Journal of Applied Microbiology, 2024, 135, lxae145
https://doi.org/10.1093/jambio/lxae145
Advance access publication date: 14 June 2024
Research Article

JOURNAL OF APPLIED MICROBIOLOGY
OXFORD UNIVERSITY PRESS

Molecular and aflatoxigenicity analyses of *Aspergillus flavus* isolates indigenous to grain corn in Malaysia; potentials for biological control

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Abstract

Aims: The present work aimed to distinguish the indigenous *Aspergillus flavus* isolates obtained from the first (pioneer) grain corn farms in Terengganu, Malaysia, into aflatoxigenic and non-aflatoxigenic by molecular and aflatoxigenicity analyses, and determine the antagonistic capability of the non-aflatoxigenic isolates against aflatoxigenic counterparts and their aflatoxin production *in vitro*.

Methods and results: Seven *A. flavus* isolates previously obtained from the farms were characterized molecularly and chemically. All isolates were examined for the presence of seven aflatoxin biosynthesis genes, and their aflatoxigenicity was confirmed using high performance liquid chromatography with fluorescence detector. Phylogenetic relationships of all isolates were tested using ITS and β-tubulin genes. Of the seven isolates, two were non-aflatoxigenic, while the remaining were aflatoxigenic based on the presence of all aflatoxin biosynthesis genes tested and the productions of aflatoxins B_1 and B_2. All isolates were also confirmed as *A. flavus* following phylogenetic analysis. The indigenous non-aflatoxigenic isolates were further examined for their antagonistic potential against aflatoxigenic isolates on 3% grain corn agar. Both non-aflatoxigenic isolates significantly reduced AFB_1 production of the aflatoxigenic isolates.

Conclusion: The indigenous non-aflatoxigenic *A. flavus* strains identified in the present work were effective in controlling the aflatoxin production by the aflatoxigenic *A. flavus* isolates *in vitro* and can be utilized for *in situ* testing.

Impact Statement

To control the aflatoxin produced by *Aspergillus flavus* in corn pre-harvest, the utilization of cultural and physical controls is often insufficient, while chemical control is environmentally hazardous. Therefore, biocontrol, specifically using the indigenous non-aflatoxigenic *A. flavus* against aflatoxigenic *A. flavus*, has provided the most promising results. The findings in the present work can be used to develop novel and indigenous non-aflatoxigenic *A. flavus* biocontrol agents against indigenous aflatoxigenic *A. flavus* and aflatoxin contamination in Malaysian grain corn agro-ecosystems.

Keywords: aflatoxin; aflatoxigenic; antagonist; *Aspergillus flavus*; biocontrol; non-aflatoxigenic

Introduction

Aspergillus section *Flavi* harbors many closely related species (e.g. *A. flavus*, *A. parasiticus*, *A. nomius*, and *A. oryzae*) that produce various secondary metabolites, among which the most important are the mycotoxin aflatoxins (Uka et al. 2019). Among the many members of this section, *A. flavus* is the most notable and receives considerable attention. *Aspergillus flavus* is a cosmopolitan (having worldwide distribution) fungus and has been found in soils, debris, tissues of agricultural crops, and on various postharvest products such as nuts, coffee, corn, soybean, and wheat (Klich 2002, Bailly et al. 2018, Jallow et al. 2021, Kapeua-Ndacnou et al. 2023). Upon infection, this mycotoxigenic species can produce the deleterious aflatoxins in food and food products under favorable conditions, thus presenting hazardous threats to consumers. Aflatoxins are carcinogenic, hepatotoxic, may exhibit immunosuppressive effects, and even cause death. They are classified as Group 1 carcinogens to humans (sufficient evidence with identified mechanism) by the International Agency for Research on Cancer (IARC, Ostry et al. 2017). Among the identified aflatoxin analogs are AFB_1, AFB_2, AFG_1, and AFG_2, with AFB_1 being the most abundant and toxic.

Like many species in *Aspergillus* section *Flavi*, *A. flavus* displays intra-species diversity with regard to morphological characteristics, genetic materials, and secondary metabolite profiles (Uka et al. 2019). In fact, members of *Aspergillus* section *Flavi* comprise of species complexes (i.e. closely related species) that could be difficult to differentiate without proper approaches, while correct species identification is important from food safety perspective. In 2006, Samson and colleagues

Received 30 March 2024; **revised** 21 May 2024; **accepted** 13 June 2024

used polyphasic approach in *Aspergillus* species identification in order to minimize error that typically occurs when using only morphological (i.e. phenotypic) or chemical (i.e. extrolite) approach. This approach extends from the traditional fungal identification, which exclusively utilizes morphological and chemical profiles, and includes molecular (genotypic) characterization, such as the sequence analysis of multiple genomic regions. This extended approach complements the traditional ones, thus helping resolve fungal species into its valid taxa (Samson et al. 2006). Beyond strict classification of species, this approach was also being used to differentiate aflatoxigenic and non-aflatoxigenic *A. flavus* isolates (Rodrigues et al. 2009).

Aspergillus flavus genotypes vary between aflatoxigenic and non-aflatoxigenic strains. In general, following DNA amplification, the presence of all aflatoxin biosynthesis genes within the gene cluster indicates the ability of any *A. flavus* isolate to produce aflatoxins; hence, the isolate is termed aflatoxigenic. Conversely, the absence of one or more genes often indicates *vice versa*, and the isolate is termed non-aflatoxigenic. Unlike their aflatoxigenic counterpart, non-aflatoxigenic *A. flavus* is unable to produce aflatoxins due to deletion, either small or large, or single nucleotide polymorphism (SNP) across the gene cluster of the aflatoxin biosynthesis pathway (Adhikari et al. 2016). This allows the latter to be used as excellent biological control agent against aflatoxin contamination. In the 1980s, the first biocontrol agent utilizing non-aflatoxigenic *A. flavus* was discovered to control aflatoxin production in cotton fields (Cotty 1994, Moore 2022). Henceforth, non-aflatoxigenic biocontrols have been studied and utilized as biocontrol agents against aflatoxins in many African countries and Italy with considerable success (Moral et al. 2020). In fact, non-aflatoxigenic *A. flavus* remains, to date, as the most effective and promising biocontrol agent introduced to control aflatoxin contamination in agricultural crops (Moore et al. 2022) since both aflatoxigenic and non-aflatoxigenic strains share similar ecophysiological requirements like growth conditions, nutrients, and habitats/niches.

