JARQ 58 (2), 83-91 (2024) https://www.jircas.go.jp

Occurrence Evaluation of Aflatoxigenic *Aspergilli* in Thai Corn Using Dichlorvos-ammonia and Whole-agar Extraction Methods

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Abstract

Aflatoxins and aflatoxigenic fungi are hazardous to food security and safety since mycotoxins and related fungi in cereals significantly affect animal and human health. The relatively high frequency of aflatoxigenic *Aspergilli* isolates in corn samples remains a concern. Accordingly, we randomly collected corn samples from 10 farms in northern and central Thailand (TM1-TM10) and aimed to detect aflatoxigenic fungi using our recently developed methods: dichlorvos-ammonia (DV-AM) and whole-agar extraction methods. When we placed 100 grains from each sample on 20 agar dish cultures (five grains per dish) to monitor the emergence of fungal colonies, the presence of *Aspergillus niger* and *A. flavus,* with an emergence frequency of 1-8 and 1-7 per 100 grains, respectively, was detected. Some isolates of *A. flavus* produced aflatoxin B_1 and B_2 in the culture media, indicating typical features of aflatoxigenic *A. flavus*, whereas the non-aflatoxin-producing isolates produced kojic acid, thereby suggesting that they belong to *Aspergillus* section *Flavi*. Chemical analysis revealed aflatoxin B_1 and B_2 contamination in some grains and sporadic contamination with fumonisin B_1. Therefore, continuous monitoring and surveillance are required owing to the prevalence of mycotoxigenic fungi in corn.

Discipline: Food
Additional key words: aflatoxin, *Aspergillus*, colony, grain, section *Flavi*

Introduction

Mycotoxins are toxic low-molecular-weight natural compounds produced by filamentous fungi. Among mycotoxins, aflatoxins (AFs) are the most notorious causative compounds of mass turkey mortality in the U.K. in the 1960s (Richard 2008). Given their robust toxicity, regulatory limits have been set in many countries for four major AFs: aflatoxin B_1 (AFB1), aflatoxin B_2 (AFB2), aflatoxin G_1 (AFG1), and aflatoxin G_2 (AFG2), or for AFB1 alone (Bennett & Klich 2003). Contamination of agricultural commodities by AFs severely threatens food safety and global trade.

AFs are produced as secondary metabolites by over ten aflatoxigenic species of the fungal genus *Aspergillus*, included in section *Flavi* (Frisvad et al. 2019). Aflatoxigenic *Aspergillus* fungi in the environment, such as field soils, contaminate agricultural products (Pfliegler et al. 2019). Although the presence of aflatoxigenic fungi does not directly indicate AFs contamination of food, it suggests a potential hazard. Aflatoxigenic *Aspergillus* is prevalent globally, notably in tropical and subtropical

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Received 9 May 2023; accepted 22 August 2023.

areas with favorable climatic conditions for fungal propagation and AFs production. Manabe & Tsuruta reported that the isolation frequency of aflatoxigenic fungi in soils of Thailand and Japan, similar to several other countries, increased in the order from subtropical to tropical zone (Manabe & Tsuruta 1978).

Various culture methods have been developed to visually select aflatoxigenic fungal colonies. These methods include dichloran rose-bengal chloramphenicol, Aspergillus flavus and parasiticus agar (AFPA), and coconut cream agar (Khan et al. 2020). Among these, the most well-known method is the AFPA (Pitt et al. 1983). It detects the red color of aspergillic acid, commonly produced by *Aspergillus flavus* and *A. parasiticus*, the two major aflatoxigenic *Aspergilli* species. However, in Southeast Asia and Japan, several *A. oryzae* strains (atoxigenic *Aspergillus* species used to ferment "sake" or Japanese wine) also express red color for the AFPA method, thus hindering its use owing to false positives. Saito and Machida (1999) developed an ammonia (AM) vapor method that detects the red color of aflatoxigenic fungal colonies on AM exposure. This method was drastically improved by adding dichlorvos (DV), a selective pesticide for esterase enzymes involved in AFs biosynthesis (Yabe et al. 2015). The DV-AM method has been proven to be highly sensitive and selective for screening aflatoxigenic fungi in field soils (Kushiro et al. 2017, Kushiro et al. 2018a, Kushiro et al. 2018b, Kushiro et al. 2020). Another application of the AM method, the whole-agar extraction method, has also been proven effective in assessing the population of aflatoxigenic fungi in various soil samples (Kishimoto et al. 2023).

