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Fungal Population and Aflatoxin Contamination on Stored Gamma-Irradiated Nutmeg (*Myristica fragrans*) Kernels

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ABSTRACT

A study on the effectiveness of gamma irradiation at doses of 5 and 10 kGy on fungal population, *Aspergillus flavus* strains, and aflatoxin B_1 contamination on stored nutmeg kernels was conducted. The kernels were collected from seeds in a period of one week from the ground at North Sulawesi Province, Indonesia. Dried shelled kernels with ± 10 % moisture content, packed in polyethylene bags at 2.1 kg/bag, were irradiated at 5 and 10 kGy and stored at ambient temperature (28 °C) for 2 and 4 months. Kernel moisture content, fungal population, and aflatoxin B_1 were determined before and after irradiation, and after 2 and 4 months of storage. Results showed that fungal population was reduced with the increasing irradiation dose. Five species of fungi were isolated, *i.e.*, *Aspergillus flavus*, *A. niger*, *Cladosporium cladosporioides*, *Eurotium chevalieri*, and *Penicillium citrinum*. A total of twelve *A. flavus* strains were isolated, five strains from unirradiated kernels and five and two strains from irradiated kernels at doses 5 and 10 kGy, respectively. Among these strains, 58 % were capable of producing L sclerotia and 25 % were identified as toxigenic. Kernel moisture during storage was 7.3 % and no aflatoxin B_1 was detected before and after irradiation, and after 2 and 4 months of storage duration.

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INTRODUCTION

Nutmeg (*Myristica fragrans* Houtt.) is one of the tropical spices that are often infected with fungi. High aflatoxin contaminations in nutmeg often indicate that the commodity is infected by *Aspergillus flavus* [1]. The humid tropical climate strongly supports the growth of mycotoxin-producing fungi [2]. Most of the fungal infections occur during nutmeg development, harvesting, and postharvest handling. Moreover, a majority of nutmeg is grown and harvested traditionally by subsistence farmers where harvest and storage conditions are insufficiently controlled [3]. Most nutmeg was harvested by collecting the seeds from the ground, drying them in open air on the ground, and storing them in warm humid conditions. During the drying process, the temperature and relative humidity (RH) are beyond the control of the majority of farmers. As a result, inapropriate storage of nutmegs is susceptible to attacked by fungi [3]. Dried-stored nutmeg is also hygroscopic and tends to absorb water vapor from the environment. This process leads to an increase in kernel moisture content that results in accelerated fungal infection and mycotoxin contamination.

Gamma irradiation is a physical method that can be used to reduce microbial contamination and as an alternative treatment to chemical fungicides on agricultural commodities including crops, herbs, and spices. Presently the physical treatment can be used as preservation method [4] and has been developed and approved by national legislations in

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over 60 countries worldwide and can be used to ensure food safety [5]. However, the sensitivity of fungi to irradiation depends on fungal strains [6]. At present, there are a few studies concerning the presence of field and storage fungi on nutmeg kernels. The objective was to investigate the effects of gamma irradiation on fungal population, *A. flavus* strains and aflatoxin B_1 (AFB_1) contamination on post-harvest nutmeg kernels after 2 and 4 months storage duration.

EXPERIMENTAL METHODS

Collecting nutmeg seeds, shelling and drying

Nutmeg seeds were obtained from plants cultivated in subsistence farms at Kauditan subdistrict, North Minahasa Regency, North Sulawesi Province, Indonesia. The seeds were collected in a period one week from the ground (naturally matured). Nutmegs with their shells was dried under sun drying followed by smoke drying (45 °C) until their moisture content declined to ±10 %. Shelling and sortation of damaged kernels were conducted manually. Only intact kernels were used in this experiment.

