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AFLATOXIN OF NUTMEG IN INDONESIA AND ITS CONTROL

Aflatoksin pada Pala di Indonesia dan Pengendaliannya

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ABSTRACT

Indonesia produces the largest amount of nutmeg in the world, accounted for 66-77% of the world market. Most nutmeg plantations (99.3%) are cultivated by small holders, mainly in five provinces, i.e. North Moluccas, Moluccas, Aceh, North Sulawesi, and West Papua. Ironically, during the last 17 years (2000-2016), exported nutmeg are detected to be contaminated with aflatoxins, especially those entering the European Market, as the result 53 out 80 (62%) cases of imported nutmegs were rejected. Aflatoxin contaminating nutmeg is found in every level of market chains in the country, from the farmers, collectors and exporters, representing that aflatoxin in nutmeg is common and serious. Aflatoxins are produced mainly by two species of fungi, i.e. *Aspergillus flavus* and *A. parasiticus*. Five groups of aflatoxins are known, i.e. aflatoxin B1, B2, G1, G2, and M1, but the international legislations are concerned on the maximum limit of aflatoxin B1 and total aflatoxin (B1+B2+G1+G2) that should not exceed of 5 and 10 μg/kg, respectively. Aflatoxin in agriculture products including nutmeg can be detected by various methods, mainly by a thin layer chromatography (TLC), high performance thin layer chromatography (HPLC), andenzyme-linked immonosorbent assay (ELISA). Minimizing aflatoxin in the nutmeg should be properly managed at every level of the production processes from harvesting, drying, and packaging. Drying is the most critical one; nutmeg should be dried as soon as being harvested to keep its water content below 10%, then it be kept in a very dry condition (10^{o}C and air humidity< 65%).

Keywords: *Myristica fragrans*, aflatoxin, detection, control.

ABSTRAK

Indonesia merupakan penghasil pala terbesar di dunia yang memasok sekitar 66-77% pasar dunia. Sebagian besar perkebunan pala (99,2%) dibudidayakan oleh petani kecil, terutama di lima provinsi, yaitu Maluku Utara, Maluku, Aceh, Sulawesi Utara, dan Papua Barat. Ironisnya, selama 17 tahun terakhir (2000-2016), ekspor pala, terutama ke pasar Eropa, terdeteksi mengandung aflatoksin sehingga 53 dari 80 (62%) kasus pala dari Indonesia ditolak. Biji pala yang tercemar aflatoksin ditemukan pada setiap tingkat rantai pasar dalam negeri, mulai dari petani, pengumpul, dan eksportir. Hal ini menunjukan bahwa aflatoksin pada pala sudah umum dan sangat serius. Aflatoksin diproduksi terutama oleh *Aspergillus flavus* dan *A. parasiticus*. Dikenal ada 5 kelompok aflatoksin, yaitu aflatoksin B1, B2, G1, G2, dan M1, tetapi peraturan perundang-undangan Inernational hanya fokus pada batas maksimum aflatoksin B1 dan jumlah aflatoksin (B1 + B2 + G1 + G2) yang masing-masing tidak boleh melebihi dari 5 dan 10 μg/kg. Aflatoksin dalam produk pertanian, termasuk biji pala, dapat dideteksi dengan berbagai metode, terutama kromatografi lapis tipis (TLC), HPLC, dan ELISA. Upaya meminimalkan aflatoksin pada biji pala harus dilakukan secara baik pada setiap tingkat proses produksi, mulai dari panen, pengeringan, dan kemasan. Pengeringan adalah proses paling penting, oleh karena itu biji pala harus dikeringkan segera setelah dipanen untuk menjaga kadar airnya di bawah 10%. Selanjutnya biji pala kering harus disimpan dalam kondisi yang sangat kering (10^{o}C dan kelembaban udara <65%).

Kata kunci: *Myristica fragrans*, aflatoksin, deteksi, pengendalian.

