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Research Article

Fungal Contamination and Toxigenicity of *Aspergillus flavus* on Postharvest Cacao Beans in Northern Sumatera, Indonesia

Kiki NURTJAHJA[*1], Liana DWI SRIHASTUTI[2], Novia PURNAMASARI[3], Greaceuli Novelina SILITONGA[4]

[1,2,3,4]Universitas Sumatera Utara, Faculty of Mathematic and Natural Sciences, Biology Department, 20155, Medan, North Sumatera Indonesia

[1]https://orcid.org/0000-0003-4250-8403, [2]https://orcid.org/0000-0002-1150-8999, [3]https://orcid.org/0000-0002-6331-1848
[4]https://orcid.org/0000-0002-7549-0839

*Corresponding author e-mail: kiki2@usu.ac.id

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Abstract: The study was carried out to enumerate fungal contamination, and toxigenicity of *Aspergillus flavus* strains on cacao beans during drying and storage. As many as 3500 g cacao beans during drying and storage were purchased from smallholder farmers on the plantation areas at Karo Regency, Northern Sumatera. The percentage of the beans contaminated by fungi was conducted using direct plating. Fungal populations on soil and beans were determined using dilution followed by pour plated in dichloran 18% glycerol agar (DG18) and *Aspergillus flavus* and *parasiticus* agar (AFPA). The mycological evaluation was carried out based on morphological characteristics. Results showed eighteen genera of soil fungi were isolated at the cacao plantation; genera of *Aspergillus* sp., *A. niger, A. flavus,* and *Penicillium citrinum* were the most important contaminants. Six species of the fungi were associated with contamination on cocoa beans during drying *i.e. Aspergillus* sp*., Candida tropicalis, Saccharomyces* sp*., A. niger, Penicillium* spp*.,* and *Fusarium* spp. Whereas three fungal species were associated during storage *i.e. A. niger*, *A. flavus,* and *P. citrinum*. The percentage of cacao contaminated during drying and storage was dominated (>40%) by *Aspergillus* sp. *Fusarium* sp. *A. niger, A. flavus,* and *P. citrinum,* respectively. Among 21 strains of *A. flavus*, 3 strains (15%) were isolated from soil, and 18 strains (85%) were isolated from beans during storage. Among toxigenic *A. flavus*, both strain scaf6 isolated from soil and strain cbaf5 isolated from beans during storage were the highest aflatoxin producers (30.0 ppb). Preventing soil contamination during harvesting, drying, and storage of cacao beans was a prerequisite to minimise fungal contamination.

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1. Introduction

Cacao (*Theobroma cacao* L.) is one of the most important commodities in North Sumatera, Indonesia. According to Directorate General of Estate Crops (2021) area of cacao plantations in North Sumatera was estimated at 53397 hectares and most of the plantations were dominated by small scale farmers where harvest and warehouse storage condition out of control and often damage quality of the beans (Martins et al., 2010). High relative humidity promote improper stored dried beans absorb water

vapour from the environment. This process leads to an increase in beans moisture that results in accelerated fungal growth. Among microorganisms, fungi are the most contaminated on dried-stored commodities, cause physical damage, are contaminated by mycotoxins (Martins et al., 2010; Copetti et al., 2014; Dharmaputra et al., 2015). Fungal contamination occurred during preharvest, harvesting, and postharvest handling. Traditional drying is common in tropical countries, particularly among subsistence farmers where postharvest cacao beans were poor processing and lack of proper facilities. During sun-drying the beans were exposed to the air wind, dust and may be contaminated by fungi. Fagbohun et al. (2011) reported that mouldy cacao beans during storage were contaminated by *Aspergillus niger*, *A. flavus*, *Botryodiplodia theobromae*, *Fusarium* spp., *Mucor* spp., *Neurospora* spp., *Penicillium* spp. and *Phytophthora palmivora*. Genus *Aspergillus* section *Flavi* particularly *A. flavus* is a natural mycobiota in cacao beans and has the potential to produce aflatoxins (Sánchez-Hervás et al., 2008). The fungal propagules germinate when the bean moisture content is suitable for their growth. Soil is the main source of fungal inoculums causing disease or contamination on agricultural products (Ehrlich, 2014; Dharmaputra et al., 2018). Other environmental factors that affect fungal infection on crop products include fungal strains and substrates (Daou et al., 2021). Therefore, an integrated approach that starts in the field prior to harvest and throughout the whole chain is required, so good agricultural practices such as preharvest and postharvest handling minimised fungal contamination in every step to deliver safe cacao commodities to consumers. The purpose of the current study was to enumerate the fungal contamination, and toxigenicity of *Aspergillus flavus* strains on cacao beans during drying and storage.