Malaysia is one of Southeast Asian countries with tropical weather of warm and humid all year round, thus predisposing the agricultural commodities to mycotoxigenic fungi and the subsequent mycotoxin contamination. The earliest recorded incidence of aflatoxin contamination and aflatoxicosis in humans was in 1988, which killed 13 children (Cheng 1992) who consumed *lau shu fun/loh shi fun* (rat tail noodles/silver needle noodles made from corn starch or tapioca starch or rice starch). Although no newer cases of similar magnitude have been reported, members of *Aspergillus* section *Flavi* and aflatoxins are continuously present in local and imported agricultural crops and postharvest products (Khayoon et al. 2012, Nithiyaa et al. 2012, Yazid et al. 2020). This constantly exposes humans to the imminent health- and life-threatening threats of mycotoxins, either by direct consumption of the contaminated food (primary exposure), or indirectly by consuming the milk of ruminants feeding on the contaminated feed (secondary exposure). In particular, ruminants such as cows, camels, sheep, and goats feeding on AFB_1-contaminated feeds will metabolize the aflatoxin and excrete its biotransformed metabolite, namely AFM_1 into milk. Although not actually toxic toward adults, children are particularly sensitive toward AFM_1 which could lead to stunted growth (Azman et al. 2021). Since aflatoxins present health hazards through the interconnected web of life (plants–animals–humans) and along food production chain, aflatoxins have become a One Health issue that requires multisectoral and transdisciplinary approach to address the appropriate solution (Frazzoli et al. 2017, Nwaji et al. 2022). As if the ever-presence of airborne fungal contaminants and the production of their dangerous mycotoxins on crops and crop products are not enough, the population especially the agricultural community is faced by the imminent threat of climate change scenarios that positively affect fungal proliferation and mycotoxin production in habitats otherwise unfavorable for the fungal contaminants (Paterson and Lima 2010). Therefore, a proper control method, especially biological-based, is urgently warranted.

In Malaysia, research on biocontrol against mycotoxigenic fungi and mycotoxins is still in its infancy (Yazid et al. 2020). Recently, grain corn, which is mainly used as an ingredient in animal feed, is mass cultivated for commercialization in Malaysia to reduce import dependency with the ultimate objective of achieving food security. In our previous works, we have determined the total fungal community structure and their multi-mycotoxin production from the first (pioneer) grain corn farms in Terengganu, Malaysia (Yazid et al. 2021) and multi-mycotoxin reduction by potential indigenous fungal antagonists (Yazid et al. 2023). In the present work, we complemented the previous works by (1) identifying indigenous aflatoxigenic and non-aflatoxigenic *A. flavus* isolates based on their molecular and chemical characteristics, (2) establishing the phylogenetic relationship to confirm the taxonomic status of the putative *A. flavus* isolates, and (3) examining the antagonistic potential of indigenous non-aflatoxigenic isolates to reduce aflatoxin production using dual-culture assay *in vitro* on grain corn agar (GCA).

Materials and methods

Chemicals and growth media

Aflatoxin standard containing AFB_1, AFB_2, AFG_1, and AFG_2 was purchased from Sigma-Aldrich (St. Louis, USA). Acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained from Elga PURELAB® Classic UV MK2 (Lane End, UK). Fungal growth media, namely potato dextrose agar (PDA), potato dextrose broth (PDB), dichloran rose bengal chloramphenicol (DRBC) agar, Dichloran-Glycerol 18 (DG-18) agar, and malt extract agar (MEA), and technical agar were purchased from Oxoid (Basingstoke, UK). All solvents used were of HPLC grade. The qualitative examination of aflatoxin production by *A. flavus* was performed on 50% coconut cream agar (CCA). The CCA was prepared by adding commercial coconut cream (Kara, Puchong, Malaysia) in distilled water in 1:1 ratio (v/v). Next, 15 g of technical agar was added to the mixture, autoclaved at 121°C for 15 min, poured into Petri plates, and left to solidify overnight (Dyer and McCammon 1994). Dual-culture of aflatoxigenic and non-aflatoxigenic *A. flavus* was carried out on 3% semi-synthetic GCA (w/v). The GCA was prepared by mixing 30 g of ground grain corn with 15 g of technical agar in 1 l of distilled water, before autoclaved at 121°C for 15 min, poured into Petri plates, and left to solidify overnight (Yazid et al. 2021). The use of GCA was to mimic the actual crop commonly contaminated by *A. flavus* in nature (Yazid et al. 2018).

Aspergillus flavus isolates

Seven *A. flavus* isolates assessed in the present work were previously isolated from kernel, soil, and tassel samples from pioneer grain corn farms (Kampong Dadong and Rhu Tapai) in Terengganu, Malaysia during 2017 cropping season (Yazid et al. 2021). Both farms were the first (pioneer) to be gazetted by the Malaysian government for mass cultivation and commercialization of grain corn. Isolation from soil sample was done by dilution plating (at dilution 10^4) on DRBC agar. Isolation from kernel and tassel was done by direct plating on PDA and DG-18 supplemented with 1 mg l^{-1} chloramphenicol to suppress bacterial growth. All media were incubated for 7 d at 30°C. Following incubation, *A. flavus* isolates were sub-cultured onto fresh PDA for further identification. All *A. flavus* isolates appeared as yellow–green obverse and pale brown reverse on PDA, with spherical/subspheroidal conidia (Samson et al. 2010). A representative isolate was also molecularly identified using polymerase chain reaction (PCR) of the internal transcribed spacer (ITS) region with primer pair of ITS 1/ITS 4 (product size: 600 bp; White et al. 1990) and annealing temperature of 52°C, submitted for sequencing to local service provider (MyTACG Bioscience Enterprise, Kajang, Malaysia), and the sequenced isolate was compared with the sequences previously published in the National Center for Biotechnology Information (NCBI) database. All isolates were deposited at Laboratory of Food Mycology, Department of Food Science, Faculty of Food Science and Technology, UPM, and maintained on PDA for further analyses. The aflatoxin production potential of all isolates was also tested on 3% GCA using high-performance liquid chromatography with fluorescence detector (HPLC-FLD).

Molecular analyses

DNA extraction

DNA of *A. flavus* isolates was extracted from 1-d-old mycelia in 10 ml of PDB, and incubated by shaking at 2 × *g* in 30°C. Following incubation, the mycelia were filtered using sterile Whatman filter paper No. 1 (Kent, UK), washed with sterile distilled water, and left to dry under laminar flow. The dried mycelia were crushed using liquid nitrogen to obtain fine powder. Thereafter, genomic DNA was extracted following manufacturer's instruction of Qiagen DNeasy plant mini kit (Germantown, USA), and kept at −20°C until further analyses.