Corn is one of the most susceptible crops to the adhesion and invasion of aflatoxigenic *Aspergillus*. Naturally occurring aflatoxigenic fungi in field soils or storehouses cause AFs pollution in corn grains, resulting in frequent reports of slight to severe contamination. A previous collaborative study between Thailand and Japan, under Thailand Law B.E.2550 in the 1980s, identified the causative agent of AF contamination in Thai corn as *Aspergillus flavus*, present in soil samples all over Thailand (Saito & Tanaka, personal communication). Based on these results, the objectives of this study were: 1) to apply the recently developed DV-AM and whole-agar extraction methods to detect aflatoxigenic fungi on corn grains; 2) to compare the occurrence of aflatoxigenic fungi among areas and grades in Thailand; and 3) to analyze the relationship between the emergence of aflatoxigenic fungal colonies and AFs' contamination level on grains. In the first experiment, corn grain samples were randomly collected from the northern and central parts of Thailand and analyzed for the presence of aflatoxigenic fungi using the improved DV-AM method with DV-coated yeast extract-sucrose-deoxycholate-chloramphenicol (YES-DOC-CP) agar (Yabe et al. 2018). Owing to difficulties involved in DV usage in Japan, the second experiment included the whole-agar extraction method to assess the occurrence of aflatoxigenic fungi using DV-free YES-DOC-CP agar (Kishimoto et al. 2023). Chemical analysis of the major mycotoxins (AFs and fumonisin B_1 (FB1)) was also conducted to evaluate the relationship between AFs contamination and the occurrence of aflatoxigenic fungi.

Materials and methods

1. Samples and media

Dried corn grain samples (ca. 100 g) were randomly collected from ten farms (TM1-TM10) in northern and central Thailand (Fig. 1, Table 1) in November and December of 2020 and sent to Kasetsart University, Thailand. The samples were exported to Japan under plant quarantine and stored at room temperature until further analysis.

2. Measurement of water content

Water content was analyzed using an official Japanese method for water content measurement in grains. Water content was analyzed by drying the ground corn grains in an oven for 1 h at 135°C; the weight difference before and after drying was considered the water content. All measurements were conducted in triplicates.

3. DV-AM method

YES-DOC-CP agar medium (Yabe et al. 2018) was primarily used for fungal colony detection. Approximately 15 ml of YES-DOC-CP agar (2% yeast extract [YE], 10% sucrose [S], 0.1% sodium deoxycholate [DOC], 0.01% chloramphenicol [CP] (Fujifilm Wako Pure Chemical Co., Osaka, Japan), and 2% agar (Becton, Dickinson, and Company, NJ, USA) was poured into a plastic petri dish (φ 9 cm). After solidification for at least one day, 10 µL of 250-fold diluted dichlorvos [DV] (Fujifilm Wako Pure Chemical Co.) methanol solution was spread on the surface of each dish. Five randomly selected corn grains from TM1-TM10 were monitored to identify the emergence of fungal species. The grains were surface-sterilized with 70% ethanol for 30 s, rinsed three times with sterile water, placed on DV-coated YES-DOC-CP agar medium, and set in the dark at 25°C for 7 d. After 7 d of incubation, dishes were placed upside-down, and 200 µL of ammonium hydroxide (AM)

Fig. 1. Sampling areas for ten corn grain samples
TM1 and TM2: from northern part of Thailand (Phayao province; average annual temperature 25.4℃)
TM3 and TM4: from unknown
TM5 to TM10: from central part of Thailand (around Nakhon Sawan province; average annual temperature 27.8℃)

Table 1. Moisture content of tested corn grain samples

Sample ID	Grade[a]	Area	Moisture content (%)
TM1	Premium	North	11.5
TM2	Premium	North	12.1
TM3	Grade 1	unknown	11.7
TM4	Grade 1	unknown	11.5
TM5	Grade 1	Central	11.3
TM6	Grade 2	Central	11.3
TM7	Grade 2	Central	11.9
TM8	Grade 2	Central	11.5
TM9	Grade 2	Central	11.6
TM10	Grade 2	Central	11.1

[a] Judged by appearance: Premium (best), Grade 1 (second), Grade 2 (third). All grain samples are for commercial purposes.

solution (28%, reagent grade, Fujifilm Wako Pure Chemical Co.) was added inside the lid of each dish. Since AM vapor changed the color of the colonies of aflatoxigenic fungi from yellow to red (Fig. 2) (Kushiro, 2019), red colonies (aflatoxigenic candidates) were picked with a sterile toothpick within 10 min and inoculated onto a new GY (2% glucose, 0.5% yeast extract, and 2% agar) dish. These isolates were used to identify the fungal species. Others (non-aflatoxigenic candidates) were also isolated for fungal species identification.