INTRODUCTION

Indonesia produces the largest amount of nutmeg in the world. The nutmeg plants (*Myristica fragrans*) initially grow in the Banda and the Moluccas Islands, but now they have spread to other parts throughout Indonesia, mainly Aceh, Papua, Java, and North Sulawesi. At the moment, the nutmegs are mostly produced in five provinces, i.e. North Moluccas, Molucca, Aceh, North Sulawesi, and West Papua

Table 1. The five largest areas producing nutmeg in Indonesia predicted in 2017*

No	Province	Acreage (ha)	Production (ton)
1	North Molucca	42,747	7,957
2	Moluccas	30,913	4,641
3	Aceh	22,078	8,507
4	North Sulawesi	19,399	4,398
5	West Papua	15,070	4,747
Total Indonesia		169,587	34,602

*Source: (Ditjenbun 2016).

(Table 1)(Ditjenbun 2016). Nutmeg is mostly (99.3%) cultivated in small farms.

In 2017, the total acreage of nutmeg plantations was predicted to reach 169,587 ha; total production was 34,516 tonnes (Ditjenbun 2016). Nutmeg plantations slightly increased in 2014 up to 147,377 ha with total production of26,468 tonnes. Since 2014, the Indonesian government planted more nutmeg trees in the Molucca Provinces, such as the East Seram (200 ha), Central Molucca (400 ha), South Buru (200 ha), West Molucca (175), Southeast Molucca (175 ha), West Seram (250 ha), and Ambon (100 ha). These efforts are aimed to increase the national production of nutmeg in the next five years.

The majority of the nutmeg produced is exported to various countries, especially in the European Union and the USA. In 2012, the total amount of export was 12,849 tonnes, with value of US $ 140,018. Therefore, the Indonesian nutmeg shared 66-77% of the world market, followed by Sri Lanka (8-13%) and Grenada (3-26%). Indonesian nutmeg entered the European market mainly through the Netherlands (27%) and Germany (23%), then the nutmegs are re-exported from these two countries to the other European countries, such as Italy, Belgium, France, and the UK. Other importing countries outside Europe were the USA and Japan, as well as the United Arab Emirates, Singapore, and Vietnam. The last three countries re-exported nutmeg to world markets. The world market of nutmeg in 2014 worth US$ 173,838,723. The main exporters of nutmeg were Indonesia (43.39% of world exports), India (17.93%), Netherlands (9.12%), Sri Lanka (7.95%), and Italy (4.22%)

Table 2. World exports value of nutmeg in 2014*

Country	Trade value (US$)	Growth rate (%)	World share (%)
Indonesia	80,594,167	-9.79	43.39
India	33,302, 005	-3.76	17.93
Netherlands	16,944,758	-37.53	9.12
Sri Lanka	14,761,331	-6.04	7.95
Italy	7,832,435	-13.60	4.22
Germany	7,205,059	-18.31	3.88
France	4,714,239	-24.47	2.54
Belgium	3,309,032	-28.43	1.78
Singapore	2,872,135	-1.42	1.55
Spain	2,303,562	-10.76	1.24
Total	173,838,723		

*Data Source: http://data.trendeconomy.com/commodities/Export/090810

[http://data.trendeconomy.com/commodities/Export/090810] (Table 2).

According to the trend economy data, the share and value of exported nutmeg from Indonesia to various countries, such as Vietnam were (9.56%;$ 17.8 M), the USA (7.60%; $ 14.1 M), the Netherlands (6.09%; 11.3 M), the United Arab Emirates (4.71%; $ 8.74 M), Germany (3.84; $7.13M), Japan (3.44%; $6.40), and Italy (3.39%; 6.30M).

AFLATOXIN AS THE KEY PROBLEM IN NUTMEG QUALITY

Although nutmeg contains a variety of antioxidant and antimicrobial components, such as sabinene (28.61%), β-pinene (10.26), α-pinene (9.72), myristicin (4.30%), isoeugenol (2 , 72%), p-cymene (1.81%), carvacrol (1.54%), eugenol (0.89%) and β-caryophellene (0.82%) (Gupta *et al*. 2013; Shazia et al., 2015), the fresh nutmeg beans are vulnerable to the infection of harmful fungi, such as *Aspergillus* spp., that can produce aflatoxins. Therefore, nutmeg beans must be dried as soon as being harvested. The ideal moisture content of dried nutmeg is less than 10% to prevent from mold infection. The general quality standard of nutmeg of Indonesia is regulated in the National Standardization of Indonesia or BSN number 0006-2015, which states that the maximum water content is 10%, free of

