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Mycotoxigenic fungal growth inhibition and multi-mycotoxin reduction of potential biological control agents indigenous to grain maize

Siti Nur Ezzati Yazid[1] · Nur Izzah Tajudin[2] · Nur Aina Aribah Razman[2] · Jinap Selamat[1,2] · Siti Izera Ismail[3,4] · Maimunah Sanny[1,2] · Nik Iskandar Putra Samsudin[1,2]

Abstract

The present work investigated the potential of fungal species from grain maize farms in Malaysia as antagonists against the indigenous mycotoxigenic fungal species and their subsequent mycotoxin production. Dual-culture assay was conducted on grain maize agar (GMA) with 12 strains of potential fungal antagonists namely *Bjerkandra adusta*, *Penicillium janthinellum*, *Schizophyllum commune*, *Trametes cubensis*, *Trichoderma asperelloides*, *Trichoderma asperellum*, *Trichoderma harzianum*, and *Trichoderma yunnanense* against seven mycotoxigenic strains namely *Aspergillus flavus*, *Aspergillus niger*, *Fusarium verticillioides*, and *Fusarium proliferatum* producing aflatoxins, ochratoxin A, and fumonisins, respectively. Based on fungal growth inhibition, *Trichoderma* spp. showed the highest inhibitory activity (73–100% PIRG, Percentage Inhibition of Radial Growth; 28/0 I_D, Index of Dominance) against the tested mycotoxigenic strains. Besides, *B. adusta* and *Tra. cubensis* showed inhibitory activity against some of the tested mycotoxigenic strains. All fungal antagonists showed varying degrees of mycotoxin reduction. Aflatoxin B_1 produced by *A. flavus* was mainly reduced by *P. janthinellum*, *Tra. cubensis*, and *B. adusta* to 0 ng/g. Ochratoxin A produced by *A. niger* was mainly reduced by *Tri. harzianum* and *Tri. asperellum* to 0 ng/g. Fumonisin B_1 and FB_2 produced by *F. verticillioides* was mainly reduced by *Tri. harzianum*, *Tri. asperelloides*, and *Tri. asperellum* to 59.4 and 0 µg/g, respectively. Fumonisin B_1 and FB_2 produced by *F. proliferatum* were mainly reduced by *Tri. asperelloides* and *Tri. harzianum* to 244.2 and 0 µg/g, respectively. This is the first study that reports on the efficacy of *Tri. asperelloides* against FB_1, FB_2, and OTA, *P. janthinellum* against AFB_1, and *Tra. cubensis* against AFB_1.

Keywords Aflatoxins · Antagonist · Dual-culture in vitro · Ochratoxins · Fumonisins

✉ Nik Iskandar Putra Samsudin
   nikiskandar@upm.edu.my

1 Laboratory of Food Safety and Food Integrity, Institute of Tropical Agriculture and Food Security, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

2 Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

3 Laboratory of Climate-Smart Food Crop Production, Institute of Tropical Agriculture and Food Security, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

4 Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

Introduction

Mycotoxigenic fungi and their toxic secondary metabolites, mycotoxins, are important agricultural contaminants, and their presence poses a significant threat to global food safety and security. Crop commodities, especially cereal grains, infected by mycotoxigenic fungi and their mycotoxins will decrease in yield, quality, and safety, as well as affecting livestock productivity, and trade rejection due to existing legislations. Furthermore, human exposure to mycotoxins, either as primary or secondary infection, also contributes to human morbidity and mortality. In countries where foods prone to mycotoxin contamination are dietary staples, and that no or minimum appropriate control measures or regulations are in place, high mycotoxicosis rates have been observed (Darwish et al. 2014).

Among the cereal grains, maize, also known as "*Queen of Cereals*" (Jaidka et al. 2019), ranks second globally in

terms of yield and acreage, and is widely grown in both developing and developed countries (Erenstein et al. 2021). In maize, the most common mycotoxigenic fungal species reported are certain species of the genera *Aspergillus*, *Alternaria*, *Fusarium*, and *Penicillium* (Oldenburg et al. 2017; Munkvold et al. 2019). These mycotoxigenic fungal species exist as plant pathogens by infecting damaged (*e.g.*, as opportunistic pathogen) or healthy (*e.g.*, as endophyte) maize plant parts, before proliferating and producing mycotoxins under suitable environmental conditions. Although diverse mycotoxins have been reported from these mycotoxigenic fungal species in maize or their products, only several are classified as economically relevant, and pose health concern. According to the International Agency for Research on Cancer (IARC), aflatoxins from *Aspergillus* section *Flavi* (*Aspergillus flavus*, *Aspergillus parasiticus*) are Group 1 carcinogens (carcinogenic to humans), while fumonisins from *Fusarium* section *Liseola* (*Fusarium verticillioides*, *Fusarium proliferatum*), and ochratoxin A from *Aspergillus* section *Nigri* (*Aspergillus niger*, *Aspergillus carbonarius*) are Group 2B (possibly carcinogenic to humans) (Ostry et al. 2017).

As an excellent source of carbohydrate and energy, maize is typically divided into sweet maize for human food, and grain maize for animal feed. For decades, Malaysia has been dependent on grain maize importation, particularly from Argentina and Brazil (Zahari and Wong 2009). This has caused a burden to the country's economy, and spawned a threat to the country's food security, especially following COVID-19 pandemic and the Russia-Ukraine War. To lessen the country's dependency on importation, the Ministry of Agriculture and Food Industries has gazetted grain maize as one of the five new wealth-generating crop commodities alongside coconut, durian, dairy, and culinary industries (Bank Negara Malaysia 2018). This entails more land exploitation for the mass-cultivation of grain maize, following which, two pioneer grain maize plantations have been established in Kampong Dadong (13 hectares cultivated area) and Rhu Tapai (3.8 hectares cultivated area) in Terengganu, Malaysia (Yazid et al. 2021).

To combat mycotoxigenic fungi and mycotoxin production, various control approaches have been employed including physical, chemical, and biological controls. Of these, biological control is regarded as an alternative for the harmful usage of chemical pesticides (Yazid et al. 2020). At present, efforts on the development of new biological control agents (BCA) are targeted on the species / strains obtained from the native / indigenous environmental niches of the fungal pathogens, as they could be more effective than introducing foreign / exotic BCA. Among the advantages of using indigenous BCA is that they could reduce the risk of non-target effect, and preserve the ecological balance of the ecosystem (De Clercq et al. 2011). Besides, indigenous BCA

could also adapt better to the niche's microclimates which assures their efficacy (Moore 2022).

Although the application of BCA against mycotoxigenic fungal species in other parts of the world is already advanced, the same is still in infancy in Malaysia (Yazid et al. 2020). Among the primary challenges in developing and applying BCA is ensuring mycotoxin inhibition instead of mycotoxin stimulation which is common among mycotoxigenic fungal species in the face of habitat / nutrient competition with the applied BCA. In a previous work (Yazid et al. 2021), we isolated and identified morphologically and molecularly several BCA candidates as well as characterising the mycotoxin potentials of several mycotoxigenic fungal species from two pioneer grain maize farms in Terengganu, Malaysia. In the present work, we therefore examined the antagonistic potential of those BCA candidates in vitro using the dual-culture assay on grain maize agar. The findings reported in the present work would provide basic information and data for further development of efficient biological control strategies in planta / in situ.

Materials and methods

Chemicals, reagents, mycotoxin standards, and growth media

All solvents and reagents used in the present work were of HPLC grade unless otherwise stated. Mycotoxin standards of aflatoxin B_1 (AFB_1), fumonisins (FB_1, FB_2), and ochratoxin A (OTA) were purchased from Sigma-Aldrich (St. Louis, USA). Acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Sodium dihydrogen phosphate and sodium tetraborate were purchased from Chemiz (Selangor, Malaysia). *Ortho*-phthalaldehyde (OPA) and 2-mercaptoethanol were purchased from Sigma-Aldrich (St. Louis, USA). Glacial acetic acid was purchased from RandM Chemicals (London, UK). Ultrapure water was obtained from Elga PURELAB® Classic UV MK2 (Lane End, UK). Potato Dextrose agar (PDA) was purchased from Oxoid (Basingstoke, UK).

