Received: 31 March 2023 | Revised: 30 May 2023 | Accepted: 1 July 2023

DOI: 10.1002/jobm.202300163

RESEARCH PAPER

Journal of Basic Microbiology

Cocoa-associated filamentous fungi for the biocontrol of aflatoxigenic *Aspergillus flavus*

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Funding information
Building a New Generation of Academics in Africa (BaNGA-Africa) project, Carnegie Corporation of New York; Division of Cancer Prevention, National Cancer Institute; Fogarty Global Health Training Program NIH, Grant/Award Number: D43 TW010543

Abstract

Aflatoxin and other mycotoxin contamination are major threats to global food security and present an urgent need to secure the global food crop against spoilage by mycotoxigenic fungi. Cocoa material is noted for naturally low aflatoxin contamination. This study was designed to assess the potential for harnessing cocoa-associated filamentous fungi for the biocontrol of aflatoxigenic *Aspergillus flavus*. The candidate fungi were isolated from fermented cocoa beans collected from four cocoa-growing areas in Ghana. Molecular characterization included Internal Transcribed Spacer (ITS)-sequencing for identification and polymer chain reaction (PCR) to determine mating type. Effects of the candidate isolates on growth and aflatoxin-production by an aflatoxigenic *A. flavus* isolate (BANGA1) were assessed. Aflatoxin production was monitored by UV fluorescence and quantified by enzyme-linked immunosorbent assay (ELISA). Thirty-six filamentous fungi were cultured and identified as *Aspergillus, Cladosporium, Lichtheimia*, or *Trichoderma* spp. isolates. The isolates generally interacted negatively with BANGA1 growth and aflatoxin production. The *Aspergillus niger* and *Aspergillus aculeatus* biocontrol candidates showed the strongest colony antagonism (54%–94%) and reduction in aflatoxin production (12%–50%) on agar. In broth, the *A. niger*

Abbreviations: DRBC, dichloran Rose Bengal chloramphenicol; ELISA, enzyme-linked immunosorbent assay; ITS, Internal Transcribed Spacer; MAT, mating type; nBLAST, nucleotide Basic Local Alignment Search Tool; PCR, polymerase chain reaction; PDA, potato dextrose agar; RCA, relative colony antagonism; RT, room temperature; WACCBIP, West African Centre for Cell Biology of Infectious Pathogens; YES, yeast extract sucrose.

isolates reduced aflatoxin production by up to 97%. Metabolites from the *A. niger* isolates showed the strongest inhibition of growth by BANGA1 and inhibited aflatoxin production. Four of the candidate isolates belonged to the MAT1-1 mating type and 12 identified as MAT1-2. This may be indicative of the potential for genetic recombination events between fungi in the field, a finding which is particularly relevant to the risk posed by *A. flavus* biocontrol measures that rely on atoxigenic *A. flavus* strains.

KEYWORDS
aflatoxins, antagonism, *Aspergillus flavus*, *Aspergillus niger*, biocontrol

1 | INTRODUCTION

Sub-Saharan Africa is one of the world's regions most vulnerable to the effects of food supply instability [1, 2]. With the looming threat of climate change, one of the anticipated consequences is a serious threat to food safety and security, with far-reaching adverse impacts on crop and livestock production [3–5]. In tandem with increased food production, guarding against postharvest crop losses are a key consideration in efforts toward the achievement of enhanced global food safety and security. Across the world, and particularly in Sub-Saharan Africa, postharvest losses are estimated to be up to 50% of annual crop production [6, 7]. The growth of microorganisms, mainly filamentous fungi, is a prominent factor that renders crops unsuitable for human consumption; generally causing accumulation of mycotoxins, unsightly appearance, changes in organoleptic properties, and spoilage [8, 9]. Mycotoxins cause a range of deleterious adverse health effects [10–12] and are currently estimated to contaminate up to 80% of the global food crop [13]. Aflatoxins, produced by strains of *Aspergillus flavus* and *Aspergillus parasiticus*, are a group of mycotoxins that are widely reported to contaminate foods and so are considered a major threat to food safety [10, 14–16]. *A. flavus* is a commonly encountered microbial contaminant of stored foods [17, 18]. Key to food security in the face of climate change, effective strategies aimed at controlling fungal and mycotoxin contamination of crops are integral to reducing losses and improving the quality and availability of food for human consumption.

A recently developed aflatoxin control strategy, which involves the use of nonaflatoxigenic *A. flavus* as a biocontrol agent, is reported to yield promising results [19, 20]. Although concerns have been raised regarding this strategy [21], one problem that has not been highlighted is the possibility of gene transfer between the aflatoxigenic *A. flavus* target isolates and the nonaflatoxigenic control agents. Already, it had been suggested that secondary metabolite gene clusters may be transferred horizontally between filamentous fungi [22–25]. Such horizontal gene transfer is a significant risk to the widespread deployment of the indicated control strategy. The use of non-*Aspergillus* filamentous fungi which interact negatively with growth and aflatoxin production by toxigenic *A. flavus* may be a safer alternative.

A previous study involving cocoa-associated filamentous fungi from Ghana failed to identify *A. flavus* among the fungi isolated from cocoa material [26]. Subsequently, a study on aflatoxin content in cocoa material from Ghana has indicated subregulatory aflatoxin levels, below 0.15 ppb [27]. These findings support previous indications of a naturally low aflatoxin contamination cocoa material [28, 29] and suggest that (1) cocoa-associated compounds may be inhibitory to aflatoxin contamination or (2) cocoa-associated microorganisms may interact negatively with *A. flavus* growth and aflatoxin contamination. This study was designed to assess the potential of cocoa-associated filamentous fungi from Ghanaian cocoa material for use in the biocontrol of aflatoxin contamination by aflatoxigenic *A. flavus*.