Packaging, irradiation and storing of nutmeg kernels

Nutmeg kernels with initial moisture content of ± 10 % were packed in polyethylene bags (2.1 kg/bag) and sealed using a mechanical sealer. The kernels were irradiated at doses of 5 and 10 kGy using a ^{60}Co source emitting gamma rays with an activity of 878.339 kCi at the Center for Isotope and Radiation Application, National Nuclear Energy Agency, Jakarta, Indonesia. As control, nutmeg kernels were not irradiated. Three replicates (= 3 bags) were used for each treatment. The bags containing nutmeg kernels were then placed randomly on wooden shelves and stored for 2 and 4 months under warehouse conditions. Each bag was used to pack nutmeg kernels with different doses of gamma irradiation and different storage duration. Therefore, the number of bags containing nutmeg kernels was 27, derived from 3 (irradiation doses) × 3 (storage duration) × 3 (replicates).

Sampling method to obtain working sample

Samples of nutmeg kernels were collected from each bag directly after irradiation (before storage) and subsequently after 2 and 4 months of storage. Sampling method to obtain working sample was according to Dharmaputra *et al*. [3]. Each sample was divided into four parts manually. One part was used as a reserved sample, while the other three parts were ground for 30 seconds at 25 000 rpm using a Mill Powder Tech Model RT-04 grinder. They were then mixed homogenously and devided into eight parts manually to determine moisture content, fungal population, and aflatoxin B_1 content.

Determination of kernel moisture content and fungal identification

Moisture contents of kernels (based on wet basis) were determined by using distillation method. Three replicates were used for each sample. Fungi were isolated and enumerated by using serial dilution method, followed by pour plate method on dichloran 18 % glycerol agar (DG18) which consisted of 10 g/L glucose, 5 g/L peptone, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$, 220 g/L glycerol, 15 g/L bacto agar, 2.0 mg/L dichloran, and 100 mg/L chloramphenicol). Plates were incubated for 7 days at room temperature (28 °C). A separate colony of each fungal species was isolated and cultured for 7 days at 28 °C in potato dextrose agar (PDA), Czapek yeast extract agar (CYA), or Czapek yeast extract agar with 20 % sucrose (CY20S).

Aspergillus flavus isolation and identification

Every single separate colony *A. flavus* in every sample was isolated on aspergillus flavus and parasiticus agar (AFPA), consisting of 10 g peptone, 20 g yeast extract, 0.5 g ferric ammonium citrate, 100 mg chloramphenicol, 18 g bacto agar, 2 mg dichloran, and 1 L distilled water.

Sclerotial production

Each *A. flavus* strain was cultured in petri dishes (9 cm diameter) containing PDA (Difco Laboratories, Sparks, US). The plates (three replicates for each strain) were incubated for 14 days at ambient temperature (28 °C). Sclerotia were harvested by adding 10 mL sterile distilled water containing 1 mL 0.05 % Tween 80 into the surface of the colony in a petri dish. The colony was scrapped off using a small sterilized brush over a No. 2 Whatman filter paper. The sclerotia were placed in a beaker glass. They were then rinsed several times using tap water and then air-dried.

Determination of toxigenic and non-toxigenic *A. flavus* strains

For determination of qualitative ability of AFB_1 production, all *A. flavus* strains were inoculated on 10 % coconut agar medium (CAM) (36 g bacto agar, 900 mL distilled water, and 100 mL coconut cream extracted from fresh shredded coconut endosperm). The medium was adjusted to pH 7.0 using 2 N NaOH and sterilized for 20 minutes at 15 lb, 120 °C. A small fragment of *A. flavus* colony was inoculated onto the center of a CAM culture plate in petri dish (9 cm diameter) and incubated for 5 days at 28 °C. The presence of yellow pigment on the reverse side of each plate was observed. The presence or absence of blue fluorescence surrounding the growing fungus colony was examined using long-wave (365 nm) UV light and expressed as positive or negative toxigenicity. An uninoculated plate was used for references.