Isolation and identification of indigenous antagonist fungi and mycotoxigenic fungi

The isolation and identification (morphological and molecular) of antagonist fungi and mycotoxigenic fungi from two pioneer grain maize farms in Terengganu, Malaysia during 2017 cropping season have been described in our previous work (Yazid et al. 2021). The two maize farms were Kampong Dadong and Rhu Tapai. Sampling was done from grain maize kernel, tassel, debris, and soil. All fungal strains were isolated from the samples by conventional dilution and

direct plating methods. Dilution plating of all samples was performed on dichloran rose bengal chloramphenicol agar (DRBC; Oxoid, Basingstoke, UK), while direct plating of kernel and tassel was performed on dichloran-glycerol 18 agar (DG-18; Oxoid, Basingstoke, UK) and PDA supplemented with 1 mg/L chloramphenicol (Merck, Darmstadt, Germany). Next, fungal strains were morphologically identified and distinguished based on colony size, colour, texture, mycelial characteristic, pigmentation, and spore formation. All strains of distinct morphology and obtained from different samples were subcultured onto fresh PDA to obtain axenic colony. Thereafter, DNA of fungal isolates of different morphology was amplified at internal transcribed spacer (ITS) region by polymerase chain reaction (PCR). Following isolation and identification, a total of 131 fungal strains were obtained, of which 30 distinct fungal species were identified and deposited into the Culture Collection of Laboratory of Food Microbiology, Faculty of Food Science and Technology, UPM, and their DNA sequences were deposited in GenBank. Further, seven mycotoxigenic fungal strains commonly found in maize were characterised; two *A. flavus* strains (Af6KR and Af7KR) producing AFB_1, two *A. niger* strains (An1KD and An3KD) producing OTA, two *F. verticillioides* strains (Fv21KD and Fv92SR) producing FB_1 and FB_2, and one *F. proliferatum* strain (Fp9DD) producing FB_1 and FB_2. Aflatoxin B_2 was not detected from the aflatoxigenic strains. All fungal strains were maintained on PDA, and incubated at 30 °C for 7 d prior to the dual-culture assay.

In silico selection of antagonist fungi

Apart from the mycotoxigenic fungal species, the remaining fungal species isolated from the pioneer grain maize farms in Terengganu, Malaysia were further screened and assessed in silico using established scientific databases (Google Scholar, ScienceDirect, Scopus) and published reports to select for suitable potential BCA, and to avoid the inclusion of risky isolates in the development and commercialisation of BCA. The complete stepwise screening procedures in the development of a novel BCA is outlined by Köhl et al. (2011). Inclusion screening criteria included reported antagonistic activity against pathogenic fungi or mycotoxigenic fungi. Exclusion screening criteria included mycotoxigenic fungi and pathogenic fungi infecting animals, humans, or plants. The search keywords included "mycotoxin", "mycotoxigenic fungi", "corn / maize pathogen", "corn / maize disease", "animal pathogen", "plant pathogen", "human pathogen", "antagonist", and "biological control / biocontrol". For maize pathogens, a reference list by Munkvold (2017) was also used. Following in silico screening, fungal species that matched with the selection criteria were selected and used in the dual-culture assay against the mycotoxigenic fungal species.

Preparation of grain maize agar as semi-synthetic growth medium for dual-culture assay

To simulate the natural condition of maize colonisation by the mycotoxigenic fungi (Samsudin and Magan 2016), and to avoid over- / underproduction of mycotoxins on commercial fungal growth media (Yazid et al. 2018), 3% grain maize agar (GMA) was prepared. Briefly, 30 g of ground (Waring blender; Waring, Torrington, CT, USA) grain maize and 15 g of technical agar (Oxoid, Basingstoke, UK) as a solidifying agent were added to 1 L of distilled water. The GMA medium was autoclaved at 121 °C and 15 psi for 15 min, cooled down to ≈50 °C, poured into 90 mm Ø Petri plates, and left to solidify. Solidified GMA plates were refrigerated at 4 °C until further use in the dual-culture assay.
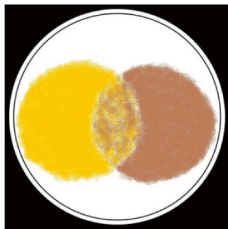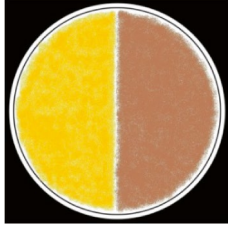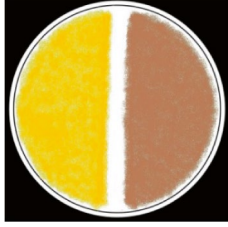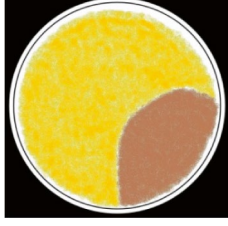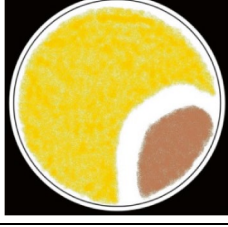
Dual-culture assay

Dual-culture assay of both mycotoxigenic fungi and antagonist candidates was performed on fresh GMA. A 7-d old sporulating hyphal plug (5 mm Ø) of mycotoxigenic fungus and antagonist candidate were aseptically inoculated 60 mm apart from each other, and 15 mm from the plate's periphery. Thereafter, inoculated plates were incubated at 30 °C for 7 d for *Aspergillus* spp., and 10 d for *Fusarium* spp. At the end of incubation period, the types of interaction (Table 1) were scored using numerical scores namely mutual intermingling (1/1), mutual antagonism upon contact (2/2), mutual antagonism at a distance (3/3), dominance of one species upon contact (4/0), and dominance of one species at a distance (5/0) (Magan and Lacey 1984; Samsudin and Magan 2016). The Percentage Inhibition of Radial Growth (PIRG, %) was also recorded by measuring the colony radii of mycotoxigenic fungi 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 mycotoxigenic fungus in control plate, and R_2 was radius of mycotoxigenic fungus in treatment plate (Rahman et al. 2009). The radii of mycotoxigenic fungi in control and treatment plates were measured from the inoculation point towards the direction of antagonist colony (treatment) or the supposed area (control).

Quantification of aflatoxin B_1 inhibition using high performance liquid chromatography

Extraction and quantification of AFB_1 was performed following the procedures described by Bragulat et al. (2001). Following incubation, five to seven hyphal plugs of mycotoxigenic *A. flavus* strains were transferred into pre-weighed Eppendorf tubes, and the weight were recorded. Next, approximately 1 mL of absolute methanol was added to the Eppendorf tubes before vortexed using a vortex mixer (LMS Co. Ltd., Japan). The tubes were incubated for 30 min

Table 1 Index of Dominance (I_D) as explained by the interaction score of a dual-culture assay between a pathogen and an antagonist. Descriptions and scores are adapted from Magan and Lacey (1984). Diagrammatic representations of colony growth are produced in-house. Standard diameter (Ø) of a Petri dish is 90 mm

Colony growth (qualitative)	Interaction score (quantitative)	Description of interaction
	1 / 1 *mutual intermingling*	The growth of one species (the pathogen) and that of another species (the antagonist) intermingle mutually, and without inhibition.
	2 / 2 *antagonism (mutual inhibition) upon contact*	The growth of one species is mutually inhibited by another species upon contact. Space exists between colonies at < 2 mm.
	3 / 3 *antagonism (mutual inhibition) at a distance*	The growth of one species is mutually inhibited by another species at a distant. Space exists between colonies at > 2 mm.
	4 / 0 *dominance (strong inhibition) of one species upon contact*	Inhibitor species continues to grow unchanged / unchallenged, or at a reduced rate, through / over the inhibited species.
	5 / 0 *dominance (strong inhibition) of one species at a distance*	Inhibitor species continues to grow unchanged / unchallenged, or at a reduced rate, through a clear zone away from the inhibited species.

at room temperature, and centrifuged (Sartorius, Germany) at 5,590 g for 5 min. Thereafter, the extract was filtered through nylon syringe filter (0.22 μm; Macherey–Nagel, Germany) into HPLC vials (Thermo Scientific, USA). Separation of AFB$_1$ was performed by reversed-phase HPLC system (Waters 600, Haverhill, USA), attached to a fluorescence detector (Waters 2475, Haverhill, USA) and a Gemini® C$_{18}$ column (5 μm, 250 × 4.6 mm; Phenomenex, Washington, USA), with a mobile phase of methanol:acetonitrile:water (55:35:10, v/v/v) at 40 °C and 0.6 mL/min flow rate. The injection volume was 20 μL. Derivatisation of AFB$_1$ was performed using a post-column (*i.e.*, Photochemical Reactor

for Enhanced Detection, PHRED; Aura Industries, USA). The excitation and emission wavelengths were 365 nm and 435 nm, respectively (Afsah-Hejri et al. 2011).

Quantification of ochratoxin A inhibition using high performance liquid chromatography

Extraction and quantification of OTA was performed following the procedures described by Mohale et al. (2013). Briefly, five to seven hyphal plugs of mycotoxigenic *A. niger* strains were transferred into pre-weighed Eppendorf tubes, and the weight were recorded. Next, approximately 1 mL

of absolute methanol was added to the Eppendorf tubes before shaken for 60 min. Thereafter, the extract was filtered through nylon syringe filter (0.22 μm) into HPLC vials. Separation of OTA was performed by Waters 600 reversed-phase HPLC and the Gemini® C_{18} column (5 μm, 250 × 4.6 mm) with a mobile phase of acetonitrile:water:glacial acetic acid (57:41:2, v/v/v) at 1.0 mL/min flow rate. The injection volume was 20 μL. Detection of OTA was performed using the Waters 2475 fluorescence detector at the excitation and emission wavelengths of 360 nm and 440 nm, respectively.