2 | MATERIALS AND METHODS

2.1 | Materials

A sample of aflatoxin-contaminated cornflour was obtained from the Mycotoxin and Histamine Laboratory of Ghana Standards Authority. Fermented cocoa beans were collected from the Cocoa Research Institute of Ghana and from farmers' fermentation setups on four cocoa farms in southern Ghana.

2.2 | Isolation of candidate filamentous fungi

Fermented cocoa beans were collected at the end of the standard 6-day heap or tray fermentation process at each

sampling site. The beans were collected in sterile, transparent zip-lock bags and transported with ice packs. Storage was at 4°C for up to 3 days within which inocula were prepared for the isolation of filamentous fungi. Long-term storage was at −20°C. From each sample, aflatoxins were extracted [28] but without immunoaffinity clean up, and measured using a MaxSignal Total Aflatoxin ELISA kit (Bioo Scientific) according to the manufacturer's guidelines. To isolate the cocoa-associated filamentous fungi, five beans from each sample were washed in 25 mL peptone water at room temperature (RT) and 100 strokes/min on a reciprocating shaker for 30 min. Aliquots of 10, 100, or 500 µL were spread on Dichloran Rose Bengal Chloramphenicol agar (DRBC; Scharlab S.L.) plates and incubated at a 30°C for up to 10 days. Morphologically distinct colonies of filamentous fungi were transferred onto fresh DRBC plates, followed by single-sporing [30] on potato dextrose agar (PDA; Scharlab S.L.). For aflatoxin-contaminated cornflour, approximately 5 g were resuspended in 25 mL peptone water for the isolation of fungi. *A. flavus* was presumptively identified by colony morphology.

Pure cultures of the isolates were transferred onto PDA slants and incubated for the preparation of spore suspensions. Spores from each isolate were harvested with 1% tween solution and stored in 15 mL falcon tubes at 4°C. Where there was no sporulation after 14 days, bits of hyphae were rubbed-off into the tween solution.

2.3 | Molecular characterization of candidate isolates

2.3.1 | Fungal DNA extraction

For each isolate, 5 mL of potato dextrose broth were inoculated with 10 µL spore suspension and incubated at RT. Fungal biomass was harvested after 5 days into 15 mL centrifuge tubes and frozen overnight at −20°C. Genomic DNA was extracted using a Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research) according to the manufacturer's guidelines. DNA were eluted in 50 µL elution buffer and stored at −20°C.

2.3.2 | Molecular identification

The isolates were identified by nuclear ribosomal DNA ITS sequencing. The extracted DNA was used as a template for PCR with the ITS1/ITS4 primer pair [31]. PCRs were done in 25 µL reaction mixtures containing 0.2 µM of each oligonucleotide primer, 0.5 µL of DNA template, and 12.5 µL OneTaq 2× master mix (New England Biolabs). PCR conditions [26] were as indicated in Table 1. Amplicons were separated on 2% w/v agarose gel, stained with ethidium bromide, and visualized under UV transillumination. The PCR products were submitted for sequencing at the WACCBIP NextGen sequencing facility. Sequence data were analyzed with Clustal X 2.0

TABLE 1 PCR primers and conditions for ITS, mating type, and aflatoxin-synthesizing genes.

	Temperature (°C)	Duration
Singlet target: ITS		
Forward primer (ITS-1): CC GTA GGT GAA CCT GC		
Reverse primer (ITS-4): TCC TCC GCT TAT TGA TAT GC		
Denaturation	94°C	5 min
Thermal cycling (35 cycles):		
Stage 1	94°C	45 s
Stage 2	50°C	60 s
Stage 3	72°C	60 s
Final extension	72°C	10 min
Duplex target: MAT1-1 and MAT1-2		
Forward primer (MAT1-1-F): ATTGCCCATTTGGCCTTGAA		
Reverse primer (MAT1-1-R): TTGATGACCATGCCACCAGA		
Forward primer (MAT1-2-F): GCATTCATCCTTTATCGTCAGC		
Reverse primer (MAT1-2-R): GCTTCTTTTCGGATGGCTTGCG		
Denaturation	95°C	5 min
Thermal cycling (40 cycles):		
Stage 1	95°C	30 s
Stage 2	57°C	60 s
Stage 3	72°C	45 s
Final extension	72°C	5 min
Duplex target: VER and AFLR1		
Forward primer (VER1): GCCGCAGGCCGCGGAGAAAGGTGGT		
Reverse primer (VER2): CCGCAGTCAATGGCCATGCAGCG		
Forward primer (AFLR1F): AACCGCATCCACAATCTCAT		
Reverse primer (AFLR1R): AGTGCAGTTCGCTCAGAACA		
Denaturation	95°C	5 min
Thermal cycling (35 cycles):		
Stage 1	95°C	30 s
Stage 2	57°C	60 s
Stage 3	72°C	45 s
Final extension	72°C	5 min

Abbreviations: ITS, Internal Transcribed Spacer; PCR, polymerase chain reaction.

software. For identification, the generated sequences were matched by nBLAST to library sequences on GenBank database.

2.3.3 | Other molecular characterization

DNA extracts from all the study isolates were used as template to characterize fungal mating type using the MAT1-1F/MAT1-1R and MAT1-2F/MAT1-2R primer pairs [32]. DNA extracts from isolates that had been identified by ITS sequencing as *A. flavus* were used as template in PCR with the ver1/ver2 [33] and aflR1-F/aflR1-R [34] for the a*flR1* and *ver* genes which are involved in aflatoxin biosynthesis [35–37]. In all cases, amplifications were in 25 μL reaction mixes containing 0.5 μL of DNA template, 12.5 μL OneTaq 2× master mix, and 0.2 μM of each forward or reverse primer. Cycling conditions were as indicated in Table 1. Amplicons were separated on 2% w/v agarose gel, stained with ethidium bromide, and visualized under UV transillumination.