Quantitative analysis aflatoxin B_1

Aflatoxin B_1 analysis was conducted using high-performance liquid chromatography (HPLC) (Agilent 1260 Infinity Isocratic, Agilent Technologies, Waldbronn, Germany). Five mililiters of filtered extract in a clean vessel was diluted with 20 mL 15 % Tween 20 solution and mixed well for 1 minute. The mixture was filtered through glass microfibre into a clean vessel. The mixture was loaded onto the AflaTest® affinity column at a rate of 1-2 drops/second until air comes through column and was then washed once with 10 mL deionized water at a rate of 2 drops/second. A glass cuvette (VICAM # 34000) under AflaTest® column was loaded with 1.0 mL HPLC grade methanol into glass syringe barrel. The AflaTest® column was eluted at a rate of 1 drop/second by passing the methanol through the column. One milliliter of eluated sample collected in a glass cuvette was added with 1 mL deionized water. Afterward, 20-100 µL of the result was injected into HPLC.

RESULTS AND DISCUSSION

Nutmeg kernels collected in a period of one week on the ground were infected by field and storage fungi. *Cladosporium cladosporioides* is a field fungus, while *Aspergillus flavus*, *Aspergillus niger*, *Eurotium chevalieri*, and *Penicillium citrinum* are storage fungi. *Cladosporium cladosporioides* and *Penicillium citrinum* were the most predominant field and storage fungi, respectively. Both of those species were still viable after 4 months storage duration at irradiation 10 kGy (Table 1).

Table 1. Fungal population on non-gamma irradiated and gamma-irradiated on-storage nutmeg kernels

Fungal species	Gamma irradiation doses (kGy)/storage duration (months)/fungal population (log cfu g^{-1})								
	No irradiation			5 kGy			10 kGy		
	0	2	4	0	2	4	0	2	4
Aspergillus flavus	0.5	1.1	0	0.5	0.8	0.8	0.5	0.5	0
A. niger	0.5	1.0	1.1	0	0	0.5	0	0	0
Cladosporium cladosporioides	1.8	0	0.8	0	0.5	1.2	0	0	1.1
Eurotium chevalieri	1.3	0.8	1.4	0	0	0	0	0	0
Penicillium citrinum	2.0	0.8	2.0	0	0	0	0	0	0.8

Irradiation at doses of 5 and 10 kGy reduce the population of all fungal species. However, each species has different viability. Irradiation dose of 10 kGy is effective to kill all fungi except *A. flavus*. The presence of *A. flavus* on irradiated kernels after 4 months of storage indicate their resistance on irradiaton. Allam and El-Zaher [7] reported that *Aspergillus flavus* is one of the Aspergilli that are relatively resistant to gamma ray. The dry condition of kernels during irradiation process suggested their spores' resistance on irradiation.

Among storage fungi that might produce aflatoxin B_1, we found twelve strains of *A. flavus*. They consist of five strains isolated from unirradiated kernels before and after 2 months of storage and the other five and two strains were isolated on stored kernels after 2 and 4 months at irradiation doses of 5 and 10 kGy, respectively. The codes of the strains are presented on Table 2.

Table 2. Codes of *A. flavus* strains isolated from unirradiated and gamma-irradiated at doses 5 and 10 kGy during 0, 2 and 4 months of storage

Gamma irradiation doses (kGy)	Storage duration (months) /*A. flavus* strains code		
	0	2	4
0 (no irradiation)	AFS0R0	$AFS2_1R0$ $AFS2_2R0$ $AFS2_3R0$ $AFS2_4R0$	
5	AFS0R5	$AFS2_1R5$ $AFS2_2R5$	$AFS4_1R5$ $AFS4_2R5$
10	AFS0R10	AFS2R10	

AF = *A. flavus*; S = storage duration: 0, 2, 4 months and number strains found (subscript); R = irradiation doses: no irradiation (0), 5 and 10 kGy

Each *A. flavus* strain (Table 3) varies in morphological characteristics such as sclerotial production, seriation of conidial head, and toxigenicity.

