J. Crop Prot. 2021, 10 (2): 375-390

Research Article

Identification of an *Aspergillus* isolate with potential for biocontrol of *Phytophthora palmivora,* causal agent of black pod disease of cocoa

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Abstract: The black pod disease of cocoa in Ghana caused by *Phytophthora palmivora* and *P. megakarya* is traditionally managed with fungicides. Because of challenges associated with fungicide use, biological control options, if available, are worth trying. A fungus with proven usefulness in suppressing *P. palmivora* and *P. megakarya* in dual plate cultures and cocoa pods has partly been identified as an *Aspergillus* (designated AI_1). However, its exact identity has been unknown, requiring specific identification by comparing it with known *Aspergillus flavus* strains (designated AI_2, AI_3, AI_4, and AI_5). It was retested against *P. palmivora* to confirm the potency of AI_1. The putative *A. flavus* isolates were also tested for the first time against *P. palmivora*. Morphological features were determined on carrot agar (CA), potato dextrose agar (PDA), and malt extract agar (MEA). Genomic DNAs from the *Aspergillus* isolates were subjected to the ITS region and β-tubulin gene sequencing. All the *Aspergillus* isolates inhibited *P. palmivora* in assay plates by levels ranging from 89.33 to 95.33% (Experiment 1) and 46.67 to 60.33% (Experiment 2). Generally, the AI_1 produced culture features similar to those of the putative *Aspergillus flavus* isolates. ITS region sequence analysis grouped all isolates as *A. flavus* and beta-tubulin also grouped AI_1, AI_2, AI_3, and AI_4 as *A. flavus* but differentiated AI_5 as *A. flavus* var. *parvisclerotigenus*. AI_3 recorded the highest inhibition zone and prevented black pod development of inoculated pods as well. The previously unknown *Aspergillus* isolates AI_1 is now conclusively identified as *A. flavus*.

Keywords: cocoa, biocontrol, *Aspergillus*, gene sequencing, *Phytophthora palmivora*

Introduction

Cocoa *Theobroma cacao* L. is an essential crop in Ghana. In 2014, approximately 1 million MT of beans was produced by Ghana, making Ghana the second most important global producer of the crop (FAOSTAT, 2014). One of the most challenging cocoa diseases in Ghana is black pod disease caused by *P. palmivora* and *P. megakarya* (Dakwa, 1987; Luterbacher and Akrofi, 1993; Opoku *et al*., 1999). *Phytophthora megakarya* is more destructive than *P. palmivora* (Brasier and Hansen, 1992). In areas attacked by *Phytophthora megakarya,* 60-100% pod losses

Handling Editor: Naser Safaie

* Corresponding author: josh_139@yahoo.com
Received: 21 January 2021, Accepted: 13 April 2021
Published online: 18 April 2021

[DOR: 20.1001.1.22519041.2021.10.2.15.1]

were detected compared to 5-20% pod losses usually attributed to *P. palmivora* (Dakwa, 1987; Opoku *et al*., 2007).

In Ghana, copper-based fungicides are required to effectively manage black pod, a costly practice for farmers (Opoku *et al*., 2007). Even though fungicides are a helpful method to manage plant diseases, it is also accompanied by some undesirable consequences (Garry, 1996; Ranasinghe *et al.,* 2003; Cloyd, 2007; Ragsdale *et al*., 2008; Chaube and Pundhir, 2009). Accumulation of any pesticide in cocoa fat may change the beans' flavor and the chocolate produced (Akrofi, 2015). Many cocoa and cocoa products importing countries have introduced maximum residue limits (MRLs) permissible in cocoa beans and their products. Japan, for instance, introduced new legislation on MRLs in 2006; the European Union (EU) has since September 2008 enacted new MRLs (EC 148/2008). An alternative/complementary disease management approach that is either devoid of or minimizes the above potential shortcomings of pesticides on cocoa would be desirable.

Koranteng (2017) reported a fungal isolate that inhibited *P. palmivora* and *P. megakarya* on dual plate cultures and separate cocoa pods. This antagonistic fungus was isolated seven years earlier and had been in refrigerated storage since then. The fungus was tentatively identified to be *Aspergillus,* but its exact identity remains unknown. Dorner (2009) evaluated nontoxigenic (atoxigenic) strains of *A. flavus* to control toxigenic strains and reported 80-90% aflatoxin contamination reduction when sterilized grain coated conidia of atoxigenic strain were applied in the field. Several products have been produced to control maize aflatoxin contamination (AF 36) and peanuts (Afla-guard®). Several research types have been conducted on atoxigenic strains of *A. flavus,* and outcomes are promising (Yin *et al*., 2008; Ehrlich, 2014).

Moreover, genetically and phenotypically, *A. flavus* populations are disparate (Geiser *et al*., 2000), atoxigenic strains L morphotype occurs in Ghana, and a product named aflasafe has been developed manage aflatoxin contamination in maize and groundnut (Agbetiameh *et al*., 2017; 2018; 2019). For the *Aspergillus* isolate to be accepted and developed further as a biocontrol agent, its accurate identification to the species level is critical. Morphologically, some *Aspergillus* species can be differentiated from other related species by colony characteristics such as diameter, color, texture, and some micromorphological features, including sizes and structure of metulae, phialides, conidia, and conidiophores (Klich, 2002). Morphological features may help identify fungi; however, they have limitations since environmental factors may influence morphological characteristics. For certainty, molecular characterization is more practical.

Fungal identification based on DNA sequences has been categorized into DNA barcoding and DNA taxonomy approaches (Yahr *et al*., 2016; Raja *et al*., 2017). DNA barcoding entails identifying an unknown fungus based on sequence similarity to a reference sequence database of known classification (Toju *et al*., 2012; Raja *et al*., 2017). The official barcode marker for species-level fungi identification as proposed by a consortium of mycologists is the internal transcribed spacer (ITS) region due to its fastest evolving rate and highest sequence variability among fungal species (Schoch *et al*., 2012; Yahr *et al*., 2016; Raja *et al*., 2017). However, observed insufficient ITS variability in some fungal species, particularly within the phylum Ascomycota (Yahr *et al*., 2016), necessitates the use of sequence information of other protein-coding genes for building phylogenetic trees using the DNA taxonomy method of fungi identification (Yahr *et al*., 2016; Raja *et al*., 2017). Protein-coding genes such as β-tubulin (*tub2*), *RPB1* and *RPB2* (respectively encode first and second-largest subunits of RNA polymerase II), and *TEF1* (translation elongation factor EF-1 alpha) have been used in conjunction with the ITS region to identify specific fungi (Yahr *et al*., 2016; Raja *et al*., 2017).

