JSM Mycotoxins **70** (2) , 51 - 56 (2020)
http://doi.org/10.2520/myco.70-2-4

JSM Mycotoxins

Note

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Detection of *Aspergillus novoparasiticus* from Japanese sugarcane field by the dichlorvos-ammonia (DV-AM) method with single colony AM assay

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Keywords
aflatoxin; diversity; soil

Abbreviations
dichlorvos, DV; ammonia, AM; aflatoxin, AF; high-performance liquid chromatography, HPLC

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(Received 30 April, 2020,
revised 18 June, 2020,
accepted 3 July, 2020)

Abstract

The dichlorvos-ammonia (DV-AM) method followed by single colony AM assay was applied for re-screening of aflatoxigenic fungi from soil samples from sugarcane field in Okinawa, Japan. In previous study, we obtained two aflatoxigenic isolates of *Aspergillus pseudonomius* located in *Aspergillus nomius* clade from one of soil samples. In this study, we re-screened the same soil sample and found an isolate with different morphology and toxigenic profile. Molecular phylogenic analyses of the isolate indicated that it is a closely related species of *A. novoparasiticus* located in *Aspergillus flavus* clade. This is the first report of this species of *Aspergilli* in Japan. Thus, the DV-AM method with single colony AM assay can be used as a tool to elucidate the diversity of aflatoxigenic fungi colonizing in the field soil.

1. Introduction

Mycotoxins are low-molecular-weight natural products produced as secondary metabolites by filamentous fungi[1),2)]. The Food and Agriculture Organization of the United Nations estimates that 25% of the world's food crops are affected by mycotoxins, of which the most notorious are aflatoxins (AFs)[3)]. In many countries, regulatory limits are set for four major AFs; aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2), or only for AFB_1, which is classified as a group 1 carcinogen (carcinogenic to humans) by International Agency for Research on Cancer (IARC)[4)].

AFs are produced by more than ten aflatoxigenic species of fungal genus *Aspergillus*[5)]. AFs contamination is often derived by aflatoxigenic fungi existing in environments including field soils[6)]. Although the presence of aflatoxigenic fungi does not directly indicate AFs contamination, there is an increased risk[7)]. Aflatoxigenic fungi have been found mainly in tropical and sub-tropical areas, where are favorable climate conditions for fungal propagation and AFs production. In Japan, a previous surveillance study indicated that aflatoxigenic fungi prevails in the areas where average annual temperature is over 18 degree such as Okinawa island[8)].

Sugarcane is widely cultivated in Okinawa and major aflatoxigenic *Aspergilli* (*A. flavus* and *A. parasiticus*) were reported in sugarcane field[9),10)]. Most of these aflatoxigenic fungi were screened using AFPA (Aspergillus flavus/parasiticus agar) medium method[11)]. Previously we adopted a newly developed dichlorvos-ammonia (DV-AM) method to screen aflatoxigenic *Aspergilli* from soil samples of Okinawan sugarcane field in the process to

elucidate the applicability of the method[12),13),14),15),16)]. We succeeded in isolation of a minor aflatoxigenic *Aspergillus* species such as *A. pseudonomius* located in *Aspergillus nomius* clade[14)]. At that time, no other aflatoxigenic *Aspergilli* were obtained.

In this study, we tried to isolate another aflatoxigenic species of *Aspergilli* using the same soil sample which emerged two of aflatoxigenic *A. pseudonomius* isolates. We conducted intensive re-screening using DV-AM method followed by single colony AM assay, which is an assay using the original AM method[17)].

2. Materials and Methods

2.1. Sample and media

A soil sample (K22) collected in sugarcane field, Okinawa, Japan[14)] was kept at 4 °C until use. For screening by DV-AM method, GYD-DV agar medium (= GYD agar medium (2% glucose, 0.5% yeast extract, 0.05% sodium deoxycholate and 2% agar) supplemented with dichlorvos (DV; Fujifilm Wako, Osaka, Japan)(methanolic DV solution (4,000 ppm, 20 µL) was spread onto a 9 cm-diam plate of GYD agar medium and kept for at least 60 min) was used. For single colony AM assay and purification of screened colonies, GY agar medium (2% glucose, 0.5% yeast extract and 2% agar) was used.

