Successive growth of gamma irradiated *Aspergillus flavus* strains isolated from nutmeg kernels (*Myristica fragrans*)

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**Abstract:** *Aspergillus flavus* is one of the important fungus which can cause nutmeg deterioration during storage. The study aimed to investigate the effect of gamma irradiation 5 and 10 kGy on conidial germination and successive growth *Aspergillus flavus* strains isolated from nutmeg kernels up to 20 generations. Thirteen strains of the *A. flavus* were sub-cultured in potato dextrose agar (6 days, 28 °C). Conidia of each strain were harvested and one mL (10⁷ conidia per mL) in 1.5 mL eppendorf tube were centrifuged 6000 ×g, air-dried (24 h, 28 °C) and irradiated at 5 and 10 kGy. Experiments were done in triplicate for each irradiation dose. Conidia with no irradiation used as control. After irradiation, conidia were re-suspended in 1 mL sterile distilled water. Serial dilutions were made and plated onto *Aspergillus flavus* and *Parasiticus* agar. Morphological characteristics such as colony diameter, mycelial branches, conidiophore and mycelial growth up to 20 generations were compared between irradiated and non-irradiated strains. Results showed that gamma irradiation up to 10 kGy inhibit conidial germination and mycelial growth of surviving strains. Successive growth of irradiated *A. flavus* up to 20 generations showed that their colony tend to grow and recover to their normal growth.

1. Introduction

Nutmeg (*Myristica fragrans* Houtt.), native to Banda Island is an economically important commodity of Indonesia. Since 2011 over 75% the world export of nutmeg was supplied from Indonesia. High content of nutmeg oil (myristicin) approximately 13.5%, strong taste and very aromatic make Indonesia is a world leading nutmeg producer and exporter [1]. As tropical spices, nutmeg mainly cultivated by small-scale farmers where the method of post-harvest handling is not conducted appropriately, consequently it affects the quality of the kernels. High rainfall and relative humidity promote improper stored nutmeg absorb water vapor from the environment. This process leads to an increase of kernel moisture content that results in accelerated fungal infection and mycotoxin production.
Aspergillus flavus is a fungus that can infect nutmeg and produces aflatoxin [2,3]. The infection occurred during preharvest, harvesting and postharvest handling. The infected conidia germinate while kernel moisture content were suitable for their growth.

The use of gamma irradiation up to 10 kGy is considered as an effective physical method to prevent A. flavus infection and to reduce its population [4]. However, the effectiveness of the irradiation was determined by the kind of commodity, fungal strains, their population, moisture content of the substrate and the development phase of fungal growth [5]. Not much research conducted on conidial germination and the effect of gamma irradiation on mycelial growth of A. flavus strains isolated from nutmeg kernels. The objectives of the research were to investigate conidial germination and the effect of gamma irradiation 5 and 10 kGy on mycelial and successive growth of A. flavus strains up to 20 generations.

2. Materials and Methods

2.1 Aspergillus flavus strains
Thirteen strains of A. flavus were obtained from culture collection of Phytopathology Laboratory, Southeast Asian Regional Centre for Tropical Biology (SEAMEO BIOTROP) Bogor, Indonesia. They were isolated from nutmeg kernels collected from small scale nutmeg farmers in North Sulawesi Province, Indonesia. Each strain was sub-cultiured in a petri dish (9 cm in diameter) containing potato dextrose agar (PDA, Difco Laboratories, Spark, MD) for 7 days (28 °C).

2.2 Harvesting conidia
Asexual spores (conidia) were harvested by adding 10 mL sterile distilled water containing 1 mL 0.05% sterile Tween 80 into the surface of the colony in a petri dish (diameter 9 cm). The colony was scrapped off using a small sterilized brush then 1 mL of conidial suspension (10⁷ conidia per mL) was transferred onto 1.5 mL eppendorf tube. In order to prevent radiolysis, the suspension was filtered through sterile filter paper and the conidia were removed to another eppendorf tube. The tube were then centrifuged at 6000 ×g for 15 min to remove remaining water. The supernatant was discarded and conidia were air dried for 24 h at ambient temperature (28 °C).