This study seeks to determine the precise identity of the *Aspergillus* isolate (AI_1) and compare other isolates using morphological and molecular means, and retest its anti-*Phytophthora* properties. The study also tests the antagonism of some known *Aspergillus flavus* isolates towards *P. palmivora*.

Materials and Methods

Sources of *Aspergillus* species and *P. palmivora*. The *Aspergillus* isolate (AI_1) with anti-*Phytophthora* property and whose specific identity needs determination was a laboratory contaminant and formed part of a refrigerated microbial collection at the Plant Pathology Laboratory, Kwame Nkrumah University of Science and Technology, Kumasi. It had been in storage (with periodic sub-culturing) for seven years. It is designated AI_1 in the current study. Four other *Aspergillus flavus* isolates, designated AI_2, AI_3, AI_4, and AI_5 used for comparison, were isolated from soil and constituted part of the Plant Pathology Laboratory microbial collection. All aspergilli cultures were established on PDA through single sporing. A pure culture of *Phytophthora palmivora* (Gh-16-ER 417) was obtained from the Cocoa Research Institute of Ghana, New Tafo, and maintained on Green Cocoa Mucilage Agar (GCMA) (Awuah and Frimpong, 2007). It was sub-cultured weekly to maintain its pathogenicity and refrigerated at 5 °C until needed.

Media preparation. For morphological studies, Oxoid potato dextrose agar (PDA), carrot agar (CA) (200 g carrot pieces boiled in 500 ml distilled water, filtered and 20 g of agar powder added), and Oxoid malt extract agar (MEA) were used. Antagonism of *P. palmivora* by all the *Aspergillus* isolates was conducted on GCMA: PDA (1: 1) mixture. The GCMA was formulated with the mucilage of cocoa beans according to the method of Awuah and Frimpong (2007). All media were sterilized by autoclaving at 121 °C at 0.98 kg/cm^2 for 15 minutes. On cooling, they were dispensed into 9-cm-diameter Petri dishes at aliquots of 20 ml and kept *in situ* for five days before use.

***In vitro* screening of *Aspergillus* isolates against *P. palmivora*.** The *Aspergillus* isolates AI_1, though known to possess antifungal activity towards *Phytophthora palmivora* (Koranteng, 2017), was retested to confirm its antagonism by modification of the zone of inhibition method (Akrasi and Awuah, 2012). The four other *Aspergillus* isolates *viz*. AI_2, AI_3, AI_4, and AI_5 were tested for antagonism for the first time. Single spores from a 7-day-old culture of each fungus were centrally placed on Petri plates containing GCMA: PDA (1: 1 ratio) and incubated for 24 h. Four 7-mm-diameter mycelial plugs of *P. palmivora* (7-day-old) were then placed top-down at four equidistant positions (25 mm) from the centrally placed *Aspergillus* isolates. Plates without *Aspergillus* served as controls. Three replicate plates for each *Aspergillus*: *Phytophthora* combination was established. The plates were incubated for seven days at 29 ± 2 °C, and the lengths of inhibition, if any, were measured from the underside of plates from the center of the central antagonist to the edge of the inhibited *P. palmivora* colony with a ruler. The average zone of inhibition for the four *P. palmivora* colonies on a plate was calculated as A – B ÷ 4 (Koranteng, 2017) and

$$\text{The percentage inhibition per plate} = \frac{(A - B)/4}{25\ \text{mm}} \times 100$$

Where A is the initial distance between the centrally placed aspergilli (antagonist) and pathogen (*P. palmivora*) (25 mm); B is the distance of the pathogen's growth towards the *Aspergillus*.

Data from all three replicate plates were averaged and analyzed. The experiment was repeated with a slight modification in that, this time, the antagonistic aspergilli were placed on plates 24 hr after the *P. palmivora* plugs were placed on the plates.

[DOR: 20.1001.1.22519041.2021.10.2.15.1]

Pathogenicity of *P. palmivora* cultures overrun on plate cultures by antagonistic *Aspergillus*. Seven-mm-diameter mycelial plugs from *P. palmivora* cultures (7-day-old) overrun by the different *Aspergillus* antagonists (AI_1 to AI_5) were placed separately into cork borer wounds on detached cocoa pods. The inoculants were covered with the cocoa tissue and pods incubated in a humidified transparent polyethylene bag at 29 ± 2 °C. Inoculation sites were observed for black pod lesions after seven days. The experiment was repeated once.

Morphological studies. A single spore of each of the five *Aspergillus* isolates was centrally placed on each plate medium described above (three plates per isolate) and incubated at 29 ± 2 °C on a laboratory bench with diffused sunlight during the day and darkness during the night. Colony diameters were measured from the reverse side of plates with a ruler (average of two diagonal measurements per plate) from day 4 to day 7. Colony colors (top and underside) were observed and described on day 7 using PanPastel's Artiste Colour Chart (www.panpastel.com). Photographs of both top and underside plates were taken on day 7 with a Canon PowerShot digital camera (8x). Sclerotia production was qualitatively scored at day 10, their sizes measured with the Amscope's microscope software, and their colors characterized as above.

Microscopic studies on the Aspergilli were done using a modified slide culture technique (Riddell, 1950). For each isolate, mycelial bits from a 7-day-old culture were placed at the four sides of a 1 cm^3 agar block of the respective medium and placed on a microscope slide suspended on a bent glass rod. The setup was placed in a Petri dish lined with moistened filter paper and incubated at 29 ± 2 ^{o}C on a laboratory bench. After 48 h, fungal growths from the agar blocks were examined *in situ* with an Amscope microscope (400x). Measurements on conidia, conidial heads, phialides, metulae, and conidiophores were done with the Amscope microscope and software. For vesicle diameter measurement, microscope slides were prepared by teasing mycelium from peripheries of 5-day-old actively growing cultures and the vesicles measured.