2.2. Screening and single colony AM assay

A subsample of soil (each 0.3 g) was suspended in 1 mL of 0.05% Tween 80 solution, and each 50 µL of soil suspension (containing ca. 15 mg of soil) was spread onto GYD-DV agar medium plates with a wide bore pipette tip (truncated yellow tip) and cultured for 3-5 days at 25 °C. After cultivation, plates were observed from backside and aliquots of 25% ammonia (AM) solution (200 µL, Fujifilm Wako) were added onto each lid of the plate. AM-induced red colonies were named using the prefix OKI-2nd- (Fig. 1). Each colony was picked up and transferred onto a new GY agar medium plate to make a giant colony for single colony AM assay (Fig. 2).

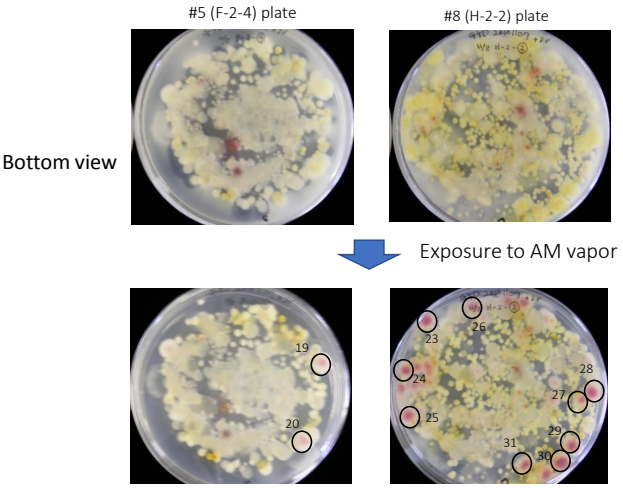

Fig. 1 Detection of aflatoxigenic fungi by the DV-AM method. The fungi were cultured on GYD-DV plate and the colors of colonies on the underside of each plate were observed before and after AM treatment.