4. Fungal species determination by genetic analysis

Isolated colonies on GY were exposed to AM vapor for >30 min to inactivate the fungi (Hess et al. 2006). Approximately 0.05 g of agar debris with fungal colony were scratched using toothpicks and transferred to

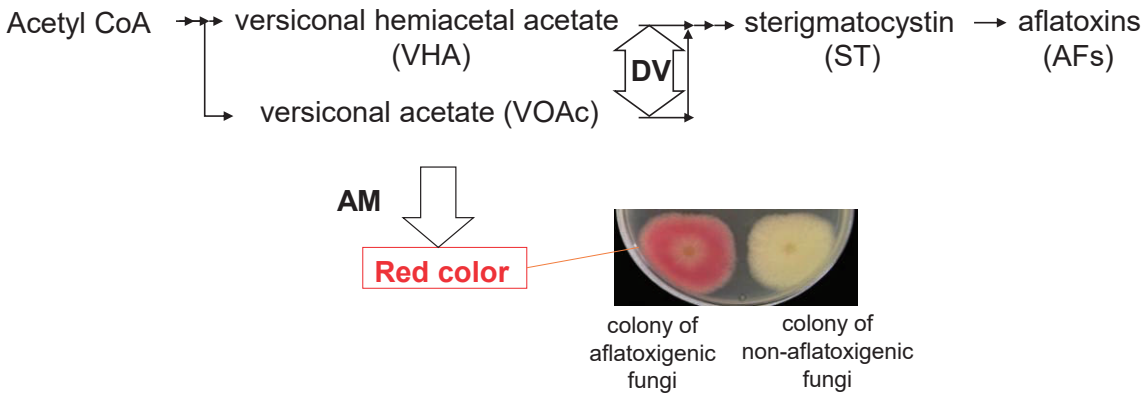

Fig. 2. Outline of DV-AM (dichlorvos-ammonia) method
DV inhibits esterase (thick arrows). The accumulation of VHA and VOAc (yellow-pigmented precursors of aflatoxigenic fungi) causes sensitive color change under alkaline conditions by AM exposure. Modified from Kushiro (2019).

microtubes. Genomic DNA was extracted using a DNA extraction kit (Kaneka Easy DNA extraction kit version 2; Kaneka Co., Hyogo, Japan) according to the manufacturer's instructions. The extracted DNA was quantified using Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). PCR was performed using primers for the calmodulin gene and ITS region (Table 2). The DNA sequences of the purified PCR products were obtained from Fasmac Co., Ltd. (Kanagawa, Japan), and then subjected to the database search. If the top species identified by the calmodulin gene primers were the same as those identified by the ITS region primers, the top-hit species was considered the identified fungus.

5. AF production analysis of isolates

Approximately 0.1 g of agar debris with fungal colonies was scratched using toothpicks, transferred to microtubes, and added with a fivefold volume of methanol. The AFs were extracted by vortexing for 1 min and sonicated for 3 min. After centrifugation, 5 µL of supernatant was evaporated under gentle nitrogen flow, and 0.05 mL of trifluoroacetic acid (TFA) was added for derivatization of AFs and vigorously shaken for 10 s. After incubation at room temperature for 15 min, 0.45 mL of acetonitrile/water (1:9, v/v) was added, and the resultant samples were directly injected into the HPLC-FL system (Shimadzu, Kyoto, Japan). Isocratic analysis was conducted using a column CAPCELL PAK C18 UG120 (Φ 3.0 × 250 mm, 5 µm particle size) (Osaka Soda, Osaka, Japan); the mobile phase comprised acetonitrile: methanol:water (1:3:6, v/v) at 0.3 mL/min flow rate. The column temperature was set to 40°C with an injection volume of 10 µL. The AFs were detected by excitation at 365 nm and emission at 450 nm. Data analysis was conducted using LabSolutions software (Shimadzu).

