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Isolation and Identification of *Aspergillus* Species Producing Ochratoxin A in Arabica Coffee Beans

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Abstract Isolation of fungi in Arabica parchment coffee and green coffee were collected from in Omkoi district, Chiang Mai, Thailand. The coffee beans were isolated by direct plating method. The postharvest fungi of Arabica parchment coffee and green coffee were assessed for the presence of *Aspergillus* species, the ochratoxigenic potential of the isolates and ochratoxin A levels. Contamination by *Aspergillus* and *Rhizopus* species was found on 25.46% and 38.88% respectively, of 12 samples. Sixteen isolates were identified to species comprised *Aspergillus* section *Ochraceus (3), Ostianus (2), Candidus (2), Sclerotiorum, Awamori, Parasiticus, Terreus* and 5 isolated of *Aspergillus* spp. not subsequently species. The fungi group of *Aspergillus* were isolated to preliminary determine the Ochratoxin A (OTA) on Coconut agar (CA) incubated at 28 C^o for 7 day, then observed the fluorescence under UV light with a wavelength of 365 nm. The result showed that 12 isolates of *Aspergillus* species produced fluorescence blue on CA medium. Confirmed by High Performance Liquid Chromatography (HPLC) found that 10 isolates produced OTA in the range of 0.004 to 12,937.50 ng/g. *Aspergillus ochraceus* isolates produced the maximum OTA observed in agar with toxigenic species.

Keywords: Ochratoxin A (OTA), Arabica coffee beans, Fluorescence, *Aspergillus* group, HPLC

Introduction

The importance of coffee in the world economy, coffee growing and drinking spread around the world. The most important strain of coffee economically are *Coffee Arabica* L. which accounts for over 70% of global production. The Ochratoxin A (OTA) has been reported from temperate to tropical climates mainly on coffee and their products. The OTA a nephrotoxic and possible carcinogen has been detected in a number of foods. In the experiment was done by Batista *et al.* (2003), processed (green) coffee beans in Brazil were assessed both before and after surface sterilization contamination

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by *Aspergillus* and *Penicillium* species was found on 96% and 42% respectively. This is in agreement with data presented by Noomim *et al*. (2008) *Aspergillus steynii* (13/13) was the best ochratoxin producer, because all strains consistently produced both OTA and OTB in large amounts. Isolates of *Aspergillus westerdijkiae* (42/42) were also consistent and 100% of them produced ochratoxin. There are also reports by Frisrad *et al.* (2004) the fungus Yellow *Aspergillus* sp. have been produce of Ochratoxin A toxin such as *Aspergillus cretensis, Aspergillus flocculosus, Aspergillus neobridgeri, Aspergillus steynii, Aspergillus pseudoelegans, Aspergillus roseoglobulosus* and *Aspergillus westerdijkiae*. Robusta coffee beans were infected by fungi more than Arabica coffee. *Aspergillus niger* infected 89% of Robusta beans, whereas *Aspergillus carbonarius* and yellow Aspergilli each infected 12–14% of beans. The OTA was not produced by *A. niger* (98 isolates) or *A. ochraceus* (77 isolates), but was detected in 110 of 113 isolates of *A. carbonarius*, 10 isolates of *A. westerdijkiae* and one isolate of *A. steynii* (Leong *et al.*, 2007). Postharvest diseases may significantly lower the quality and quantity of this produces, most postharvest fungi on chili are storage fungi such as the fungal genera *Aspergillus, Penicillium, Rhizopus, Cladosporium* and *Trichoderma* (Suwan and Akarapisan, 2012). The reported incidence of toxigenicity among strains of *A. carbonarius* isolated from grapes ranges between 58% and 97% of strains surveyed (Battilani *et al.*, 2003) The objective of study was to investigate the distribution of fungi with the incidence and toxigenicity of OTA-producing *Aspergillus* species infecting Arabica parchment coffee.

Materials and methods

Isolation of fungi from Arabica coffee beans

The first step was performed using the method of Leong *et al.* (2007) as follows. The Arabica coffee beans were collected from Omkoi district, Chiang Mai. The Postharvest fungi were isolated by direct plating method. Fifty beans per sample were plated on water agar (WA) (10 beans/plate) incubated at 28 C^o for 7 days. Fungi were identified under the stereomicroscope.