2. Material and Methods

2.1. Soil sample and cacao beans

Soil samples were obtained from the area of one hectare of a smallholder cacao plantation located in Karo Regency, North Sumatera, Indonesia. Sampling was conducted randomly by dividing each areas into 100 sampling plots. Each plot (1×1 m^2) was divided into 10 points, and 20 g of soil sample was obtained for each point. All of the soil composite samples obtained were mixed thoroughly and placed into a sterile bag, then stored in the cold for further use. At the same time as soil sampling, 3500 g of cacao beans during drying (following harvesting) and storage (±1 month after sun-drying) were purchased from five smallholder farmers at the site of the plantation.

2.2. Beans moisture content

The drying oven method was conducted to determine cacao beans' moisture according to Standard Nasional Indonesia (2008).

2.3. Determination of the percentage of beans contaminated by fungi

The percentage of cacao beans contaminated by fungi was conducted by direct plating technique in dichloran 18% glycerol agar (DG18, NEOGEN®, Lansing, MI, USA) medium. The beans were superficially disinfected to remove the surface contaminants using sodium hypochlorite (NaOCl) for 1 minute. Beanswere then aseptically placed on DG18 medium (pH 5.6) in a petri dish (9 cm diameter) with 5 cacao beans per petri. All plates were incubated for 7 days at 29ºC. Ten replications were made for each sample.

2.4. Fungal population on soil and beans

Fungal population contaminated on beans and soil was determined using dilution technique followed by pour plated in DG18 medium. As much as 25 g of soil sample in erlen meyer 1000 ml was diluted in sterilised distilled water until the volume was up to 250 ml. Then, the soil suspension was homogenised using shaker Kottermann 4020, Hanigsen, W. Germany 250 rpm for 2 minutes, and a serial dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) was made. As much as 1 ml of each of the suspension in a petri dish (9 cm diameter) was pour plated DG18 medium. Three replicate plates were made for each dilution.

The fungal population on beans was determined as follows: every 3500 g of cacao beans was ground for 30 seconds (25 000 rpm) using Mill Powder RT-04 no Serie 980923, Taiwan. The ground

beans were then divided into sub-samples for the determination of moisture content and fungal population. As much as 25 g of the ground bean in a flask of 1000 ml was diluted in distilled water until the volume was up to 250 ml. The suspension was then homogenised using shaker Kottermann 4020, Hanigsen, W. Germany 250 rpm for 2 minutes, and a serial dilution was made. As much as 1 ml of each of the suspension in a petri dish (9 cm diameter) was then pour plated with DG18 medium. Three replicate plates were made for each dilution. All plates were incubated for 7 days at 29ºC. The colony of each fungal species was counted, isolated, and cultured for 7 days at 28ºC in potato dextrose agar (PDA), czapek yeast extract agar (CYA), or czapek yeast extract agar with 20% sucrose (CY20S). Fungal identification was made based on morphological characteristics according to Samson et al. (2002) and Pitt and Hocking (2009).

2.5. Isolation of *Aspergillus flavus*

To isolate *A. flavus* strains, the homogenised suspensions of soil and cacao beans were inoculated in a*spergillus flavus* and *parasiticus* agar (AFPA) medium in petri dishes (9 cm diameter). All plates were incubated for 7 days at 29°C. The presence of an orange color on the reverse side of the medium was indicated as *A. flavus* (Pitt and Hocking, 2009).