6. Whole-agar extraction method with AF accumulation analysis in an entire dish

Albeit without the DV, YES-DOC-CP was used in the whole-agar extraction method. One hundred grains from each sample (TM1-TM10), totaling 1,000 grains, were monitored to identify the emergence of fungal species and underwent chemical analysis. Corn grains were surface-sterilized with 70% ethanol for 30 s, rinsed three times with sterile water, placed on DV-free YES-DOC-CP agar medium in 20 replicates (5 grains/dish × 20 dishes), and set in the dark at 25°C for 7 d. The cultured dishes were exposed to AM vapor until the fungi were inactivated (> 30 min of exposure). The whole culture (agar with fungi) was scratched with chopsticks and transferred to 50 mL centrifuge tubes for further analyses. DV-free YES-DOC-CP dish cultures harvested in 50 mL centrifuge tubes were extracted with threefold volume of methanol, followed by vortexing for 1 min and sonication for 3 min. After centrifugation, 5 µL of supernatant was processed and analyzed by HPLC-FL as described in **5**.

7. Kojic acid (KA) production in isolates and whole dish extract

Kojic acid (KA) levels were assessed using the same methanol extract described in **5**. and **6**. Approximately 100 µL of the supernatant was diluted with 900 µL of 0.1% aqueous acetic acid and directly injected into an HPLC-UV system (Shimadzu,). Isocratic analysis was conducted using an L-column ODS (Φ 4.6 mm × 250 mm, 5 µm particle size) (CERI, Tokyo, Japan); a mobile phase comprised methanol:0.1% acetic acid (1:9, v/v) at 0.3 mL/min flow rate. The column temperature was set at

Table 2. Identification and AF/KA production analysis of the isolates

Isolate ID	Colony color	Color change by ammonia	Identified species	Identities of calmodulin gene[a]	Identities of ITS region[b]	Production		
						AFB1	AFG1	KA
TM1-C	greenish-yellow	−	*A. flavus*	500/500 (100%)	291/291 (100%)	−	−	+
TM1*-D	greenish-yellow	−	*A. flavus*	506/506 (100%)	290/290 (100%)	+	−	+
TM2-A	greenish-yellow	−	*A. flavus*	515/515 (100%)	295/295 (100%)	−	−	+
TM2-B	greenish-yellow	+	*A. flavus*	510/510 (100%)	288/288 (100%)	+	−	+
TM2-C	greenish-yellow	+	*A. flavus*	508/508 (100%)	290/290 (100%)	+	−	+
TM2-D	greenish-yellow	−	*A. flavus*	515/515 (100%)	290/290 (100%)	+	−	+
TM4-A	black	−	*A. niger*	505/505 (100%)	302/302 (100%)	−	−	−
TM5-A	greenish-yellow	+	*A. flavus*	522/522 (100%)	294/294 (100%)	+	−	+
TM5-C	greenish-yellow	+	*A. flavus*	523/524 (99%)	291/291 (100%)	+	−	+
TM5-D	greenish-yellow	+	*A. flavus*	500/500 (100%)	290/290 (100%)	+	−	+
TM5-E	greenish-yellow	+	*A. flavus*	512/512 (100%)	290/290 (100%)	+	−	+

[a] PCR was performed using primers for calmodulin gene (cmd5: 5′-CCGAGTACAAGGARGCCTTC -3′, cmd6: 5′-CCGATRGAGGTCATRACGTGG -3′).
The identities of the PCR products of the calmodulin gene primers with the calmodulin gene sequences registered at NCBI.
[b] PCR was performed using primers for ITS region (ITS3: 5′-GCATCGATGAAGAACGCAGC -3’, ITS4: 5′-TCCTCCGCTTATTGATATGC -3′).
The identities of the PCR products of the ITS3 and ITS4 primers with the ITS region sequences registered at UNITE.
*Dichlorvos-free agar (Fig. 4, right)
AF: aflatoxin; KA: kojic acid; AFB1: aflatoxin B_1; AFG1: aflatoxin G_1

40°C, and the injection volume was 10 µL. KA was detected by measuring the UV absorbance at 270 nm. Data were analyzed using LabSolutions software (Shimadzu).