Quantification of fumonisin inhibition using high performance liquid chromatography

Extraction, derivatisation, and quantification of FB_1 and FB_2 were performed following the procedures described by Visconti et al. (2001). Briefly, five to seven hyphal plugs of mycotoxigenic *Fusarium* strains were transferred into pre-weighed Eppendorf tubes, and the weight were recorded. Next, approximately 1 mL of methanol:acetonitrile:water (25:25:50, v/v) was added to the Eppendorf tubes before vortexed, shaken for 60 min, and filtered through nylon syringe filter (0.22 μm) into HPLC vials. Next, the filtrate was evaporated to dryness in a stream of nitrogen before re-dissolved in the same mobile phase used for HPLC analysis. Prior to the analysis, 50 μL of fumonisin standards and samples were derivatised with 100 μL of OPA for 1 min. The derivatisation reagent was prepared fresh by dissolving 40 mg of OPA in 1 mL of absolute methanol before adding 5 mL of 0.1 M disodium tetraborate and 50 μL of 2-mercaptoethanol in a capped amber vial. Separation of FB_1 and FB_2 was done by Waters 600 reversed-phase HPLC and the Gemini® C_{18} column (5 μm, 250 × 4.6 mm), in a mobile phase of methanol:0.1 M sodium dihydrogen phosphate (77:23, v/v) adjusted to pH 3.35 using phosphoric acid, at 1.0 mL/min flow rate. The injection volume was 30 μL. Detection of FB_1 and FB_2 was performed using the Waters 2475 fluorescence detector with the excitation and emission wavelengths of 335 nm and 440 nm, respectively.

Prior to all mycotoxin quantification, their mobile phases were filtered using nylon membrane filter (0.45 μm; Merck, Darmstadt, Germany), and sonicated using ultrasonic bath (Power Sonic 420, Korea) for 30 min to degas. Following mycotoxin detection, data were processed using Empower 2 Chromatography Data Software (Waters, Haverhill, USA). The percentage of mycotoxin inhibition was calculated using the equation % inhibition $= (B_0 - B_1 / B_0) \times 100$, where, B_0 was the concentration of mycotoxin in control plate, and B_1 was concentration of mycotoxin in treatment plate (Rahman et al. 2022). Data of % inhibition are given in Supplementary 4.

Determination of linearity, limit of detection, and limit of quantification

Linear calibration curves (Supplementary 1) were constructed for each of the tested mycotoxins by plotting the peak area (response) obtained from HPLC analysis against the concentrations of each calibrant solutions to calculate the actual mycotoxin production of the mycotoxigenic fungal species. The calibrant solutions were prepared and diluted in different solvents: AFB_1 (0.5 – 100 ng/mL) and OTA (5 – 100 ng/mL) in methanol, while FB_1 and FB_2 (0.05 – 25 μg/mL) in acetonitrile:water. Next, linear regression was performed to establish the correlation coefficient (R^2). The limit of detection (LOD) and limit of quantification (LOQ) of each chromatographic analysis were estimated following the method prescribed by the International Conference on Harmonisation (ICH) using the formula; LOD = 3σ/s, and LOQ = 10σ/s, where σ was the standard deviation of blank responses, and s was the slope of the calibration curve (Shrivastava and Gupta 2011). The linearity, LOD, and LOQ of AFB_1 was R^2 = 0.9992, 0.01 ng/mL, and 0.02 ng/mL, respectively. The linearity, LOD, and LOQ of OTA was R^2 = 0.9961, 0.2 ng/mL, and 0.8 ng/mL, respectively. The linearity, LOD, and LOQ of FB_1 was R^2 = 0.9955, 0.0003 μg/mL, and 0.001 μg/mL, respectively. While, the linearity, LOD, and LOQ of FB_2 was R^2 = 0.9901, 0.0004 μg/mL, and 0.001 μg/mL, respectively.

Statistical analysis

All experiments were conducted in four replicates ($n = 4$). Data of PIRG (%) were then averaged and presented as mean ± SE (standard error). Data of multi-mycotoxin production following dual-culture assay were tested for normality based on Kolmogorov–Smirnov and Shapiro–Wilk. All data violated the normality assumption (Supplementary 3), even after transformation. Therefore, non-parametric Kruskal–Wallis was employed to compare the distribution between groups for each mycotoxigenic strains tested. All analyses were statistically significant at $p < 0.05$ (Supplementary 3). The subsequent post hoc Dunn's multiple comparisons were conducted and each resulting p-values were corrected with Benjamini–Hochberg False Discover Rate (FDR). The mycotoxin concentrations between pairs were considered as statistically significant based on the corrected p-value at < 0.05. Data of multi-mycotoxin were reported in median with interquartile range (IQR) as error bars. The statistical software SPSS version 29.0 (SPSS Inc., Chicago, IL) was used to perform the analysis.

Results

Antagonist candidates

Supplementary 2 shows the fungal isolates obtained from grain maize farms in Terengganu, Malaysia, and the rationale for their selection or rejection (in silico screening) as antagonist candidates for dual-culture assay. Only eight species (12 strains) matched with the selection criteria *i.e.*, neither a plant, human, or animal pathogen, nor a mycotoxigenic fungal species.

Growth inhibition of mycotoxigenic fungal strains co-cultivated with antagonist candidates in dual-culture assay

The growth inhibition of the mycotoxigenic fungal strains co-cultivated with the antagonist candidates were quantified by (a) Percentage Inhibition of Radial Growth (PIRG; %), and (b) Index of Dominance (I_D). Table 2 shows the PIRG (%) of seven mycotoxigenic fungal strains co-cultivated with antagonist candidates. The PIRG (%) shows varying trends of radial growth inhibition of the mycotoxigenic fungal strains, which can be divided into three classes: high, medium, or weak antagonists (Soytong and Quimio 1989). Overall, high antagonistic activity (> 80%) was shown by all *Trichoderma* spp. against all mycotoxigenic fungal strains, except for *Trichoderma harzianum* Th33SR against *A. flavus*, of which the PIRG was moderate (60 – 80%). Besides, *Bjerkandera adusta* Ba42DD also showed high antagonistic

activity against *A. flavus*, while *Trametes cubensis* Tc28DD showed high antagonistic activity against *F. verticillioides* and *F. proliferatum*. Further, *B. adusta* Ba42DD and *Schizophyllum commune* Sc40DD showed a moderate antagonistic activity against both *F. verticillioides* and *F. proliferatum*, while *Penicillium janthinellum* Pj46SD showed a moderate antagonistic activity against *A. flavus* Af6KR. Others were mostly weak antagonists (< 60%).

Table 3 shows the interaction scores and index of dominance (I_D) between seven mycotoxigenic fungal strains and antagonist candidates isolated from the grain maize farms in Terengganu, Malaysia. Meanwhile, Table 4 shows the in vitro fungal interaction as explained by the interaction score of co-cultivated antagonists and mycotoxigenic strains on 3% grain maize agar and isolated from grain maize farms in Terengganu, Malaysia. Interaction scores and the subsequent I_D utilise numerical notation to indicate the nature of interaction between the antagonist candidates and the mycotoxigenic strains, as well as the possible mechanism of antagonism (Magan and Lacey 1984). Antagonists are deemed highly competitive and become dominant when they score a 4 / 0 or 5 / 0 against the mycotoxigenic species, and not as competitive when they score a 1 / 1, 2 / 2, or 3 / 3 (Table 1). Besides, the 4 / 0 score indicates strong inhibition of the antagonist against the mycotoxigenic species "*upon contact*", which could mean that the former is mycoparasites and the possible mechanism of antagonism is the production of cell wall-degrading enzymes that could inhibit the mycotoxigenic fungal growth. The 5 / 0 score indicates strong inhibition of the antagonist against the

Table 2 Percentage Inhibition of Radial Growth (PIRG, %) of seven mycotoxigenic fungal strains co-cultivated with antagonist candidates isolated from grain maize farms in Terengganu, Malaysia

Antagonist candidate	Percentage Inhibition of Radial Growth (PIRG, %)						
	Af6KR	Af7KR	An1KD	An3KD	Fp9DD	Fv21KD	Fv92SR
Ba42DD	83 ± 3.3	91 ± 1.4	33 ± 1.3	40 ± 3.9	78 ± 1.1	74 ± 6.8	62 ± 1.2
Pj46SD	67 ± 4.6	46 ± 5.2	0.7 ± 0.7	10 ± 8.1	38 ± 1.2	36 ± 1.0	45 ± 0.4
Pj81SR	64 ± 1.7	63 ± 1.3	12 ± 1.7	19 ± 1.9	38 ± 2.3	42 ± 1.8	46 ± 1.0
Sc40DD	52 ± 2.4	59 ± 1.9	34 ± 2.2	37 ± 2.3	63 ± 3.5	65 ± 1.3	71 ± 1.0
Tc28DD	45 ± 7.0	44 ± 1.3	26 ± 10.8	27 ± 8.2	87 ± 5.3	83 ± 2.5	92 ± 1.1
Ta39KR	100 ± 0	100 ± 0	100 ± 0	97 ± 3.4	100 ± 0	97 ± 2.8	99 ± 1.2
Ta31KR	98 ± 1.7	99 ± 1.1	90 ± 3.5	96 ± 4.0	100 ± 0	94 ± 1.3	98 ± 1.6
Th20SR	91 ± 3.0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Th24KD	87 ± 3.0	88 ± 2.3	100 ± 0	100 ± 0	99 ± 1.3	100 ± 0	100 ± 0
Th33SR	73 ± 3.8	92 ± 4.1	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Th36SR	85 ± 1.6	91 ± 2.2	94 ± 5.5	96 ± 4.0	100 ± 0	100 ± 0	100 ± 0
Ty34KD	87 ± 2.2	81 ± 1.1	85 ± 1.5	83 ± 0.7	93 ± 2.8	99 ± 0.4	100 ± 0