2.4 | Aflatoxin production

All the isolates that were identified as *Aspergillus flavus* by ITS sequencing were screened for aflatoxin production on yeast extract sucrose (YES) agar with cyclodextrin [38]. Blue-green fluorescence under UV was used to mark the aflatoxin-producing isolates. Aflatoxin production by the control aflatoxigenic *A. flavus* (BANGA1) was confirmed by broth culture in YES followed by enzyme-linked immunosorbent assay (ELISA) for total aflatoxin content. Cocoa-associated candidate isolates that had been identified as *A. flavus* and/or produced aflatoxins, indicated by fluorescence on agar, were excluded from further experiments.

2.5 | Competition assays

Both growth and aflatoxin production by BANGA1 were used as markers to assess competition between aflatoxigenic *A. flavus* and the cocoa-associated candidate isolates.

2.5.1 | Competition on agar

Interactions on agar were assessed by a modification of previously described methods [39, 40]. On the bottom of a 10 cm Petri dish containing YES agar with cyclodextrin, two spots for inoculation were marked, each 2 cm away from the center and 3 cm from the edge. For each selected candidate isolate, plates were inoculated in triplicate with 10 μL of spore suspension on one of the marked spots. After incubation at RT and normal ambient conditions of light for 24 h, 10 μL BANGA1 spore suspension was used to inoculate the second spot. Incubation continued for up to 7 days.

Effect of candidate isolate on the growth of BANGA1

The plates were monitored for daily measurements of colony diameter. A modified scoring scheme [40] describing the colony interactions between the isolates was applied as shown in Table 2. Relative colony antagonism (RCA) was calculated as:

$$\mathrm{RCA} = \frac{(\text{Competitor score} - \text{BANGA1score})}{(\text{Highest possible score} - \text{Lowest possible score})} \times 100,$$

where the highest possible score = 6 and lowest possible score = 1 as seen in Table 2.

The final antagonism score for each isolate was determined as the mean RCA for three replicates.

Effect of candidate isolate on aflatoxin production by BANGA1

The culture plates were observed daily for aflatoxin production, marked by the appearance of green fluorescence under UV light. A subjective scoring scheme representing the extent of aflatoxin production by BANGA1 was applied and the isolates were scored daily until day 7. Scoring was such that isolates with no fluorescence (–) scored 0, isolates that may be fluorescing ($✓_M$) scored 1; isolates that showed slight fluorescence ($✓_S$) scored 2, clear fluorescence (✓) scored 3, and strong fluorescence ($✓_↑$) gave a score of 4. The total score was calculated as the mean of the aflatoxin scores from days 3–7 for three replicates.

TABLE 2 Scoring scheme for colony interactions between BANGA1 and candidate isolates.

Interaction	Score for competitor isolate
Mutually intermingling growth	1
Overgrowth by BANGA1	2
Overgrowth by competitor	3
Contact, no mixing	4
Slight mutual inhibition	5
Overt mutual inhibition	6

Candidate isolates which indicated an overall negative interaction with growth or aflatoxin production on agar were assessed for interactions in broth coculture with BANGA1, and effects of secreted metabolites on aflatoxin production.

2.5.2 | Interactions in broth

Aflatoxin production

For each isolate, four 50 mL centrifuge tubes containing 20 mL YES broth were inoculated with 20 μL of spore suspension. After incubation in a slanted position at RT and ambient light conditions for 24 h, the tubes were inoculated with 20 μL of BANGA1 spore suspension, briefly vortexed to mix, and returned to incubation. Culture tubes for BANGA1 were treated similar to the candidate isolates. At the end of the 5-day incubation period, the tubes' contents were vigorously vortexed to mix, and samples of the vortexed spent media were filtered, diluted where necessary, and analyzed for aflatoxin content by ELISA.

2.6 | Effect of fungal metabolites on BANGA1

2.6.1 | Growth on metabolite agar

Four 50 mL centrifuge tubes containing 20 mL YES broth were inoculated with 20 μL of spore suspension. After incubation in a slanted position at RT and ambient light conditions for 72 h, the tubes were inoculated with 20 μL of BANGA1 spore suspension, briefly vortexed to mix, and returned to incubation for a further 24 h. Culture tubes for BANGA1 were treated in the same way. At the end of incubation, the tubes' contents were vigorously vortexed to mix, centrifuged for 10 min at 8000*g* and 4°C to pellet fungal mass, and the spent media were filtered through a 0.22 μm sterile syringe filter. The sterile filtrate from each tube was added at the proportion of 9 mL filtrate: 3 mL molten YES agar that had been composed to 4× the normal concentrations of constituents. The mixture was poured in a 6 cm Petri dish, allowed to set and inoculated at the center with 20 μL of BANGA1 spore suspension. The colony diameter of BANGA1 on each plate was measured daily. The effect of competitor isolates' metabolites on the growth of BANGA1 was measured by the mean colony growth index for three replicates, which was calculated from the colony daily growth rate (the daily increase in colony diameter) as follows:

$$\text{Daily growth rate} = \text{current day diameter} - \text{previous day diameter}.$$

For each replicate, growth index for each day was calculated as:

$$\text{Growth index} = \text{Daily growth rate} \times \text{Colony diameter on day 3}.$$

2.6.2 | Aflatoxin production on metabolite agar

The colonies were observed daily for 5 days under UV for the appearance of green fluorescence to mark aflatoxin production. The experiment was repeated for each plate which did not show any aflatoxin fluorescence.