8. Chemical analysis of AFs and fumonisin B_1 (FB1)

Chemical analyses of AFs and FB1 in grains were conducted using spike and recovery tests in triplicate, following the Japanese Analytical Standards of Feeds (FAMIC), with slight modifications. The spike level for AFs was 2 µg/kg, whereas that for FB1 was 1,000 µg/kg. For AFs, powdered grains (5 g) were extracted with 20 mL of acetonitrile:water (86:14, v/v), vortexed for 1 min, sonicated for 3 min, and then loaded onto a MultiSep 226 AflaZon + column (Romer Labs, MA, USA) for purification. The flow-through was dried under gentle nitrogen flow, TFA-derivatized, and analyzed using HPLC-FL. For FB1, powdered grains (5 g) were extracted with 25 mL of methanol:water (75:25, v/v), vortexed for 1 min, sonicated for 3 min, and loaded onto a Bond Elut SAX column (Varian, CA, USA) for purification. Liquid chromatography with a tandem mass spectrometer (LC-MS/MS) was employed to analyze the resulting solution (4500 Qtrap, AB Sciex, MA, USA).

Results and discussion

The moisture content of the corn grain samples ranged from 11.1% to 12.1%, without differences between the areas and grades (Table 1). The drying procedures seemed to be performed and standardized in Thailand.

In the first experiment, aflatoxigenic fungi were screened using the DV-AM method on a small scale (five grains from each sample). Fungal colonies appeared at high frequencies on the DV-coated YES-DOC-CP agar (Fig. 3). In some cases, the growth of *Zygomycota* was rapid, preventing the screening of *Aspergillus* (TM3 in Fig. 3). Several greenish-yellow colonies (e.g., TM5-A) and one black colony (TM4-A) with morphological features of *Aspergillus* fungi were picked before inactivating the fungi using AM (within 10 min of exposure). Some colonies, such as TM5-A, C, D, and E, were assumed as aflatoxigenic fungi. Table 2 shows that all the greenish-yellow isolates were identified as *A. flavus*, whereas a single black isolate was identified as *A. niger*. The isolates were chemically analyzed for mycotoxin production.

Chemical analysis of the AFs revealed the co-existence of aflatoxigenic *A. flavus* and atoxigenic *A. flavus* (Table 2), as observed previously (Kushiro et al. 2018a). The aflatoxigenic isolates exclusively produced group B AFs (AFB1 and AFB2) and not group G AFs (AFG1 and AFG2), which are typical features of

aflatoxigenic *A. flavus*. Chemical analysis indicated that all isolates, except TM4-A, produced KA, regardless of AFs production (Table 2), which are typical features of *Aspergillus* section *Flavi*. *Aspergillus* section *Flavi* contains other minor aflatoxigenic species, such as *A. pseudonomius* and *A. novoparasiticus,* present in soil samples from subtropical areas (Kushiro et al. 2018b, 2020). In the first (small scale) screening of corn grains, only *A. flavus*, the predominant aflatoxigenic species, was found among *Aspergillus* section *Flavi*, suggesting that the diversity of aflatoxigenic fungi in grains may be lower than that in soil.

The DV-AM method is one of the most sensitive detection methods for aflatoxigenic fungi. However, using DV has been recently restricted since it was designated as one of the specified chemical substances by Japanese law (Ministry of Health, Labour and Welfare, Japan). Our previous control experiments using autoclaved soil suggested that DV did not affect the recovery of fungal spores from an *A. flavus* strain added artificially (Kishimoto et al. 2023). In naturally occurring *A. flavus* on corn grain, a comparison experiment using DV-coated or DV-free YES-DOC-CP agar revealed that both methods helped effectively isolate *A. flavus*. Moreover, DV did not affect or inhibit the emergence of *Zygomycota* (Figs. 3, 4).

Therefore, in the second experiment, large-scale screening of aflatoxigenic fungi was conducted using the agar extraction method on DV-free YES-DOC-CP agar (Kishimoto et al. 2023) (Fig. 5). As listed in Table 3, the colony-forming units (CFU) of the fungi were substantially high, ranging from 53 to 98 (from 53 to

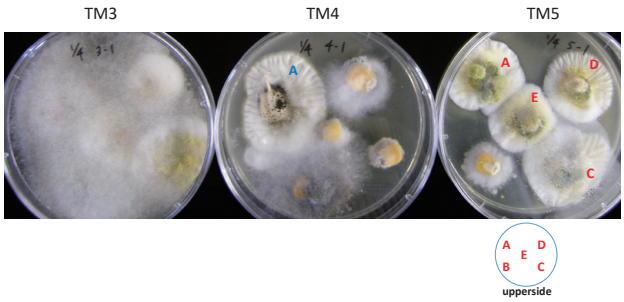

Fig. 3. First screening of aflatoxigenic fungi by DV-AM method
Five grains were set on a DV-coated YES-DOC-CP agar dish.
Emerged colonies of *Aspergilli* were picked up within 10 min of AM exposure.