Data are means of four replicates ($n = 4$), and expressed as mean ± standard error (± SE)

Mycotoxigenic fungal strains: *Af6KR Af7KR Aspergillus flavus* strains, *An1KD, An3KD Aspergillus niger* strains, *Fp9DD Fusarium proliferatum* strain, *Fv21KD, Fv92SR Fusarium verticillioides* strains. Antagonist candidates: *Ba42DD Bjerkandera adusta*, *Pj46SD, PJ81SR Penicillium janthinellum*, *Sc40DD Schizophyllum commune*, *Tc28DD Trametes cubensis*, *Ta39KR Trichoderma asperelloides*, *Ta31KR Trichoderma asperellum*, *Th20SR Th24KD, Th33SR, Th36SR Trichoderma harzianum*, *Ty34KD Trichoderma yunnanense*

Table 3 Interaction scores and Index of Dominance (I_D) of seven mycotoxigenic fungal strains co-cultivated with antagonist candidates isolated from grain maize farms in Terengganu, Malaysia

Antagonist candidate	Mycotoxigenic fungal strain							I_D
	Af6KR	Af7KR	An1KD	An3KD	Fp9DD	Fv21KD	Fv92SR	
Ba42DD	4/0	4/0	1/1	1/1	4/0	4/0	4/0	22/2
Pj46SD	1/1	2/2	2/2	2/2	2/2	2/2	2/2	13/13
Pj81SR	1/1	2/2	2/2	2/2	2/2	2/2	2/2	13/13
Sc40DD	1/1	1/1	1/1	1/1	1/1	1/1	4/0	10/6
Tc28DD	4/0	4/0	1/1	1/1	4/0	4/0	4/0	22/2
Ta39KR	4/0	4/0	4/0	4/0	4/0	4/0	4/0	**28/0**
Ta31KR	4/0	4/0	4/0	4/0	4/0	4/0	4/0	**28/0**
Th20SR	4/0	4/0	4/0	4/0	4/0	4/0	4/0	**28/0**
Th24KD	4/0	4/0	4/0	4/0	4/0	4/0	4/0	**28/0**
Th33SR	4/0	4/0	4/0	4/0	4/0	4/0	4/0	**28/0**
Th36SR	4/0	4/0	4/0	4/0	4/0	4/0	4/0	**28/0**
Ty34KD	4/0	4/0	4/0	4/0	4/0	4/0	4/0	**28/0**

Bold I_D values indicate the highest I_D scores exhibited by the antagonist candidates

Mycotoxigenic fungal strains: *Af6KR, Af7KR Aspergillus flavus* strains, *An1KD, An3KD Aspergillus niger* strains, *Fp9DD Fusarium proliferatum* strain, *Fv21KD, Fv92SR Fusarium verticillioides* strains. Antagonist candidates: *Ba42DD Bjerkandera adusta, Pj46SD, PJ81SR Penicillium janthinellum, Sc40DD Schizophyllum commune, Tc28DD Trametes cubensis, Ta39KR Trichoderma asperelloides, Ta31KR Trichoderma asperellum, Th20SR, Th24KD, Th33SR, Th36SR Trichoderma harzianum, Ty34KD Trichoderma yunnanense*

mycotoxigenic species "*at a distance*" which could mean that the possible mechanism of antagonism is the production of antifungal compounds by the antagonist that could inhibit the pathogenic growth. Taken together (*i.e.*, cumulative of different interaction scores from similar antagonist against mycotoxigenic species), these interaction scores provide the Index of Dominance (I_D) in which the higher the I_D the higher the antagonism potential of an antagonist candidate. Overall, *Trichoderma* spp. exhibited the highest I_D, while *S. commune* Sc40DD exhibited the lowest I_D. The antagonist candidates *Trichoderma* spp. against all tested mycotoxigenic fungal strains, *B. adusta* Ba42DD against *A. flavus*, *F. proliferatum*, and *F. verticillioides* strains, and *Tra. cubensis* Tc28DD against *A. flavus*, *F. proliferatum*, and *F. verticillioides* strains all showed strong antagonism upon contact (4 / 0), while *S. commune* Sc40DD was only able to strongly antagonise one mycotoxigenic fungal strain (*F. verticillioides* Fv92SR). Other antagonist candidates were less aggressive upon contact (< 2 mm zone of inhibition; *P. janthinellum* Pj46SD and Pj81SR), or did not show antagonism at all (1 / 1). None of the antagonist candidates could inhibit mycotoxigenic fungal strains at a distance (5 / 0).

Multi-mycotoxin reduction of mycotoxigenic fungal strains co-cultivated with antagonist candidates in dual-culture assay

Following the dual-culture assay, all antagonist candidates were tested for their ability to reduce mycotoxins produced by each of the tested mycotoxigenic fungal strains using HPLC.

Overall, all antagonist candidates did not show consistent pattern of mycotoxin reduction. Although most of the antagonist candidates could reduce the tested mycotoxins, some candidates could only reduce the mycotoxin in moderate amounts, while some even induced / stimulated mycotoxin production, which is a poor trait for a potential BCA. Therefore, the best antagonist candidates were selected based on their ability to reduce the highest amount of individual mycotoxin produced by all the tested mycotoxigenic fungal strains. For example, the best antagonist candidate against two tested aflatoxigenic *A. flavus* strains should be able to reduce AFB_1 in both strains, instead of in only one strain. Figure 1 shows the AFB_1 production (ng/g) by two aflatoxigenic *A. flavus* strains co-cultivated with antagonist candidates isolated from the grain maize farms in Terengganu, Malaysia. No significant difference was observed between the productions of AFB_1 by Af6KR co-cultivated with all antagonists against control. Nevertheless, several antagonist candidates could reduce the AFB_1 production by strain Af6KR by more than 50% (Supplementary 4). Against *A. flavus* strain Af7KR, most antagonists could significantly reduce the AFB_1 production by approximately 100%. For both tested aflatoxigenic *A. flavus* strains, three antagonist candidates that could reduce the highest amount of AFB_1 were *P. janthinellum* Pj46SD (median; 50.6 and 9.6 ng/g), *Tra. cubensis* Tc28DD (median; 72 and 0 ng/g), and *B. adusta* Ba42DD (median; 115.1 and 0 ng/g) by approximately 75% and 89%, 50% and 100%, and 24% and 100% (Supplementary 4), respectively. In contrast, *S. commune* Sc40DD and almost all *Trichoderma* spp. stimulated AFB_1 production by the *A. flavus* strains.

Table 4 In vitro fungal interaction as explained by the interaction score of co-cultivated antagonists (right) and mycotoxigenic strains (left) on 3% grain maize agar and isolated from grain maize farms in Terengganu, Malaysia

Mycotoxigenic strain (Control)	Interaction score		
	1/1 Antagonist	**2/2** Antagonist	**4/0** Antagonist
Aspergillus flavus	Sc40DD	Pj46SD	Th24KD
A. niger	Sc40DD	Pj81SR	Ty34KD
Fusarium proliferatum	Sc40DD	Pj81SR	Ta39KR
Fusarium verticillioides	Sc40DD	Pj46SD	Tc28DD

Antagonist candidates: *Pj46SD, PJ81SR Penicillium janthinellum, Sc40DD Schizophyllum commune, Tc28DD Trametes cubensis, Ta39KR Trichoderma asperelloides, Th24KD Trichoderma harzianum, Ty34KD Trichoderma yunnanense*

Figure 2 shows the OTA production (ng/g) by two *A. niger* strains co-cultivated with antagonist candidates isolated from the grain maize farms in Terengganu, Malaysia. Similarly, no significant difference was observed between OTA productions of all co-cultivated *A. niger* An1KD with antagonists, against control. Nevertheless, six antagonist strains could reduce OTA production by An1KD at > 80% (Supplementary 4). In contrast, OTA production by *A. niger* strain An3KD was significantly reduced by five *Trichoderma* spp. The three best antagonist candidates that could reduce the highest amount of OTA were *Tri. harzianum* Th24KD (median; 83.2 and 0 ng/g), *Tri. harzianum* Th33SR (median; 0 and 356.8 ng/g), and *Trichoderma asperellum* Ta31KR (median; 130.3 and 53.7 ng/g) by approximately 98% and 100%, 100% and 95%, and 97% and 98% (Supplementary

4), respectively. In contrast, the basidiomycete *B. adusta* Ba42DD, *S. commune* Sc40DD, and *Tra. cubensis* Tc28DD stimulated OTA production by the *A. niger* strains.