DNA extraction. Genomic DNA was extracted at the Biotechnology Laboratory, CRIG, Akim Tafo from single spored 7-day-old *Aspergillus* isolates cultured on PDA with the CTAB method. For each isolate, 50 mg of the fungal mycelial mat was ground in liquid nitrogen with a sterilized pestle and mortar. Nine hundred µl pre-warmed extraction buffer (65 °C) was added, mixed, and incubated at 65 °C for 1 h. 900 µl of Phenol: Chloroform (1: 1) was added and vortexed. The mixture was centrifuged at 10,000 rpm for 10 minutes, and the supernatant was transferred into new 2 ml Eppendorf tubes. Two µl RNAse (10 mg/µl) was added and incubated at 37 °C for 30 minutes. Seven hundred and fifty µl of chloroform was added, vortexed, and centrifuged at 10,000 rpm for 10 min. Five hundred µl of the supernatant was transferred into 2 ml Eppendorf tubes, and 250 µl of 7.5 M NH_4OAc and 1 ml of ethanol (99%) added. The mixture was kept on ice for over an hour, centrifuged at 12,000 rpm for 15 minutes. The liquid phase was poured out and centrifuged quickly to remove the remaining liquid. The tubes were dried in the laminar flow for 60 minutes. The DNA pellets were dissolved in 40 µl TE buffer and the quantity checked by NanoDrop spectrophotometer at Functional Bioscience, USA.

Polymerase Chain Reaction. The ITS region was amplified using primers ITS 1 (5-TCCGTAGGTGAACCTGCGG-3) and ITS 4 (5-TCCTCCGCTTATTGATATGC-3) (White et al. 1990). A segment of the β - tubulin gene was amplified using primers bT2a (5-GGTAACCAAATCGGTGCTGCTTTC-3) and bT2b (5-ACCCTCAGTGTAGTGACCCTTGG C-3) (Glass and Donaldson 1995). Promega's Go Taq Hot Start Polymerase was used. Each 15 µl of PCR mixture contained 9.15 µl of

[DOR: 20.1001.1.22519041.2021.10.2.15.1]

water, 0.6 µl of fungal DNA, 3 µl of 5x Buffer (Promega), 0.3 µl of dNTP's, 0.5 µl of forward primer and 0.5 µl of reverse primer, 0.75 µl of $MgCl_2$ (Promega) and 0.2 µl of Go Taq polymerase. The PCR mixtures were first heated at 95 °C for 10 mins, followed by 40 cycles of 95 °C for 20 s, 56 °C for 30 s and, 72 °C for 30 s, and a final extension of 72 °C for 10 min in an Eppendorf MasterCycler. The samples were run on a 2% agarose gel (Seakem LE agarose) to check for amplification. Samples that displayed a band on the gel were cleaned up using ExoSAP-IT (ThermoFisher Scientific).

ITS and β-tubulin gene sequencing. One (1) µl of purified PCR product was used directly in dideoxy-termination sequencing reactions using Big Dye Terminator v3.1 (Applied Biosystems) and run on an ABI 3730xl DNA analyzer. Both strands of the PCR products were sequenced twice. The sequences were quality checked and proofread with Sequencher v. 5.0 (Gene Codes). The sequences of the PCR products were aligned using muscle in MegAlign (DNASTAR), and specific homologies for the sequence were searched in the GenBank database through the NCBI n- BLAST (Altschui *et al*., 1990) analysis. The sequenced PCR products (query sequence) were compared with sequences in the database to obtain identity value in percentage.

Statistical analysis. For *in vitro* screening of antagonists against the pathogen, a completely randomized design with three replications was used. The data was analyzed by GenStat statistical package 12th edition, and the means were compared with Fisher's protected Least Significant Differences at 5%. A phylogenetic tree was constructed with the maximum likelihood method in MEGA 7.0.26 (Tamura and Nei, 1993; Kumar *et al*., 2016). Phylogenetic analyses included 485 nucleotide positions of the ITS region and 429 nucleotide positions of the beta-tubulin gene, and sequenced data were deposited into GenBank (NCBI) (Table 1).

Table 1 Accession numbers of *Aspergillus* isolates generated from internal transcribed spacer (ITS) region and beta-tubulin gene sequences.

Isolate ID	Origin	Accession numbers	
		Internal Transcribed Spacer region	Beta Tubulin gene
AI_1	Ghana	MT093446	MT105371
AI_2	Ghana	MT093447	MT105372
AI_3	Ghana	MT093448	MT105373
AI_4	Ghana	MT093449	MT105374
AI_5	Ghana	MT093450	MT105375

Results

Antagonism of the *Aspergillus* isolates towards *P. palmivora*. All the five *Aspergillus* isolates inhibited *P. palmivora* in a manner significantly different from the control. Inhibition zone lengths ranged from 22.67 (AI_5) to 23.83 mm (AI_3) that is 89.33 to 95.33% inhibition. Inhibition of the *P. palmivora* was again obtained when the experiment was repeated (Table 2, Fig. 1).

Table 2 Length of inhibition zones and % inhibition obtained with *Aspergillus* isolates against *Phytophthora palmivora*.

Aspergillus / Pp combination[1]	Experiment 1		Experiment 2	
	Length of inhibition (mm)[2]	Inhibition (%)[3]	Length of inhibition (mm)[2]	Inhibition (%)[3]
AI_1 + Pp	23.5	94.00	12.33	49.33
AI_2 + Pp	22.33	89.33	15.08	60.33
AI_3 + Pp	23.83	95.33	14.92	59.67
AI_4 + Pp	23.58	94.33	13.17	52.67
AI_5 + Pp	22.67	90.67	11.67	46.67
Pp alone (Control)	0	0	0	0
CV (%)	2.20		8.70	
LSD (0.05)	0.75		1.74	

[1] *Aspergillus* isolates: AI_1 (Partly identified *Aspergillus* isolate); AI_2 (*A. flavus* strain 1); AI_3 (*A. flavus* strain 2); AI_4 (*A. flavus* strain 3); AI_5 (*A. flavus* strain 4); Pp = *Phytophthora palmivora*.

[2] Data was taken after seven days. Values are mean lengths of inhibition zones from four replicate plates.

[3] Percentage inhibition was calculated from values of mean lengths of inhibition zones.

Experiment 1: Antagonists were placed 24 h before the Pp plugs were placed on the media.

Experiment 2: Antagonists were placed 24 h after the Pp plugs were placed on the media.