3 | RESULTS

Overall, 36 morphologically distinct filamentous fungal isolates were recovered from the fermented cocoa beans collected from four cocoa-farming areas in southern Ghana: Agona Asafo (5.5548° N, 0.6577° W), Osiem (6.2495° N, 0.4278° W), Suhyen (6.2012° N, 0.3121° W), and New Tafo-Akim (6.2162° N, 0.3708° W). The isolates marked as "other" were from successive subcultures of a previously isolated candidate or from an aflatoxin-contaminated cornflour sample which yielded the aflatoxin-producing *Aspergillus flavus* control isolate (BANGA1). Isolation of BANGA1 from cornflour was based on both colony morphology and green fluorescence on YES-cyclodextrin agar (Figure 1a). The isolate showed strong fluorescence (Figure 1a) and was, therefore, used as the control in the study. Overall, the cocoa-associated *A. flavus* isolates showed less fluorescence on agar: no fluorescence (Figure 1b) or varying degrees of fluorescence (Figure 1c,d). The isolates shown in Figure 1d demonstrated clear differences in aflatoxin production—although there was clear fluorescence from each isolate, fluorescence from (iii) extended well beyond the colony boundary unlike (ii) and (iv). None of the cocoa bean samples from any site, including those which yielded *A. flavus* isolates, showed detectable levels of aflatoxin contamination by the ELISA kit used. One of the 11 *A. flavus* isolates indicated the presence of the *aflR1* gene but did not amplify with the ver primer pair, nor produce green aflatoxin fluorescence on agar (Table 3).

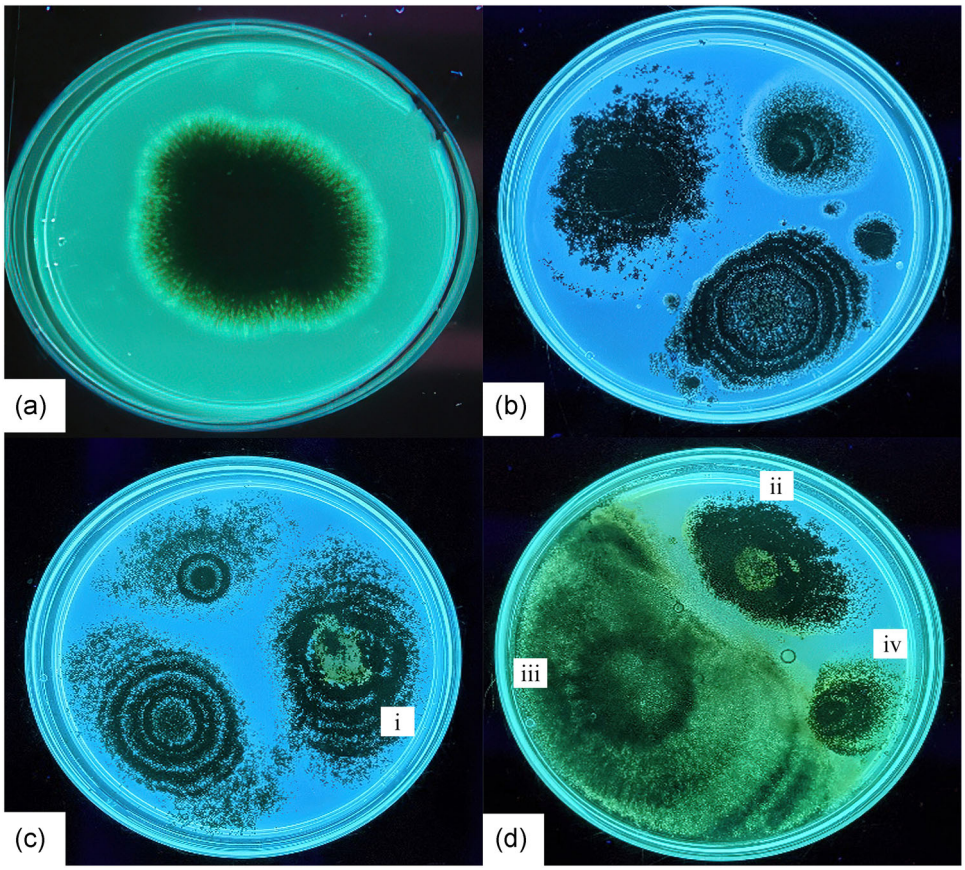

FIGURE 1 Candidate filamentous fungi and aflatoxigenic *Aspergillus flavus* BANGA1 on yeast extract sucrose agar.

3.1 | Identification and molecular characterization of study isolates

Amplicon sizes of the ITS-PCR products ranged between 400 and 800 bp. nBLAST on the GenBank database identified the isolates as belonging to the *Aspergillus*, *Cladosporium*, *Lichtheimia*, and *Trichoderma* genera (Table 3). The DNA sequences have been deposited in the NCBI GenBank database with accession numbers OP597655–OP597674. All but one of the isolates that were identified as *A. flavus* produced PCR product with both the aflR1 and ver primer pairs (Table 3). AC3 gave product for *aflR1* but not *ver* and did not fluoresce under UV.

Twenty of the isolates did not amplify with either the MAT1-1 or MAT1-2 primer pair (Table 3). Of those that gave PCR product, four isolates (*A. flavus* and *A. fumigatus*) amplified with the MAT1-1 primer and 12 isolates (*A. niger*, *A. flavus*, *A. aculeatus*, *A. fumigatus*, *Trichoderma virens*, *L. ramosa*) identified as MAT1-2 mating type. The control *A. flavus* isolate (BANGA1) amplified with the MAT1-2 primer. Only one *A. niger* isolate (AC8) produced amplicon with a MAT primer.