Figures 3 and 4 show the FB_1 and FB_2 production (μg/g) by two *F. verticillioides* strains co-cultivated with antagonist candidates isolated from the grain maize farms in Terengganu, Malaysia. Against *F. verticillioides* strain Fv21KD, three *Tri. harzianum* strains could significantly reduce FB_1 and FB_2 production. These antagonists were *Tri. harzianum* strain Th20SR that could significantly reduce both FB_1 and FB_2, strain Th24KD that could significantly reduce FB_1, and strain Th33SR that could significantly reduce FB_2. Besides, the FB_1 concentration produced by Fv21KD against *Tri. harzianum* Th20SR and Th24KD were significantly lower than those against the antagonists *P. janthinellum* strains
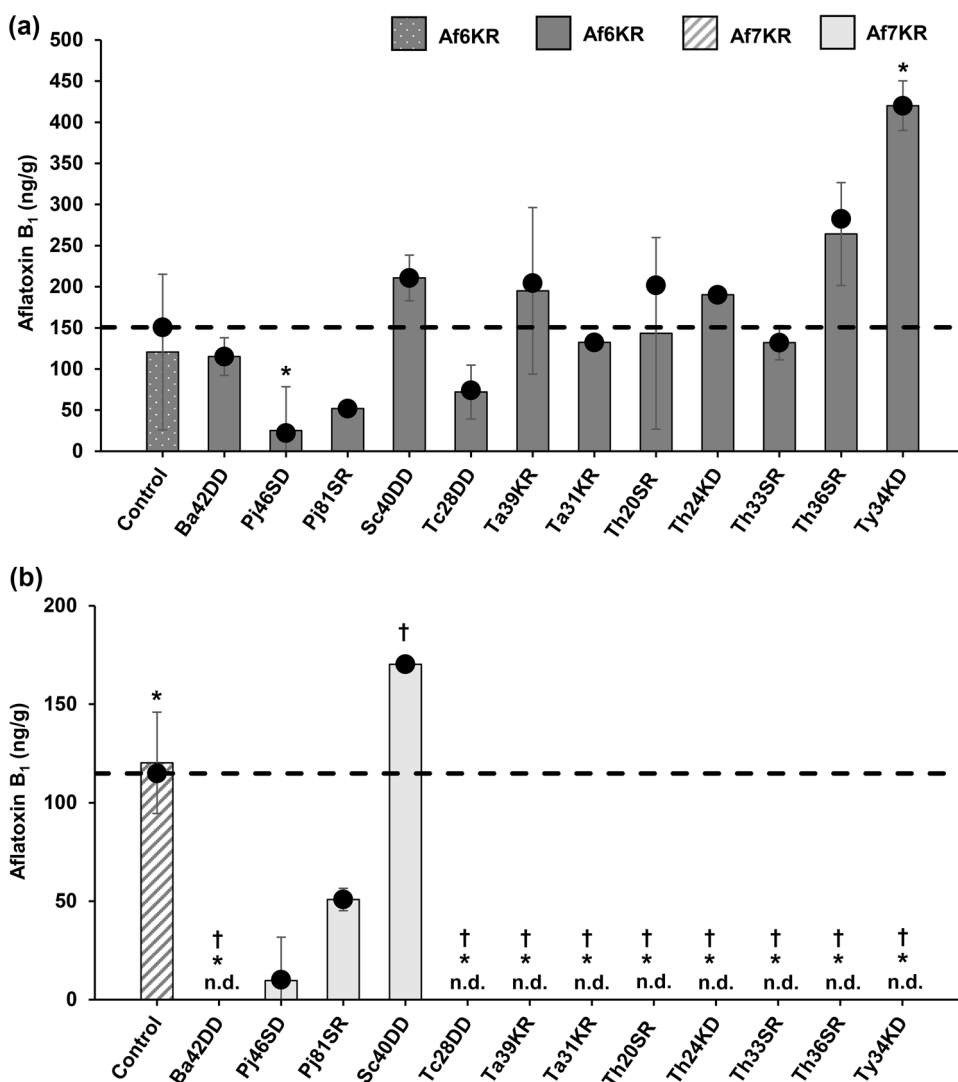
Fig. 1 Aflatoxin B$_1$ (AFB$_1$; ng/g) produced by *Aspergillus flavus* strain (**a**) Af6KR and (**b**) Af7KR co-cultivated with antagonist candidates isolated from grain maize farms in Terengganu, Malaysia. Data are median $\pm$ IQR. Black circles indicate the means of AFB$_1$ concentration of both control (mycotoxigenic strain) and treatments. Dashed line is the cut-off point (based on the control value) by which AFB$_1$ was considered as inhibited (black circles below line) or stimulated (black circles above line) by the antagonists. Asterisk (*) and dagger (†) indicate statistically significance at a corrected *p*-value < 0.05 following *post-hoc* Dunn's multiple comparisons with Benjamini–Hochberg FDR correction. Symbol for bars in (**a**) – *statistically significant between each other. Symbol for bars in (**b**) – *statistically significant as compared to control, †statistically significant as compared to Sc40DD. Ba42DD – *Bjerkandera adusta*; Pj46SD, PJ81SR – *Penicillium janthinellum*; Sc40DD – *Schizophyllum commune*; Tc28DD – *Trametes cubensis*; Ta39KR – *Trichoderma asperelloides*; Ta31KR – *Trichoderma asperellum*; Th20SR, Th24KD, Th33SR, Th36SR – *Trichoderma harzianum*; Ty34KD – *Trichoderma yunnanense*. n.d. – not detected

and *Tra. cubensis* Tc28DD. Against *F. verticillioides* strain Fv92SR, almost all antagonists could significantly reduce the FB$_2$ production. However, no significant difference was observed between the FB$_1$ productions by Fv92SR control strain with all other strains co-cultivated with antagonist candidates. Nevertheless, these antagonists could reduce FB$_2$ production by Fv92SR to approximately 86% (Supplementary 4). *Tri. asperellum* Ta31KR, *Tri. harzianum* Th36SR, and *Trichoderma asperelloides* Ta39KR could reduce the highest amount of FB$_1$ production (median; 278.1 and 59.4; 166.2 and 81.2; 317.5 and 97 µg/g, respectively) by *F. verticillioides* by approximately 94% and 86%, 86% and 81%, and 94% and 78%, respectively. *Tri. harzianum* Th20SR, Th33SR, and Th24KD could reduce FB$_2$ production by *F. verticillioides* at almost 100% (median; 3.8 and 0; 3.8 and 0; 5.9 and 0 µg/g, respectively). In contrast, *Trichoderma yunnanense* Ty34KD stimulated FB$_1$ production by the *F. verticillioides*.
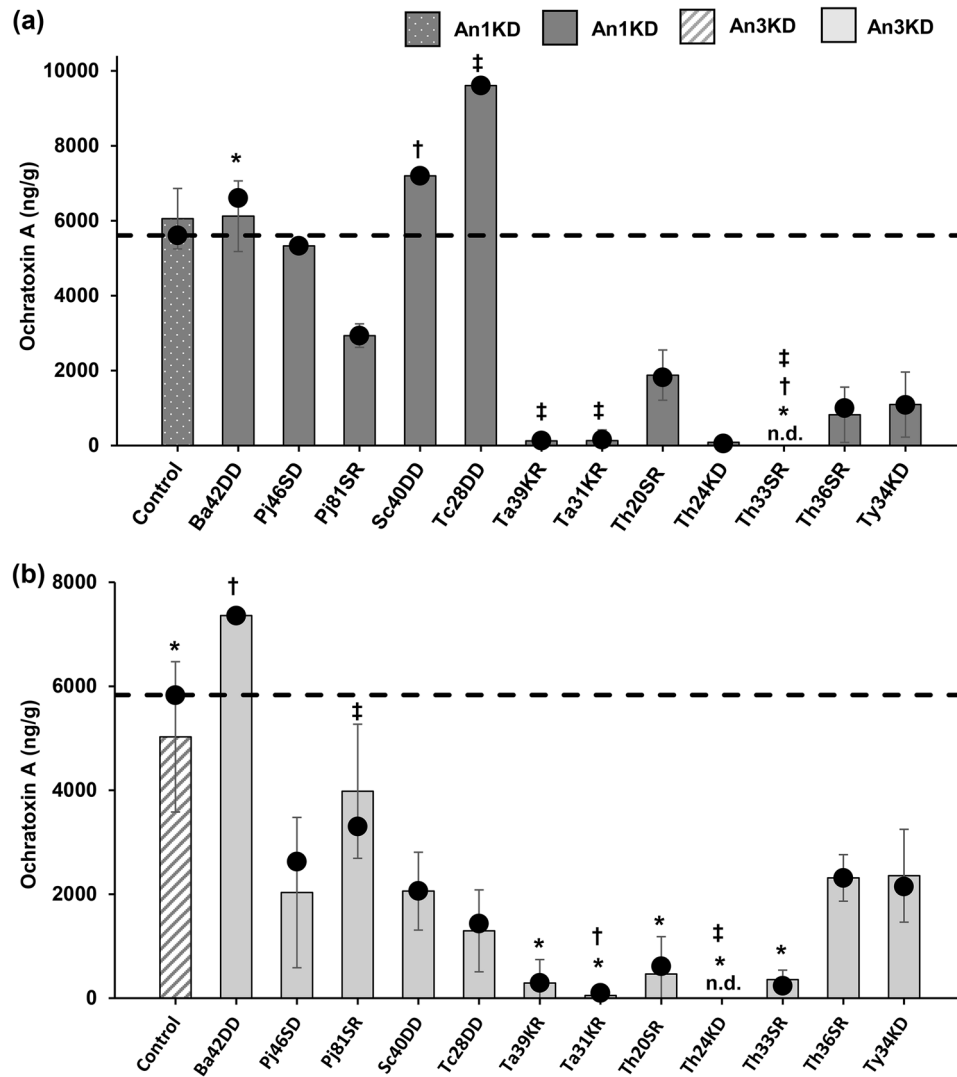
Fig. 2 Ochratoxin A (OTA; ng/g) produced by *Aspergillus niger* strain (**a**) An1KD and (**b**) An3KD co-cultivated with antagonist candidates isolated from grain maize farms in Terengganu, Malaysia. Data are median ± IQR. Black circles indicate the means of OTA concentration of both control (mycotoxigenic strain) and treatments. Dashed line is the cut-off point (based on the control value) by which OTA is considered as inhibited (black circles below line) or stimulated (black circles above line) by the antagonists. Asterisk (*), dagger (†), and diesis (‡) indicate statistically significance at a corrected p-value < 0.05 following post hoc Dunn's multiple comparisons with Benjamini–Hochberg FDR correc-