Table 3. The maximum limit of aflatoxin in the spice products allowed in the European Market*

Types of food products	B1 Aflatoxin Content (µg/kg)	Aflatoxin total (B1+B2+G1 +G2) (µg/kg)
Spices herbs: hot chilli, white and black peppers, nutmeg ginger, and curcuma	5	10
Consumable rice for food products	2	4
Corn	5	10

Sumber: *CIR (2016)

fungus and insects (both living and dead), and contains no foreign objects (BSN 2015).

A specific rule on the maximum limit of aflatoxin in nutmeg bean has not been set, but it can refer to the general guidelines of processed nut products, i.e. the BPOM No. HK.00.06.1.52.4011, stating that the maximum limit of aflatoxin B1 of food products and spices powder is 15 µg/kg, while the total aflatoxin is 20 µg/kg (BPOM 2009). The provision is similar to that adopted in the United States, that the maximum limit of total aflatoxins (B1 + B2 + G1 + G2) for all types of food products, except for milk, is 20 µg/kg, while Brazil sets a maximum limit of aflatoxin total (B1 + B2 + G1 + G2) on the spice products is 20 µg/kg(Dors *et al*. 2011). However, European countries impose the Commission Regulation EC No. 1881/2006 stating the maximum limit of the aflatoxin, i.e. 5µg/kg for aflatoxin B1 and for the aflatoxin total (B1 + B2 + G1 + G2) is 10 µg/kg (CIR 2016)(Table 3).

Based on the study conducted by Dharmaputra *et al*. (2015), from 76 samples of nutmeg taken from the farmers, collectors and exporters in the North Sulawesi, it reveals that aflatoxin in nutmeg is common and serious; about 55.83% of the nutmeg samples have been contaminated with aflatoxins (Table 4). The study also reveals that although the moisture content of the nutmeg samples in the samples is lower than that the maximum limit set in the regulation of theNational Standardization Agency of Indonesia (BSN) number 0006-2015, hence the nutmegs are infested by *Aspergillus niger* and *E. fibuliger* (Dharmaputra et al., 2015). It means that the minimum water content of the nutmeg beans need to be adjusted in the state regulatory should be lower than 10%.

Based on the Rapid Alert System for Food and Feed (RASFF) search data for the period 2000-2016, nutmegs imported from Indonesia (80 cases) and India (50 cases) were frequently detected to be contaminated by aflatoxin, whereas from Sri Lanka (4 cases) and Grenada (2 cases) were lessdetected [https://webgate.ec.europa.eu/rasff-window/portal/?event=searchResultList]. The majority (62%) of the Indonesian contaminated with aflatoxin were rejected (Figure 1). This finding was quite alarming for Indonesian nutmeg exporters and they should take necessary efforts to minimize it.

Table 4. Aflatoxin contamination on nutmeg taken from the farmers, collectors and exporters in the North Sulawesi*

Delivery chain level	Sample number	Moisture content (%)	Damaged (%)	Total fungal population (cfu/g wet sample)	Number of aflatoxin (%)
Farmer	25	9.80 ± 2.62	70.55 ± 29.66	3.9×10^5	60.00
Collector	22	10.49 ± 2.25	76.70 ± 32.39	1.3×10^6	45.45
Exporter	29	9.33 ± 1.13	71.75 ± 30.27	9.9×10^3	62.06
Average		**9.77 ±1.97**			**55.83**

*Source: Dharmaputra *et al*. (2015)