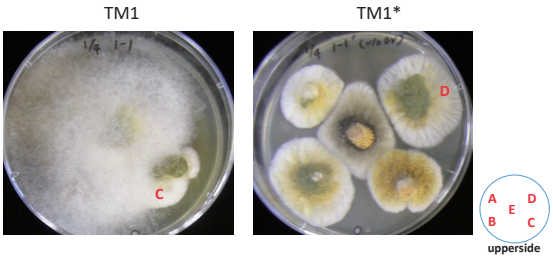

Fig. 4. Comparison between DV-coated and DV-free YES-DOC-CP agar
Five grains were compared on DV-coated (left) or DV-free (right) YES-DOC-CP agar dish.
Emerged colonies of *Aspergilli* were picked up within 10 min of AM exposure.
* DV-free agar

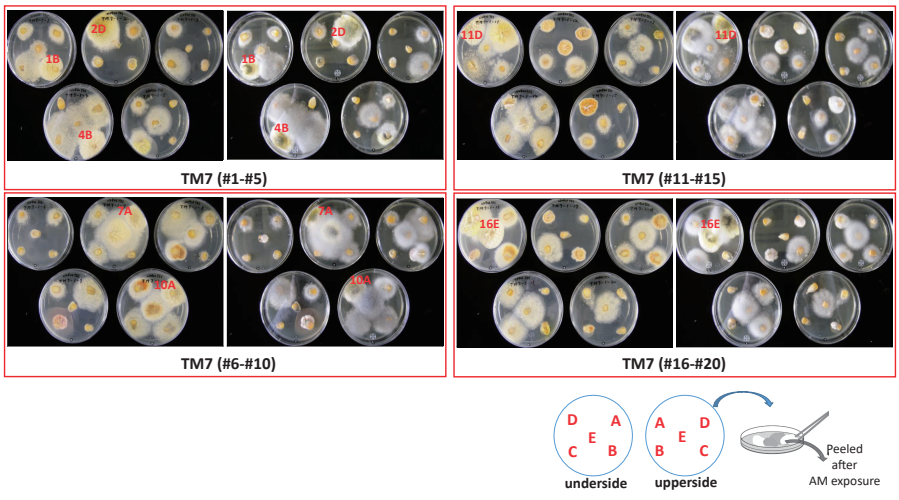

Fig. 5. Second screening using 100 grains and whole-agar extraction method
Five grains were set on a DV-free YES-DOC-CP agar dish.
Emerged colonies of *Aspergilli* were picked up within 10 min of AM exposure.
The whole-agar dish culture was peeled by chopsticks after >30 min of AM exposure.

Table 3. CFU, *A. niger* and *A. flavus* occurrence in corn grain samples

Sample ID	Number of grains	CFU	*A. niger*	*A. flavus*	
				Total	AFB1 producer
TM1	100	81	2	1 (0 ～ 5)*	1 (0 ～ 5)
TM2	100	85	1	3 (1 ～ 8)	0 (0 ～ 4)
TM3	100	53	3	2 (0 ～ 7)	0 (0 ～ 4)
TM4	100	75	4	1 (0 ～ 5)	0 (0 ～ 4)
TM5	100	85	3	4 (1 ～ 10)	2 (0 ～ 7)
TM6	100	93	8	7 (3 ～ 14)	3 (1 ～ 8)
TM7	100	95	4	7 (3 ～ 14)	5 (2 ～ 11)
TM8	100	88	2	7 (3 ～ 14)	5 (2 ～ 11)
TM9	100	98	2	1 (0 ～ 5)	0 (0 ～ 4)
TM10	100	54	2	2 (0 ～ 7)	0 (0 ～ 4)
sum	1,000	807	31	35	16

* 95% confidence limits are shown in parentheses (from'Methods of Analysis in Health Science 2020').
AFB1: aflatoxin B_1