Aflatoxin biosynthesis gene identification

Seven aflatoxin biosynthesis genes, namely *pksA*, *nor-1*, *ver-1*, *verA*, *aflR*, *aflJ*, and *omtA*, were tested to distinguish *A. flavus* isolates into aflatoxigenic or non-aflatoxigenic genotypes (Table 1). These genes are located at early, middle, and late regions of the aflatoxin biosynthesis cluster. The PCR reaction for all genes was 20 µl. The reaction mixture contained 10 µl of EconoTaq Plus Green 2 × Master Mix, 0.4 µl of each primer (0.2 µM), 3 µl of DNA template, and 6.6 µl of RNase-free water. The amplification was carried out using Kyratec SuperCycler SC-200 (Victoria, Australia). Next, the amplicons were separated using 1.5% agarose gel stained with 3 µl of ethidium bromide. The gel electrophoresis was running for 80 min at 90 V and 400 mA in 1 × Tris-acetate-ethylene diamine tetra acetic acid (TAE), and amplified genes were observed under UV light using gel documentation. The amplicon size was estimated using Gel Pilot® 100 bp Plus DNA ladder.

Genomic DNA amplification and sequencing

PCR and sequencing of *A. flavus* isolates' DNA were performed on two genomic regions: ITS (product size: 600 bp) with primer pair ITS1 5′TCC GTA GGT GAA CCT GCG G 3′ and ITS4 5′ TCC TCC GCT TAT TGA TAT GC 3′ (White et al. 1990); and partial *β*-tubulin (*benA*, product size: 495 bp) with primer pair Bt2a 5′ GGT AAC CAA ATC GGT GCT GCT TTC 3′ and Bt2b 5′ ACC CTC AGT GTA GTG ACC CTT GGC 3′ (Glass and Donaldson 1995). The PCR reaction for both regions was 25 µl. The reaction mixture contained 12.5 µl of EconoTaq Plus Green 2 × Master Mix (Lucigen Corporation, Wisconsin, USA) and 3 µl of DNA template. For amplification of ITS and *benA* regions, 1.25 µl of each ITS primers (0.5 µM) and 2.5 µl of each *benA* primers (1 µM) were added to the mixture. The remaining volume for both reaction mixtures was top-upped with RNase-free water. The EconoTaq Plus Green contained 0.1 units μl^{-1} EconoTaq DNA Polymerase, PCR buffer of pH 9.0, 3 mM magnesium chloride ($MgCl_2$), 400 µM of each dNTPs, PCR enhancer/stabilizer, and blue and yellow tracking dyes.

The amplification was performed as follows. For ITS region: pre-denaturation at 95°C for 2 min; 30 cycles of denaturation at 95°C for 45 s, annealing at 52°C for 30 s, and extension at 72°C for 1 min and 20 s; followed by final extension at 72°C for 10 min. For *benA* region: pre-denaturation at 95°C for 1 min; 35 cycles of denaturation at 95°C for 1 min, annealing at 61°C for 1 min, and extension at 72°C for 1 min; followed by final extension at 72°C for 5 min (Norlia et al. 2019a). Thereafter, the amplicons were separated by gel electrophoresis using 1.5% agarose gel (Promega, Wisconsin, USA) stained with 3 µl of ethidium bromide (Promega, Wisconsin, USA), and ran for 80 min at 90 V and 400 mA in 1 × TAE. The amplified genes were observed under ultraviolet light using gel documentation (Syngene, Bangalore, India), and the size was estimated using Qiagen Gel Pilot® 100 bp Plus DNA ladder (Germantown, USA). The amplicons were then submitted to local service provider (MyTACG Bioscience Enterprise, Kajang, Malaysia) for DNA purification and sequencing. A commercial aflatoxigenic type strain, *A. flavus* NRRL 3357, was obtained from New South Wales (Australia), maintained axenically on PDA at 30°C until sporulation, and served as positive control.

Sequence alignment, model selection, and phylogenetic analysis

The sequences obtained were edited and trimmed using BioEdit v. 7.2.5 (Hall 1999). Phylogenetic tree was built based on the sequences obtained in the present work and the published sequences, including reference strains of *Aspergillus* section *Flavi* species obtained from the NCBI database (Table 2). The species provided in the list are closely related to *A. flavus* and could also produce aflatoxins (Norlia et al. 2019b). The subsequent sequence alignment, model selection, concatenation of ITS and *β*-tubulin genomic regions, and building of the phylogenetic tree were done using Mega v. 11 software (Tamura et al. 2021). All sequences were aligned using MUSCLE algorithm. Maximum likelihood (ML) tree was developed for individual

Table 1. Targeted genes, primers, and PCR conditions for amplification of targeted aflatoxin biosynthesis genes of indigenous *A. flavus* isolates.

Set	Target gene	Primer pair	Primer sequence	[a]Size (bp)	Annealing temperature (°C)	Reference
[b]M1	**aflR** *aflR*	*aflr1*	5′TAT CTC CCC CCG GGC ATC TCC CGG 3′	1032		
		aflr2	5′CCG TCA GAC AGC CAC TGG ACA CGG 3′			
	nor-1 *aflD*	*nor1*	5′ACC GCT ACG CCG GCA CTC TCG GCA C 3′	400	67	Criseo et al. (2001), Norlia et al. (2019a)
		nor2	5′GTT GGC CGC CAG CTT CGA CAC TCC G 3′			
	ver-1 *aflM*	*ver1*	5′GCC GCA GGC CGC GGA GAA AGT GGT 3′	537		
		ver2	5′GGG GAT ATA CTC CCG CGA CAC AGC C 3′			
	pksA *aflC*	*pksa1*	5′GCT GGG ATT CTG CAT GGG TT 3′	536		
		pksa2	5′CAG TTG CTC CCA AGG AGT GGT 3′			
M2	**omtA** *aflP*	*omt1*	5′CAG GAT ATC ATT GTG GAC GG 3′	594	61	Yin et al. (2009), Norlia et al. (2019a)
		omt2	5′CTC CTC TAC CAG TGG CTT CG 3′			
	glcA	*glca1*	5′GTA CGA TGC AAA TGG CGT CC 3′	851		
		glca2	5′GAA GCT CTG TGT CGT TGG GA 3′			
[c]S1	**aflJ** *aflS*	*aflj1*	5′CTT CAA CAA CGA CCC AAG GTT 3′	435	55	Chang et al. (2005)
		aflj2	5′AGA TGA GAT ACA CTG CCG CA 3′			
S2	**verA** *aflN*	*vera1*	5′CCG CAA CAC CAC AAG TAG CA 3′	423	55	Chang et al. (2005)
		vera2	5′AAA CGC TCT CCA GGC ACC TT 3′			

In set, the number after symbol indicates the number of sets. In target gene, original gene names are in bold, and new gene names are in italic (Yu et al. 2004).
[a]Amplicon size.
[b]M: multiplex PCR.
[c]S: singleplex PCR.

