Genetic Diversity of *Aspergillus flavus* Isolated from Pepper at North Sumatera

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Abstract: *Aspergillus flavus* is one of the postharvest fungi that often infects white and black pepper (*Piper nigrum* L.). Fungal infection on pepper not only spoiled physically but also produce mycotoxins. The aim was to study *Aspergillus flavus* toxigenicity and their genetic diversity on dried-stored white and black pepper sold at traditional markets in North Sumatera. Fungal population was determined based on colony forming unit per mililiter (cfu/mL) using serial dilutions and pour plated in dichloran 18% glycerol agar (DG18) medium. Toxigenicity of *A. flavus* was determined culturally using coconut agar medium (CAM) 10%. Phylogenetic between *A. flavus* strains were carried out by isolating genome of representative toxigenic and non-toxigenic *Aspergillus flavus* strains according to the MiniKit Promega protocol and amplified using 10 Random Amplified Polymorphic DNA (RAPD) primers. The amplified bands were scored and translated to be biner data, then analyzed using Numerical Taxonomy and Multivariate System (NTSys) and clustered by Unweighted Pair Group Method with Arithmatic Average Algorithm (UPGMA). Results showed that a total of thirty one *A. flavus* strains were isolated. Based on toxigenicity determination found that twelve strains of *A. flavus* were toxigenic (aflatoxin producers) and nineteen strains were non-toxigenic (non-aflatoxin producers). Dendogram of similar bands was constructed and showed the highest similarity coefficient of *A. flavus* strains was 0.84 (84%) which means that the strains were similar even though they were isolated from different traditional markets.

1 INTRODUCTION

Pepper (*Piper nigrum* L.) *i.e* black and white pepper, is one of spices that commoly produced by tropical countries with high temperature, hummidity. Most of the commodity is cultivated conventionally and lack of good agricultural practices (Pickova et al. (2020). Similar to crops, dried pepper is susceptible contaminated by moulds. the infection can cause spoiled physically and chemically, loss of aroma, taste and contaminated by mycotoxins. Fungal infection and aflatoxin contamination on black and white pepper were previously studied. The infection of pepper by *Penicillium* sp was reported by Bokhari (2007) and Pitt and Hocking (2009). Black and white pepper sold by retailer at traditional markets were contaminated by *Aspergillus chevalieri*, *A. flavus*, *A. niger* and *A. sydowi* (Nurtjahja et al. (2019). Fungal infection and mycotoxin contamination might occur during preharvest and postharvest handling. Inappropriate drying, storing might increase fungal population. As a soil fungi, *Aspergillus flavus* contaminate crops in field such as leaves, flowers and fruits crops (Hererra et al. 2014). There are many strains and genetic variability of *Aspergillus flavus* in field, some of the strains are toxigen (aflatoxin producer) and the other are non-toxigen (non-aflatoxin produces) (Midorikawa et al. 2008; Ehrlich, 2014). The genetic variability of *Aspergillus flavus* strains on field was studied by Solorzano et al. (2014). The objectives of the current study was to determine genetic diversity of *A. flavus* strains isolated from dried-stored black and white pepper sold at traditional markets in Medan, North Sumatera.

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2 MATERIALS AND METHODS

2.1 Sample Collection

A total of 250 g samples of dried white and black pepper were obtained from 5 retailers at five traditional markets. Only intact seeds used in this experiment. Each sample then was placed into a sterile polyethylene bag and stored in a refrigerator at ± 12°C for further use.

2.2 Determination Fungal Population

The population of *A. flavus* was determined by a dilution and pour plated in dichloran 18% glycerol agar (DG18) medium. Each sample was ground and 25 g were put into a 500 ml flask and suspended with 250 ml of sterile distilled water and then homogenized to obtain a 10^{-1} suspension. Dilution was carried out on 10^{-2}, 10^{-3} and 10^{-4}. One ml of dilution suspension was cultured on DG18 medium. Each dilution was repeated 3 times. All plates were incubated for 5 days (29ºC). Population of *A. flavus* per gram of pepper (cfu/g) was determined using the formula:

$$\text{Fungal population} = \frac{1}{XY} \cdot Z \ \text{(cfu/g)}$$

X = volume of suspension transferred to each petri dish (1 ml)
Y = dilution which gives the fungus colonies separately
Z = average number of colonies of each fungal species from 3 petri dishes

2.3 Fungal Identification

Each colony of *A. flavus* was cultured on potato dextrose agar (PDA) medium then all of the isolates were identified according to Pitt and Hocking (2009).

2.4 Toxygenicity Determination of *Aspergillus flavus* Strains

The toxigenicity of each *A. flavus* was determined qualitatively by culturing in 10% coconut agar medium (CAM) in petri dish (9 cm in diameter) according to Lin and Dianese (1978). Toxigenic strains was indicated by the presence of yellow pigment at the reverse side of the medium.

