Coating formulas protect shelled nutmeg seeds from 
*Aspergillus* colonization

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Abstract. Coating of nutmeg seeds may prevent the colonization of *Aspergillus* contamination. The study aimed to evaluate the effect of coating formulas to *A. flavus* on shelled nutmeg. Shelled nutmeg seeds were coated with aqueous (TT) and gel (GM+) formulas containing propylparaben (0.1%), potassium sorbate (0.8%), and clove oil (1.25%), and the aqueous GM- (0.65% clove oil and 0.4% potassium sorbate). The coated seeds were then sun-dried and sprayed with *A. flavus* conidia. The untreated control was only inoculated with *A. flavus*. The colonization of *A. flavus* on the seeds was visually observed. The aflatoxins, propylparaben, and potassium sorbate in the treated seeds were analyzed with HPLC. The results showed that seeds treated with GM+, GM-, and TT formulas were visually free from *A. flavus*. The total aflatoxins were not detected in the seeds treated with the GM+, but in the coated with GM- and TT was 0.95 µg/kg and 12.6 µg/kg, respectively. In the uncoated seeds, the total aflatoxins were 695.9 µg/kg. Propylparaben and potassium sorbate residues in the coated seeds were 11 mg/kg and 415 mg/kg, respectively. The coating formula effectively minimized *A. flavus* colonization and aflatoxin. Therefore, the coating formula could be used for reducing *Aspergillus* contamination.

1. Introduction

The nutmeg plant (*Myristica fragrans* Hout) is one of Indonesia's strategic export commodities because it generates foreign exchange, and many farmers are involved, thus contributing to employment. In 2013, foreign exchange generated from nutmeg exports was US$ 122,000,000, with the farmers involved amounting to 168,658 households [1]. Indonesia is the largest producer and exporter of nutmeg globally, followed by Grenada, India, Malaysia, Sri Lanka, and Papua New Guinea [2]. The world's demand for nutmeg is 70% in Indonesia's supply, followed by Grenada at 25%, and the rest by India and Papua New Guinea [3, 4]. However, the value of a dollar received by nutmeg exporting countries in 2016, the highest was obtained by India (US$107,906 thousand), and Indonesia was the second (US$96,672 thousand) [5].

One of the critical issues in nutmeg exports from Indonesia is the aflatoxin contamination, which exceeds the European market limit [6]. The maximum limit for aflatoxin B1 in powdered spices is 15 µg/kg, and the total aflatoxin is 20 µg/kg [7]. In almost every nutmeg supply chain, aflatoxin contamination was detected, starting from farmers, collectors, and exporters [8]. Due to limited drying facilities, the nutmeg seeds' moisture content is still >10%. The high-water content in the nutmeg seeds will stimulate the colonization of various fungi, including aflatoxin-producing fungi, such as *Aspergillus flavus* and *A. parasiticus*. One of the efforts to protect nutmeg from fungal contamination is through
coating with chemical compounds that can inhibit or prevent the growth of *Aspergillus* spp. Several types of chemicals can be used, such as essential oils [9], *Cerbera odorollam* plant extracts, cloves, and mahogany (*Swietenia macrophylla*) [10], and food preservatives, such as methyl parabens, propylparaben, and potassium sorbate [11, 12]. This study aimed to test the effectiveness of the coating formula containing clove oil, propylparaben, and potassium sorbate to *A. flavus* on shelled nutmeg.

2. Materials and methods
The study was conducted in the laboratory of Plant Disease of the Indonesian Spice and Medicinal Crops Research Institute in 2020.

2.1. Nutmeg seed
The nutmeg seeds were purchased from a wholesaler in the Bogor area. The nutmeg seeds were physically characterized with blackish-brown shells. The seeds were then soaked overnight in water to facilitate separating the mace from the nutmeg shell. The maces were then removed, and the seeds were washed under tap water. While washing, the seeds were rubbed by hand to clean all debris on the seeds' surfaces. Next, the seeds were placed on trays and dried for 2–3 days in the sun until the seeds were dried. The dried seeds were characterized by shaking with the hand; if sound, the inner seeds had separated from the shell. Finally, the seed shells were broken with a hammer, and the inner shelled seeds were ready for treatments.

2.2. Preparation of *Aspergillus flavus*
An isolate of aflatoxin-producing *A. flavus* fungi was obtained from the microbe culture collection of BIOTROP, IPB (Dr. Okky Dharmaputra). *A. flavus* isolate was propagated on potato agar media and incubated at room temperature.