Table 2. *Aspergillus* section *Flavi* with published sequences in NCBI database and their accession number used for phylogenetic tree analysis of individual and combined (ITS, *benA*) genomic regions.

Species	Strain	GenBank accession number	
		ITS	*benA*
A. arachidicola	CBS 117610	MF668184	EF203158
A. arachidicola	CBS 117614	KY937923	KY924665
A. flavus	NRRL 21882	HQ856223	MG825981
A. flavus	NRRL 3357	MF966967	M38265
A. minisclerotigenes	CBS 117635	KY937925	KY924667
A. minisclerotigenes	NRRL 29000	KY937929	KY924668
A. niger	CBS 113.46	FJ629351	FJ629302
A. nomius	NRRL 13137	AF027860	AF255067
A. nomius	NRRL 25393	AF027864	AF255068
A. novoparasiticus	AFc31	KC964099	KY924669
A. novoparasiticus	AFc32	KC964100	KY924670
A. oryzae	CBS 100925	MF668185	EF203138
A. parasiticus	CBS 100308	KJ175436	KJ175496
A. parasiticus	NRRL 492	KY937934	KY924674
A. parvisclerotigenus	AFc36	KC964102	KC954604
A. parvisclerotigenus	CBS 121.62	EF409240	EF203130
A. pseudotamarii	NRRL 25518	KY937937	KY924675
A. pseudotamarii	NRRL 443	AF004931	EF661476
A. tamarii	CBS 118098	KJ175442	KJ175500
A. tamarii	CBS 121599	KJ175443	KJ175501

and combined (concatenated) datasets. *A. niger* strain CBS 113.46 was used as the outgroup taxon. The best-fit nucleotide substitution model was determined by the lowest Bayesian information criterion score, and indicated as follows: ITS dataset = T92 + G (Tamura-3-parameter + discrete gamma distribution), length alignment = 418; β-tubulin dataset = K2 + G (Kimura-2-parameter + discrete gamma distribution) length alignment = 350 bp; combined dataset = T92 + G, length alignment = 822 bp. The robustness of phylogeny was assessed by bootstrap method with 1000 replicates, and bootstrap value of lower than 70% were not shown.

Aflatoxigenicity test

Qualitative assessment by CCA

Approximately 10 µl of *A. flavus* spore suspensions (10^6 spores ml^{-1}) were prepared in sterile distilled water with

0.01% v/v Tween-80, counted using a Neubauer hemocytometer, inoculated centrally on the 50% CCA, and incubated in the dark at 30°C for 7 d. Following 2, 5, and 7 d of incubation, the reverse side of the colony was observed for the presence of blue-fluorescence ring under ultra-violet light (UV light; λ 365 nm), which indicated aflatoxin biosynthesis (Dyer and McCammon 1994).

Quantitative assessment by HPLC-FLD

For HPLC-FLD quantification, ~10 µl of the spore suspension (10^6 spores ml^{-1}) of each isolate were inoculated centrally on PDA and MEA. Thereafter, all plates were incubated in the dark for 7 d at 30°C. Following incubation, five agar plugs were transferred into a pre-weighed 2-ml Eppendorf tube (Hamburg, Germany) and weighed. Approximately 1 ml of methanol was then added into the plugs before vortexed and left in ambient temperature for 30 min (Bragulat et al. 2001). Next, the extract was filtered using 0.22 µm nylon syringe filter (Macherey-Nagel, Düren, Germany) into HPLC vials (Thermo Scientific, Waltham, USA). Aflatoxin separation was carried out using reversed-phase HPLC (Waters 600, Haverhill, USA), with a mobile phase of water: methanol: acetonitrile (55:35:10, v/v/v) at 40°C and 0.6 ml min^{-1} flow rate. A Gemini® C_{18} column with particle size of 5 µm and 250 × 4.6 mm dimension was used for the separation. The injection volume was 20 µl. Aflatoxin derivatization was performed using a post-column (i.e. Photochemical Reactor for Enhanced Detection, PHRED; Aura Industries, San Diego, USA). Aflatoxin detection was performed using a fluorescence detector (Waters 2475, Haverhill, USA) with the excitation and emission wavelengths of 365 and 435 nm, respectively (Afsah-Hejri et al. 2011). The solvents used for mobile phases were firstly filtered using nylon membrane filter (0.45 µm; Merck, Darmstadt, Germany), and sonicated using ultrasonic bath (Power Sonic 420, Shanghai, China) for 30 min to degas. Data were accessed and processed using Empower 2 Chromatography Data Software (Waters, Haverhill, USA). Thereafter, linear calibration curves were developed from 7-point working standard solutions diluted from stock solution of 1000 ng ml^{-1} in absolute methanol: AFB_1 and AFG_1 (2, 5, 10, 25, 50, 75, and 100 ng ml^{-1}); AFB_2 and AFG_2 (0.6, 1.5, 3, 7.5, 15, 22.5, and 30 ng ml^{-1}). The correlation coefficient (R^2) was established from linear regression. The limit of detection (LOD) and limit of quantification (LOQ) of each chromatographic analysis were calculated following the method prescribed by the International Conference on Harmonization (ICH) using the formula; $LOD = 3\sigma/s$ and $LOQ = 10\sigma/s$, where σ was the standard deviation of blank responses, and s was the slope of the calibration curve (Shrivastava and Gupta 2011). For AFB_1, the obtained R^2, LOD, and LOQ were 0.9864, 0.02 ng ml^{-1}, and 0.1 ng ml^{-1}, respectively. For AFB_2, the obtained R^2, LOD, and LOQ were 0.9855, 0.003 ng ml^{-1}, and 0.1 ng ml^{-1}, respectively.