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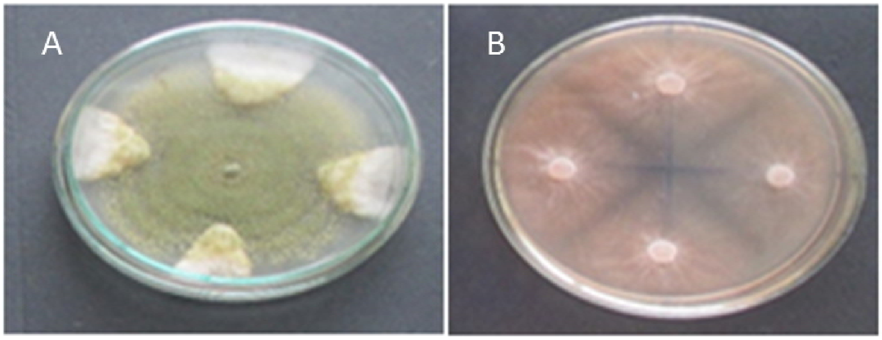

Figure 1 *Phytophthora palmivora* colonies overran by antagonistic *Aspergillus* isolate (A) and control (B; plate without antagonist) at day 7.

Pathogenicity *of P. palmivora* cultures on detached cocoa pods. Most of the *P. palmivora* mycelial plugs from cultures overrun by antagonist *Aspergillus* isolates could not cause lesions on detached cocoa pods (Table 3). The most effective *Aspergillus* isolates were AI_2 and AI_3, which caused one lesion at 12 inoculated sites. Mycelial plugs from *P. palmivora* cultures without any *Aspergillus* isolate produced black pod lesions at all 12 inoculated sites (Table 3, Fig. 2).

Morphological Identification of *Aspergillus* isolates. Generally, all five *Aspergillus* isolates produced features such as colony growth and pigmentation, concentric rings, sclerotia, conidial head sizes, vesicle sizes, conidia sizes, phialides, etc. on the media. These features, except a few, were similar for all the *Aspergillus* isolates on all three media (Table 4a, b and Table 5, Fig. 3 and 4).

Table 3 Pathogenicity of *P. palmivora* obtained from cultures overrun by *Aspergillus* strains on detached cocoa pods.

Aspergillus/Pp combination[1]	No. of pods with lesion / No. of pods inoculated		Total[4]	Inhibition (%)
	Experiment 1[2]	Experiment 2[3]		
AI_1 + Pp	2/6	0/6	2/12	83.33
AI_2 + Pp	0/6	1/6	1/12	91.67
AI_3 + Pp	1/6	0/6	1/12	91.67
AI_4 + Pp	1/6	2/6	3/12	75.00
AI_5 + Pp	1/6	3/6	4/12	66.67
Pp alone (Control)	6/6	6/6	12/12	0

[1] *Aspergillus* isolates: AI_1 (Partly identified *Aspergillus* isolate); AI_2 (*A. flavus* isolate 1); AI_3 (*A.flavus* isolate 2); AI_4 (*A. flavus* isolate 3); AI_5 (*A. flavus* isolate 4); Pp = *Phytophthora palmivora.*
[2]Experiment 1: Cocoa pods inoculated with Pp mycelial plugs overrun by antagonists placed 24 h before the Pp plugs on the culture media.
[3]Experiment 2: Cocoa pods inoculated with Pp mycelial plugs overrun by antagonists placed 24 h after the Pp plugs on the culture media.
[4] Total is the outcome of the two experiments.

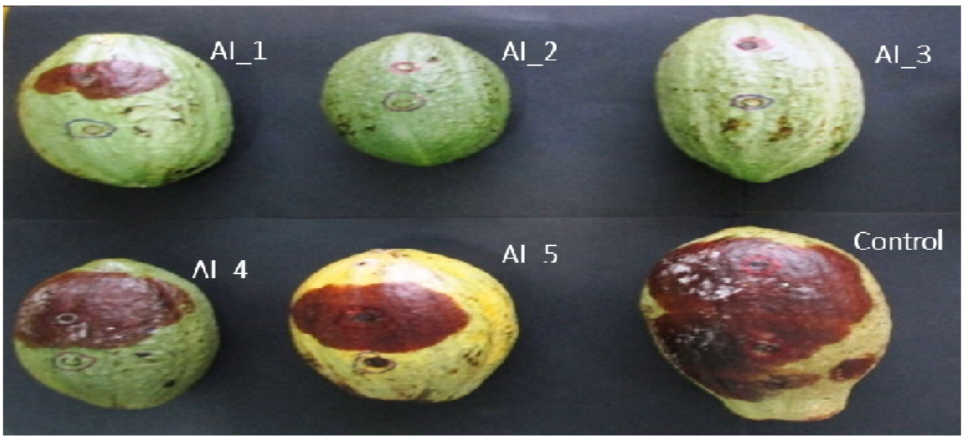

Figure 2 Inoculated cocoa pods showing black pod lesions after seven days. Refer to table 2 for narration of AI_1 – AI_5.

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Table 4a Macroscopic features of *Aspergillus* isolates at day 7.

Aspergillus Isolates	Culture medium	Colony color (Top)	Colony color (Reverse)	Denseness of mycelium	Shape of concentric rings
AI_1	CA	Hansa Yellow Extra Dark	Bright Yellow Green Tint	Dense Concentric rings	Regular
	PDA	Hansa Yellow Extra Dark	Bright Yellow Green Extra Dark	Sparse Concentric rings	Regular
	MEA	Hansa Yellow Extra Dark	Orange	Dense Concentric rings	Irregular
AI_2	CA	Hansa Yellow Extra Dark	Bright Yellow Green Tint	Sparse Concentric rings	Regular
	PDA	Hansa Yellow Extra Dark	Bright Yellow Green Tint	Sparse Concentric rings	Irregular
	MEA	Hansa Yellow Extra Dark	Orange	Dense Concentric rings	Irregular
AI_3	CA	Hansa Yellow Shade	Hansa Yellow Tint	Sparse Concentric rings	Regular
	PDA	Hansa Yellow Extra Dark	Bright Yellow Green Extra Dark	Sparse Concentric rings	Regular
	MEA	Hansa Yellow Extra Dark	Orange Shade	Dense Concentric rings	Regular
AI_4	CA	Hansa Yellow Extra Dark	Bright Yellow Green Tint	Dense Concentric rings	Regular
	PDA	Hansa Yellow Extra Dark	Bright Yellow Green Tint	Dense Concentric rings	Regular
	MEA	Hansa Yellow Extra Dark	Bright Yellow Green Tint	Dense Concentric rings	Regular
AI_5	CA	Hansa Yellow Extra Dark	Burnt Sienna	Sparse Concentric rings	Irregular
	PDA	Hansa Yellow Extra Dark	Yellow Ochre	Sparse Concentric rings	Irregular
	MEA	Hansa Yellow Extra Dark	Yellow Ochre Shade	Dense Concentric rings	Irregular

Table 4b Macroscopic features of *Aspergillus* isolates.