98%). The high frequency of CFU may be attributed to the tropical climate of Thailand and its similar moisture contents (Fig. 1, Table 1). All samples contained *Aspergilli* of *A. niger* and *A. flavus* regardless of the area and grade. *A. niger* isolates frequently coexist with *A. flavus* (Yabe et al. 2015), which may act as biocontrol agents for aflatoxigenic fungi. The number of *A. niger* and total *A. flavus* were 31 and 35, respectively (per 100 × 10 = 1,000 grains). The average percentage of aflatoxigenic fungi was 1.6% (16 isolates per 1,000 grains) (Table 3). Among the corn grain samples, TM6, TM7, and TM8 exhibited a higher occurrence of *A. flavus* (7, 7, and 7, respectively) and AFB1 production (3, 5, and 5, respectively) than the other seven samples (Table 3). These three samples were of a low grade (grade 2) from the central part of Thailand. These grade differences may be attributed to the occurrence and frequency of aflatoxigenic fungi in the environment where the corn was harvested.

This study examined DV-AM (Figs. 3, 4; Table 2) and whole-agar extraction (Fig. 5, Table 3) methods for their application to Thai corn grains. In the DV-AM method, the columns of "Color change by ammonia" and "Production-AFB1" in Table 2 indicate a false-negative (TM2-D) among *A. flavus* isolates. This suggests a lower production of AFB1 in TM2-D than in the other six AFB1 producers, which was confirmed by HPLC-FL analysis. No false-positive results were obtained by the DV-AM method. These results demonstrate the effectiveness of the DV-AM method for screening aflatoxigenic fungi from corn grains. In contrast, as presented in Table 4, for the whole-agar extraction method using DV-free agar medium, "Color change by ammonia" was frequently unclear for each isolate. However, here, the "Production-AFB1" of w (whole-agar extract) became an indicator of AFB1 producers, such as TM7-11w, even if the dish was covered by *Zygomycota.* Therefore, both methods effectively detect aflatoxigenic and atoxigenic *Aspergillus* in corn grains. Since the YES-DOC-CP medium can easily discriminate greenish-yellow colonies with or without DV (Tables 2, 4), these two methods can be applied to other grains, such as peanuts or Job's tears.

AFs and FB1 are two major mycotoxin corn contaminants. In addition to AFs, the FB1 contamination of Thai corn was reported by Yoshizawa et al. (1996). In this study, AFs and FB1 contamination levels in corn grain samples were validated using spike and recovery tests as part of the chemical analysis (Table 5). Recovery (%) was acceptable within the AOAC criteria (70% - 120%), except for AFB1 in one sample (TM8), which exhibited substantially high contamination, thus suggesting their overall reliability. While AFB1, ranging from trace (below 0.1 ppb (μg/kg)) to high (> 20 ppb, the regulatory limits for total AFs in Thailand), was detected in all samples. High sporadic FB1 contamination was detected in TM4 and TM5 (> 1,000 μg/kg). In these samples, the AF levels were below the limit of quantification. The causal agent of FB1 contamination differs from *A. flavus,* and it is assumed to be *Fusarium* species, such as *F. proliferatum* and *F. verticillioides*. Therefore, they may act as natural competitors of aflatoxigenic fungi in these samples. Thus, further screening for FB1-producing fungi is required.

High levels of AFB1 were detected in TM6, TM7, and TM8 (10.6, 0.77, and 30.4 μg/kg, respectively). This tendency was correlated with the higher occurrence of *A. flavus* and aflatoxigenic fungi in these samples (Table 3). These mycological observations and chemical data are

Table 4. Production analysis of the isolates or whole-agar extracts

Isolate / dish ID	Colony color	Color change by ammonia	Production		
			AFB1	AFG1	KA
TM7-1B *	greenish-yellow	+	+	−	
TM7-1w **			+	−	+
TM7-2D	greenish-yellow	+	+	−	
TM7-2w			+	−	+
TM7-4B	greenish-yellow	+	+	−	
TM7-4w			+	−	+
TM7-7A	greenish-yellow	unclear	+	−	
TM7-7w			+	−	+
TM7-10A	greenish-yellow	unclear	−	−	
TM7-10w			−	−	+
TM7-11D	greenish-yellow	unclear	−	−	
TM7-11w			+	−	+
TM7-16E	greenish-yellow	unclear	+	−	
TM7-16w			+	−	+