Preliminary screening with Ochratoxin A (OTA) on Coconut agar

The isolates fungi group of *Aspergillus* were single point inoculated on Coconut agar (CA) incubated at 28 C^o for 3 days in the dark. To detect fluorescence, the reverse side of the CA plates was viewed under UV light with a wavelength of 365 nm. An uninoculated CA plate was used as a control.

Species that do not produce OTA were also inoculated on CA plates as negative controls.

Determination Ochratoxin A (OTA) of fungi by HPLC

All of identification the isolates of *Aspergillus* group which produced OTA were inoculated in Yeast Extract Sucrose (YES) agar. The strains were three-point in YES agar incubated at 28 C^o for 7 day in the dark. Three agar plugs were removed from area of the colony, 1 g of YES agar were added in 2 ml H_2O beaker and mashed homogenization. Then, the 1ml solutions were filtered through a syringe filter into a brown bottle. The injection volume was 100 µl onto the HPLC (model SCL-10AVP SHIMADZU).

Identification of Aspergillus group

All isolate of *Aspergillus* group which produced OTA were analyzed species *Aspergillus* by using Biolog FF microplate. (Prima Scientific Co.,Ltd)

Results and discussion

Fungal infection and Identification of fungi isolates

Infection with one or more fungi, with Yellow *Aspergillus* and Black *Aspergillus* being the dominate species, disease incidence of fungi on Arabica parchment coffee (CNK1a) and green coffee (CNK1b) showed that percent infection with 63.9% and 45.8%. The contaminated with *Aspergillus* species and *Rhizopus* sp. respectively. For isolate Arabica parchment coffee (CNK2) disease incidence of fungi showed percent infection with 69.4 % and isolate Arabica parchment coffee (CNK3) disease incidence of fungi showed the highest percent infection with 100 %. Perrone *et al*. (2003) reported that Arabica coffee bean samples from the North Thailand, two types of samples, parchment coffee bean and green coffee beans were showed that approximately 75 % of the samples were contaminated by black aspergilli and similar levels of contamination were observed for isolates belonging to *Aspergillus* section Circumdati. As well as Taniwaki *et al.* (2003) reported that *A. ochraceus* was the dominant yellow *Aspergillus* species in Brazilinan coffee.

Contaminating coffee samples were isolated and identified. *Aspergillus* specie producers Ochratoxin A (OTA) in Yellow *Aspergillus*, Black *Aspergillus* and Green *Aspergillus* Groups. *Aspergillus* species were subsequently identified by Biolog, as three isolates of *Aspergillus ochraceus*, two isolates of *Aspergillus ostianus*, two isolates of *Aspergillus candidus*, one isolates of

Aspergillus sclerotiorum, one isolates of *Aspergillus awamori*, one isolates of *Aspergillus parasiticus*, one isolates of *Aspergillus terreus*. Five isolates of *Aspergillus* sp. was not subsequently identified to species. Noonim *et al.* (2003) reported that *A. sclerotiicarbonarius* is related to *A. carbonarius* and *A. ibericus* and was found only in the Southern region of Thailand. The *A. ochraceus* and *A. ostianus* were two predominant species, comprising 18.75% and 12.5% on Arabica parchment coffee. Morello *et al.* (2007) reported that by using sequences of the β-tubulin gene, which most isolates from Brazilian coffee (84%) previously identified as *Aspergillus ochraceus* were actually *A. westerdijkiae*.

Screening of Aspergillus isolates for Ochratoxin A (OTA) on Coconut agar

A simple and rapid screening method was developed for the detection of OTA in fungi cultures using Coconut agar (CA). Three groups of *Aspergillus* as yellow, black and green were tested preliminary determine produced OTA on CA. The plates were incubated at 28 C^{o} for 3 days then, examined for pigmentation and fluorescence under UV light (365 nm). Twelve isolates of Yellow *Aspergillus* which, OTA produces a blue fluorescence usually covering the whole colony on CA. *Aspergillus parasiticus* produced a yellow green fluorescence usually covering the colony on CA not produced OTA (Fig. 1a) and *Aspergillus* sp. (Yellow) which, OTA produces a blue fluorescence medium on CA (Fig. 1b). *Aspergillus ochraceus* which, OTA produced a blue fluorescence strong on CA (Fig. 1c) and *Aspergillus terreus* that did not produce pigmentation a blue fluorescence on CA were considered to be OTA-negative (Fig. 1d). Ochratoxigenic *Aspergillus* were common found in Arabica parchment coffee. The dominant species, *Aspergillus ochraceus* and *Aspergillus ostianus* produced OTA. Heenan *et al.* (1998) previous reported OTA produced a blue green fluorescence usually covering the colony on coconut cream agar. As well as Mantle and Chow (2000) reported that Asian coffee isolates produced ochratoxin A the range 400 mg kg-1, on coconut agar OTA appears as an intense blue fluorescence.

