Detection of aflatoxigenic fungi in imported raw nuts using the dichlorvos-ammonia (DV-AM) method

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Abstract

The dichlorvos-ammonia (DV-AM) method is a simple and sensitive visual method for detecting aflatoxigenic fungi. Since aflatoxin contamination has been frequently reported in ground nuts and tree nuts in many countries, we herein used the DV-AM method for the detection of aflatoxigenic fungi in imported raw nuts and seeds: peanuts from South Africa, macadamia nuts from Australia, pistachio nuts from Iran, macadamia nuts from Kenya, almond nuts from California, U.S., cashew nuts from Viet Nam, hazelnuts from Turkey, pumpkin seeds from China, sunflower seeds from Bulgaria, and walnuts from the U.S. Using the DV-AM method revealed that yellow fungal colonies surrounding the peanuts and the macadamia nuts were changed to red by ammonia treatment. Thin-layer chromatography analyses showed that the red fungi from the peanuts produced mainly aflatoxin B₁ and the red fungi from the macadamia nuts produced mainly aflatoxins B₁ and G₁. Based on their calmodulin gene sequences, the peanuts’ fungi were identified as Aspergillus flavus, and those from macadamia nuts from Australia were Aspergillus parasiticus. Aflatoxigenic fungi were not detected in the other nuts and seeds. These results demonstrate that the DV-AM method can be used to detect aflatoxigenic fungi in various imported foods. This method will also be useful for the determination of critical control points for food safety at different stages of the food chain: harvesting, sorting, washing, various food processes, and transporting.

Keywords

aflatoxin; Aspergillus flavus; Aspergillus parasiticus; edible nuts, macadamia nuts; peanuts, pistachio nuts.

Introduction

Aflatoxins (AFs) are highly toxic and carcinogenic secondary metabolites produced by fungi¹. The contamination of agricultural commodities with AFs is not only a serious health hazard to humans and animals but also a cause of huge economic losses worldwide². Aflatoxins are produced mainly by Aspergillus flavus Link and Aspergillus parasiticus Spear,¹ and these fungi have been thought to be distributed mostly in tropical and subtropical regions³,⁴, but aflatoxigenic fungi have also been reported in temperate regions such as the United States and Japan⁵,⁶.

Aflatoxigenic fungi may be widely distributed around the world. There have been no reports of crops grown on the mainland of Japan that are heavily contaminated with AFs, whereas aflatoxigenic fungi have been detected in the Kanto and the Hokuriku areas, which are the central parts of Japan’s mainland⁶,⁷. The origin and dynamic distribution of the aflatoxigenic fungi in Japan is thus important to prevent future possible contaminations of AFs in crops in Japan.

Edible seeds such as peanuts and pistachio nuts are some of the crops that are the most susceptible to fungal attacks⁵,⁹. In fact, aflatoxicosis was first identified in the 1960s in England when approx. 100,000 turkeys died due to peanut meal that was contaminated by AFs produced by Aspergillus flavus. Among nuts, peanuts are grown on a large scale in almost all of the tropical and subtropical countries such as India, China, and West Africa¹⁰. Aflatoxin contamination can occur at the pre-harvest growth stage and at the post-harvest stage including during drying, storage, and food processing. Peanuts are cultivated in many regions in Japan, among which Chiba and Ibaraki are the main prefectures for peanut production. AF contamination has not been
detected in peanuts in Japan, but aflatoxigenic fungi have been reported in peanut fields in Japan\(^6\).

Many countries have set up both regulations to control the contaminants in foodstuffs to protect human health and specific maximum residues limits for AFs in different agricultural commodities\(^{10,11}\). Imported nuts are also analyzed for AF contamination, and nuts can be imported when their AF values are under the regulatory limit of Japan. Suzuki et al. reported that the fungi in the \(A. \text{flavus}\) group were detected in many imported foods (i.e., peanuts, pistachio nuts, yellow corn, and popcorn) by using Aspergillus Flavus Parasiticus Agar (AFPA) medium, which is a selection medium for \(A. \text{flavus}\) and \(A. \text{parasiticus}\)\(^1\), and that 21%–86% of the \(A. \text{flavus}\) fungi produced AFs\(^{13}\).