tion. Symbol for bars in (**a**) – *statistically significant between each other, †statistically significant between each other, ‡statistically significant as compared to Tc28DD. Symbol for bars in (**b**) – *statistically significant as compared to control, †statistically significant between each other, ‡statistically significant between each other. Ba42DD – *Bjerkandera adusta*; Pj46SD, PJ81SR – *Penicillium janthinellum*; Sc40DD – *Schizophyllum commune*; Tc28DD – *Trametes cubensis*; Ta39KR – *Trichoderma asperelloides*; Ta31KR – *Trichoderma asperellum*; Th20SR, Th24KD, Th33SR, Th36SR – *Trichoderma harzianum*; Ty34KD – *Trichoderma yunnanense*. n.d. – not detected

Figure 5 shows the FB$_1$ and FB$_2$ production (µg/g) by *F. proliferatum* co-cultivated with antagonist candidates isolated from the grain maize farms in Terengganu, Malaysia. Four *Trichoderma* spp. could significantly reduce FB$_1$ and FB$_2$ produced by the mycotoxigenic strain. These antagonists were *Tri. harzianum* Th20SR that could reduce both FB$_1$ and FB$_2$, *Tri. asperelloides* Ta39KR that could reduce the FB$_1$ production, and *Tri. harzianum* strain Th24KD and Th36SR that could reduce the FB$_2$ production. *Tri. harzianum* Th20SR

could also significantly reduce the FB$_2$ production *by F. proliferatum* as compared to *S. commune* Sc40DD. The fungal antagonists that reduced FB$_1$ produced by *F. proliferatum* in high amount were *Tri. asperelloides* Ta39KR (median; 244.2 µg/g), *Tri. harzianum* Th20SR (median; 314.2 µg/g), and Th36SR (median; 337.9 µg/g), by approximately 94%, 94%, and 92%. The antagonist candidates that could reduce the FB$_2$ in high amount were *Tri. harzianum* Th20SR (median; 0 µg/g), Th36SR (median; 0.9 µg/g), and Th24KD (median; 1 µg/g), by
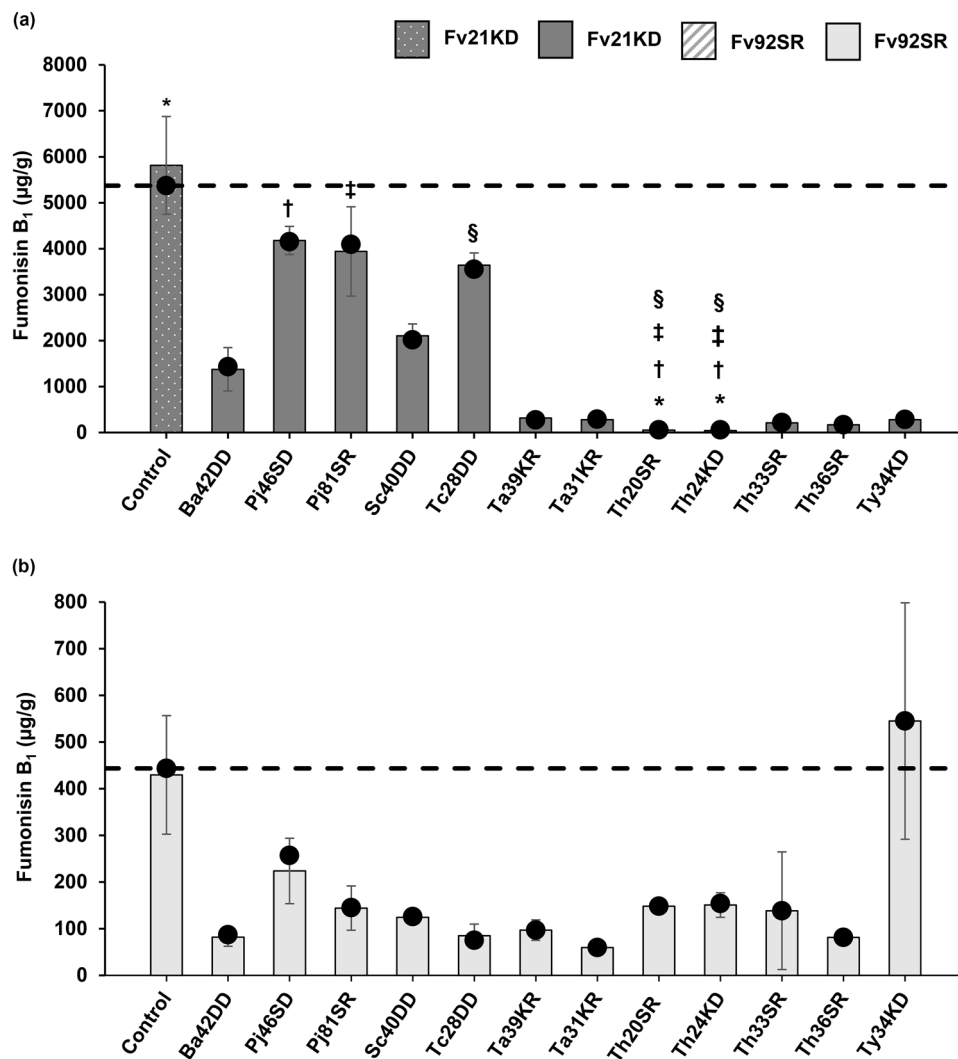
Fig. 3 Fumonisin B$_1$ (FB$_1$; μg/g) produced by *Fusarium verticillioides* strain (**a**) Fv21KD and (**b**) Fv92SR co-cultivated with antagonist candidates isolated from grain maize farms in Terengganu, Malaysia. Data are median ± IQR. Black circles indicate the means of FB$_1$ concentration of both control (mycotoxigenic strain) and treatments. Dashed line is the cut-off point (based on the control value) by which FB$_1$ is considered as inhibited (black circles below line) or stimulated (black circles above line) by the antagonists. Asterisk (*), dagger (†), diesis (‡), and silcrow (§) indicate statistically significance at a corrected *p*-value < 0.05 following post hoc Dunn's multiple comparisons with Benjamini–Hochberg FDR correction. Symbol for bars in (**a**) – *statistically significant as compared to control, †statistically significant as compared to Pj46SD, ‡statistically significant as compared to Pj81SR, §statistically significant as compared to Tc28DD. Bars in (**b**) are not statistically significant to each other. Ba42DD – *Bjerkandera adusta*; Pj46SD, PJ81SR – *Penicillium janthinellum*; Sc40DD – *Schizophyllum commune*; Tc28DD – *Trametes cubensis*; Ta39KR – *Trichoderma asperelloides*; Ta31KR – *Trichoderma asperellum*; Th20SR, Th24KD, Th33SR, Th36SR – *Trichoderma harzianum*; Ty34KD – *Trichoderma yunnanense*

approximately 100%, 99%, and 99%, respectively (Supplementary 4). In contrast, *B. adusta* Ba42DD stimulated the fumonisins production by *F. proliferatum*.