Aspergillus Isolates	Culture medium	Sclerotia production[1]			Sclerotia diameter (μm)[2]	Sclerotia color[2]	Colony diameter (mm)[3]
		Day 4	Day 7	Day 10			
AI_1	CA	_	_	_	_	_	85 – 89
	PDA	_	_	_	_	_	90
	MEA	_	+	+	470 – 680	Burnt sienna	70 – 75
AI_2	CA	_	_	_	_	_	90
	PDA	_	_	+	480 – 670	Burnt sienna	84 – 90
	MEA	_	_	+	500 – 640	Burnt sienna	77 – 84
AI_3	CA	_	_	_	_	_	85 – 87
	PDA	+	+ +	+ +	436 – 630	Burnt sienna	90
	MEA	+	+ +	+ +	538 – 620	Burnt sienna	87 – 90
AI_4	CA	_	_	_		_	86 – 90
	PDA	_	+	+	495 – 565	Burnt sienna	90
	MEA	_	+	+	470 – 680	Burnt sienna	85 – 90
AI_5	CA	+ +	+ + +	+ + +	90 – 170	Burnt sienna	73 – 85
	PDA	+ +	+ + +	+ + +	140 – 275	Burnt sienna	51 – 57
	MEA	+ +	+ + +	+ + +	160 – 210	Burnt sienna	50– 60

[1] □ = Absent; + = Low; + + = Medium; + + + = High.
[2] At day 10.
[3] At day 7.

Table 5 Microscopic characters of *Aspergillus* cultures on the three different media.

Aspergillus isolates[1]	Culture medium[2]	Conidial head size (L × W) (µm)	Conidiophore (L × W) (µm)	Vesicle diameter (µm)	Conidia diameter (µm)	Phialide (L × W) (µm)	Metulae (L × W) (µm)
AI_1	CA	58 - 118 × 56 – 135	300 – 720 × 8 – 12	17-23	3-4	4.2-7.5 × 3-3.5	7.3-11 × 3-4
	PDA	90 - 140 × 75 – 160	380 – 530 × 8 – 12	16-23	3-7	7-10 × 3-4	_
	MEA	88 - 128 × 44 – 137	293 – 430 × 6.5 – 9	24-27	3.8-5.5	5-7 × 3-4	_
AI_2	CA	50 - 160 × 53 – 143	445 – 520 × 7.5 – 9	20-26	3-4	7-8 × 3-4	_
	PDA	44 - 75 × 35 – 75	300 – 550 × 7 – 10	12-18	3-4	3-5 × 2-3.5	_
	MEA	89 - 164 × 47 – 106	460 – 520 × 7.6 – 10	12-18	3-4.5	7-9 × 3-3.5	7-11 × 3-5
AI_3	CA	64 - 110 × 50 – 125	370 – 400 × 5 – 10	19-27	3.5-4.5	10-12 × 3-4	_
	PDA	100 - 160 × 75 – 87	400 – 650 × 6.5 – 11	14-24	3-5	6-13 × 3-4	_
	MEA	90 - 171 × 47-101	420 – 550 × 6.6 – 10	14-18	3-4.5	3-4.3 × 2.5-3.6	_
AI_4	CA	57 - 120 × 65 – 150	380 – 550 × 6 – 9.5	20-22	3.5-5	5-6 × 3-4	6-9 × 3-4
	PDA	90 - 135 × 80 – 100	400 – 600 × 5 – 9	15-19	3.7-5	4.3-7 × 2-3.5	_
	MEA	69 - 186 × 61 – 107	260 – 580 × 5 – 8.5	12-25	3.6-4.2	6-11 × 3-3.7	_
AI_5	CA	95 - 150 × 100 – 160	570 – 1120 × 6.5 – 11	17-24	3-4	7-8 × 2.6-3	_
	PDA	57 - 101 × 54 – 145	390 – 900 × 3 – 8	12-20	2.7-5	10-12 × 3-5	_
	MEA	145 - 180 × 157 -191	430 – 640 × 6 – 10	20 – 26	3 – 4.1	5.8 – 10 × 2 – 3.7	_

[1] See table 1 for the narration of AI_1 – AI_5.
[2] CA = carrot agar; PDA = potato dextrose agar; MEA = malt extract agar.

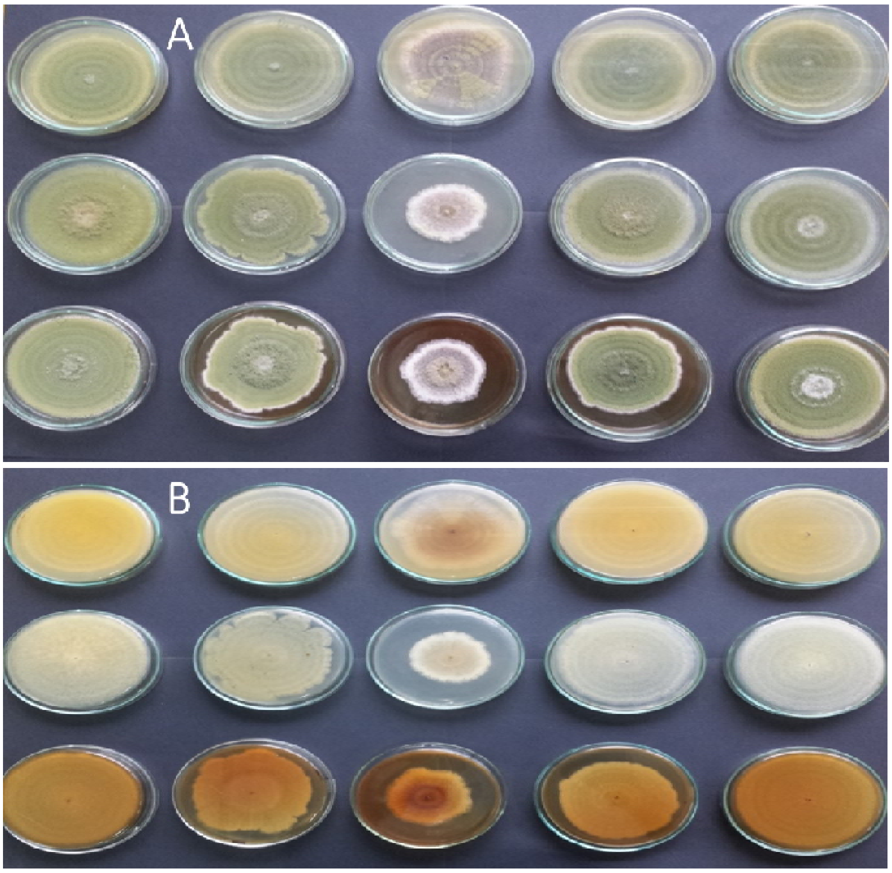

Figure 3 Seven-day-old colonies of the *Aspergillus* isolates on CA (top row), PDA (middle row), and MEA (bottom row). For all rows, Left to right: AI_3, AI_2, AI_5, AI_1, and AI_4. **A.** Top of culture plates; **B.** Underside of culture plates.