2.3. Coating formula
Viscous (GM + and GM-) and liquid (TT) coating formulas were made. The GM + formula contained active ingredients of clove oil (1.25%), potassium sorbate (0.8%), and propylparaben (0.1%) with carriers, including Arabic gum (0.25% w/w), CMC (0.5% w/w), hydrolyzed starch (1% w/w), glycerin (0.04% v/v), and Tween 80 (0.05% v/v). The GM-coating formula was made the same as GM+, but the clove oil content concentration reduced to 0.6% and potassium sorbate 0.4%. The TT liquid formula contained the same active ingredients as GM (Table 1), but added with another substances, such as arabic gum (0.5% w/w), sago flour (3%), tapioca flour (1%), glycerin (0.8% v/v), and Tween 80 (1.0% v/v).

| Coating formula | Clove oil (%) | Potassium sorbate (%) | Propylparaben (%) |
|----------------|--------------|-----------------------|-------------------|
| GM-            | 0.62         | 0.4                   | 0.1               |
| GM+            | 1.25         | 0.8                   | 0.1               |
| TT             | 1.25         | 0.8                   | 0.1               |

2.4. Coating treatment
The dried shelled nutmeg seeds were dipped in the coating formulas (GM+, GM-, and TT) for a few minutes, then taken out and placed on trays, dried in the sun for 1–2 hours or until the coating layers dried. The coating treatment was repeated twice. As a control, shelled nutmeg seeds were not treated with a coating formula. Instead, the coated nutmegs were placed in a plastic box lined with moist tissue papers. One box contained 30 coated nutmeg seeds. Furthermore, the treated seeds were inoculated by spraying the conidia suspension of *A. flavus* using a mini perfume sprayer. The treatments were repeated
three times.

The parameters observed were the percentage of *A. flavus* colonization, aflatoxin content, and potassium sorbate and paraben residue in the seeds. Fungal colonization on the inoculated nutmeg seeds was visually observed daily for one month. In addition, the percentage of the seed surface colonized by *A. flavus* were counted. Colonization of *A. flavus* on the seeds was characterized by a greenish-yellow color of conidia of the fungus. For aflatoxins, potassium sorbate, and paraben analyses, the seeds were sampled from each box and pulled together as a bulk sample. The seeds were then sent to a standardized laboratory in Bogor and were chromatographically analyzed using High-Performance Liquid Chromatography (HPLC) method code 18-5-30/MU/SMM-SIG. The detection level of the HPLC code 18-5-30/MU/SMM-SIG for aflatoxin B1 was 0.1623 µg/kg, aflatoxin G1 was 0.1591 µg/kg, aflatoxin B2 was 0.0392 µg/kg, and aflatoxin G2 was 0.0392 µg/kg.

3. Results and discussion

3.1. Colonization of *Aspergillus flavus* in shelled nutmeg and peeled nutmeg

The three coating formulas made and tested were GM+ (standard), GM- (reduced concentration of clove oil and potassium sorbate), and TT liquid formula, which contained the same active ingredients as the GM formula. The results showed that the shelled nutmeg coated with the formulas did not show the colonization of *A. flavus* (Table 2, Figure 1), whereas the untreated control was heavily colonized (Figure 2). These results indicated that the GM+ and GM- (gelform) were effective in protecting the seeds, but in GM- formula might be more efficient since it contained less clove oil (0.6%) than GM+ (1.2% clove oil). The liquid coating formula TT was also able to protect the fungal colonization, similar to the GM+ and GM- formula. In the future, TT might be further developed since it is easier to apply by spraying.

| Treatment       | Colonization (%) | Disease reduction (%) |
|-----------------|------------------|-----------------------|
| GM (+) [gel]    | 0.00a            | 100.00                |
| GM (-) [gel]    | 0.00a            | 100.00                |
| TT [liquid]     | 0.00a            | 100.00                |
| Untreated control | 100.00b         | 0.00                  |

Note: GM+ and TT contained clove oil (1.25%), potassium sorbate (0.8%), and propylparaben (0.1%). GM- contained less clove oil (0.6%), potassium sorbate (0.4%), and propylparaben (0.1%)
Figure 1. Shelled nutmeg seeds treated with the GM- (left) and TT (right) formula are free from the colonization of *A. flavus*.

Figure 2. Untreated shelled nutmeg seeds that were heavily colonized with *Aspergillus flavus*. The yellow-green color indicated *A. flavus* growth.
3.2. Aflatoxin contamination level

The study showed that aflatoxin contamination on the nutmeg seeds coated with the GM formulas (GM+ and GM-) was minimal, i.e., <1 µg/kg (Table 3). The TT coating treatment was less effective than GM. The aflatoxin level in the TT coated seeds was higher, i.e., 12.6 µg/kg and 11.64 µg/kg for aflatoxin total and aflatoxin B1, respectively, than in the GM treated (0.95 µg/kg). However, the aflatoxin content of nutmeg treated with TT was still below the maximum level of 20 µg/kg [7]. In contrast, the uncoated nutmeg seeds were high, i.e., 696.91 µg/kg of the total aflatoxins and 693.96 µg/kg of the aflatoxin B1. The study suggests that GM+, GM-, and TT could be developed further to coat shelled nutmeg seeds. For practical reasons, TT’s spraying formulae would be more suitable than dipping (GM formula).