* Isolates (shown in Fig. 5)
** w = whole-agar extract
AFB1: aflatoxin B_1; AFG1: aflatoxin G_1; KA: kojic acid

Table 5. Chemical analysis of AFs and FB1 in corn grain samples

Sample ID	AFB1 (μg/kg)	Recovery (%) at 2 μg/kg spike	AFB2 (μg/kg)	Recovery (%) at 2 μg/kg spike	AFG1 (μg/kg)	Recovery (%) at 2 μg/kg spike	AFG2 (μg/kg)	Recovery (%) at 2 μg/kg spike	FB1 (μg/kg)	Recovery (%) at 1,000 μg/kg spike
TM1	0.27	95	<LOD **	97	<LOD	95	<LOD	96	290	
TM2	0.25	109	<LOD	111	<LOD	116	<LOD	109	290	
TM3	0.44	91	<LOD	110	<LOD	106	<LOD	110	20	
TM4	trace *	107	<LOD	113	<LOD	112	<LOD	114	2,100	
TM5	trace	109	<LOD	110	<LOD	113	<LOD	108	9,400	
TM6	10.6	85	0.72	108	<LOD	115	<LOD	108	350	75
TM7	0.77	105	<LOD	116	<LOD	114	<LOD	115	540	
TM8	30.4	−22	1.55	118	<LOD	99	<LOD	111	250	
TM9	0.16	106	<LOD	110	<LOD	114	<LOD	111	290	
TM10	0.14	106	<LOD	108	<LOD	112	<LOD	108	140	

* trace = between LOD (limit of detection, 0.05 μg/kg) and limit of quantification (0.10 μg/kg), ** <LOD = below LOD
AFB1: aflatoxin B_1; AFB2: aflatoxin B2; AFG1: aflatoxin G_1; AFG2: aflatoxin G_2; FB1: fumonisin B_1

consistent since these three samples were all of Grade 2. AFB1 levels in the other seven samples were sufficiently low (< 0.5 μg/kg), below one-fortieth of the regulatory limits for total AFs in Thailand.

Thus, our findings indicate the existence of naturally occurring aflatoxigenic fungi in Thai corn. As shown in Table 5, the percentage of AF-contaminated corn (over LOD) was 100%. AF contamination of corn has been recognized as a serious problem in Thailand (Pitt et al. 1993). Waenlor and Wiwanitkit (2003) summarized that AF-contaminiated corn was 0% - 72%, depending on the reported year between 1967 and 1996. Recently, Songsermsakul (2015) summarized that only one report from 2000 to 2010 indicated the percentage of AF-contaminated corn to be 85.7%. The increase in AF-contaminated corn may be derived from the higher sensitivity (low LOD) of recent detection methods, such as ELISA, HPLC-FL, or LC-MS/MS, than that of previous thin-layer chromatography. Since the number of samples was limited in this study, further surveillance must compare the past and present AF-contamination levels of Thai corn.

The causative agent was mainly *A. flavus* in the 1980s (Saito & Tanaka, personal communication), as confirmed in this study. Pitt et al. (1993) reported that the major *Aspergillus* fungi on corn grain was *A. flavus*

(85%), followed by *A. niger* (64%). The tendency agrees with this study, but the percentages were less than those in this study, as shown in Table 3. The decrease in *Aspergilli* occurrence could be due to the storage and transport period from Thailand to Japan, affecting the samples' freshness. Nevertheless, this study suggests that *Fusarium* fungi is responsible for FB1 accumulation. The co-contamination of Thai corn with AFs and FB1 was first reported by Yoshizawa et al. (1996), but the interaction between AF-producers and FB1-producers remains inconclusive. The increasing global warming effects warrant further monitoring and surveillance of the fungal and other mycotoxin-producing species responsible for the contamination.

Conclusions

The occurrence ratio of fungi in post-harvest corn grains was comparable across Thailand. The DV-AM and whole-agar extraction methods effectively assess the occurrence of aflatoxigenic fungi in corn grains. The emergence of aflatoxigenic fungi was high among Grade 2 samples, and their occurrence and AFs contamination levels were relatable.

Acknowledgements

We thank the Development Strategy Center, NARO (NDSC), for financially supporting this international collaborative study. We also thank Dr. Kenji Tanaka (Masayo Kushiro's former supervisor) for valuable information and to the Research Center of Advanced Analysis, NARO, for providing the LC-MS/MS apparatus for chemical analysis.

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