Discussion

In the present work, fungal isolates indigenous to maize agro-ecosystems (grain maize farms in Terengganu, Malaysia) that were not pathogenic and mycotoxigenic were screened for their potentials to inhibit mycotoxigenic fungi and reduce mycotoxin production in vitro using the dual-culture assay on grain maize agar. In developing a potential BCA for commercialisation, these selection criteria are of utmost importance, other than the economic value of the crop on which the BCA is to be applied (Köhl et al. 2011). For this approach, a Malaysian guideline, that is, the "Guidelines for Biopesticide Registration" GP 7/2016 (data requirements for microbial registration) (DOA 2016), was used as the basis for the selection of suitable antagonists.
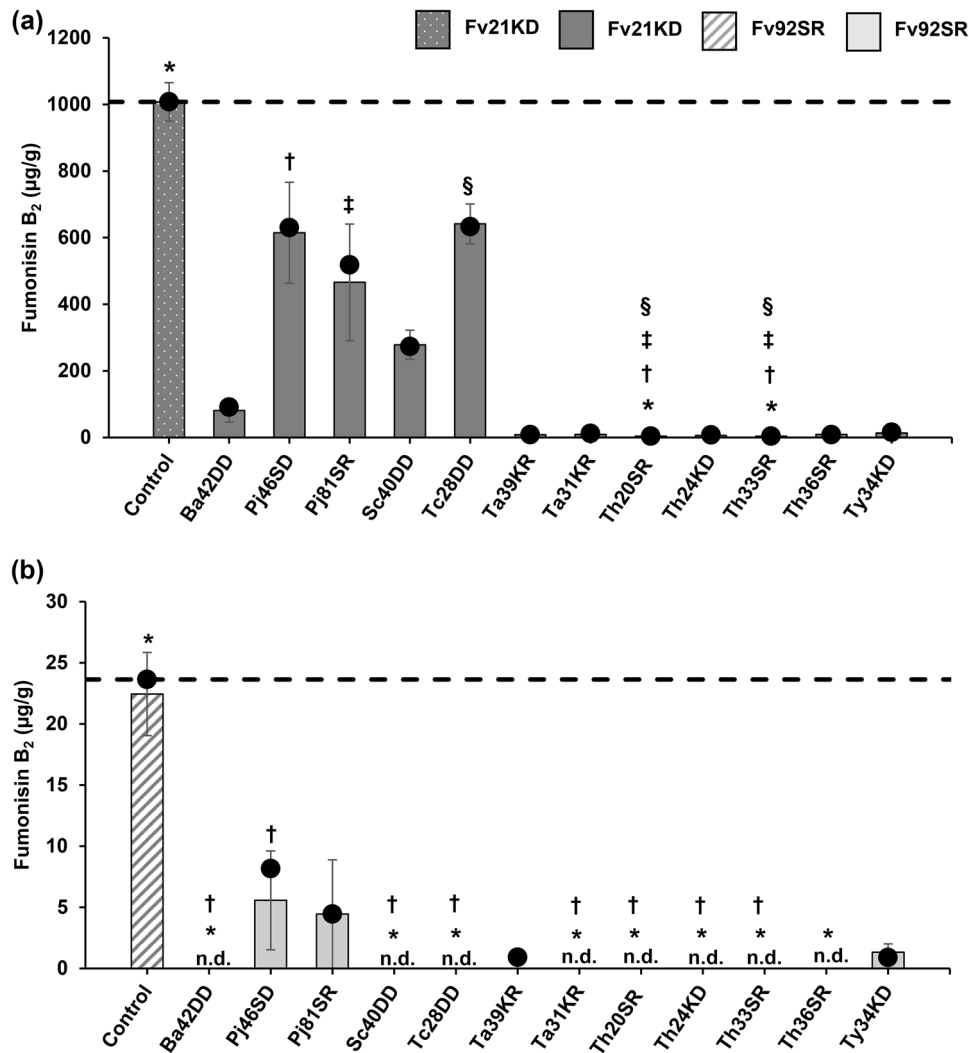
Fig. 4 Fumonisin B_2 (FB_2; µg/g) produced by *Fusarium verticillioides* strain (**a**) Fv21KD and (**b**) Fv92SR co-cultivated with antagonist candidates isolated from grain maize farms in Terengganu, Malaysia. Data are median±IQR. Black circles indicate the means of FB_2 concentration of both control (mycotoxigenic strain) and treatments. Dashed line is the cut-off point (based on the control value) by which FB_2 is considered as inhibited (black circles below line) or stimulated (black circles above line) by the antagonists. Asterisk (*), dagger (†), diesis (‡), and silcrow (§) indicate statistically significance at a corrected *p*-value<0.05 following post hoc Dunn's multiple comparisons with Benjamini–Hochberg

FDR correction. Symbol for bars in (**a**) – *statistically significant as compared to control, †statistically significant as compared to Pj46SD, ‡statistically significant as compared to Pj81SR, §statistically significant as compared to Tc28DD. Symbol for bars in (**b**) – *statistically significant as compared to control, †statistically significant as compared to Pj46SD. Ba42DD – *Bjerkandera adusta*; Pj46SD, PJ81SR – *Penicillium janthinellum*; Sc40DD – *Schizophyllum commune*; Tc28DD – *Trametes cubensis*; Ta39KR – *Trichoderma asperelloides*; Ta31KR – *Trichoderma asperellum*; Th20SR, Th24KD, Th33SR, Th36SR – *Trichoderma harzianum*; Ty34KD – *Trichoderma yunnanense*. n.d. – not detected

The guideline was promulgated in reference to international guidelines such as those prescribed by the Food and Agricultural Organisation (FAO 2012) "Guidance for Harmonising Pesticide Regulatory Management in Southeast Asia".

During the in silico screening, it was found that some fungal isolates assessed in the present work might behave as pathogens to plants, humans, or animals, but also exhibit beneficial characteristics that could be exploited by the industry (*e.g.*, production of degrading enzyme for organic pollutant, or synthesis of silver nanoparticles for biotechnological

applications; Arun et al. 2014; Kang et al. 2019). This is especially the case for the basidiomycete strains *B. adusta* and *S. commune* which are wood-rot fungi, and have been implicated with systemic mycoses such as allergic reaction in humans (Chowdhary et al. 2014). In fact, it is not uncommon for certain fungi to exhibit dual-nature of existence; both beneficial and detrimental. Despite the pathogenic nature of these species, some publications have also reported their efficacy as BCA either in vitro (Naidu et al. 2016; Choo et al. 2021; Burnevica et al. 2022) or *in planta* (Feng et al.
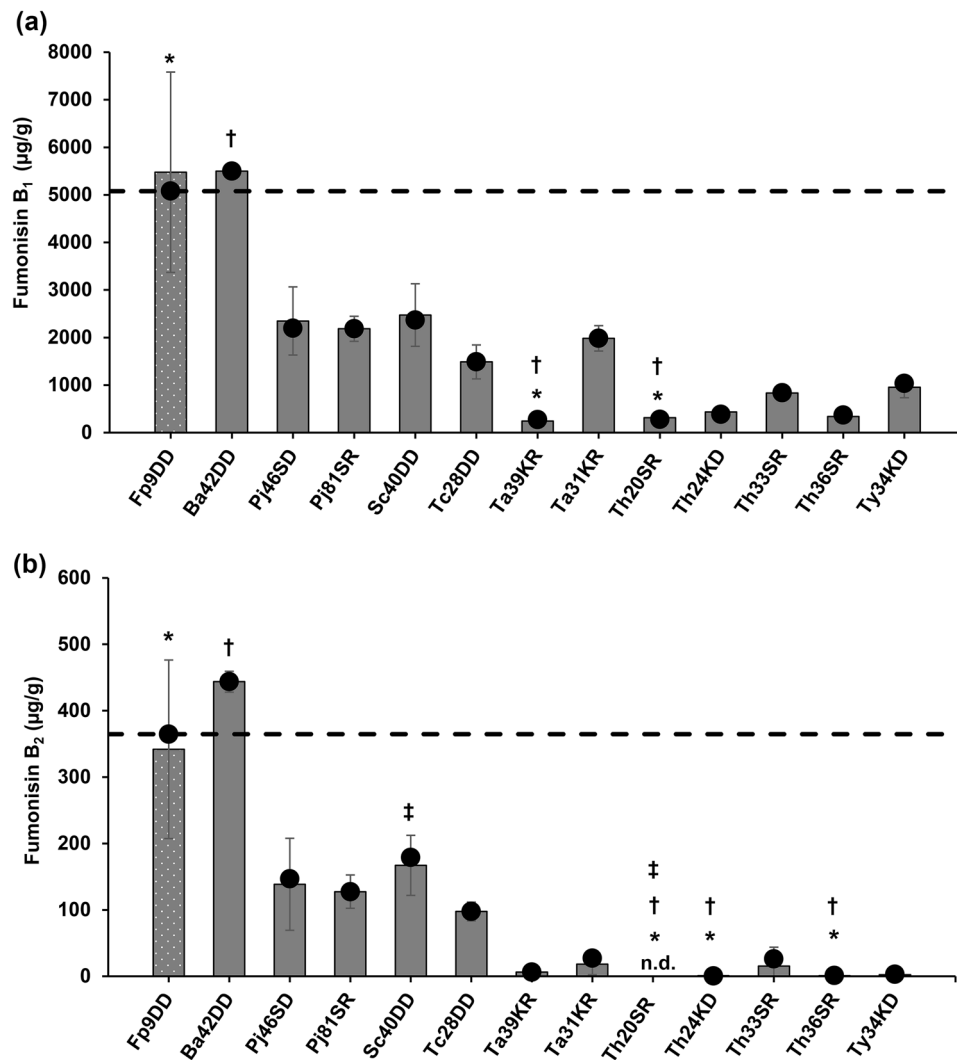
Fig. 5 **a** Fumonisin B_1 (FB$_1$ µg/g) and (**b**) fumonisin B_2 (FB$_2$; µg/g) produced by *Fusarium proliferatum* strain Fp9DD co-cultivated with antagonist candidates isolated from grain maize farms in Terengganu, Malaysia. Data are median $\pm$ IQR. Black circles indicate the means of FB$_1$ and FB$_2$ concentration of both control (mycotoxigenic strain) and treatments. Dashed line is the cut-off point (based on the control value) by which FB$_1$ and FB$_2$ are considered as inhibited (black circles below line) or stimulated (black circles above line) by the antagonists. Asterisk (*), dagger (†), and diesis (‡) indicate statistically significance at a corrected *p*-value < 0.05 following post hoc Dunn's multiple comparisons with Benjamini–Hochberg FDR correction. Symbol for bars in (**a**) – *statistically significant as compared to control, †statistically significant as compared to Ba42DD. Symbol for bars in (**b**) – *statistically significant as compared to control, †statistically significant as compared to Ba42DD, ‡statistically significant between each other. Ba42DD – *Bjerkandera adusta*; Pj46SD, PJ81SR – *Penicillium janthinellum*; Sc40DD – *Schizophyllum commune*; Tc28DD – *Trametes cubensis*; Ta39KR – *Trichoderma asperelloides*; Ta31KR – *Trichoderma asperellum*; Th20SR, Th24KD, Th33SR, Th36SR – *Trichoderma harzianum*; Ty34KD – *Trichoderma yunnanense*. n.d. – not detected