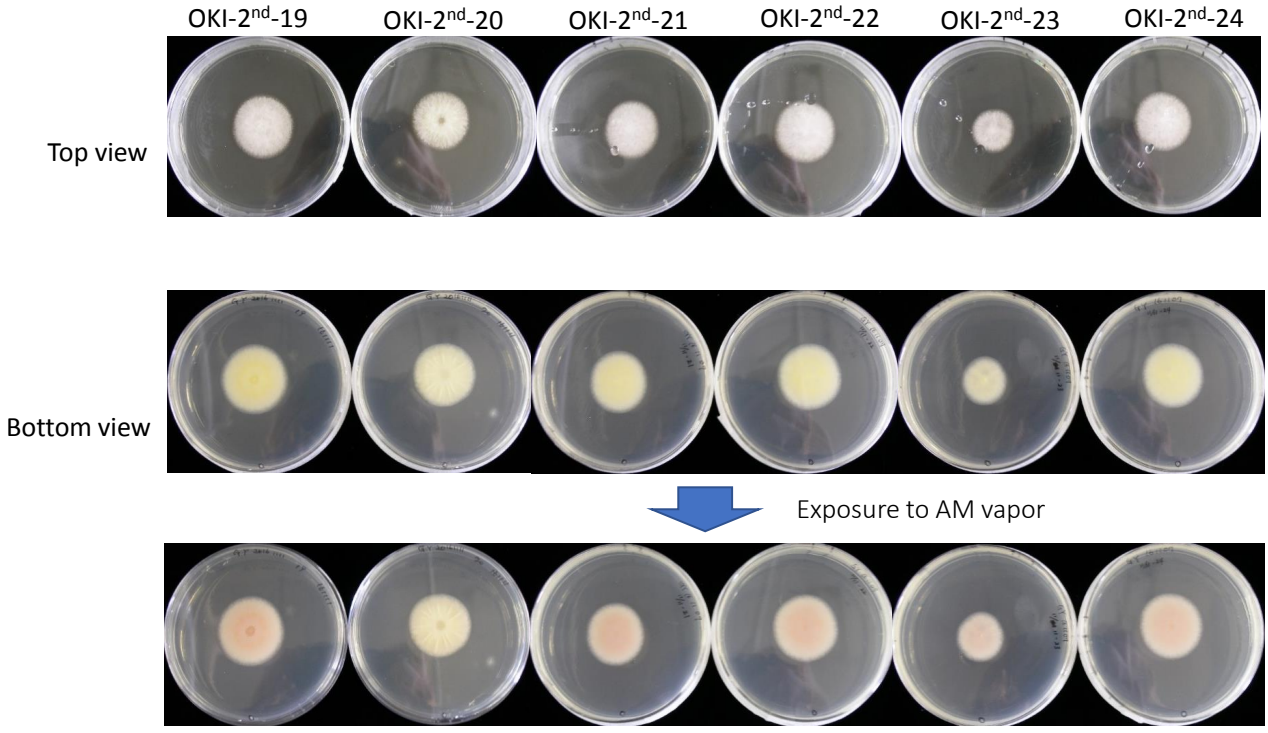

Fig. 2 Single colony AM assay. A giant colony was cultured on GY plate and the color of colony on the underside of each plate was observed before and after AM treatment.

2.3. Morphological observation

Possible aflatoxigenic fungal colonies were purified to prepare conidiospore suspension in 0.05% Tween 80[13]. The spore suspension (2 µL) of each isolate was used for three-point fashioned inoculations onto -GY agar plates (Fig. 3). Previously obtained isolates OKI-12 and OKI-16 were also cultivated as positive controls (both aflatoxigenic *A. pseudonomius*)[14]. Plates were observed for macroscopic features (colony features and color) and two representative isolates (OKI-2nd-20 and OKI-2nd-21) were further analyzed microscopic details at Techno Suruga Laboratory (Shizuoka, Japan).

2.4. Chemical analysis

Possible aflatoxigenic fungal colonies were harvested after 3-5 days and treated by the exposure to AM vapor. To confirm AF production, approximately 1 g of GY agar with each colony was scratched and extracted with 5 mL of methanol in 50 mL centrifuge tubes by 1 min of vortex and 3 min of sonication. After centrifugation, 0.01 mL of supernatant was evaporated under gentle nitrogen flow and 0.05 mL of trifluoroacetic acid was added for derivatization of AFB_1 and AFG_1 to become AFB_{2a} and AFG_{2a} with enhanced fluorescence. After kept at room temperature for 15 min, 0.45 ml of acetonitrile - water (1:9, v /v) was added, and the dissolved samples were directly injected into the HPLC-FL system (Shimadzu, Kyoto, Japan). LC analysis was conducted in the same way as reported previously[14].

2.5. Genetic analysis and deposit

Genomic DNA of OKI-2nd-20 and OKI-2nd-21 were extracted and calmodulin (*cmd*) gene was then analyzed at Techno Suruga Laboratory (Shizuoka, Japan) in the same way as reported previously[14]. The phylogenic tree was depicted by Neighbor-Joining method. The genomic DNA sequence data for *cmd* genes of OKI-2nd-20 and OKI-2nd-21 were deposited in the DDBJ, EMBL and GenBank nucleotide sequence data under accession numbers of LC529205 and LC529941.

3. Results and Discussion

K22 soil sample, which contained *A. pseudonomius* isolates (OKI-12 and OKI-16) at previously conducted screening[14], was used for this study. The soil was cultured on 10 GYD-DV agar plates (#1-10) and analyzed by DV-AM method. Finally, 39 red colonies were observed (Fig. 1, Table 1). Each colony was then picked up and transferred to a new GY agar plates for single colony AM assay to observe its morphology and AF production (Table 1, Fig. 2). As summarized in Table 1, among 39 colonies, 6 colonies failed to grow on GY agar medium (indicated judge "F") and 9 colonies was non-*Aspergillus* fungi (indicated judge "N"). The rest 24 colonies of putative *Aspergilli* revealed the existence of two groups; the minor group (1 isolate, named OKI-2nd-20) exerted pale-pink color (indicated judge "P") while the majority (23 isolates including OKI-2nd-19 and OKI-2nd-21) exerted red color (indicated judge "R") after exposure to AM vapor.