Dual-culture assay on GCA and determination of aflatoxin reduction

Approximately 5 µl of the aflatoxigenic and non-aflatoxigenic *A. flavus* spore suspensions were aseptically inoculated on fresh 3% semi-synthetic GCA at 60 mm apart from each other and 15 mm from the plate's periphery. The inoculated plates were then incubated for 7 d at 30°C. Following incubation, the types of interaction between each of aflatoxigenic and non-aflatoxigenic pairs were scored using numerical scores, namely mutual intermingling (1/1), mutual inhibition upon contact (2/2), mutual inhibition at a distance (3/3), dominance of one species upon contact (4/0), and dominance of one species at a distance (5/0). The first integer of the numerical score denotes the antagonist, while the second integer denotes the pathogen (Magan and Lacey 1984, Magan et al. 2020). The percentage inhibition of radial growth (PIRG, %) was also recorded by measuring the colony radii of aflatoxigenic isolate from both treatment and control plates and computing them into the equation of $\%PIRG = (R_1 – R_2/R_1) \times 100$, where R_1 was radius of aflatoxigenic isolate in control plate, and R_2 was radius of aflatoxigenic isolate in treatment plate (Rahman et al. 2009). Thereafter, five agar plugs of the aflatoxigenic isolates were randomly collected at the region between the center and the periphery of the colonies for aflatoxin analysis as previously described (Priesterjahn et al. 2020).

Statistical analysis

All experiments were conducted in four replicates ($n = 4$). Measurements were averaged and reported as mean ± SE. Data of AFB_1 reductions following dual-culture assay were examined for normality using Kolmogorov–Smirnov and Shapiro–Wilk tests. The statistical analysis for AFB_1 reductions was done using one-way analysis of variance (ANOVA) with 95% confidence interval. P-value of <.05 was accepted as significant difference. Next, *post hoc* Tukey's Honest Significant Difference (Tukey's HSD) was carried out with P-value of <.05 to compare the significant difference between groups. The statistical software SPSS v. 29.0 (SPSS Inc., Chicago, USA) was used to perform all statistical analyses.

Results

Detection of aflatoxin biosynthesis genes and aflatoxin production potentials of indigenous *A. flavus* isolates

To confirm the aflatoxigenicity of *A. flavus* isolates, they were subjected to PCR analysis, and the targeted aflatoxin biosynthesis genes at early (*pksA* and *nor-1*), middle (*ver-1* and *verA*, including regulatory genes *aflR* and *aflJ*), and late (*omtA*) regions of the cluster were tested. Table 3 depicts the amplification of targeted aflatoxin biosynthesis genes of *A. flavus* isolates. All five aflatoxigenic *A. flavus* isolates, namely Af3SD, Af6KR, Af7KR, Af2SR, and Af4TR, exhibited all the targeted genes. The two non-aflatoxigenic *A. flavus* isolates, Af1KD and Af5TD, failed to yield amplicons of the targeted genes located at early and middle regions of the gene cluster and were only able to amplify the targeted gene located at the late region of the cluster (*omtA*). A gene in the sugar cluster, *glcA*, is a positive marker for *A. flavus*, and was amplified by all the *A. flavus* isolates tested. In addition, all targeted genes in the type strain (positive control) were also amplified.

Table 4 depicts the production of aflatoxins (µg g^{-1}) by *A. flavus* isolates obtained from pioneer grain corn farms in Terengganu, Malaysia using HPLC-FLD. As expected, HPLC-FLD data paralleled those of molecular data (Table 3). Among the seven tested *A. flavus* isolates, five produced AFB_1 on the media tested (aflatoxigenic), and the other two did not (non-aflatoxigenic). Of the five aflatoxigenic *A. flavus* isolates, only three produced both AFB_1 and AFB_2. No AFG_1 and AFG_2

Table 3. Amplification of targeted aflatoxin biosynthesis gene and one gene in sugar cluster of indigenous *A. flavus* isolates by PCR.

Isolate	Aflatoxin biosynthesis gene cluster							[a]Sugar cluster
	Early		Middle				Late	
	pksA *aflC*	nor-1 *aflD*	aflR *aflR*	aflJ *aflS*	ver-1 *aflM*	verA *aflN*	omtA *aflP*	glcA
[b]NRRL 3357	+	+	+	+	+	+	+	+
Af1KD	–	–	–	–	–	–	+	+
Af3SD	+	+	+	+	+	+	+	+
Af5TD	–	–	–	–	–	–	+	+
Af6KR	+	+	+	+	+	+	+	+
Af7KR	+	+	+	+	+	+	+	+
Af2SR	+	+	+	+	+	+	+	+
Af4TR	+	+	+	+	+	+	+	+

Original aflatoxin gene names are above and in bold letters. New aflatoxin gene names are below and in italic letters. + target gene present, – target gene absent.
[a]A gene in the sugar utilization cluster.
[b]*Aspergillus flavus* type strain (positive control).

Table 4. Production of aflatoxins (μg/g) on different growth media by *A. flavus* isolates obtained from Kampong Dadong and Rhu Tapai pioneer grain corn farms in Terengganu, Malaysia.

Isolate	[a]CCA	[b]PDA (μg/g)		[c]MEA (μg/g)	
		AFB_1	AFB_2	AFB_1	AFB_2
[d]NRRL 3357	+	4.6 ± 1.9	n.d.	2.9 ± 0.5	n.d.
Af1KD	–	n.d.	n.d.	n.d.	n.d.
Af3SD	–	10.3 ± 3.5	n.d.	15.7 ± 1.5	n.d.
Af5TD	–	n.d.	n.d.	n.d.	n.d.
Af6KR	+	61 ± 7.2	0.5 ± 0.1	37 ± 4.3	0.1 ± 0.04
Af7KR	–	72 ± 6.9	0.1 ± 0.02	10 ± 0.8	n.d.
Af2SR	+	62.6 ± 3.6	0.5 ± 0.1	39.3 ± 4.1	0.2 ± 0.1
Af4TR	–	7.6 ± 0.8	n.d.	10 ± 0.7	n.d.

Data are means of triplicate ($n = 3$) and expressed as mean ± standard error (± SE). AFB_1: aflatoxin B_1; AFB_2: aflatoxin B_2. +: fluoresce; −not fluoresce. n.d.: not detected.
[a]Coconut cream agar. [b]Potato Dextrose Agar. [c]Malt extract agar. [d]*Aspergillus flavus* type strain (positive control).

were detected from all the five aflatoxigenic isolates tested. With regard to aflatoxin concentrations, the production varied based on the growth media they grew on. Generally, all five aflatoxigenic *A. flavus* isolates produced AFB_1 at more than 10 μg g^{-1}. AFB_2 was produced much lesser, at <1 μg g^{-1}. CCA analysis, however, showed varying correlation with HPLC-FLD analysis. Three aflatoxigenic *A. flavus* isolates fluoresced on CCA, and showed parallel result with HPLC-FLD analysis, while two aflatoxigenic *A. flavus* isolates showed negative result on CCA.