2.5 Extraction Genome *Aspergillus flavus* Strains

As much as 40 mg fungal mycelia in a microtube containing 600 µl nuclei lytic was homogenised and extracted using procedure Mini Kit (Promega, Madison, WI, USA). Deoxyribonucleic acid concentration obtained was determined using nanophotometer (IMPLEN, Munich, Germany). Electrophoresis of the DNA was conducted using 1.2% agarosa gel (SCIE-PLAS, Cambridge, England) and stained by 1µl ethidium bromide (EtBr) and visualise using Gel Doc (Uvitecc, Cambridge, Serial) under UV light (303 nm).

2.6 Amplification of DNA and PCR-RAPD

Amplification of DNA was conducted using 10 primers (Macrogen, Korea) as follows:

Primers	Nukleotides sequence (5'⟶ 3')
OPA04	AATCGGGCTG
OPB10	CTGCTGGGAC
OPD10	GGTCTACACC
OPD20	ACCCGGTCAC
OPF10	GGCTGCAGAA
OPF13	GGAAGCTTGG
OPK20	GTGTCGCGAG
OPO20	ACACACGCTG
OPQ20	TCGCCCAGTC
OPT20	GACCAATGCC

PCR was performed as follows: a preincubation step at 94ºC for 10 minutes followed by 35 cycles of denaturation at 94ºC for 1 minute, annealing at 65ºC for 2 minutes, extension at 72ºC for 2 minutes. The PCR products were analyzed by electrophoresis (SCIE-PLAS. Ltd, Cambridge, England) using 1.5% agarose gels in 1 × TAE [40 mM Tris-acetate, 1 mM EDTA (pH 8)]. Gels were stained with 0.5 µg μl^{-1} ethidium bromide and visualized using Gel Doc (Uvitec, Cambridge, Serial no. 13200263) under UV light (303 nm). Polymorphic bands produced were read and genetic diversity were analysed using *Numerical Taxonomy and Multivariate Analysis System*, version 2.1. (NTSYSpc21). Dendrogram analysis was described by *Unweighted Pair Group Method with Arithmatic Average Algorithm* (UPGMA).

3 RESULTS AND DISCUSSION

All samples, dried black and white pepper sold by retailers at traditional markets, were stored in closed jars.

3.1 Toxygenicity of *Aspergillus flavus* Strains

Thirty one strains of *A. flavus* were successfully isolated and the strains consisted of 10 strains isolated from black pepper and 21 strains were isolated from white pepper (Table 1).

Table 1.Total number of *Aspergillus flavus* strains isolated from black and white pepper sold by retailers at traditional markets

	Aspergillus flavus strains			
	toxigen	non-toxigen	population (cfu/ml)	total
black pepper	3	7	0.5×10^3	10
white pepper	9	12	0.9×10^3	21

The population of *A. flavus* in black pepper was less than that of white pepper, in addition to toxigenic strains were less contaminated in black pepper than that of white pepper. The presence of *A. flavus* in black and white pepper indicate that the fungal species was able to grow in dried pepper during storage. We assumed that high fungal population might occur during inappropriate storage or cross contamination. Previous study by Leger et al. (2000) stated that no specific host for *A. flavus* and they are able to grow at minimum a_w 0.78 (Pii and Hocking, 2009). For phyllogenetic study, among of the 31 toxigenic and non-toxigenic *A. flavus*, only 8 genomes of representative traditional markets and toxigenic and non-toxigenic *A. flavus* strains were extracted as shown in Table 2.

Table 2. Representative toxigenic and non-toxigenic *A. flavus* strains isolated from black and white pepper obtained from each traditional market for phylogenetic study

A. flavus code	isolate sources	toxigenicity
HPB3	black pepper	toxigen
HPR2	black pepper	toxigen
HSB1	black pepper	non-toxigen
HSB2	black pepper	non-toxigen
PSL2	white pepper	non-toxigen
PPR2	white pepper	toxigen
PSB3	white pepper	non-toxigen
PSS7	white pepper	toxigen

Genome extraction of 8 representative toxigenic and non-toxigenic *A. flavus* strains is shown in Figure 1. The purity each of the genome was between 1.6 to 1.8. Previous study by Sambrook et al. (1989) reported that the purity of genome for molecular study at ratio A_{280}/A_{280} was 1.8 to 2.0.