2.3 Gamma irradiation
Air-dried conidia in the eppendorf tube were irradiated using a Cobalt-60 source emitting gamma rays at doses 5 and 10 kGy at The Center for Isotope and Radiation Application, National Nuclear Energy Agency (BATAN), Jakarta, Indonesia. Non-irradiated conidia were used as control. The treatment as well as the control were done in triplicate.

2.4 Conidial germination
Conidia for germ tube observation was conducted immediately after irradiation. Two or three drops of barely molten PDA (±45 °C) were put on the surface of sterilized object glass using a serological pipette. One ose conidia were spread sparsely and a cover slip was put on the surface of the medium. The culture was incubated at ambient temperature (28 °C). The emerging germ tube was observed after 4 h incubation and subsequently every 1 h up to the tenth hours using Olympus CH2 Japan light microscope.

2.5 Successive growth of Aspergillus flavus
Irradiated and non-irradiated conidia in eppendorf tube were re-suspended by adding 1 mL of sterile distilled water. After serial dilution (10⁻¹ to 10⁻⁴ per mL), 1 mL of the conidial suspension were cultured on Aspergillus Flavus and Parasiticus Agar (AFPA) and incubated for 6 days at 28 °C. Using cork borer (diameter 5 mm), the mycelial plug was cultivated at the center of PDA plate on a petridish (diameter 9 cm). Each culture was incubated for 6 days and regenerated every 6 days (28 °C) on PDA up to 20 generations. The experiments were conducted in three replicates for each strain. Colony growth, macroscopic and microscopic characteristics of each strain were observed at each generation.
2.6 Sclerotial production
Sclerotial production was observed according to Novas and Cabral [6] with a slight modifications. Each strain of *A. flavus* was grown in a petri dish (9 cm diameter) on PDA plates. 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 onto the plate. The surface of the colony was scrapped off over a No. 2 Whatman filter paper using a small brush. The sclerotia were placed in in a beaker glass, they then rinsed 2 to 3 times using tap water and then air-dried.

3. Results and Discussion
Among 13 strains *A. flavus*, AF4, AF6, AF11 and AF12 were survive after irradiation 5 kGy. Whereas only one strain (AF3) was found to survive after irradiation at 10 kGy. Codes of non-irradiated and irradiated strains were presented on Table 1.

| Code of non-irradiated strains | Code of irradiated strains at doses 5 and 10 kGy |
|--------------------------------|--------------------------------------------------|
| AF4                            | 5AF4                                             |
| AF6                            | 5AF6                                             |
| AF11                           | 5AF11                                            |
| AF12                           | 5AF12                                            |
| AF3                            | 10AF3                                            |

3.1 Microscopic characteristics
Characteristics of each *A. flavus* strain were presented in Table 2. Most strains produced large (L) sclerotia (diameter >400 µm). Conidial head with vesicle in diameter 25 µm, the conidial head consist of either uniseriate or biseriate. Rough wall conidiophores particularly close to their vesicle were found in all strains. Conidiophores, metulae and phialides of irradiated strains shorter than those of non-irradiated strains.

| *A. flavus* strains | Sclerotia (µm) | Conidiophore (µm) | Vesicle (µm) | Seriation | Phialides (µm) | Metulae (µm) | Conidial wall |
|---------------------|----------------|-------------------|--------------|------------|----------------|--------------|---------------|
| AF3                 | 450            | 350-600           | 25           | u          | 7.8            | -            | rough         |
| 10AF3               | 500            | 300-350           | 25           | u          | 6.7            | -            | rough         |
| AF4                 | 500            | 350-600           | 25           | u          | 7.3            | -            | rough         |
| 5AF4                | 500            | 300-500           | 25           | u          | 7.0            | -            | rough         |
| AF6                 | 500            | 500-700           | 25           | b          | 7.3            | 5.2          | rough         |
| 5AF6                | 500            | 250-500           | 25           | b          | 6.8            | 3.4          | rough         |
| AF11                | NF             | 400-600           | 25           | b          | 7.8            | 5.0          | rough         |
| 5AF11               | 450            | 250-400           | 20           | u          | 7.3            | -            | rough         |
| AF12                | 500            | 650-700           | 25           | b          | 7.8            | 5.0          | rough         |
| 5AF12               | 500            | 500-550           | 25           | u          | 7.7            | -            | rough         |