Phylogenetic analysis

Figure 1 depicts the phylogenetic relationships between fungal species in *Aspergillus* section *Flavi* based on the combined datasets of ITS and β-tubulin. Phylogenetic analysis based on the ITS region alone (Supplementary 1) gave poor resolution and could not discriminate most of the species in *Aspergillus* section *Flavi*. This gene clustered together all seven tested sequences with other reference species of *A. flavus*, *A. oryzae*, *A. minisclerotigenes*, and *A. parvisclerotigenus*. Therefore, inference on valid nomenclature for the tested isolates was not possible. The phylogenetic analysis based on β-tubulin gene (Supplementary 2) provided better resolution, and could resolve most of the species in this section. The exception was for *A. oryzae* that shared the same clade with *A. flavus*, and likewise *A. novoparasiticus* with *A. parasiticus*. The *A. flavus*/*A. oryzae* clade was supported by high bootstrap value (>70%), and clustered all the seven tested isolates. The combined set also showed similar result as the individual β-tubulin phylogeny, with even higher statistical support (98% bootstrap value) in *A. flavus*/*A. oryzae* clade.

Indigenous non-aflatoxigenic *A. flavus* as potential biocontrol agents to control aflatoxin production

Two indigenous non-aflatoxigenic *A. flavus* isolates identified in the present work were tested against the aflatoxigenic *A. flavus* isolates in dual-culture assay on GCA. Initially, five aflatoxigenic *A. flavus* isolates were inoculated and incubated on GCA for 7 d at 30°C to determine the baseline aflatoxin production prior to co-cultivation. However, only two isolates produced AFB_1 (Af6KR, mean = 124.4 ng g^{-1}, and Af7KR, mean = 114.9 ng g^{-1}, Fig. 2). Therefore, the subsequent dual-culture assay was conducted with only these two aflatoxigenic *A. flavus* isolates.

In vitro fungal interaction of co-cultivated aflatoxigenic and non-aflatoxigenic *A. flavus* isolates exhibited sparse growth and conidial formation on GCA (Table 5). Furthermore, both non-aflatoxigenic *A. flavus* isolates were less aggressive against each of the aflatoxigenic *A. flavus* isolates (interaction score; 2/2 and 3/3), and displayed weak antagonistic activity (PIRG < 50%).

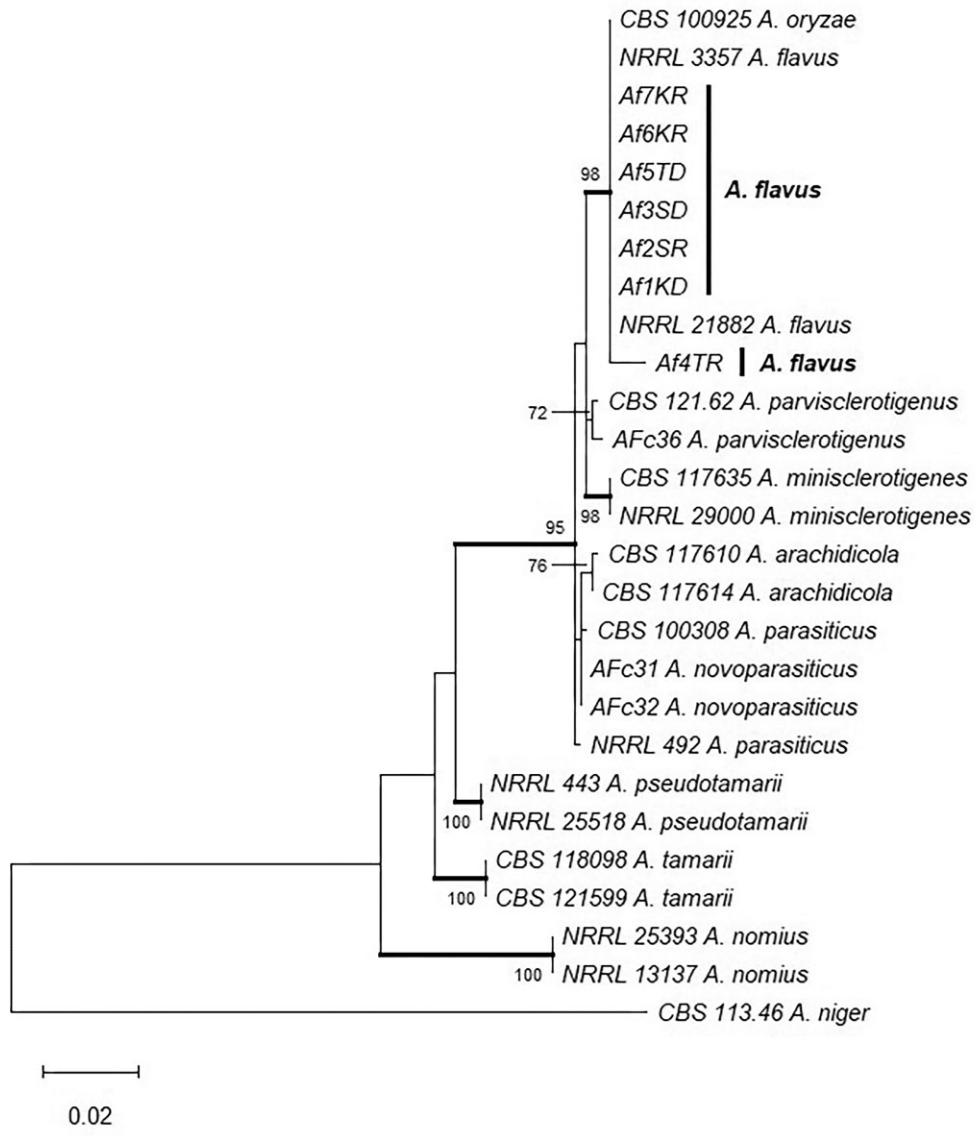


Figure 1. ML tree of *Aspergillus* section *Flavi* based on the combined datasets of ITS and β-tubulin. Isolates with the initial code *Af* labelled as *A. flavus* in black are isolates tested in the present work. Branches in bold are highly supported by bootstrap values of >90%. Bootstrap values of <70% are not shown. *Aspergillus niger* strain CBS 113.46 is the outgroup taxon. Scale bars are the number of substitutions.