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

All *A. flavus* strains were further isolated on potato dextrose agar (PDA, Oxoid Ltd, Basingstoke, Hants, UK) in petri dishes (diameter 9 cm) and incubated for 7 days at 29ºC. The toxigenicity of each *A. flavus* strain was determined using 10% coconut agar medium (CAM) according to Lin and Dianese (1976). The medium was sterilised for 20 minutes at 120 ºC. Each plate was then inoculated by *A. flavus* strain. All plates were incubated for 5 days at 29ºC. The presence of yellow pigment on the reverse side of the medium indicates aflatoxin producer strains (Lin and Dianese, 1976; Davis et al., 1987).

2.7. Aflatoxins determination

Assessment of aflatoxin B_1 (AFB_1) production was done using the thin layer chromatography (TLC) method. A colony of *A. flavus* (from a CAM plate incubated at 29ºC for 5 days) was mixed with 50 ml of ethanol in a waring blender; the suspension was extracted for 30 minutes and filtered using filter paper (Whatman #1). The filtrate was then transferred to a 250 ml separating funnel and extracted twice with 50 mL of n-hexane, and cleaned with 50 ml of chloroform. The extract was then dehydrated in a vial and filtered using anhydrate sodium sulfate (Na_2SO_4). Using a microsyringe, 10 µl of the residue was spotted onto a TLC plate (MERCK # 1.05554, Silica gel 60, F254) and ran for 20 minutes. The developing solvent was chloroform: acetone (9: 1). Commercially available aflatoxins were used as standards (Sigma-Aldrich Chemical Company, USA).

3. Results and Discussion

3.1. Soil fungi and cacao beans

A total of 18 fungal species were isolated from the soil. However, only six of the species contaminate cacao beans during drying, and three fungal species contaminate during storage (Table 1). *Trichoderma* spp. followed by *Aspergillus chevalieri, A. flavus, Aspergillus* sp*., Drechslera tritici-repentis, A. Sydowii*, *Mucor circinelloides,* and *P. Corylophilum* were among the soil fungi commonly isolated. During drying (sun-drying) (bean moisture content 39.16%), the fungi most frequently isolated were *Aspergillus* sp., *Candida tropicalis, Saccharomyces* sp., *A. niger*, *Penicillium* spp*.,* and *Fusarium* spp. Whereas during storage, beans' moisture content declined to 7.02%, and the fungal contaminations were dominated by *A. niger, A. flavus*, and *P. citrinum,* respectively. As shown in Table 1, more fungal species were observed on soil than on cacao beans. The population of *Aspergillus* sp. (7×10^7 cfu g^{-1}) was the most contaminant cacao beans during sun-drying followed by *Candida tropicalis* (4.3×10^7 cfu g^{-1}). Whereas, during storage, *Aspergillus niger* was the most found (6×10^3 cfu g^{-1}), followed by *A. flavus* (6×10^2 cfu g^{-1}) and *P. citrinum* (1.4×10^3cfu g^{-1}). It seems that *A. niger*, *A. flavus,* and *P. citrinum*

are the most important contaminant on cacao beans produced by smallholder cacao beans at Karo Regency.

Fungal contamination on the cacao during drying and storage seemed to originate from the soil of the plantation or might cross-contamination occurred due to the practice of sun-drying by traditional farmers using tarpaulin or cemented pavement in an open area close to the ground. A previous study by Fagbohun et al. (2011) reported that *A. flavus*, *A. niger,* and *Rhizopus* spp. were the most contamination occurred on dried cacao beans stored on the bare floor. Dharmaputra et al. (2018) reported more fungal infection and aflatoxin contamination on storage nutmeg kernel collected from falling on the ground than that of nutmeg by picking from the tree. A similar finding was reported by Copetti et al. (2011), who reported the highest numbers of fungi contaminating cacao beans during drying and storage in comparison to during processing cacao into chocolate.