Table 3. Aflatoxins, potassium sorbate, and propylparaben contents in coated nutmeg.

| Treatment   | Total aflatoxins (µg/kg) | B1 (µg/kg) | G1 (µg/kg) | B2 (µg/kg) | G2 (µg/kg) | Potassium sorbate (mg/kg) | Propyl paraben (mg/kg) |
|-------------|--------------------------|------------|------------|------------|------------|--------------------------|------------------------|
| GM (-) (gel)| 0.95                     | 0.95       | nd         | nd         | nd         | 95.46                    | 741.32                 |
| GM (+) (gel)| nd                       | nd         | nd         | nd         | nd         | 116.41                   | 415.76                 |
| TT (liquid) | 12.16                    | 11.64      | nd         | 0.53       | nd         | 66.41                    | 213.74                 |
| Control     | 696.91                   | 693.96     | nd         | 2.95       | nd         | nd                       | nd                     |

Note: The detection level of HPLC code 18-5-30/MU/SMM-SIG:
Aflatoxin B1 0.1623 µg/kg; aflatoxin G1 0.1591 µg/kg; aflatoxin B2 0.0392 µg/kg; aflatoxin G2 0.0392 µg/kg.
Nd= not detected

Aflatoxin contamination of agricultural products, including nutmeg seeds, is common and has become the main concern of all nations. Indonesia has set the maximum limit of aflatoxin total (B1 + B2 + G1 + G2) in food products and spices powder for 20 µg/kg [7], similar to that applied in the USA and Brazil [13]. However, European countries impose strict regulations, i.e., the aflatoxin total maximum limit is 10 µg/kg [6]. Factors that affect aflatoxin contamination varied, one of the most critical is the water content of the agriculture products. The higher the water content the most vulnerable it is to Aspergillus contamination. As found in the previous study, that the moisture contents of nutmeg kernels at farmers, collectors, and exporters in the North Sulawesi Province were 8-18% (10.88%), 7.5-15.50% (11.07%), and 7.00-11.50% (9.48%), respectively [8]. This means that commercial nutmeg seeds have higher water content: a better way to minimize aflatoxin is needed by applying a proper drying process by farmers. Therefore, harvested nutmegs must be dried as soon as possible to reduce water content since Aspergillus spp. as the causal agent of aflatoxin cannot develop in a very dry condition (10°C) and relative humidity <65%) [14]. The drying process could be used by exposing nutmeg seeds under the sun for 3-4 weeks [15].

Recently, Sembiring et al. [16] have found several drying types that could be used to dry nutmeg seeds. The drying types evaluated were the fiber-house, the electric hot-box, and the traditional rack types. The study showed that the fiber-house and electric hot-box drying types were faster in drying the nutmeg seeds (2-3 days) compared with the traditional rack methods either uncovered with a black cloth (6-7 days) or covered (5-6 days). The moisture content of the dried seeds was less than 6% and the aflatoxins contamination was less than 5 µg/kg. The oil and oleoresin of the dried seeds have also fulfilled the standard. One of the drying models, especially the fiber-house, is promising to be further developed on a larger scale because it is ecofriendly and required no electricity.

The use of food preservatives in coating nutmeg-shelled seeds has not been reported, therefore, the method could be further developed. However, the present study showed that the potassium sorbate and propylparaben residue were still high as shown in Table 3. The highest propylparaben was found in the nutmeg seeds coated with GM+ formula, i.e., 741.32 mg/kg. The propylparaben residue in the GM- was
415.76 mg/kg and in the TT was 213.74 mg/kg. The potassium sorbate residue was the highest in the nutmeg seed coated with the GM- formula (116.41 mg/kg). In contrast, the least was from TT (66.41 mg/kg). The potassium sorbate residue in the coated shelled nutmegs is still safe because the maximum limit is 1000 mg/kg body weight [17].

In general, the study highlighted the importance of treating shelled nutmeg seeds with a coating formula. The coating formula containing a mixture of clove oil, potassium sorbate, and propylparaben can protect the nutmeg seeds from Aspergillus contamination. However, since the propylparaben limits its use due to safety concerns, therefore, more safely food preservatives or botanical materials need to be investigated for controlling Aspergillus spp. affecting nutmeg seeds.

4. Conclusion
Seed coating formulas (GM+, TT, and GM-) containing a mixture of clove oil, potassium sorbate, and propylparaben effectively protected the shelled nutmeg seeds from colonization of A. flavus. Aflatoxin contamination in the coated nutmeg seeds was minimal and below the standard limit of 20 μg/kg. As a result, the coated seeds contained below the level of aflatoxins. GM+ gel formula is the most effective, but the liquid formula of TT might be more practical for use.

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