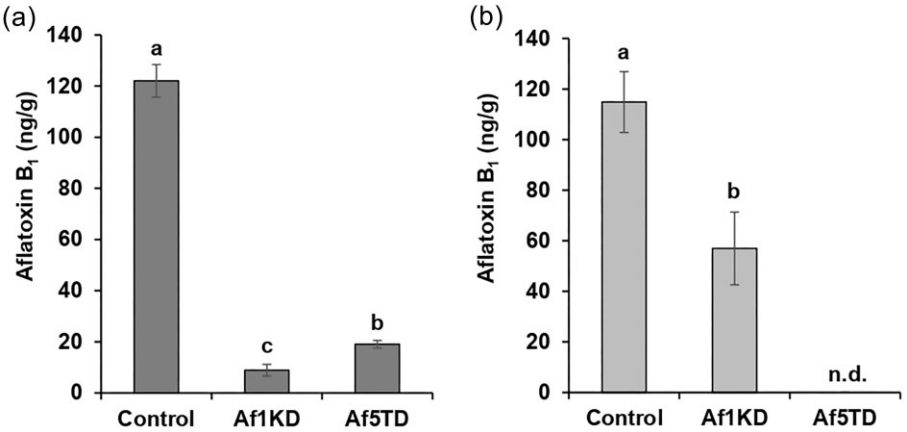
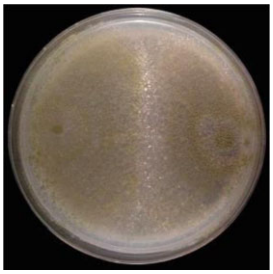
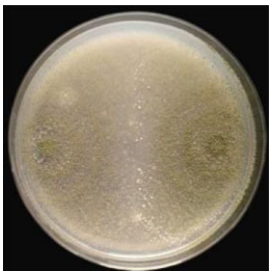
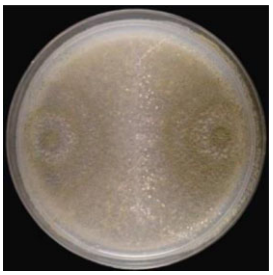


Figure 2. Aflatoxin B_1 (ng g^{-1}) produced by aflatoxigenic *A. flavus* isolates (a) Af6KR and (b) Af7KR on 3% semi-synthetic GCA after co-cultivation with non-aflatoxigenic *A. flavus* isolates Af1KD and Af5TD obtained from pioneer grain corn farms in Terengganu, Malaysia. Data are mean ± SE of four replicates ($n = 4$). Different lowercase letters indicate significant difference ($P < .05$) between groups using Tukey's Honest Significant Difference (Tukey's HSD). n.d.—not detected.

Table 5. *In vitro* fungal interaction as explained by interaction score and percentage inhibition of radial growth (PIRG, %) of co-cultivated aflatoxigenic (right) and non-aflatoxigenic (left) *A. flavus* isolates obtained from pioneer grain corn farms in Terengganu, Malaysia on 3% semi-synthetic GCA.

Non-aflatoxigenic isolate	Aflatoxigenic isolate	
	Af6KR	**Af7KR**
Af1KD		
Interaction score	2/2	3/3
PIRG	41 ± 4.1	44 ± 2.1
Af5TD		
Interaction score	2/2	2/2
PIRG	43 ± 0.6	41 ± 1.2

Data of PIRG (%) are means of four replicates ($n = 4$), and expressed as mean ± standard error (± SE).
Aflatoxigenic isolates: Af6KR, Af7KR. Non-aflatoxigenic isolates: Af1KD, Af5TD.

Figure 2 depicts the AFB_1 (ng g^{-1}) production by two aflatoxigenic *A. flavus* isolates Af6KR and Af7KR on GCA after co-cultivation with non-aflatoxigenic *A. flavus* isolates Af1KD and Af5TD. AFB_1 production by aflatoxigenic *A. flavus* isolate Af6KR was significantly reduced by the non-aflatoxigenic *A. flavus* isolates Af1KD and Af5TD by ∼93% and 85%, respectively, while AFB_1 production by aflatoxigenic *A. flavus* isolate Af7KR was significantly reduced by the non-aflatoxigenic *A. flavus* isolates Af1KD and Af5TD by ∼50% and 100%, respectively.

Discussion

Based on the phylogenetic analysis using ITS and β-tubulin, the tested isolates were validated as *A. flavus*. The utilization of ITS, although enough to differentiate between fungal species of different genera and the congeneric species (fungi in the same genus), may not be sufficient to resolve the species identification especially for cryptic (closely related) species such as *A. flavus*. Therefore, secondary barcode is necessary to delineate the species (Houbraken et al. 2014, Lücking et al. 2021). β-tubulin is appropriate for this purpose since it could accurately discriminate closely related species within the section *Flavi* (Kim et al. 2020). In the present work, the β-tubulin and the combined sets resolved the tested isolates to *A. flavus*. *A. oryzae*, which resides in the same clade as *A. flavus* is considered as the domesticated species of *A. flavus* without the aflatoxin-producing gene, has been used for koji fermentation of fermented foods, and not found in agricultural fields (Frisvad et al. 2019), thus is not the same species as *A. flavus*. This was also corroborated by Norlia et al. (2019a).

The rationale behind *A. flavus* taxonomic validation in the present work lies on certain requirements for the registration of new non-aflatoxigenic strain as biocontrol agent, namely a validated species name, as well as risk assessment analysis, including the toxicity study (Braverman et al. 2010). In the section *Flavi* and besides *A. flavus*, many other species could also produce aflatoxins (Norlia et al. 2019b). This warranted proper species identification in order that proper and adequate control could be achieved. In the case of misidentification, Uka and colleagues (2019) published a study delineating a misidentified strain that had long been known and utilized as *A. flavus*. This strain has been renamed into its closely related species *A. nomius* (Uka et al. 2019). They also strongly emphasized the importance of having molecular analysis (such as phylogenetic) along with the phenotype-based analysis when dealing with fungal species identification.

Of the two non-aflatoxigenic *A. flavus* isolates identified, both had the same amplification pattern, that is, only one of the seven targeted genes was amplified, which was located at the end of the cluster. This indicated a deletion of the genes at early and middle regions of the cluster, thus suggesting large gene deletions (>1 kb, Adhikari et al. 2016) across the cluster of 70-kb region. Other studies have shown similar, or even diverse amplification pattern within the aflatoxin gene cluster of *A. flavus* (Chang et al. 2005, Mauro et al. 2013, Adhikari et al. 2016). These patterns explain the deletion/mutation events that occurred, rendering the isolates unable to produce aflatoxins, thus becoming non-aflatoxigenic.

