressure) than when composing 80% of the aflatoxigenic inoculum (as in the 4:1 disease pressure) (Table 3). Treatment efficiencies of the tested isolates in this study

Table 3 Influence of non-aflatoxigenic and aflatoxigenic ratios of *A. flavus.* on reduction of aflatoxins produced by aflatoxigenic isolates of *A. flavus* (ZMW 0127)

	Aflatoxin B (B_1 + B_2) ($\mu g g^{-1}$)[s]		R (%)[t]		Treatment efficiency (TE)	
Non-aflatoxigenic [r]	(2:1)	(4:1)	(2:1)	(4:1)	(2:1)	(4:1)
CHv 1051	49^{a}	69^{a}	51.0	34.9	1.02^{a}	1.72^{b}
CHr 1701	44^{a}	72^{b}	56.4	32.1	1.13^{ab}	1.61^{b}
GWe 2274	46^{b}	79^{b}	54.5	25.5	1.09^{a}	1.26^{a}
CHp 1019	40^{a}	80^{b}	60.4	24.5	1.21^{c}	1.23^{a}
GKw 2471	48^{c}	59^{ab}	52.5	44.3	1.05^{a}	2.22^{c}
MRn 9932	46^{bc}	68^{a}	54.5	35.8	1.09^{a}	1.79^{bc}
MTd 0208	40^{a}	56^{c}	60.4	47.2	1.21^{c}	2.36^{c}
NRRL 21882	42^{a}	54^{c}	58.4	49.1	1.17^{ab}	2.46^{c}
ZMW 0127	101^{d}	106^{d}	...	...	...[d]	...[d]

[r]NRRL 21882: Afla-Guard active ingredient. ZMW 0127 produces aflatoxins and it represents the positive control

[s]Atoxigenic and toxigenic isolates were co-inoculated at 1×10^6 total conidia ml^{-1} in two ratios of concentrations: 100% toxigenic and 50% atoxigenic (2:1) and 80% toxigenic and 20% atoxigenic (4:1)

[t]Aflatoxin B reduction (%) = [1 − (total aflatoxin content in groundnut seed co-inoculated with both toxigenic and atoxigenic isolates of *Aspergillus flavus*/total aflatoxin content in groundnut seed inoculated with ZMW 0127)] × 100

Mean values with the same superscript letter within a column are not significantly different @ 5% significance level

at 4:1 and 2:1 ranged from 1.20 to 2.52 and from 1.02 to 1.21, respectively.

3.3 Discussion

The efficiency of the native Zimbabwean atoxigenic isolates in reducing aflatoxin contamination of groundnut seed concurs with the results reported from Ghana [19], with regard to the use of atoxigenic strains for biological competitive exclusion of the toxigenic strains in both maize and groundnuts. The degree to which the seven isolates lowered contamination of the groundnut seed was statistically at par to NRRL 21882 strain, the active ingredient of Afla-Guard biopesticide registered for use in aflatoxin management on maize and groundnuts in the USA [20]. These results indicate that there are many atoxigenic strains falling within similar vegetative compatibility groups of *Aspergillus flavus* in Zimbabwe which has the capability to be incorporated in green chemical formulations as active ingredients for management of aflatoxin contamination in groundnuts. Variance in the bio-competitive ability of the isolates under the study reflects beyond reasonable doubt adaptation differences among the isolates under laboratory and gnotobiotic conditions [21]. Those distinct differences of the studied strains can be due to native and endemic variations in *A. flavus.* survival and colonization strategies; hence, it was evident that some of the isolates in this study employed the ramify and old survival strategy, where some co-inoculations increase plant tissue invasion associated with reduced conidial sporulation.

The efficacy of the tested non-aflatoxigenic strains against the aflatoxigenic strain native to Zimbabwe (ZMW 0127) indicates that the non-aflatoxigenic strains of *A. flavus.* have sound practical applications against vast communities of aflatoxin-producing fungi across all the agro-ecological zones in Zimbabwe and, as a result, be convenient in plummeting aflatoxin contamination in groundnut. The notion of treatment efficiency (TE) introduced in this study here was to enable quantification of the comparative quantity of aflatoxin reduction attained per each toxigenic isolate tested [22]. An efficacy of 1.0 designates a 50% decrease in aflatoxins from an equivalent proportion of aflatoxigenic and non-aflatoxigenic conidia. TE values which were greater than 1.0 highlight that aflatoxin reductions per each respective co-inoculation were more than the proportionate percentage of the overall endemic non-aflatoxigenic isolate inoculated. Thus, an augmented TE value indicated a greater competence with which the non-aflatoxigenic isolate diminutions aflatoxin content during coinfection with an aflatoxigenic isolate.

In this study, higher TE values were attributed with lower proportions of co-inoculation (Table 3). This suggests non-aflatoxigenic strains perform more competently when existent at low proportions and achieve superior reductions than would be projected based on their occurrences in the environment alone [18, 23] and also had similar results which were obtained after administering lower proportions of non-aflatoxigenic strains against aflatoxigenic strains of *A. flavus*. From the current study, the native non-aflatoxigenic strains of *A. flavus.* yielded superior results which were more comparable to the approved commercial biocontrol product on the market. From the seven effective strains of non-aflatoxigenic strains in Zimbabwe, all of them did not produce cyclopiazonic (results not shown).

Therefore, since these non-aflatoxigenic strains were evaluated in the current study, it is therefore sound to incorporate them in the integrated disease management systems since they will offer or prove more advantageous over the introduced strains with including improved environmental safety and better adaptation to the target region [24–26]. Greater adaptation to the Zimbabwean agro-ecosystems should mean both increased efficacy in the target area and greater carryover between crops.

4 Conclusions

Strikingly high aflatoxin concentrations in Zimbabwe in groundnut production require an implementation of measures which harness a comprehensive and adaptive development program towards good agricultural practices, which are recent, long-lasting, and effective. Therefore, the incorporation of native endemic strains of atoxigenic *A. flavus.* in Zimbabwean agro-ecosystems will enable area-wide management of aflatoxin contamination and infection and hence curb the risks farmers, the general public, and the agrarian sector encounter in a bid to operate in farming.

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Author's contributions
AC planned and designed the project and conducted the field sampling and laboratory analysis as well as data analysis. AC wrote and discussed the manuscript. AC read and has approved the final manuscript.

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The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable

Consent for publication
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Competing interests
The author declares that there are no competing interests.

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