We previously developed a sensitive and simple visual method for detecting aflatoxigenic fungi that we named the ‘dichlorvos-ammonia (DV-AM) method’\(^{7,14}\). With this method, we can directly detect aflatoxigenic fungi as red colonies among various types of microorganisms on a selection medium. In the present study, we used the DV-AM method to search for aflatoxigenic fungi contaminating 10 types of imported raw nuts. We were able to detect the aflatoxicogenic fungi from peanuts from South Africa and macadamia nuts from Australia. Our results demonstrated that the DV-AM method is useful for the detection of the aflatoxigenic fungi contaminating various raw foods.

**Materials and Methods**

**Materials**

A total of 10 types of edible nuts imported from foreign countries were purchased in their unopened packages through two companies via the Internet (Table 1): peanuts (\(Arachis \text{hypogaea}\)) from South Africa, macadamia nuts (\(Macadamia\) spp.) from Australia, pistachio nuts (\(Pistachio\) spp.) from Iran, macadamia nuts (\(Macadamia\) spp.) from Kenya, almond nuts (\(Prunus \text{amygdalus}\)) from the U.S. (in California), cashew nuts (\(Anacardium \text{occidentale}\)) from Viet Nam, hazelnuts (\(Corylus\) spp.) from Turkey, pumpkin (\(Cucurbita\) spp.) seeds from China, sunflower (\(Helianthus \text{annu}\)) seeds from Bulgaria, and walnuts from the U.S. All the nuts had been unshelled. All samples were kept at 4°C after their purchase and until their use.

**Media**

The DV-AM method routinely uses YES-DOC-CP-DV agar medium\(^7\). After 15 ml of YES-DOC-CP agar medium (2% yeast extract, 10% sucrose, 0.1% Na DOC [Fujifilm Wako Pure Chemical Co., Osaka, Japan], 0.1 g chloramphenicol [CP]/L, and 2% agar) was poured and solidified in a plastic petri, 20 µl of 100-fold diluted DV (Wako, Osaka, Japan) methanol solution was spread onto the surface of the resulting medium. We also used GY medium (2% glucose, 0.5% yeast extract, 2% agar) for the purification of each isolated fungus\(^{15}\). Potato dextrose agar (PDA) medium (Difco Laboratories, Sparks, MD) was used for the morphological characterization of isolated fungi.

**The DV-AM method**

Whole nuts without crushing were put on YES-DOC-CP-DV agar medium in duplicate. After incubation in the dark at 28°C for 3 or 4 days, each plate was placed upside-down and added with ≥200 µL of ammonium hydroxide solution (28%, reagent grade, Wako) to the inside of the lid of each plate. When colonies’ color changes from yellow to red were observed from the underside of the plates, conidiospores of each red colony (a candidate aflatoxigenic fungus) were picked up with a sterile toothpick and inoculated onto a new GY plate. The fungal isolates were further purified by two repetitions of single conidiospore isolation.

**The characterization of the isolated fungi**

To confirm the AF productivity of each isolated fungus, an aliquot of the fungal colony together with the agar underneath the colony on the GY agar plate was collected with a toothpick and transferred to a new microtube. The metabolites in the sample were extracted with ethyl acetate, and the extract (10 µl) was then analyzed by thin-layer chromatography (TLC) using a silica gel plate (silica gel 60, #5721; Merck, Rahway, NJ, U.S.) and a developing solution of toluene-ethyl acetate-acetic acid (60:30:4, vol/vol/vol)\(^6\).