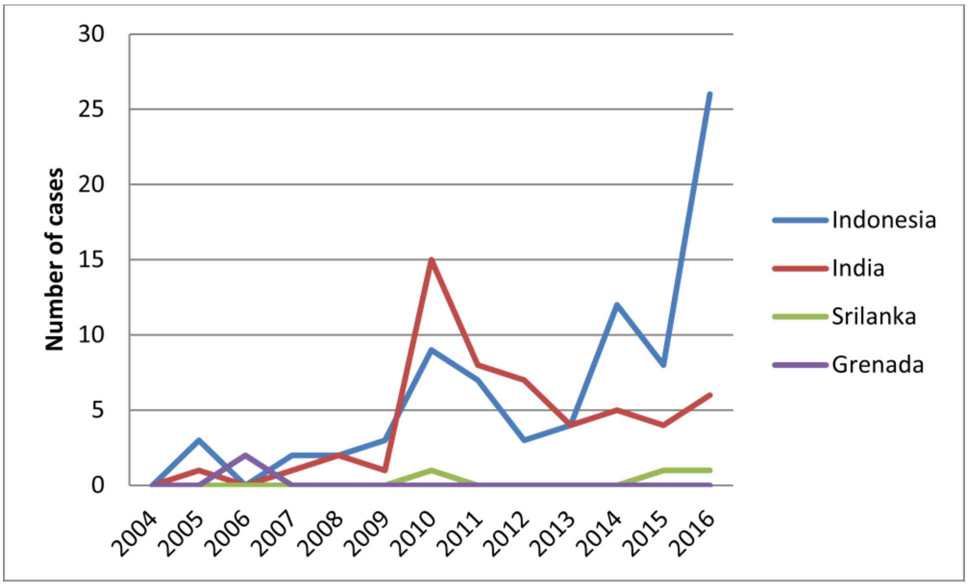

Figure 1. Number of cases of aflatoxin contaminated nutmeg from Indonesia entering the Economic Union Market reported by RASFF in 2000-2016*

*Source: RASFF Portal (https://webgate.ec.europa.eu/rasff-window/ portal/?event=searchResultList https://webgate.ec.europa.eu/rasff-window/%20portal/?event=searchResultList, downloaded at 2nd September 2016).

AFLATOXSIN TOXICITY

Five groups of aflatoxins are known, i.e. aflatoxin B1, B2, G1, G2, and M1 (AFM1). Aflatoxin B1, B2, G1 and G2 are commonly encountered in agriculture products such as maize, peanut, almond, nutmeg and kinds of nuts, whereas aflatoxin M1 is detected in milk and milk products. Aflatoxin B1 is a human carcinogen, causes genotoxic, carcinogenic, hepatotoxic, teratogenic, and immunosuppressive and aflatoxin M1 is a possible carcinogen for genotoxic, carcinogenic, hepatotoxic (Pietri and Piva, 2012). Further, Pietri and Piva (2012) stated that in most studies, they are more focused on the presence of aflatoxin B1 because it is extreme acute and chroni toxicity for human and animals. However, the legislations are concerned in the presence of aflatoxin B1 and total aflatoxin (B1+B2+G1+G2) to clarify if the targeted substances are safe or not for human consumption. Two species of Aspergillus, i.e. *A. flavus* and *A.parasiticus* are known to produce aftaoxin. The name of aflatoxin stands from Aspergillus falvus toxin. *A. flavus* produces aflatoxin B1, B2 and cyclopiazonic acid, whereas *A. parasiticus* produces aflatoxin B1, B2, G1 and G2 (Pietri and Piva, 2012).

Commission Implementing Regulation-EU 2016/24 8 January 2016(CIR (2016) imposes of official controls on imports of certain feed and food of non-animal origins, including groundnuts from Brazil and *Capsicum annum* and nutmeg from India since January 2010, whereas nutmeg from Indonesia is subjected to inspection since July 2012. The regulation requires that all consignments of groundnuts from Brazil, Capsicum annum from India and nutmeg from India and Indonesia are accompanied by a health certificate stating that the products have been sampled and analyzed for the presence of aflatoxins and have been found compliant with union legislation.