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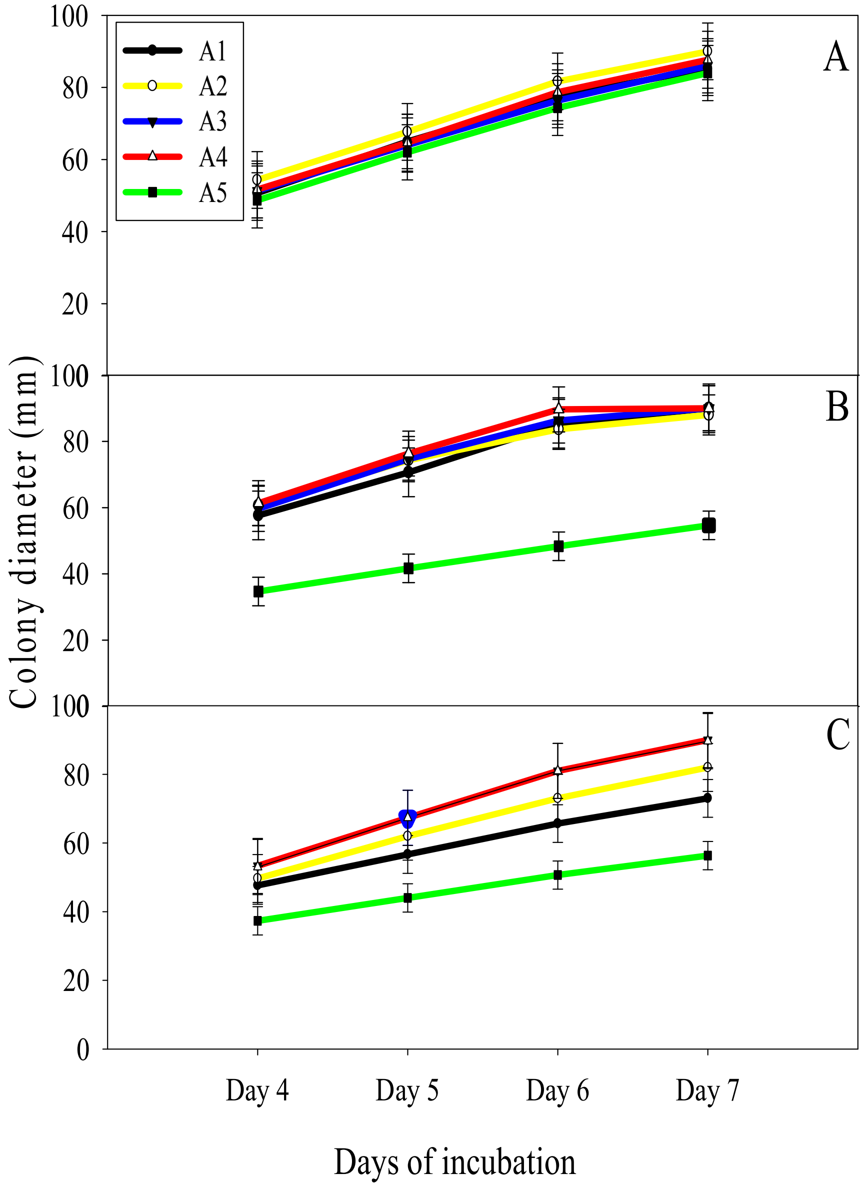

Figure 4 Colony diameters of five *Aspergillus* isolates on three different media. A = carrot agar; B = potato dextrose agar; C = malt extract agar.

Molecular identification of *Aspergillus* isolates. The concentrations of total genomic DNA of all the five *Aspergillus* isolates ranged from 85.7 to 227 µg/µl, implying the DNAs were highly concentrated in all isolates and so were diluted below 50 µg/µl before used. PCR products from the ITS region amplified around 600 base pairs while products from β – tubulin region amplified around 550 base pairs. Phylogenetic analysis of the β-tubulin showed that AI_1, AI_2, AI_3, and AI_4 clustered with other strains of *A. flavus*, whereas AI_5 clustered with *A. parvisclerotigenus* (Fig. 5). In contrast, phylogenetic analysis of the internal transcribed spacer showed that all the *Aspergillus* isolates clustered with other strains of *A. flavus,* but AI_1 and AI_5 clustering patterns indicate there are differences between the two strains and the other three isolates (Fig. 6).

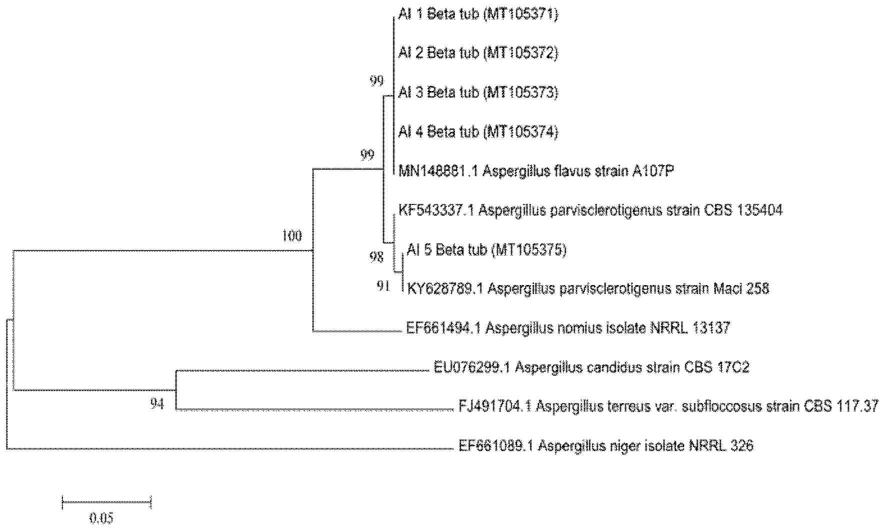

Figure 5 Phylogenetic analysis of β-tubulin region (429 nucleotide positions) of the five isolates and five closely related strains of *Aspergillus flavus*. The tree was constructed by the maximum likelihood method based on the Tamura-Nei model with 1000 bootstrap replicates. Only bootstrap values of ≥ 50 are shown. Scale bar indicates the estimated number of nucleotide substitutions per 12 nucleotides.