For the identification of the fungi, genomic DNA in an aliquot of fresh fungal mycelia on the GY agar plate was extracted using zirconium beads, Tris-EDTA-saturated phenol, and a FastPrep FP100A cell disrupter (Q-BIO 101, Thermo Fisher Scientific, Waltham, MA). After centrifugation, the upper aqueous layer containing DNA was diluted and then used in a polymerase chain reaction (PCR). The calmodulin (\(cmd\)) gene was amplified by PCR using the enzyme KOD-Plus (Toyobo, Osaka, Japan) and cmd5 and cmd6 primers\(^17\). The PCR product was sequenced, and the sequence data were analyzed by a BLAST search in the DNA Data Bank of Japan (DDBJ). The genomic nucleotide sequence data for the \(cmd\) genes of 10 aflatoxigenic fungi (named FUT-F300 and FUT-F302 through -310) were deposited in the DDBJ/EMBL/GenBank nucleotide sequence database under accession nos. LC498723 to LC498732 (Table 1).

For the determination of the morphological characteristics of the fungi, each fungus was inoculated onto PDA medium and cultured at 28°C for 3 days or 1 week. Each colony was observed using a VHX digital microscope equipped with a VHX-Z25 lens (Keyence, Osaka, Japan).
Results

The imported raw peanuts, macadamia nuts, and pistachio nuts were purchased and analyzed by the DV-AM method. After 3 days’ culture, fungal colonies appeared surrounding all nuts on the YES-DOC-CP-DV agar medium (Fig. 1A). When the same plates were treated with AM vapor, the colors of some parts of the colonies of peanuts and macadamia nuts changed from yellow to red, suggesting that these red colonies may correspond to aflatoxigenic fungi. In contrast, the fungal colonies surrounding pistachio nuts did not show any red colonies by the DV-AM method. They appeared as dark green colonies, and microscope observation showed that they were different from those of Aspergillus (data not shown).

The production of AFs by the fungi purified from the red colonies of peanuts (named FUT-F300 to FUT-F302) and macadamia nuts (FUT-F303 to FUT-F310) were analyzed by TLC. The fungi from the peanuts produced mainly AFB$_1$ and no AFG$_1$ (Fig. 1B). The fungi from the macadamia nuts produced mainly AFB$_1$ and AFG$_1$.

After genomic DNA was extracted from mycelia of each aflatoxigenic fungus, cmd gene was amplified by PCR and then sequenced. Our BLAST search analyses of the sequences indicated that FUT-F300 has 100% identity to A. flavus voucher BF9 cmd gene [MK304474], FUT-F302 to FUT-F303 and FUT-F305 to FUT-F310 showed high identity to A. parasiticus voucher APS 55 cmd gene [MH743104]. Among them, FUT-F302, 303, 309, and 310 showed 100% identity to the same gene. FUT-F304 showed 99.31% identity to A. parasiticus culture NRRL:29590 cmd gene [MK119708].

All isolates from the peanuts and macadamia nuts showed macronematous and colorless conidiophores producing monophialide nonindigenous cells on the swollen, spherical apex; these are characteristic findings for Aspergillus isolates (Fig. 1C, D). None of the isolates except FUT-F309 formed sclerotia. FUT-F309 formed some 590-nm-dia. sclerotia (Fig. 1D), indicating that this is the L (large) strain with sclerotia.

We then used the DV-AM method to analyze the other imported raw nuts (macadamia nuts, almond nuts, cashew nuts, walnuts, hazelnuts, pumpkin seeds, and pistachio nuts) that had been purchased from another company (Fig. 2). Although conidiospores were picked from some reddish colonies after AM treatment and then analyzed by TLC, no aflatoxigenic fungi were detected. Contamination of macadamia nuts with the aflatoxigenic fungi depended on either export country (Fig. 1).

Discussion

Our results demonstrated that the DV-AM method is useful for the detection of aflatoxigenic fungi contaminating imported raw nuts. Although we used the whole nuts in this work, crushing or grounding of nuts would be useful for more quantitative measurements of aflatoxigenic fungi. Various types of fungi other than aflatoxigenic fungi were also detected in all 10 types of nuts, indicating that it is important to put nuts in the refrigerator or freezer immediately after purchase. It is also advisable to roast or heat nuts.