A previous study reported that strains of *A. flavus* consist of biseriate and uniseriate strains [8]. However, most uniseriate and L strains were observed in this study. Fifty-eight percent (7 of 12) *A. flavus* strains isolated from unirradiated and irradiated kernel formed Large (L) sclerotia and 5 of 12 strains were non-sclerotial producers as it was reported in other studies [8].

Table 3. Morphological characteristics and toxigenicity of *A. flavus* strains isolated from unirradiated and irradiated nutmeg kernels

A. flavus strains	Diameter (µm) type sclerotia*	Uniseriate/ biseriate	Fluorescence in CAM	Toxigenicity
AFS0R0	NSP	b	+	toxigenic
$AFS2_1R0$	700-800 (L)	u	-	non-toxigenic
$AFS2_2R0$	520-580 (L)	u	-	non-toxigenic
$AFS2_3R0$	520-540 (L)	u	-	non-toxigenic
$AFS2_4R0$	510-800 (L)	u	-	non-toxigenic
AFS0R5	NSP	u	-	non-toxigenic
$AFS2_1R5$	NSP	u	+	toxigenic
$AFS2_2R5$	NSP	u	-	non-toxigenic
$AFS4_1R5$	NSP	u	-	non-toxigenic
$AFS4_2R5$	560-580 (L)	u	+	toxigenic
AFS0R10	520-540 (L)	b	-	non-toxigenic
AFS2R10	520-560 (L)	u	-	non-toxigenic

L= (Large) sclerotia, NSP = non-sclerotial producer, +, - = fluorescence, no fluorescence

*sclerotia were observed after incubation for 2 weeks on potato dextrose agar at 28 ºC

Odhiambo *et al*. [9] differentiate toxigenic and non-toxigenic *A. flavus* strains based on yellow pigment formation using PDA medium. Our results found that 25 % (three of twelve) *A. flavus* strains, namely AFS0R0, $AFS2_1R5$, and $AFS4_2R5$, were AFB_1 producers or toxigenic strains (Table 3) as indicated by the presence of yellow pigment on the reverse side of the colony after 5 days of incubation at 28 °C in CAM medium. In addition, toxigenic strains in CAM medium were indicated by the presence of blue fluorescence surrounding the colony and very light yellowish brown (beige) at the colony edge under 365-nm UV light.

Kernel moisture content and AFB_1 contamination before and after iradiation and after 2 and 4 months of storage to all samples are shown on Table 4. Our results showed that no AFB_1 production was detected. The presence of three toxigenic strains (AFS0R0, $AFS2_1R5$, $AFS4_2R5$) in kernels do not necessarily indicate that the strains are aflatoxin producers. We assumed that the strains were not able to metabolize under the dry condition of the kernels during storage.

Table 4. Moisture content and AFB_1 concentration on irradiated and unirradiated nutmeg kernels after 0, 2 and 4 storage duration

Kernels Parameter	Gamma irradiation dose (kGy) / storage duration (month)								
	No irradiation			5			10		
	0	2	4	0	2	4	0	2	4
Moisture content (% w.b)	7.4	7.5	6.7	7.2	7.7	6.9	6.9	7.8	7.3
AFB_1 concentration (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND

ND = not detected; limit of detection <0.92 ppb

Packing kernels in polyethylene bags maintain kernel moisture (7.0 ± 0.3 %) and inhibit fungal growth and aflatoxin production. In addition, the predominance of non-toxigenic L strains might prevent aflatoxin production by toxigenic strains [10].

CONCLUSION

A gamma irradiation dose of up to 10 kGy on dry nutmeg is effective to kill fungi except *A. flavus*. Twelve *A. flavus* strains were isolated. Among these, 25 % were characterized as aflatoxin producers. Five of the strains were found on unirradiated kernels and five and two strains were found on kernels irradiated at doses of 5 and 10 kGy, respectively. No aflatoxin B_1 was found on dried irradiated and unirradiated kernels packed in polyethylene bags during storage of up to 4 months.

Reducing fungal population by integrated established system for the safe handling such as drying and proper storage before irradiation might be required to increase the effectiveness of irradiation.

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