Macroscopic morphological observation by three-point fashioned inoculations was conducted using OKI-12 and OKI-16 (aflatoxigenic *A. pseudonomius*) as controls. It was revealed that OKI-2nd-20 showed distinct features compared to other isolates (Fig. 3). OKI-2nd-20 had relatively faster in radial growth and differed in the color of colonies. On the other hand, OKI-2nd-21 and OKI-2nd-22 had similar macroscopic appearance

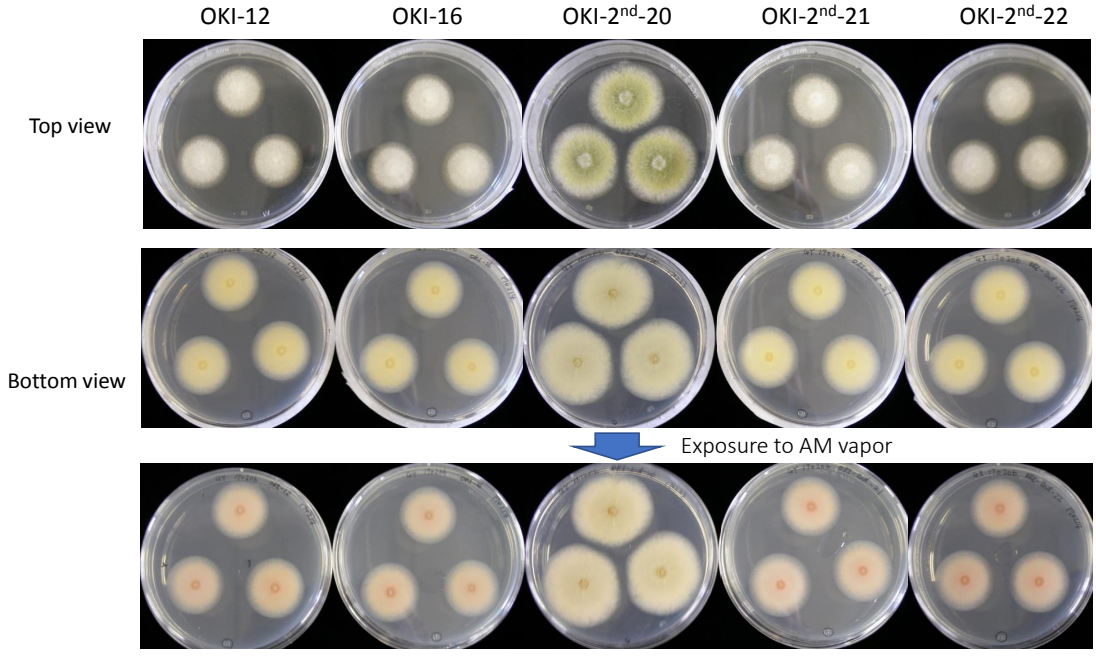

Fig. 3 Macroscopic features of selected isolates

(morphology and color) to OKI-12 and OKI-16, suggesting they were also aflatoxigenic *A. pseudonomius*.

AF production was compared between the extracts of OKI-12 and OKI-2nd-20. OKI-2nd-20 produced AFB_1, AFG_1, AFB_2 and AFG_2 in the same way as OKI-12, however; quantity was different from that of the extract of OKI-12, showing less accumulation of AFs with little production of AFG_1 (Fig. 4).

Therefore, we considered that OKI-2nd-20 might be an isolate of different species from other major isolates. Further phylogenetic analysis was carried out based on partial nucleotide sequence of *cmd* gene. Due to the morphological observation (Fig. 5A) and sequence information, this isolate was closely related to *A. novoparasiticus* located in *Aspergillus flavus* clade (Fig. 5B, left). This species was just recently reported from environmental and clinical sources in Colombia and Brazil[18]. Major four AFs production was reported in this species[18], which is consistent with our result (Fig. 4). In contrast, OKI-2nd-21 was identified as *A. pseudonomius* (Fig. 5B, right).

This work showed that the soil sample collected in the sugarcane field contained different species of aflatoxigenic fungi, *A. pseudonomius* and *A. novoparasiticus*. Previously, we reported aflatoxigenic as well as non-aflatoxigenic fungi isolated from the same Mexican maize field[19]. These are consistent with many previous findings that closely related *Aspergilli* with distinct toxigenicity co-exist in the same environment. Relationships among these *Aspergilli* as well as between aflatoxigenic fungi and other microorganisms in environment such as soils and plants remain to be studied.