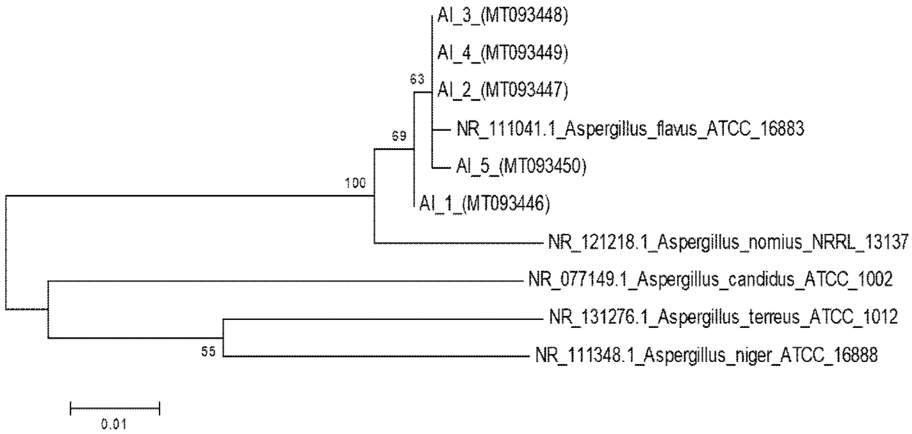

Figure 6 Phylogenetic analysis of ITS region (485 nucleotide positions) of five *Aspergillus* isolates and five *Aspergillus* species from the NCBI database. The tree was constructed by the maximum likelihood method based on Tamura 3-parameter with 1000 bootstrap replicates. Only bootstrap values of ≥ 50 are shown. Scale bar indicates the estimated number of nucleotide substitutions per 10 nucleotides.

Using a combination of the ITS and β-tubulin gene sequencing, all the isolates were identified as *A. flavus*, β-tubulin classified A5 as *A. flavus* var. *parvisclerotigenus*.

Discussion

The fungal isolate AI_1, which Koranteng (2017) reported to be an *Aspergillus* species with an inhibitory effect towards *P. palmivora,* proved to be similarly antagonistic to the Oomycetes in the current study. This isolate was still inhibitory after seven years since its isolation suggests stability, making it ideal for biocontrol purposes. All the other four putative *A. flavus* isolates used for comparison also similarly inhibited the *P. palmivora*. When placed centrally on an agar plate in dual plate culture tests with *P. palmivora* the *Aspergillus* Isolate (AI_1) prevented the *P. palmivora* cultures from growing towards it, suggesting that antibiosis could be one of the mechanisms of inhibition by the isolate. Control of plant pathogens through antibiosis is generally attributed to antifungal substances/secondary metabolites produced by the biocontrol agent and elaborated into the environment (Asaka and Shoda, 1996; Ownley and Windham, 2007). Even though some *Aspergillus* species, such as *A. flavus,* are well-known toxin producers (Klich and Pitt, 1988; Varga *et al*., 2011; Agbetiameh *et al*., 2018), also, non-toxin producing strains of *A. flavus* have been used to develop aflasafe that compete with this toxin producer to reduce aflatoxins to the barest minimum (Agbetiameh *et al*., 2018). Some like *A. flavus* var. *columnaris* have antimicrobial properties and are active against *Candida albicans*, *Escherichia coli*, *Salmonella typhimurium,* and *Shigella dysenteriae in vitro* (Fawzy *et al*., 2011). Several investigators have reported other secondary metabolites produced by A. flavus. Such metabolites include aspergillic acid (White and Hill, 1943), kojic acid (Varga *et al*., 2011), cyclopiazonic acid, norsolonic acid (Sun and Qi, 1991), 3-nitropropionic acid (Bush *et al*., 1951), paspalinine (Cole *et al*., 1981), asperflavin (Grove, 1972a, b; Grove, 1973) and aflatrem (Frisvad *et al*., 2005). Some of these metabolites have antagonistic activities toward fungi. For example, aspergillic acid (Barathova *et al*., 1969) and kojic acid (Chee and Lee, 2003) have been reported to inhibit *Botrytis cinerea* and *Candida albicans in vitro*.

In the current study, cocoa pods inoculated with mycelial plugs obtained from *P. palmivora* cultures overrun by *Aspergillus* isolates in inhibition plate cultures generally did not produce lesions after seven days, indicating possible death of mycelial/sporangia of the *Phytophthora palmivora*. AI_3 was the highest inhibitor of *P. palmivora* colonies on both *in vitro* and *in vivo* among the five isolates. Such a phenomenon could be due to the lethal action by secondary metabolites produced by the *Aspergillus* isolates and or mycoparasitism by the aspergilli. Mpika *et al*. (2009) reported that when mycelial plugs from *P. palmivora* colonies that had contacted antagonistic *Trichoderma* species were placed on detached cocoa pods, many of them could not subsequently cause lesions on the pods, corroborating the findings of the current research. This observation should, however, be further examined.

Colony appearance of the *Aspergillus* isolates AI_1, as well as its radial growth and microscopic and other characteristics, generally were similar to those of the putative *Aspergillus flavus* isolates AI_2, AI_3, and AI_4 on all the culture media, indicating that the isolate AI_1, which has been tentatively identified as an *Aspergillus* is *Aspergillus flavus*. The isolate AI_1 is classified as L morphotype based on sclerotia production since it produced few but large sclerotia measuring more than 400 µm (Saito and Tsurata, 1993; Cotty and Cardwell, 1999). On these bases, AI_2, AI_3, and AI_4 were also L morphotypes. Agbetiameh *et al*. (2018) reported that in Ghana, atoxigenic strains of *Aspergillus flavus* L morphotype occur. It cannot be ascertained whether the isolate AI_1 produces aflatoxin or not. The isolate AI_5 was characterized by high sclerotia production. Data and pictorial appearance of this *Aspergillus* isolate (AI_5) agree with the descriptions of Saito and Tsurata (1993) and Cotty and Cardwell (1999) for *Aspergillus flavus* var. *parvisclerotigenus.* So, the *Aspergillus* isolate (AI_5) is a different strain from the other four isolates, i.e., AI_1, AI_2, AI_3, and AI_4.