3.2 | Interaction on agar: Effects on growth and aflatoxin production by BANGA1

The plates for assessment of colony interactions with BANGA1 (Figure 2) showed results macroscopic ranging from varying extents of intermingling (Figure 2a,b) to mutual coexistence (Figure 2c,d), and then colony antagonism (Figure 2e,f). On the colony antagonism index, the highest-scoring candidates were *A. niger* and *A. aculeatus* isolates (Table 4). *Lichtheimia ramosa* and *T. virens* had the lowest antagonism scores. Again, only *L. ramosa* and *T. virens* isolates increased the aflatoxin fluorescence score (Table 4). Coculture with the *L. ramosa* isolates produced the earliest and most intense observation of fluorescence. All the other candidate isolates decreased the fluorescence of BANGA1 by approximately 12%–50%. Aflatoxin fluorescence due to

TABLE 3 Sample collection sites and molecular characterization of candidate isolates.

				Molecular characterization			
Sampling area	**Code**	**Isolate ID**	**Blue-green fluorescence**	***VER***	***AflR1***	***MAT1-1***	***MAT1-2***
Agona Asafo	AC2	*Aspergillus flavus*	✓	+	+	+	–
	AC3	*Aspergillus flavus*	–	–	+	–	–
	AC7	*Cladosporium* spp.				–	–
	AC8	*Aspergillus niger*				–	+
	AC9	*Aspergillus flavus*	✓	+	+	+	–
	AC10	*Aspergillus melleus*				–	–
	AC14	*Aspergillus flavus*	✓	+	+	–	+
	AC19	*Aspergillus niger*				–	–
	AO1	*Aspergillus niger*				–	–
	AO3	*Trichoderma virens*				–	–
	AO4	*Aspergillus niger*				–	–
	AO6	*Aspergillus flavus*	✓	+	+	+	–
	AO8	*Aspergillus aculeatus*				–	–
	AO9	*Trichoderma virens*				–	–
Osiem	E1	*Aspergillus flavus*	✓	+	+	–	–
	E4	*Aspergillus flavus*	✓	+	+	–	–
	E6	*Aspergillus niger*				–	–
	E7	*Aspergillus niger*				–	–
	E8	*Aspergillus aculeatus*				–	–
	E11	*Aspergillus flavus*	✓	+	+	–	+
	E13	*Aspergillus flavus*	✓	+	+	–	+
	E16	*Aspergillus niger*				–	–
Suhyen	S4	*Aspergillus aculeatus*				–	–
	S6	*Aspergillus aculeatus*				–	–
	S7	*Aspergillus aculeatus*				–	+
	BROWN	*Trichoderma virens*				–	+
New Tafo-Akim	T2	*Aspergillus fumigatus*				–	+
	T4	*Aspergillus terreus*				–	–
	T5	*Aspergillus terreus*				–	–
	T6	*Lichtheimia ramosa*				–	+
	T7	*Aspergillus flavus*	✓	+	+	–	+
	T8	*Lichtheimia ramosa*				–	+
	T10	*Aspergillus fumigatus*				+	–
	T15	*Aspergillus flavus*	✓	+	+	–	+
Other	FT14	*Aspergillus niger*				–	–
FT14-C	*Aspergillus aculeatus*				–	+	
BANGA1	*Aspergillus flavus*	✓	+	+	–	+	

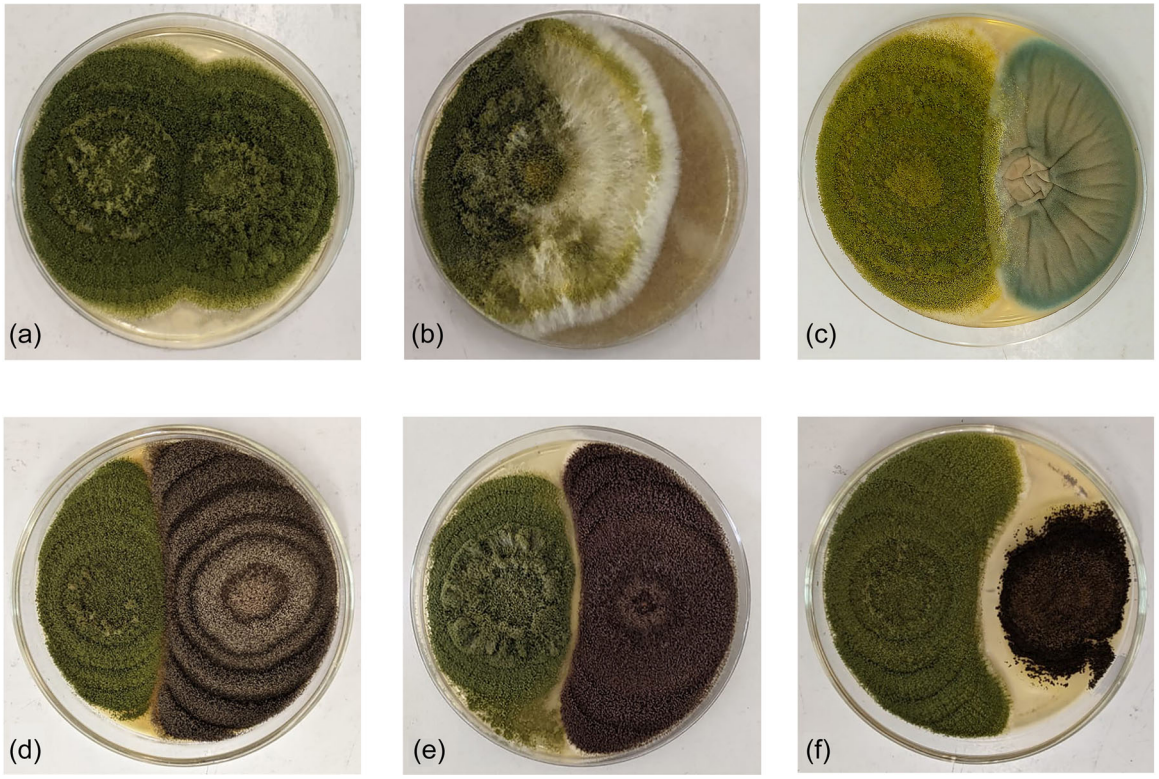

FIGURE 2 Representative colony interactions. The plates show mutually intermingling growth (a), overgrowth of one isolate by another (b), colonies growing up to each other (c, d), slight mutual inhibition (e), and overt mutual inhibition (f).