NF = not found, u = uniseriate, b = biseriate

The effect of gamma irradiation at doses 5 and 10 kGy on conidial germination to all *A. flavus* strains was presented in Figure 1. No germination to all strains before 4 h of incubation. Emerging a new germ tube occurred 5 h after incubation (28 °C) particularly on non-irradiated strains (AF6, AF11, AF12). After 6 h of incubation all non-irradiated conidia germinated. A single germ tube was indicated only one nucleus
presented in a conidium. The presence of two nuclei in single *A. flavus* conidium was reported by Runa et al. [7]. Barhoom and Sharon [8] stated that the germination of conidium either from one side or from both sides depending on external condition.

Germination is metabolisms that involved in many enzymes and proteins [9]. Gamma irradiation inhibits germination to all irradiated strain (10AF3, 5AF4, 5AF6, 5AF11, 5AF12) (Figure 2). Conidia of these strains started to germinate 7 h after incubation. Similar results also was reported that inhibition of conidial germination of *Botrytis cinerea* and *Penicillium expansum* caused by irradiation occurred at dose 3 kGy [10]. Inhibition of conidial germination using ionizing irradiation was supposed due to radiolytic products of water in conidia cytoplasm yielding hydroxyl radical or hydrogen peroxide, the radical molecule interfere with organic molecules such as enzymes or deoxyribonucleic acid (DNA) that inhibit cell division during germination [11,12]

![Germ tube (µm) of irradiated and non-irradiated *A. flavus* strains after several hours of incubation on PDA (28 °C)](image)

**Figure 1.** Germ tube (µm) of irradiated and non-irradiated *A. flavus* strains after several hours of incubation on PDA (28 °C)

### 3.2 Mycelial growth

The growth of irradiated and non-irradiated strains through successive growth up to 20 generations were presented in Figure 2. The colony diameter of irradiated strains (56.1±0.3 mm) was smaller than that of non-irradiated (65.4±0.4 mm). Inhibition of conidial germination at first successive generation lead to reduce mycelial growth to all irradiated strains. In addition, different size of conidiophores and phialides (Table 2) lead to differences in colony diameter and colony texture between irradiated and non-irradiated strains. Macroscopically, the colony of non-irradiated strains was characterized by larger diameter, loose mycelial texture and rough colony surface. In contrast to irradiated strains that have smaller diameter, dense mycelia texture and smooth colony surface.

Changes of mycelial growth such as apical and lateral branches were caused by internal and external factors [13]. More lateral branches were produced than apical branch on irradiated hyphae strains and vice versa occurred on non-irradiated strains.

Mycelial growth of irradiated strains at 20 generations tended to have similar growth to non-irradiated strains. Similar findings were reported by Ribeiro et al. [14] that subculture of irradiated filamentous fungi on nutritional media such as *Cladosporium* spp., *Curvularia* spp., *Fusarium* spp. or *Aspergillus* spp. *A. flavus* and *A. parasiticus* tended to grow and recover to normal growth pattern.
Figure 2 The growth of irradiated and non-irradiated *A. flavus* strains up to 20 generations on PDA (28 °C)

The presence of germ tube on irradiated *A. flavus* with conidial concentration $10^7$ per mL showed that gamma irradiation up to 10 kGy were not sufficiently to kill dry conidia. More dense and more mycelial branches were formed in irradiated strains than that of non-irradiated could increase their resistance to irradiation. Minimizing fungal infection during pre- and postharvest and preventing fungal growth on irradiated nutmeg kernels during storage were recommended.

4. Conclusion
Gamma irradiation doses 5 and 10 kGy inhibit of conidial germination of *A. flavus*. Successive growth of irradiated *A. flavus* up to twenty generation tend to grow similar to mycelial of non-irradiated. Reducing *A. flavus* infection were recommended during pre and postharvest to prevent fungal growth on irradiated nutmeg kernels.
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