The DV-AM method revealed aflatoxigenic fungi in the peanuts and macadamia nuts. These results indicate...
Fig. 1  The DV-AM analyses of the peanuts, macadamia nuts, and pistachio nuts. A: Nuts were incubated on YES-DOC-CP medium supplemented with DV for 3 days and then treated with ammonia solution (AM). Color changes of the undersides of the plates with AM were observed. B: TLC analyses of mycelial extract of the fungi isolated from the red colonies with AM. Lanes 1–3: Fungi isolated from peanuts. Lanes 4–11: Fungi isolated from macadamia nuts. S: A mixture of AFs. C: Morphologies of the FUT-F300 isolate from peanuts (left) and the isolated FUT-F304 (center) and FUT-F309 (right) from macadamia nuts. The upper side (top) and underside (middle) of each plate are shown. D: Microscopy photos of FUT-F300 (left) and FUT-F309 (right). Width of white bars show 100 µm.
that aflatoxigenic fungi may enter Japan from some foreign countries as parts of imported foods. We observed that most of the macadamia nuts in the same package were contaminated with aflatoxigenic fungi. In our preliminary experiments, three washes of the macadamia nuts with 0.05% Tween 80 greatly decreased the number of aflatoxigenic fungi in the nuts by the DV-AM method (data not shown), suggesting that the aflatoxigenic fungi bound mostly to the surface of the nuts. Since the raw unshelled (peeled) macadamia nuts were herein used, the aflatoxigenic fungi (probably as conidiospores) had probably mixed easily in the same bag.

The aflatoxigenic fungi isolated from the peanuts were B-group AF producers. *A. flavus* are generally known to produce only B-group AFs, and herein the *A. flavus* FUT-F300 was matched to the TLC data (Fig. 1B). However, FUT-302 isolated from peanuts was *A. parasiticus* based on its cmd gene sequence, which is generally known to produce B- as well as G-group AFs. Since only an atypical population of *A. parasiticus* that produces aflatoxins B has been reported, FUT-302 may be a similar atypical *A. parasiticus* strain. This remains to be determined.

The fungi from the macadamia nuts from Australia were all *A. parasiticus* based on cmd genes, and they...
produced both B- and G-group AFs. Interestingly, among them, only FUT-F309 formed sclerotia. These results suggested that at least two different types of A. parasiticus are contaminants in the same package of macadamia nuts. Although pistachio nuts and almonds from Iran have been known to be frequently contaminated with aflatoxins\(^{10}\), the pistachio nuts from Iran were not herein detected, whereas green colonies of different fungi were predominantly observed, and their morphologies were different from those of the Aspergillus group (data not shown). In this work, only 16 pistachio nuts were analyzed. To reveal that the pistachio nuts were not contaminated with aflatoxigenic fungi, it is necessary to find out more nuts in the same bag or in several rots. This is under investigation. Also, we observed that most of all nuts were contaminated with different kinds of fungi. Characterization of the fungi in each nut would be interesting to make the edible nuts safer.

We purchased nuts from two different shops through the Internet for this study. The peanuts and macadamia nuts that were contaminated with aflatoxigenic fungi were purchased from the same shop. This might reflect differences in their production and distribution stages such as at harvesting, sorting, washing, transporting, and so on. The DV-AM method will be practically useful for searching for the critical control points for safe food supplies and food safety.

**Conclusion**

The DV-AM method was confirmed to be useful for the detection of aflatoxigenic fungi, which have contaminated raw imported nuts in Japan. Aflatoxigenic A. flavus fungi were isolated from peanuts from South Africa, and A. parasiticus fungi were isolated from Macadamia nuts from Australia. The DV-AM method will thus be useful for improving food processing.

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