BANGA1 was absent or visibly less in areas of the agar close to all the *A. niger* and *A. melleus* isolates (Table 4).

3.3 | Interaction in broth: Effect on aflatoxin production by BANAG1

Only one isolate each of *T. virens* (BROWN) and *A. terreus* (T5) did not reduce aflatoxin production by BANGA1 (353.1 ± 32.8 ppb) after the 7-day coculture (Table 4). BROWN increased aflatoxin production by 8.4% ($p = 0.0139$) whereas the reduction by T5 was not significant ($p = 0.9502$). All the other candidate isolates significantly reduced aflatoxin production by approximately 2%–97% ($p < 0.0001$) of the BANGA1 aflatoxin level. Isolates that caused reduction of over 90% were *A. niger*, *A. fumigatus*, and *L. ramosa*. One *A. niger* isolate (E16) reduced aflatoxin level to below 10 ppb.

3.4 | Effect of metabolites on growth and aflatoxin production

BANGA1 was cultured on agar composed from spent media of the candidate isolates to assess the effects of metabolites on growth and aflatoxin production. As indicated by the mean growth index (Table 5), none of the candidate isolates caused a significant reduction in the growth of BANGA1. Isolates of *A. aculeatus*, *A. ochraceus*, *A. niger*, *A. terreus*, and *T. virens* significantly reduced the fluorescence score (Table 5). Three *A. niger* isolates completely inhibited aflatoxin production, indicated by the absence of fluorescence at the end of culture.

4 | DISCUSSION

Biocontrol of aflatoxigenic filamentous fungi is critical to global health and food security. It is acknowledged that food materials vary in their susceptibilities to spoilage by fungal and mycotoxin contamination. Mycotoxin contamination of foods is projected to worsen with the impact of climate change, with new mycotoxins becoming important to human health and food security [41–43]. Understanding the factors responsible for the apparent resistance of some foods to mycotoxin contamination would greatly aid in the design of strategies for the biocontrol of toxigenic fungi. With cocoa material, it has been noted that the apparent failure of aflatoxin-producing fungi to thrive on the substrate is unlikely to be due to the presence of methylxanthines which are the main bioactive compounds present [44, 45]. To investigate the contribution of

TABLE 4 Colony interactions and aflatoxin production on agar.

Isolate ID	Relative colony antagonism (%)	Aflatoxin fluorescence on agar					Relative score (%)	Direction	Aflatoxin in coculture (ppb)
		Day 3	Day 4	Day 5	Day 6	Day 7			
Control (*Aspergillus flavus*)									
BANGA1	0	✓	✓	✓	✓	✓↑	100	Toward	353.1 ± 32.8
Aspergillus aculeatus									
AO8	87	✓S	✓S	✓S	✓	✓	76 ± 10	Away	242.8 ± 6.7
E8	73	—	—	✓M	✓S	✓	56 ± 12*	Away	91.1 ± 15.8
S4	60	—	✓S	✓	✓	✓	71 ± 11**	Away	151.2 ± 3.9
S6	53	✓M	✓	✓	✓	✓↑	89 ± 11	Away	281.5 ± 7.0
S7	60	✓M	✓M	✓	✓↑	✓↑	87 ± 13	Toward	165.9 ± 7.6
FT14-C	66	—	—	✓S	✓	✓	55 ± 17*	Toward	345.5 ± 2.4
Aspergillus fumigatus									
T2	20	✓S	✓	✓	✓	✓	86 ± 8	Toward	302.8 ± 25.5
T10	20	—	✓M	✓	✓	✓↑	79 ± 15	Toward	311.7 ± 11.1
Aspergillus melleus									
AC10	67	✓S	✓	✓	✓	✓	74 ± 11**	Away	259.4 ± 16.6
Aspergillus niger									
AC8	70	—	✓M	✓S	✓	✓	51 ± 13*	Away	57.7 ± 4.5
AC19	80	✓S	✓	✓	✓	✓	78 ± 7	Away	222.5 ± 15.4
AO1	80	—	—	✓	✓	✓	59 ± 14**	Away	218.9 ± 6.7
AO4	80	—	—	✓S	✓	✓	59 ± 15**	Away	129.9 ± 11.3
E6	93	✓S	✓	✓	✓	✓	85 ± 4	Away	20.1 ± 1.2
E7	80	✓S	✓	✓	✓	✓	90 ± 5	Away	56.3 ± 8.3
E16	80	✓S	✓	✓	✓	✓	83 ± 6	Away	8.3 ± 2.5
FT14	80	✓	✓	✓	✓	✓	85 ± 5	Away	203.2 ± 9.7
Aspergillus terreus									
T4	50	—	—	✓	✓	✓	63 ± 14**	Toward	173.6 ± 7.0
T5	40	—	—	✓	✓	✓	71 ± 12**	Toward	341.8 ± 6.4^
Cladosporium spp.									
AC7	20	✓M	✓S	✓S	✓	✓	58 ± 10	Toward	301.3 ± 6.1
Lichtheimia ramosa									
T6	0	✓	✓↑	✓↑	✓↑	✓↑	113 ± 9	Toward	120.4 ± 3.0
T8	7	✓	✓	✓↑	✓↑	✓↑	108 ± 5	Toward	159.3 ± 3.3
Trichoderma virens									
AO3	0	✓	✓	✓	✓↑	✓↑	107 ± 6	Away	115.9 ± 6.9
AO9	0	✓S	✓	✓	✓	✓↑	95 ± 6	Away	80.7 ± 4.1
BROWN	40	—	✓M	✓	✓	✓	71 ± 13	Toward	382.7 ± 4.7

Note: The values shown for relative colony antagonism and relative fluorescence score represent mean ± SEM of n = 3. No fluorescence observed (–) = 0; May be fluorescing (✓M) = 1; Slight fluorescence observed (✓S) = 2, Clear fluorescence (✓) = 3; Strong fluorescence (✓↑) = 4. Comparisons were made for each isolate score against the score for BANGA1.