Although the amplification of the targeted genes in the present work was complementary to that of aflatoxin production data obtained by HPLC-FLD, studies have found rather conflicting results (Gallo et al. 2012, Norlia et al. 2019a). Despite having all five to seven tested genes amplified, they deduced that the lack of aflatoxin production in the *A. flavus* isolates was due to some other untested genes that most likely carried defects or deletions. This indicated that it is still necessary for a thorough examination of all the 25 genes to reveal the exact deletion pattern that could occur in the aflatoxin gene cluster, and therefore confirm their non-aflatoxigenicity status. Furthermore, among the requirements for the registration of biocontrol agents is the information on their mechanism of non-aflatoxigenicity (Grubisha and Cotty 2015). Therefore, besides the gene amplification that explains deletions, a detailed examination on SNP in the gene cluster can also be performed in future study.

Among the seven tested isolates, five were aflatoxigenic, and four of these were high aflatoxin producers (>10 000 ng g^{-1}; Norlia et al. 2018). This contradicted the result reported in our previous work (Yazid et al. 2021) in which only three isolates were deemed as aflatoxigenic based on quantification from GCA. While using agar-based natural substrates (Yazid et al. 2018) could provide nearly accurate mycotoxigenic production potential of the mycotoxigenic species on the chosen substrates, it might not accurately define the true mycotoxin potential of the mycotoxigenic strain, hence the discrepancy. Previous studies have utilized commercial media such as yeast extract agar and Czapek yeast autolysate (Rodrigues et al. 2009, Norlia et al. 2018) to boost aflatoxin production since these media contain added compounds that fit for this purpose (Yazid et al. 2018). Furthermore, in some cases, the use of actual corn kernels may be needed to complement the use of corn-based agar (Probst and Cotty 2012). Work on corn kernels is currently ongoing.

Although the indigenous non-aflatoxigenic *A. flavus* isolates could not strongly inhibit the growth of their aflatoxigenic counterparts, our findings showed that it could nevertheless significantly reduce AFB_1 production *in vitro*. Similar result was also reported by Rahman et al. (2022). The AFB_1 might have been highly reduced due to the thigmoregulation (i.e. touch inhibition) mechanism, in which the reduction occurred because of physical contact of both non-aflatoxigenic and aflatoxigenic *A. flavus* isolates. Specifically, the physical contact mediated by ligand of both non-aflatoxigenic and aflatoxigenic *A. flavus* isolates prevented aflatoxin production by the aflatoxigenic *A. flavus* isolate (Huang et al. 2011, Ojiambo et al. 2018). In the present work, the contact occurred on all co-cultivated plates (Table 5), that although all non-aflatoxigenic *A. flavus* isolates mutually inhibited their aflatoxigenic counterparts as indicated by a mycelial separation, the hyphae continued to grow and touch one another in the middle. The other possible mechanism for AFB_1 reduction is its degradation by the non-aflatoxigenic isolates. This might occur as the non-aflatoxigenic isolates utilize AFB_1 as carbon source (Maxwell et al. 2021).

However, unlike *in vitro* study in which both aflatoxigenic and non-aflatoxigenic *A. flavus* are in close contact with each other, the mechanism of aflatoxin reduction in nature, such as corn fields, relies mainly on competitive displacement (Bandyopadhyay et al. 2022). Competitive displacement occurs when the non-aflatoxigenic strains, which typically have rapid growth rate and are aggressive colonizer, are applied in excess into the targeted fields before rapidly propagating and displacing the population of the aflatoxigenic strains. This suppression in population size of the aflatoxigenic strains will reduce aflatoxin accumulation in the targeted fields, thus reducing aflatoxin contamination in the agricultural crops (Hruska et al. 2014). Besides competitive displacement, thigmoregulation, degradation, and production of extrolites and volatile compounds are regarded as the indirect mechanism of aflatoxin reduction in nature (Bandyopadhyay et al. 2022).

From the biocontrol point-of-view, effective selection of non-aflatoxigenic isolate as biocontrol candidate requires examination of several criteria, as listed by Moral et al. (2020). Among these criteria, we had only determined the mechanism of non-aflatoxigenicity of the selected indigenous *A. flavus* isolates, as well as their efficacy *in vitro* against their aflatoxigenic counterparts. However, other criteria are also prerequisite prior to their commercialization and wide applications. These include screening potential isolates for area-wide adaptation, and those obtained from a widely distributed non-aflatoxigenic vegetative compatibility group to ensure genetic stability, and therefore limiting possibility of recombination. Besides, the selected isolate as biocontrol agent must also be tested widely and for multiple times in the targeted crop regions. These could be undertaken in the future.

In conclusion, the present work confirmed the identity and the aflatoxigenicity of previously isolated indigenous *A. flavus* from two pioneer grain corn farms in Terengganu, Malaysia. The antagonistic potential of the identified non-aflatoxigenic strains was also established. Two of seven *A. flavus* isolates were confirmed as non-aflatoxigenic, which could highly reduce AFB_1 produced by both aflatoxigenic isolates tested on 3% semi-synthetic GCA, albeit having weak ability to inhibit their growth on the dual-culture assay. The non-aflatoxigenic biocontrol candidate assessed in the present work should be further tested *in planta/in situ* in the grain corn agroecosystems.

Ethical approval

The present work required neither animal nor human ethical approval.

Supplementary data

Supplementary data is available at *JAMBIO Journal* online.

Conflict of interest: None declared.

Funding

The authors would like to thank the Malaysian Ministry of Higher Education for providing financial support under the High Impact Centre of Excellence Phase I and Phase II grant schemes [grant no.: HICoE/ITAFoS/2017/FS9 and UPM.ITAFoS.800–3/8/HICoE(FasaII)/2003/5230006] awarded to the Institute of Tropical Agriculture and Food Security, UPM, and the Faculty of Food Science and Technology, UPM for the research facilities. The first author would also like to acknowledge the financial support received from the School of Graduate Studies, UPM under the Graduate Research Fellowship scheme

[grant no: UPM/SPS/GS50379/2017-2020] for her PhD candidature.

Author contributions

Siti Nur Ezzati Yazid (Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft), Jinap Selamat (Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing), Siti Izera Ismail (Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing), Maimunah Sanny (Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing), and Nik Iskandar Putra Samsudin (Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing).

Data availability

The data of the present work, other than those presented herein, will be available upon request to the corresponding author.

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Received 30 March 2024; **revised** 21 May 2024; **accepted** 13 June 2024