The Republic of Indonesia publishes the regulation of the Minister of Agriculture (MoA) 04/Permentan/PP.340/2/2015, known as the Food Safety Control on Importation and Exportation of Fresh Food of Plant Origin, MoA regulation 04/2015 (Permentan. 2015). This regulation imposes the importations and exportations of certain fresh foods should comply with food safety regulations on the maximum limit of

Table 5. Detection of fungi producing aflatoxin in nutmeg

Species	Country Reported	Reference
Penicillium glaucum	Unknown	Hansen and Jung (1973)
Aspergillus flavus and *Aspergillus parasiticus*	Unknown	Olsen *et al*. (1998) *in*(Mizakovaet al., 1993)
Aspergillus ochraceus	Unknown	Hübner *et al* (1998) in (Mizakovaet al., 1993)
A. niger and *Endomyces fibuliger*	Indonesia	Dharmaputra *et al.* (2015)
A. niger	Iraq	Abdulla and Toma (2013)
A.flavus and *A. parasiticus,*	Indonesia and India packaged in Italy	Pesavento *et al.* (2016)
A.nomius and *A.bombycic*	Indonesia	Okano *et al*.(1997)

chemical and biological contaminations and types of the contaminations concerned. The importation and exportation of the products should be accompanied with certifications or documents stating that the products have been analyzed by recognizing countries or registered laboratories in the exporting and importing countries prior to its arrival or departure at the Indonesian borders.

ASPERGILLUS SPECIES CONTAMINATING NUTMEG

Various studies reveal that nutmeg is contaminated with several fungi, not only those known as aflatoxin producers such as *A. flavus* and *A. parasiticus*, but also the others, such as *P. glaucum, A. niger, A.ochraceus*, and *Endomyces fibuliger* (Table 5). Detection of aflatoxin in nutmeg have been reported from various countries in the Europe and the Middle East since 2002, indicating that aflatoxin problem is very serious. For example, Hübner *et al*. (1998) *in* Mizakova *et al*. (1993) isolated *Aspergillus ochraceus* from coriander, marjoram, white pepper, paprika, nutmeg and thyme, whereas Olsen *et al*. (1998) *in* Mizakova *et al*. (1993) examined a number of samples of white pepper, chilli, nutmeg, and paprika. They confirmed the presence of *Aspergillus flavus* and *Aspergillus parasiticus.*

AFLATOXIN BIOSYNTHESIS

Biosynthesis of aflatoxin of *A. parasiticus* is described by Yu and Ehrlich (2011) and (Yu 2012).

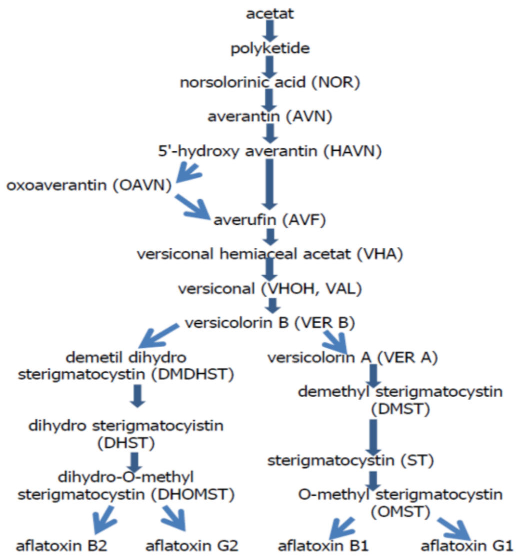

Figure 2. Biosynthesis of aflatoxin of *Aspergillus parasiticus* (Yu 2012)

There are various steps in that processes and enroling different genes. The main substrate used is acetat. In the first step, acetat is metabolized into nonsorinic acid, follows with averantin. In the further steps, versicoloninB is brokendown into demetil dyhydro sterigmatocystin as the main substance for aflatoxin B2 and G2, whereas versicolorin A becomes aflatoxin B1 and aflatoxin G1 (Figure 2).