*$p \leq 0.005$; **$p \leq 0.05$;

^$p \geq 0.05$.

TABLE 5 Growth of BANGA1 on metabolite agar.

Isolate ID	Mean growth index	Representative aflatoxin fluorescence					Relative score (%)
		Day 3	Day 4	Day 5	Day 6	Day 7	
Control (*Aspergillus flavus*)							
BANGA1	5.2 ± 1.3	✓M	✓	✓	✓	✓	100
Aspergillus aculeatus							
AO8	4.2 ± 0.4	—	—	✓S	✓	✓	57 ± 17
E8	3.9 ± 1.3	—	—	—	✓	✓	22 ± 13*
S6	6.0 ± 0.3	—	—	✓M	✓	✓	38 ± 14**
FT14-C	4.3 ± 0.3	—	—	—	✓M	✓	19 ± 11*
Aspergillus fumigatus							
T2	5.8 ± 1.8	—	✓S	✓	✓	✓	77 ± 17
T10	5.4 ± 2.2	—	—	✓	✓	✓	62 ± 18
Aspergillus melleus							
AC10	4.1 ± 0.3	—	—	✓S	✓	✓	48 ± 15**
Aspergillus niger							
AC8	3.4 ± 0.8	—	—	✓S	✓S	✓	45 ± 14**
AC19	3.4 ± 0.2	—	—	✓M	✓M	✓	31 ± 11*
AO4	3.6 ± 1.0	—	—	—	—	—	0*
E6	3.6 ± 0.4	—	—	✓M	✓S	✓	47 ± 17
E7	3.4 ± 1.4	—	—	—	—	—	0
E16	4.7 ± 2.6	—	—	✓M	✓	✓	38 ± 17**
FT14	4.0 ± 1.6	—	—	—	—	—	0*
Aspergillus terreus							
T4	5.1 ± 0.1	—	—	✓M	✓	✓	31 ± 15*
T5	5.3 ± 0.3	—	✓S	✓	✓	✓	92 ± 16
Cladosporium spp.							
AC7	4.1 ± 1.1	✓M	✓S	✓	✓	✓↑	80 ± 15
Lichtheimia ramosa							
T6	5.9 ± 2.3	—	✓M	✓	✓	✓↑	84 ± 17
T8	8.1 ± 2.9	✓M	✓S	✓	✓	✓↑	84 ± 17
Trichoderma virens							
AO3	4.3 ± 1.2	✓	✓	✓	✓↑	✓↑	130 ± 8
AO9	4.7 ± 1.0	✓S	✓	✓	✓	✓↑	115 ± 8
BROWN	4.2 ± 0.9	—	—	—	✓M	✓M	11 ± 9*

Note: The values shown for mean growth index and relative fluorescence score represent mean ± SEM of n = 3. No fluorescence observed (–) = 0; May be fluorescing (✓M) = 1; Slight fluorescence observed (✓S) = 2, Clear fluorescence (✓) = 3; Strong fluorescence (✓↑) = 4. Comparisons were made for each isolate score against the score for BANGA1.

*$p \leq 0.005$; **$p \leq 0.05$.

cocoa-associated filamentous fungi, this study identified 25 candidate isolates, mostly *A. niger* and *A. aculeatus*, from fermented cocoa bean samples.

The low fluorescence on agar shown by the cocoa-associated *A. flavus* isolates suggests that aflatoxin production by cocoa-associated aflatoxigenic fungi may be naturally low. *AflR1* and *ver* were chosen for this study because of their involvement in the start and middle, respectively, of aflatoxin biosynthesis [46]. The *aflR1* gene product is recognized as the key regulator of aflatoxin synthesis [35], although its presence is not indicative of aflatoxigenicity [47–49]. The absence of amplification with the ver primer in an *A. flavus* isolate (AC3) suggests that the isolate may be lacking the requisite complement of enzymes to enable aflatoxin synthesis. Nevertheless, the presence of the *aflR1* gene in 100% of the field *A. flavus* isolates collected in this study is indicative of the strong potential for aflatoxigenic strains to develop from atoxigenic isolates due to recombination events, for example, from mating, or from horizontal gene transfer events which are currently understudied in filamentous fungi.

While recent evidence suggests that some strains of *A. flavus* may be hermaphroditic [50], *A. flavus* has classically been noted as a heterothallic fungus [51–53]. Of the 11 *A. flavus* isolates that were collected from the study sites, three belonged to the MAT1-1 mating type whereas five identified as MAT1-2 (Table 3). All the sites produced MAT1-2 isolates whereas MAT1-1 *A. flavus* were recovered from only one site. Although the isolates collected in this study are too few to support an analysis of the functional implications of the MAT gene ratio, the presence of the two mating types in field samples from the same site is an indication of the potential for mating events which could lead to genetic recombination and consequent increases in the proportions of aflatoxigenic *A. flavus* in the field. Interestingly from one site, one isolate of *A. fumigatus* identified as MAT1-1, and another identified as MAT1-2. This was taken to strongly support our suggestion that sexual reproduction between compatible isolates, leading to recombinant offspring, was an occurrence in the field. Such a projection is not favorable for the aflatoxin biocontrol strategies which rely on the competitive advantage of large populations of atoxigenic *A. flavus* over toxigenic field isolates [19, 54–56].