Table 1. Fungal population (cfu g^{-1}) isolated from soil at cacao plantations and cacao beans during drying and storage

Fungal species	Fungal population (cfu g^{-1}) on cacao beans		
	Soil	During drying	During storage
***Acremonium* sp.**	2×10^2	0	0
***Aspergillus* sp.**	5×10^2	7×10^7	0
A. flavus	5×10^2	0	6×10^2
A. niger	0	3×10^5	6×10^3
A. sydowii	4.8×10^2	0	0
A. tamarii	1.4×10^2	0	0
A. chevalieri	1×10^3	0	0
Botrytis cinerea	1×10^2	0	0
Candida tropicalis	0	4.3×10^7	0
Fusarium oxysporum	1×10^2	0	0
***Fusarium* spp.**	1×10^2	1×10^2	0
Drechslera tritici-repentis	5×10^2	0	0
Mucor circinelloides	3×10^2	0	0
***Penicillium* spp.**	0	3×10^5	0
P. citrinum	0	0	1.4×10^3
P. corylophilum	3×10^2	0	0
***Saccharomyces* sp.**	0	1.8×10^7	0
***Trichoderma* spp.**	1.7×10^3	0	0

cfu g^{-1} = colony forming unit per gram.

As a terrestrial habitat, conidia of fungi are abundant in plantation areas, and they are dormant, disperse or grow in organic matter and contaminate agricultural products during harvesting (Krijgsheld et al., 2012). Risk of fungal infection in small scale farms might be due to improper harvest and storage methods by the farmers. Open sun-drying by spreading the beans using tarpaulin or plastic sheet and close to the ground was a high risk of cross contamination. Storage with high relative humidity and temperature might favor fungal growth. As previously studied by Sardar et al. (2019) that farm households who had low education were likely less awareness to raise knowledge of agro-ecosystems in related to their agricultural products. None of the yeasts was observed on the soil and storage beans. The highest population of yeasts (*C. tropicalis*) (4.3×10^7 cfu g^{-1}) and *Saccharomyces* sp.(1.8×10^7 cfu g^{-1} was isolated on beans only during drying. Environmental contamination by yeasts is caused by cacao pods during pod breaking, insects, fermentation boxes, equipment used, and the highest number of yeasts occurred spontaneously at the beginning of fermentation (Copetti et al., 2011; De-Vuyst & Weckx, 2016; Mota-Guiterrez et al., 2018). The other soil fungi, *A. sydowii*, *A. tamarii*, *Trichoderma* spp., and *A. Chevalieri,* decreased in their viability during storage.

3.2. The percentage of cacao beans contaminated by fungi

Moisture content of cacao beans determines the percentage of the beans contaminated by fungi. As many as 5 fungal species were contaminated with cacao bean during drying, and four species were found during storage. *Aspergillus* sp. (72%) had the highest percentage on cacao during drying followed by *Fusarium* sp. (48%), *Penicillium* spp. (24%), *A. niger* (20%) and *Saccharomyces* sp. (8%). High moisture content (39.16 %) of cacao during drying promotes the growth of field fungi, particularly *Fusarium* sp. and yeast (*Saccharomyces* sp.).

Table 2. Moisture content and the percentage of cacao beans contaminated by fungi during drying and storage

Cacao beans	Moisture content (%)	Fungal species	Percent beans contaminated by fungi
During drying	39.16	*Aspergillus* sp.	72
		Penicillium spp.	24
		Fusarium sp.	48
		Aspergillus niger	20
		Saccharomyces sp.	8
During storage	7.02	*Aspergillus niger*	100
		A. flavus	72
		P. citrinum	40