AFLATOXIN DETECTION

Several screening methods for direct visual determination of aflatoxins and ochratoxin A (OTA) production have been reported, including coconut culture media (Davis, 1987), methylated β-cyclodextrin (Fente *et al*., 2001), ammonia vapour (Kumar *et al*. 2007), and HPLC (Yazdani *et al*., 2011). A coconut culture media was first developed by (Davis, 1987). Under a long wave UV light (365 nm), 2-5 day-old cultures of *A. parasiticus* and *A. flavus* grown on the coconut media produced blue fluorescence. Adding 3% methylated β-cyclodextrin on agar media strengthened the appearace of blue fluorescence pigment produced by aflatoxin producing *Aspegillus* (Fente *et al.* 2001). *A.flavus* grown on agar media, such as Czapek Dox Agar, Yeast Extract Sucrose Agar, or Potato Dextrose Agar for 7 days then it is exposed to a fume of 2ml concentrated ammonia hydroxide solution produced pink/red color in contrast to non producing aflatoxin strain. A typical HPLC chromatogram of aflatoxin is shown in Figure 3. It is observed that the retention time for aflatoxin G2 is 12.748 min; G1 is 14.84 min; aflatoxin B2 is 16.068, aflatoxin B1 is 18.301 min (Yazdani *et al.* 2011). To have a confirming results of *Aspergillus* productng aflatoxin, the visual base methods described above need to be supported with a more reliable ones, such as a thin layer chromatography (TLC), high performance thin layer chromatography (HPLC), and ELISA (Yazdani *et al*. 2011; Nair *et al*., 2014; Sudini *et al*., 2011).

Other methods of the detection of aflatoxins, such as mass spectroscopy, electrochemical immunosensor, radioimmunoassay, and immonodipstick, are reviewed by (Wacoo *et al*., 2014). (Baranyi *et al*., 2013) reviewed modern techniques for detection of aflatoxin producing fungi. These include a multiplex PCR targeting specific genes (*aflD, aflR, aflP*), PCR assays targeting aflP, aflD, aflM genes, and PCR assays aplifying individual sequence of the aflRS, aflM, and aflO genes.

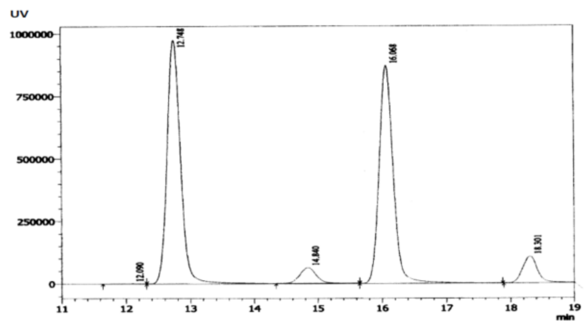

Figure 3. HPLC chromatogram of aflatoxin standards. Retention time for aflatoxin G2 is 12.748 min; G1 is 14.84 min; aflatoxin B2 is 16.068, aflatoxin B1 is 18.301 min (Yazdani e*t al.* 2011).

AFLATOXIN CONTROL

Harvesting

Nutmegs must be harvested at 9 months old where the fruits become yellowish brown, having wrecked, and fulli becomes dark brown in colour. Nutmeg fruits must be properly harvested by direct picking from the twigs using a hook or other equipment. Shaking of the branches or twigs which commonly practiced by farmers should be avoided since the fallen fruits are proned to be contaminated by soil microbes. Study byAccinelli *et al.* (2008) revealed that agricultural soil and plant residues were significant sources of *A. flavus.*

Drying

Fresh harvested nutmegs should be dried as soon as possible under the sun or fumed at 45°C to reduce their water content to 8-10%. After unshelled, the nutmegs are placed on the racks and dried again under the sun for 3-4 weeks until the water content becomes 10-12% (Ditjenbun, 2012). Dharmaputra *et al*. (2015) showed that most farmers in the North Sulawesi dried shelled and in-shell nutmegs under sun-drying method by spreading the nutmegs on tarpaulin placed on the ground and then stored in plastic bags. At the collector level, however, drying of nutmegs was conducted by using sun-drying and smoke drying methods; smoke drying was used especially during rainy season. However, based on the study of (Hansen and Jung, 1973), after drying any agriculture products such as beans should be kept in a very dry conditions (10°C and relative humidity <65%) because *Aspergillus* will cease to produce aflatoxin at 13°C. After drying, the unshelled dried nutmegs should be kept in sealed polyetilene bags and stored in a dry condition. Trenk and Hartman (1970) warned that in the case of corn, remoisted dried corn was subject to more rapid fungal deterioration and aflatoxin formation by *Aspergillus flavus* than the freshly harvest ones. Unfortunately, Dharmaputra *et al.* (2015) showed that the moisture contents nutmeg kernels at farmers, collectors and exporters in the North Sulawesi Province were higher than that of recommended by theIndonesian National Standardisation (SNI). For examples, the range and average of moisture content at farmers' level, collectors, and exporters were 8-18% (10.88%), 7.5-15.50% (11.07%), and 7.00-11.50% (9.48%), respectively. This means that a better processing of nutmeg is only adopted by exporters.