This is the first report of *A. novoparasiticus* in Japan. According to the previous report, the AFPA medium method, the most popular method for discrimination of major aflatoxigenic fungi, failed to detect *A. novoparasiticus* because of its lack of aspergillic acid production on AFPA[18]. In this study, the DV-AM method combined with single colony AM assay succeeded in discriminating *A. novoparasiticus* as an aflatoxigenic fungus. The single colony AM assay is a simple AM method originally developed for rapid screening of aflatoxigenic strains of *A. flavus* and *A. parasiticus*[17], which proved to be also useful for the discrimination of aflatoxigenic strains depending on toxin production level in this work. Thus, the DV-AM method with single colony AM assay will become a powerful tool to elucidate the diversity of aflatoxigenic *Aspergilli* diverse in their toxigenic profiles, colonizing in the field.

Table 1 Isolated colonies

Plate #	Judge*			
	F	N	P	R
#1(F-1-2)	OKI-2nd-1			
#2(F-2-1)		OKI-2nd-3		OKI-2nd-2, OKI-2nd-4, OKI-2nd-5
#3(F-2-2)	OKI-2nd-9	OKI-2nd-10		OKI-2nd-6, OKI-2nd-7, OKI-2nd-8
#4(F-2-3)	OKI-2nd-17	OKI-2nd-15, OKI-2nd-16, OKI-2nd-18		OKI-2nd-11, OKI-2nd-12, OKI-2nd-13, OKI-2nd-14
#5(F-2-4)			OKI-2nd-20	OKI-2nd-19
#6(H-1-2)				OKI-2nd-21
#7(H-2-1)				OKI-2nd-22
#8(H-2-2)	OKI-2nd-26	OKI-2nd-31		OKI-2nd-23, OKI-2nd-24, OKI-2nd-25, OKI-2nd-27
				OKI-2nd-28, OKI-2nd-29, OKI-2nd-30
#9(H-2-3)	OKI-2nd-32, OKI-2nd-34	OKI-2nd-36, OKI-2nd-38		OKI-2nd-33, OKI-2nd-35, OKI-2nd-37
#10(H-2-4)		OKI-2nd-39		

*F: fail to grow, N: non-*Aspergillus*, P: pale-pink color by AM, R: red color by AM