There is keen interest in fungal biocontrol strategies which take advantage of antagonistic colony interactions between competitor isolates and the target fungi [57–59]. Several of the cocoa-associated fungi from this study, particularly *A. niger* and *A. aculeatus* isolates, interacted negatively with growth and/or aflatoxin production by the test *A. flavus* isolate (BANGA1) (Figure 2; Table 4). In the scoring of antagonism, tolerance for coexistence with BANGA1 was prioritized over colony diameter representing growth rate. The highest antagonism scores were assigned to those isolates that demonstrated a lack of tolerance for coexisting with BANGA1 (Figure 2e,f). For the isolates that showed hyphal spread over BANGA1, lower scores were assigned for that interaction because in all cases, the overgrowths did not stop BANGA1 from continuing to grow and spread on the plate. Aspergilli are noted as some of the most bio innovative of the filamentous fungi, known for producing a range of bioactive metabolites [60–62]. In most cases for the *A. niger* isolates, there was clear mutual inhibition between the competitor and BANGA1 (Figure 2). This indicates excellent potential for the cocoa-associated *A. niger* strains to be harnessed for direct use or to be prospected for agents that will be useful in inhibiting the growth of *A. flavus*. Beyond growth inhibition, the *A. niger* isolates interacted negatively with aflatoxin production on agar (Table 4). In addition to delaying aflatoxin production by BANGA1, the absence of blue/green fluorescence in the direction toward some of the competitor isolates was a further demonstration of inhibitory effect on aflatoxin production. This could be due to the effects of metabolites and/or metabolism of the competitor isolate, leading to the secretion of inhibitory agents and substrate modifications such as changes in pH or nutrient availability. Even volatiles from competitor isolates have been shown to be capable of interacting with *A. flavus* growth and aflatoxin production [63]. Further study of the observed inhibitory effects is warranted.

The suggestion that the indicated isolates may secrete metabolites that were inhibitory to BANGA1 was tested by assessing growth and aflatoxin fluorescence on metabolite agar. Again, spent media containing metabolites from the *A. niger* isolates caused the greatest reduction in both parameters (Table 5). Beyond this, metabolites from three of the *A. niger* isolates appeared to completely prohibit aflatoxin production on agar over 5 days of observation. Only data collected up to Day 5 of incubation was used in assessing the metabolite effects because on this agar composition, BANGA1 always covered the plate surface by Day 6. Cocultures in broth indicated that four of the *A. niger* isolates reduced aflatoxin production by approximately 84%–98%. One isolate (E7) which prohibited aflatoxin fluorescence on agar reduced content in broth by 84%. The isolates present excellent potential, whether for single use or in combination, to control growth and aflatoxin production of *A. flavus*. This could be a safer and more effective alternative to the use of atoxigenic *A. flavus* because the risk of genetic recombination events between the control and target strains would be removed.

Although our previous report [26] did not identify *A. flavus* isolates from cocoa farm waste material, aflatoxigenic *A. flavus* were isolated from three of the four sampling sites in this study. Overall, the cocoa-associated isolates used in this study interacted negatively with growth and aflatoxin production by the test *A. flavus* isolate. This has provided insight into why aflatoxin contamination of cocoa-associated material is not recorded as a major challenge to the cocoa industry. The data from this study identified the potential for genetic recombination events between fungi in the field, alluding to the risk posed by *A. flavus* biocontrol measures that rely on atoxigenic *A. flavus* strains. *A. niger* isolates that can be harnessed for use in controlling growth and aflatoxin production by *A. flavus* have been identified. It is of keen interest to further describe the usefulness, molecular agents, and mechanisms responsible for the inhibitory activities observed.

AUTHOR CONTRIBUTIONS

Daniel Oduro-Mensah: Conceptualization; formal analysis; funding acquisition; investigation; methodology; resources; supervision; validation; writing—original draft. **Sammy T. Lowor**: Conceptualization; methodology; supervision. **Yahaya Bukari**: Conceptualization; methodology. **Jacob Kwaku Donkor**: Data curation; investigation. **Bismarck Minnah**: Data curation; investigation. **Abdul Hamid Nuhu**: Data curation; investigation. **Derry Dontoh**: Methodology. **Ayesha Algade Amadu**: Formal analysis; writing—review & editing. **Augustine Ocloo**: Validation; writing—original draft.

ACKNOWLEDGMENTS

The authors acknowledge the cooperation of cocoa farmer groups in the sampling sites, and contributions from Akua Ansah Larbi, Caroline Anaba, Jessica Attipoe, Nana Tawiah Ghunney, Bridget Quist, Christabel Koranteng, Doreen Akorwome, Nana Ama Yeboah, Nana Ama Peprah, Cornelius Dzikunoo, Stephanie Hammond and Najlau Zakariah to the bench work. This work was funded by the Building a New Generation of Academics in Africa (BaNGA-Africa) project, funded by Carnegie Corporation of New York. Fogarty International Center and National Cancer Institute provided support through the Fogarty Global Health Training Program NIH Grant No. D43 TW010543 for the preparation of this manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

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

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How to cite this article: Oduro-Mensah D, Lowor ST, Bukari Y, Donkor JK, Minnah B, Nuhu AH, et al. Cocoa-associated filamentous fungi for the biocontrol of aflatoxigenic *Aspergillus flavus*. J Basic Microbiol. 2023;1–14. https://doi.org/10.1002/jobm.202300163