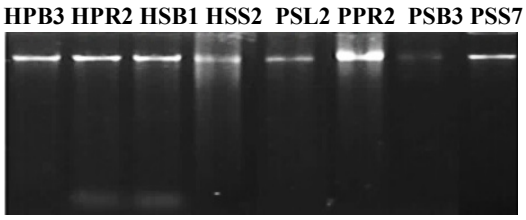
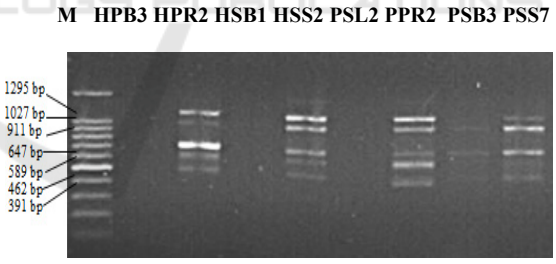


Figure 1. Electrophoresis of 8 strains of *A. flavus* genomes

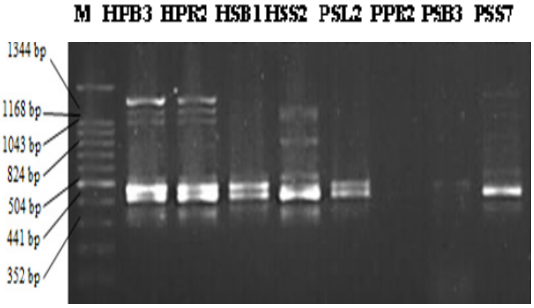
Amplification *A. flavus* genomes and PCR-RAPD

Amplification of genomes of 8 *A. flavus* strains using 10 RAPD primers (OPA04, OPB10, OPD10, OPD20, OPF10, OPF13, OPK20, OPO20, OPQ20, OPT20) showed different bands, however, among 10 primers used, only 3 primers (OPD20, OPF10, OPT20) were succesfully amplified and produce 7 bands with polymorphism 100%. The electrophoresis of genome amplification using primers were OPD20, OPF10, OPT20 is shown in Figure 2.

Primer OPD20



Primer OPF 10



Primer OPT20

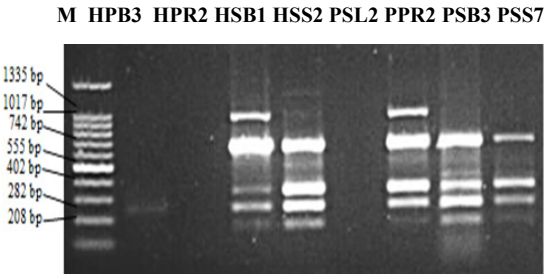


Figure 2. Electrophoresis of amplification of 8 genomes of *A. flavus* strains using primers: OPD20, OPF10, and OPT20. M= marker ladder (100 bp)

Random Amplified Polymorphic DNA (RAPD) amplification in Figure 2 showed that each primer has different bands and polymorphism percentage. Primer OPF10 has the smallest band and the highest on OPT20

3.2 Genetic Diversity Analysis of *A. flavus* Strains

Based on matrix of on Table 4 showed that the highest genetic distance with genetic similarity 0.21 occured on *A. flavus* strains: HSS2 and HPB3, PSL2 and HSS2, PPR2 and HPB3, PSB3 and HPR2. Whereas, the lowest genetic distance with genetic similarity 0.84 occured only in *A. flavus* strains PSB3 and HSB1.

Table 4: Genetic similarity of *A. flavus* strains isolated from black and white pepper sold by relailers at traditional markets based on primers OPD20, OPF10 and OPT20

	HPB3	HPR2	HSB1	HSS2	PSL2	PPR2	PSB3	PSS7
HPB3	1.00							
HPR2	0.63	1.00						
HSB1	0.63	0.26	1.00					
HSS2	0.21	0.47	0.47	1.00				
PSL2	0.78	0.52	0.73	0.21	1.00			
PPR2	0.21	0.36	0.57	0.68	0.31	1.00		
PSB3	0.57	0,21	0,84	0.52	0.68	0.52	1.00	
PSS7	0.52	0.57	0.68	0.57	0.52	0.68	0.63	1.00

red color= high similarity; yellow= low similarity

Dendrogram of all *A. flavus* strains are grouped in one cluster with similarity 44% (Figure 3).

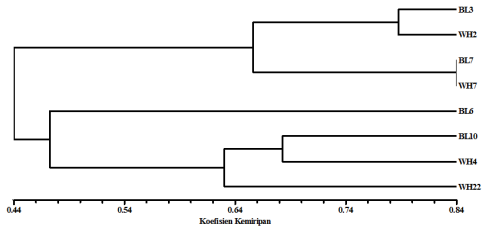


Figure 3. Dendrogram of *A. flavus* strains isolated from dried black and white pepper sold by retailers at traditional markets

Based on dendrogram in Figure 3 showed high possibility that *A. flavus* contamination on dried black and white pepper sold by retailers at 5 traditional markets took place at out of the markets, it might occure at distribution chains. Even though, their population may increase due to high relative humidity during storage.

4 CONCLUSIONS

Black and white pepper sold at traditional markets are contaminated by toxigenic and non-toxigenic *A. flavus*. Cross contamination may increase during storage, therefore, good hygienic practices particularly on distribution chain also required to reduce fungal infection and aflatoxin contamination.

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