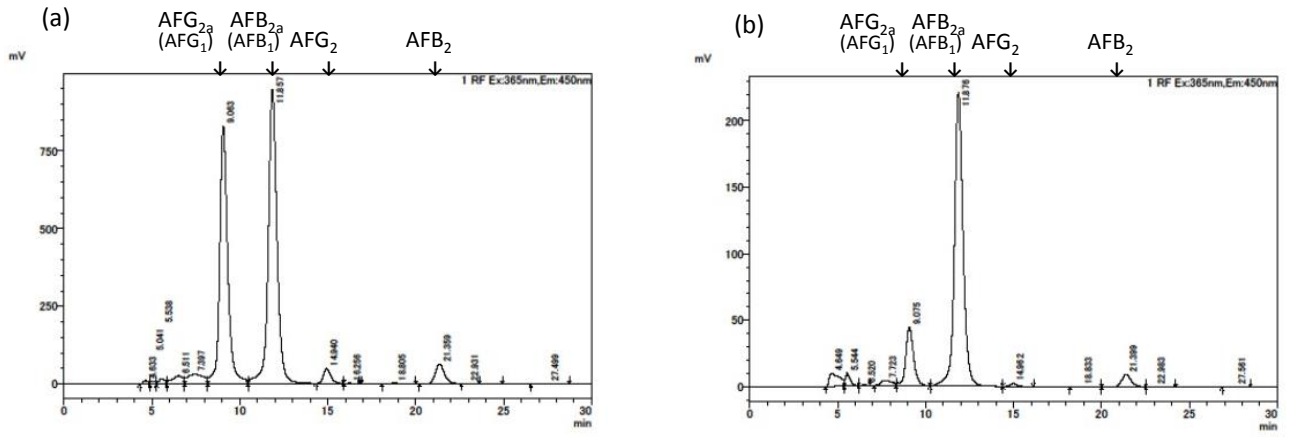

Fig. 4 HPLC chromatograms of the extracts of (a) OKI-12 and (b) OKI-2nd-20

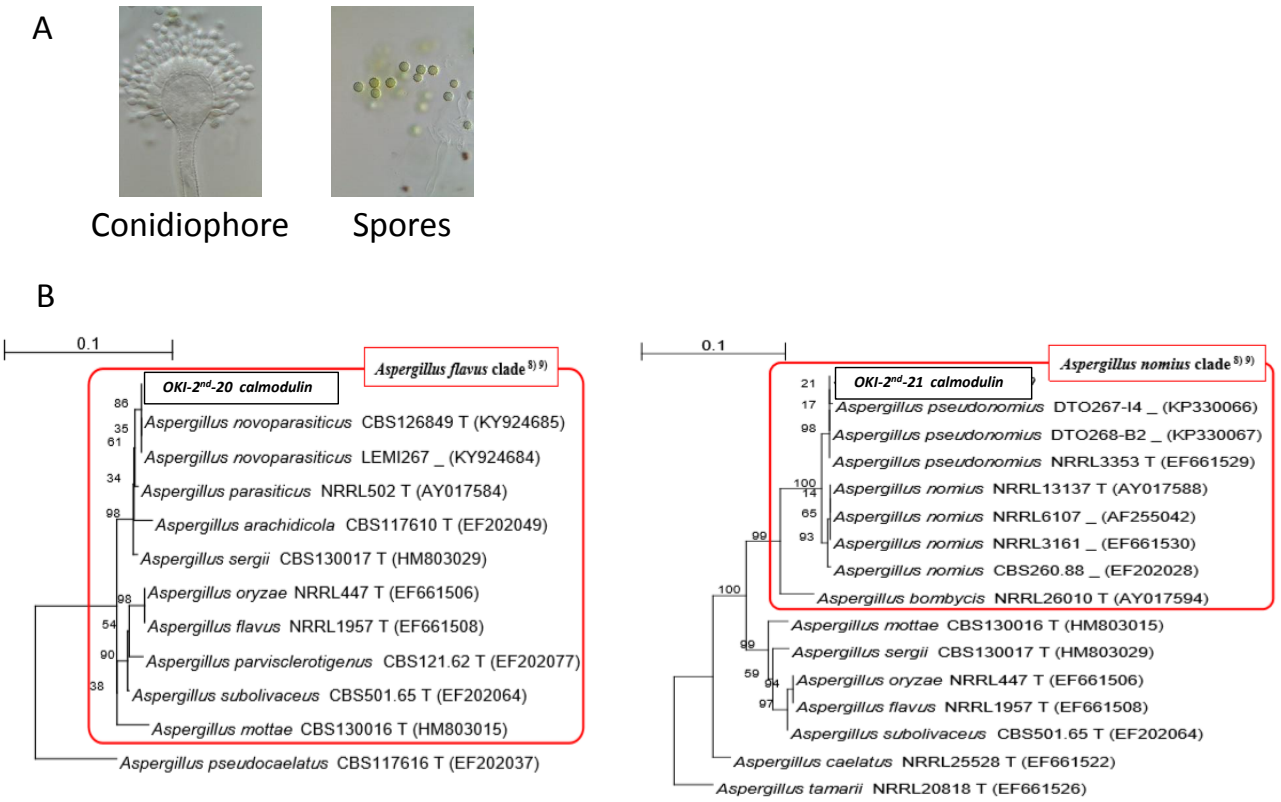

Fig. 5 Morphological observation (A) and phylogenetic analyses (NJ method) (B) of the isolated fungi. A. OKI-2nd-20. B. OKI-2nd-20 (left) and OKI-2nd-21 (right).

Acknowledgements

This work was supported by the Research Project for Improving Food Safety and Animal Health from Ministry of Agriculture, Forestry and Fisheries, Japan, and partially supported by JSPS KAKENHI Grant Number JP16H05785. We are grateful to Mr. Masahiro Fujita at Gakushuin University for assisting the screening. We thank the Advanced Analysis Center, NARO, for use of facilities.

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