High percentage of *Aspergillus* sp. (72%) on cacao during drying was consistent with the findings of Copetti et al. (2011), who reported that high diversity of fungi, especially *Aspergillus* sp. contaminated cacao beans at the farm during drying and storage. *Fusarium* sp. was the second contaminant (48%) and found only on cacao during drying while the beans' moisture content was still high. The presence of *Fusarium* sp. as a contaminant at the beginning of storage was reported on peanut (Santos et al., 2016), nutmeg (Nurtjahja et al., 2017), and maize (Carbas et al., 2021). A previous study by Yuan et al. (2020) reported that *Fusarium* is ubiquitous in terrestrial ecosystems. The occurrence of *Fusarium* on the soil at cacao plantations might cause the mold to be contaminated during harvesting and then grow on cacao beans during drying. Previously studied by Ploetz (2006) and Rosmana et al. (2013) reported that *Fusarium* caused disease in cacao. The absence of *Fusarium* sp. on the bean during storage indicated that bean moisture content (7.02%) inhibited the fungal growth. In line with this study, Nurtjahja et al., (2017) reported that *F. semitecum* and *F. verticillioides* were found only at the beginning of storage nutmeg with water activity (a_w) 0.80 and 0.97. Similar to *Fusarium*, the presence of *Saccharomyces* sp. on cacao during drying showed that the rest of the yeast might still be present after the natural fermentation process by farmers, as previously reported by De-Vuyst & Weckx (2016).

The highest percentage of cacao beans during storage was contaminated by *Aspergillus niger* (100%) followed by *A. flavus* (72%) and *P. citrinum* (40%). As shown in Table 1, the population of *A. niger* has become an important fungal contaminant on dried and stored cacao. A previous study by Fagbohun et al. (2011) showed that *A. niger* was most found on mouldy cacao beans during storage.

A total of 21 strains of *A. flavus* were isolated, which consisted of 3 strains isolated from soil at the cacao plantation, and eighteen strains isolated from cacao beans during the storage (Table 3). None *A. flavus* was found on cacao during drying. Among toxigenic (aflatoxin producers) *A. flavus*, strain scaf6, isolated from soil at cacao plantation was the highest aflatoxin producer (30 ppb). Whereas 18 toxigenic strains were isolated at cacao during storage, strains cbaf5 and cbaf11 produce aflatoxin 30.0 and 12.9 ppb, respectively.

Even though the population of *A. flavus* strains on the soil at cacao plantation was only 5×10^2 cfu g^{-1} (Table 1), certain strains of the toxigenic *A. flavus* isolated from soil (scaf6) might potential as a source of aflatoxin contamination on cacao during storage. Soil is the main source of fungal contamination, spoilage, and cause disease in agricultural products (Ehrlich, 2014; Dharmaputra et al., 2018; Winter & Pereg, 2019; Serumaga et al., 2020). The population of the toxigenic *A. flavus* on cacao beans might increase during improper preharvest, harvesting, and postharvest such as drying and storage. Even though fungal contamination on agricultural products can not be avoided (Lane et al., 2018), preventing beans from contact with soil and proper drying and storage of cacao are important to minimise deterioration and aflatoxin contamination.

Table 3. Toxigenic and non-toxigenic *A. flavus* strains on soil and cacao beans during drying and storage

	Aspergillus flavus strains	Toxigenicity on CAM	Aflatoxin B_1 (ppb) detected by TLC
Soil at cacao plantation	scaf3	-	0
	scaf6	+	30.0
	scaf12	-	0
Cacao beans during drying	0	0	0
Cacao beans during storage	cbaf1	+	< 3.01
	cbaf2	-	0
	cbaf3	-	0
	cbaf4	+	< 3.01
	cbaf5	+	30.0
	cbaf6	+	< 3.01
	cbaf7	+	< 3.01
	cbaf8	+	< 3.01
	cbaf9	+	< 3.01
	cbaf10	+	< 3.01
	cbaf11	+	12.9
	cbaf12	-	0
	cbaf13	-	0
	cbaf14	+	< 3.01
	cbaf15	-	0
	cbaf16	+	< 3.01
	cbaf17	-	0
	cbaf18	+	< 3.01

CAM = coconut agar medium; TLC = thin layer chromatography.

4. Conclusion

High population of fungi at cacao plantation was the potential to contaminate cacao beans. Some of the fungi were associated with contamination of postharvest cacao beans during drying and storage. The fungal genera such as *Aspergillus* sp, *A. niger,* and *P. citrinum* were the most important contaminants, and their population was increased in cacao beans during storage. The study results showed that proper drying and storage by smallholder farmers were required to minimize fungal infection and potential aflatoxin contamination.

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