The EU-Indonesia Support Program has recently published a manual for improving nutmeg quality for export, which especially in eliminating aflatoxins (EU-TSP, no date.). The prorgam has published the guideline for good agricultural practices of processing nutmeg from harvesting to packaging. The manual recommends to minimise wound at the time of harvesting and transportation of nutmeg. For example, using a long hook in harvesting of nutmeg from the tree, drying of nutmeg as soon as harvested to reduce water content of the nutmeg to less than 12%, stored at a relative humidity <75%, and adopt higienic practices throughprocessing. A simple oven box drying has been designed to operate at 40-45^{o}C and powered with 1 KW to dry nutmeg, especially when rainy season.

Heat treatment

Aflatoxins are resistant to heat. For example, ginger or curry seasoning contaminated by aflatoxin can not be removed by heating with microwave or oven at a temperature 180^{o}C (Menon *et al.* 2010; Mobeen *et al.* 2011). Pesavento *et al.* (2016) show that application of heat on shelled nutmeg imported from India and Indonesia, packaged in Italy are less contaminated with molds compared with those of non-heated. Steaming is also effective in reducing mold from agricultural products. Because the quantity of aflatoxin contamination in food products is low, therefore, preventive screening of the whole food chain is very important.

Packaging

Packaging aims to ensure agriculture products are safe for consumers. The nutmegs are commonly packed in bags of gunnis or plastics, and wood cases. Weights of the bags depends on their quality, i.e. 90 kgs for ABCD shelled nutmeg (Ditjenbun 2012). Based on the study by Dharmaputra *et al.* (2015) in the North Sulawesi Province, it revealed that many exporters stored nutmegs in gunny and plastic (polypropylene) bags. They occationally monitored the quality of nutmeg kernels for aflatoxin contamination using a long wave ultraviolet lamp. Aflatoxin contaminated nutmeg kernels would produce blue green yellow fluorescence. Those found to be contaminated by alfatoxins must be removed prior to export.

CONCLUSION

Nutmegs from Indonesia exported to the European Union Market in the last sixteen year (2000-2016) have been detected to be contaminated by aflatoxin with the rates of 62%, therefore, they were rejected.This finding is quite alarming for Indonesian as the main exporter of nutmeg to Europe. This is in line with the finding from North Sulawesi as one of the main producing provinces of nutmeg, revealed that aflatoxin in nutmeg is common and serious; about 55.83% of the nutmeg samples have been contaminated.This represents that aflatoxin in nutmeg is common and serious. Aflatoxins are produced mainly by two species of fungi, i.e. *Aspergillus flavus*, *A. parasiticus*, but other fungi may also produce, such as *Penicillium glaucum, A. niger, A.ochraceus*, and *Endomyces fibuliger.* Five groups of aflatoxins are known, i.e. aflatoxin B1, B2, G1, G2, and M1, but the international legislations are concerned on the maximum limit of aflatoxin B1 and total aflatoxin (B1+B2+G1+G2) that should not exceed of 5 and 10 μg/kg, respectively. Aflatoxin produced by *Aspergillus* spp. can be detected by various methods, but the most reliable ones are by a thin layer chromatography (TLC), high-performance thin layer chromatography (HPLC), and ELISA. Minimizing aflatoxin in the nutmeg should be properly managed at every level of the production processes from harvesting, drying, and packaging. The key points in minimizing aflatoxin are drying nutmeg as soon as harvested to keep its water content below 10% and sanitary practices during the whole processes. Monitoring of nutmeg quality, i.e. dryness, in every level of market chains from farmers to exporters is a must. *Aspergillus* spp. as the causal agent of aflatoxin can not develop in a very dry condition (10^{o}C and relative humidity <65%).

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