Most researchers studied colony growth of *Aspergillus flavus* using triplicate mycelial plugs on the same culture plate. They generally recorded colony diameters lower than the 84-90 mm on carrot agar, 54-90 mm on potato dextrose agar, and 56-90 mm on malt extract agar obtained in the current study where only a single conidium was centered on an agar plate. Lower colony growths would be recorded for triplicate plated mycelial plugs because such colonies would prevent each other from expanding optimally. Media type could also affect colony growth. For

[DOR: 20.1001.1.22519041.2021.10.2.15.1]

example, 30-50 mm were recorded by Samson *et al*. (1995) on malt extract autolysate (MEA) agar; 47-57 mm by Afzal *et al*. (2013) on Czapek Solution Agar and Malt Extract Agar, 35-40 mm and 50-55 mm by Nyongesa *et al*. (2015) on Czapek Dox Agar and Malt Extract Agar, respectively for *A. flavus* after seven days. Therefore, in comparative studies of *Aspergillus flavus* and fungi, the cultural conditions ought to be standardized.

The widespread use of ITS region and other sequencing has enabled scientists to categorize fungi after morphological observations (Kristensen *et al*., 2005; Manikandan *et al*., 2009; Caira *et al*., 2012). Sequenced results from the ITS region and β – tubulin gene in the current study support the assertion that AI_1 is *Aspergillus flavus*. AI_5 was also identified by molecular approaches to be *Aspergillus flavus* var. *parvisclerotigenus*. Cluster analysis using DNA sequence information from the ITS region produced one clade comprising the five *Aspergillus* isolates (AI_1 – AI_5) and *A. flavus* from the NCBI database and different from the other species. This result implies that based on the ITS regions, the isolates could not be discriminated, and are thus, closely related in this genomic region. AI_1, AI_2, AI_3, and AI_4 clustered with *Aspergillus flavus* from the NCBI database, and all the isolates had almost 100% similarity with the corresponding strain while AI_5 which formed a sister group with *A. parvisclerotigenus* strain CBS 135405 and had 99% similarity based on NCBI database using the β-tubulin gene sequences.

In conclusion, the *Aspergillus* isolate AI_1 is identified conclusively as *A. flavus* based on morphological and molecular similarities with known *A. flavus* isolates. This isolate is stable in culture and has great potential for biological control. The potential biocontrol agent *A. flavus* effect should be investigated on product quality and the aflatoxin production of these isolates.

Acknowledgments

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. We are very grateful to the late Mr. Samuel Obeng, Mrs. Cecilia Obeng, Ernest Obeng, Emmanuel Owusu Mensah, and Prince Obeng for providing financial assistance for this work. Furthermore, we appreciate the efforts of Sean Tracey and Rick Gadzinski, Functional Bioscience, Wisconsin, United States of America, Dr. Stephen Larbi Koranteng, University of Education, Ashanti Mampong, Mrs. Zipporah Appiah Kubi, Dr. Joseph Adomako, and Prof. Marian Quain, all of Council for Scientific and Industrial Research – Crops Research Institute, Ghana.

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[DOR: 20.1001.1.22519041.2021.10.2.15.1]

شناسایی جدایه *Aspergillus* با پتانسیل کنترل بیولوژیکی قارچ *Phytophthora palmivora* عامل بیماری غلاف سیاه کاکائو

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چکیده: بیماری غلاف سیاه کاکائو در غنا ناشی از قارچ *Phytophthora palmivora* و *P. megakarya* به‌طور سنتی با قارچ‌کش‌ها کنترل می‌شود. به‌دلیل چالش‌های مرتبط با استفاده از قارچ‌کش‌ها، گزینه‌های کنترل بیولوژیکی، مورد توجه می‌باشند. قارچ *Aspergillus* (AI_1) به‌عنوان قارچ مؤثر در بیوکنترل *P. palmivora* و *P. megakarya* در آزمون کشت متقابل جداسازی شد. اما، هویت دقیق آن ناشناخته بوده و نیاز به شناسایی خاص و مقایسه آن با سویه‌های شناخته شده آسپرژیلوس فلاووس (AI_2، AI_3، AI_4 و AI_5) دارد. برای تأیید قدرت AI_1 در برابر *P. palmivora* آزمایش مجدد انجام شد. جدایه‌های *A. flavus* نیز برای اولین بار در برابر *P. palmivora* مورد آزمایش قرار گرفتند. ویژگی‌های ریخت‌شناسی روی آگار هویج (CA)، آگار دکستروز سیب‌زمینی (PDA) و آگار عصاره مالت (MEA) تعیین شد. DNA ژنومی از جدایه‌های آسپرژیلوس به منطقه ITS و توالی ژن β-توبولین قرار گرفتند. تمام جدایه‌های آسپرژیلوس با غلظت‌های ۸۹/۳۳ تا ۹۵/۳۳ درصد (آزمایش ۱) و ۴۶/۶۷ تا ۶۰/۳۳ درصد (آزمایش ۲) قارچ *P. palmivora* را در پلیت‌های آزمایشی مهار کردند. به‌طور کلی، AI_1 ویژگی‌های کشت مشابه ویژگی‌های جدایه قارچ *A. flavus* را تولید می‌کند. تجزیه‌وتحلیل توالی منطقه ITS همه جدایه‌ها به‌عنوان *A. flavus* شناخته شدند و هم‌چنین بتاتوبولین جدایه‌های AI_1، AI_2، AI_3 و AI_4 را به‌عنوان *A. flavus* گروه‌بندی کرد. اما AI_5 به‌عنوان *A. flavus* var *parvisclerotigenus* شناخته شد. AI_3 بالاترین منطقه مهار را ثبت کرد و از توسعه غلاف سیاه غلاف تلقیح شده نیز جلوگیری کرد. جدایه‌های *Aspergillus* که قبلاً ناشناخته بودند، اکنون به‌طور قطعی به‌عنوان *A. flavus* شناخته می‌شوند.

واژگان کلیدی: کاکائو، کنترل بیولوژیک، آسپرژیلوس، تعیین توالی ژن، *Phytophthora palmivora*