2021; Chen et al. 2022). Hence, we deduced that both species may behave as pathogen to a narrow host range, and that their pathogenicity could be minimised by good agricultural practices (GAP). If their advantages as a strong BCA could outweigh their disadvantages as a pathogen, their elimination in the earlier antagonist screening will hinder the development of novel and effective BCA against mycotoxigenic fungal species. Of course, one of the strictest criteria for the development of effective BCA against mycotoxigenic fungal

species in maize agro-ecosystems was that no fungal isolates that have been proven as maize pathogen (of which *B. adusta* and *S. commune* are not) or mycotoxigenic species should be included in further screening by the dual-culture assay.

Based on the growth inhibition data, *Trichoderma* spp. could serve as excellent antagonist candidates. Of these, the antagonism potential of *Tri. harzianum* strains was the highest. Besides *Trichoderma* spp., *B. adusta* and *Tra. cubensis* could also serve as excellent antagonist candidates,

particularly against *A. flavus*, *F. verticillioides* and *F. proliferatum*. In terms of mycotoxin reduction, varying patterns were observed. However, *Tri. asperelloides* was observed to reduce OTA and both fumonisins by at least > 70% (Supplementary 4), thus showing the ability of a single antagonist candidate against a broad range of mycotoxins. To the best of our knowledge, no report is available on the antagonism potential of *Tri. asperelloides* against *A. niger*, *F. verticillioides*, *F. proliferatum*, OTA, and fumonisins. For the other *Trichoderma* isolates, the antagonism potentials of *Tri. yunnanense* against mycotoxigenic fungal growth and mycotoxin production was also not reported elsewhere. In general, the data on the antagonism potentials against mycotoxigenic fungal species and mycotoxin production by non-*Trichoderma* isolates such as *P. janthinellum* and *Tra. cubensis* demonstrated in the present work, are largely unknown. Therefore, these isolates could become novel antagonist candidates that may be exploited in the development of effective BCA against mycotoxigenic species and mycotoxin production.

Our results were consistent with other studies that examined the efficacy of fungal antagonists against mycotoxigenic fungal growth and the subsequent mycotoxin. For example, *B. adusta* has been reported as a good fungal agent that could reduce AFB_1 in vitro by approximately 100%. The mycotoxin removal was attributed to the binding on cell wall and cell lysates along with exopolysaccharides of *B. adusta* (Choo et al. 2021). Besides, the efficacy of *Trichoderma* spp. had also been documented. Gajera and Vakharia (2012) reported high inhibition of *Tri. harzianum* against *A. niger* growth on PDA, while, Valero et al. (2006) reported high inhibition of *Tri. harzianum* against OTA of the sister species, *A. carbonarius*, on synthetic nutrient medium. *Tri. asperellum*, *Tri. harzianum*, and *Tri. viride* were also effective against the growth of *A. niger* in onion bulb (Prajapati and Patil 2017). For *Fusarium* spp., *Tri. asperellum* could reduce *F. verticillioides* growth by approximately 100% in vitro, and could also significantly reduce the average damage of fungal disease in maize plant (Cuervo-Parra et al. 2022). Meanwhile, *Tri. asperellum* and *Tri harzianum* could reduce FB_1 and FB_2 accumulation in vitro by > 60% and > 80%, respectively (Tian et al. 2020). *T. harzianum* has been reported to induce growth reduction of *F. proliferatum* by approximately 30% on meal maize agar, as well as almost total reduction of FB_1 on both maize agar and maize kernel (Rojo et al. 2007).

Trichoderma spp. are well-established as BCA against phytopathogens. They are mycoparasitic fungi that could produce a cascade of extracellular proteins and cell wall-degrading enzymes as antagonism mechanisms against pathogens. *Tri. harzianum* could parasitise *A. flavus* by forming coils around the latter's hyphae, followed by degradation and lysis of the latter's hyphae (Kifle et al. 2017). Similar mechanism was also reported for *Trichoderma* spp. against *A. niger* (Gajera and Vakharia 2012), *F. verticillioides*, and *F. proliferatum* (Yassin et al. 2021). Besides, *Tri. harzianum* could also produce volatile inhibitory compounds and/or compete for nutrients (glucose) during the interaction with *F. verticillioides*. The mechanism of action of the basidiomycete *B. adusta* against pathogens is mycoparasitism and the production of enzymes such as catalase, superoxide dismutase, and peroxidase (Feng et al. 2021). *S. commune* is also mycoparasitic, and could produce the enzyme laccase (Puig and Cumagun 2019; Takemoto et al. 2010). Since very little is known on *Tra. cubensis* as BCA, the knowledge on its antagonism characteristic is, therefore, still new, and is open for BCA development opportunity.

Despite the excellent pathogenic growth inhibition and mycotoxin reduction exhibited by *Trichoderma* spp., *B. adusta*, and *Tra. cubensis*, certain tested antagonist candidates could also induce / stimulate mycotoxin production in the present work. The stimulation of mycotoxins following the co-cultivation with antagonist candidates, although undesirable, is a normal defence response exerted by the mycotoxigenic fungal species as a result of stress and competition for niche and/or nutrient. According to Künzler (2018), chemical defence, such as the production of chemical metabolites, is the main defence strategy of fungi. Therefore, it is assumed that the aflatoxin, OTA, and fumonisin stimulation upon competition with the antagonist candidates (*B. adusta*, *Trichoderma* spp., *Tra. cubensis*, *S. commune*) observed in the present work is the result of their defence mechanism. The mechanisms upon which defence occurs might be explained by several hypotheses: (1) physical contact between the mycotoxigenic species and the antagonist (Schroeckh et al. 2009) (2) as a reaction to the mycoparasitic behaviour of antagonist (Pellan et al. 2021) and/or (3) the induction of transcription factor of genes in biosynthetic pathways of mycotoxins (Yin et al. 2012; Künzler 2018).

In conclusion, *Trichoderma* spp. exhibited high antagonistic activity against the growth of all the tested mycotoxigenic fungal strains in the present work. Besides, *B. adusta* and *Tra. cubensis* could also inhibit the mycotoxigenic fungal growth to a certain extent. High AFB_1 reduction was shown by *P. janthinellum*, *Tra. cubensis*, and *B. adusta*. OTA was highly reduced by *Tri. harzianum* and *Tri. asperellum*. FB_1 and FB_2 produced by *F. verticillioides* was highly reduced by *Tri. harzianum*, *Tri. asperelloides*, and *Tri. asperellum*. FB_1 and FB_2 produced by *F. proliferatum* was highly reduced by *Tri. harzianum* and *Tri. asperelloides*. These antagonist candidates that exhibited excellent antagonism against fungal growth and mycotoxin production in vitro in the present work will be further tested *in planta* / in situ prior to their mass production and commercial application in maize agro-ecosystems.

Declarations

Conflict of interest None.

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