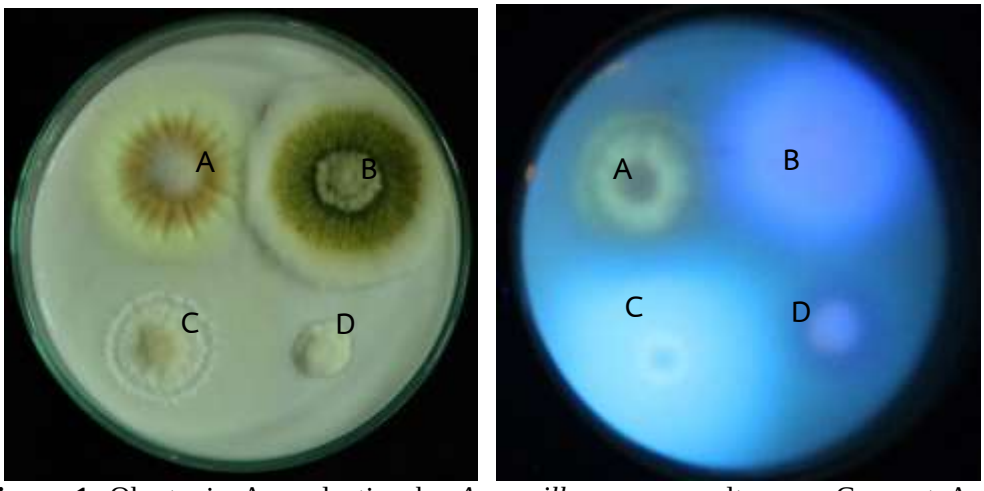

Figure 1. Ohratoxin A production by *Aspergillus* groups culture on Coconut Agar (CA), (A) *Aspergillus parasiticus*, (B) *Aspergillus* sp. (Yellow) (C) *Aspergillus ochraceus* and (D). *Aspergillus terreus,* fig right viewed under long wavelength UV (365 nm).

HPLC analysis

Three groups of *Aspergillus* as yellow, black and green were tested preliminary determine produced OTA on CA. The result showed that 12 isolates of *Aspergillus* species produced fluorescence blue on CA medium. In confirmed produced OTA using the agar plug HPLC method on Yeast Extract Sucrose (YES) agar. Twelve isolates of *Aspergillus* were detected for OTA production. The *Aspergillus ochraceus* was produced OTA levels higher than 12,937.50 ng/g (Table 1). Chen *et al.* (2013) reported that *Penicillium chrysogenum, Penicillium glycyrrhizacola, Penicillium polonicum, Aspergillus ochraceus* and *Aspergillus westerdijkiae* could produce OTA, concentration varied among the isolates from 12.99 to 39.03 µg/kg. Similar work has reported high Ochratoxin A production (400–16,000 µg/kg) in grains such as wheat, rye and barley, showing that some isolates of *Aspergillus ochraceus* are high producers of this toxin (Kononenko *et al*., 2000).

Table 1. Detection of Ochratoxin A (OTA) by Coconut agar (CA), Potato dextrose agar (PDA), Yeast extract sucrose agar (YES) direct method and by the HPLC agar plug method

Isolate	CA[1]	PDA[2]	YES[3]	HPLC (ng/g)	Biolog
Cnk2/a	B (+)	-	-	1651.34	*Aspergillus* sp. (yellow)
Cnk2/b	B (+++)	B (+)	-	152.42	*Aspergillus ostianus*
Cnk2/c	B (++)	B (+)	-	4122.44	*Aspergillus sclerotiorum*
Cnk3/a	B (++)	-	-	190.32	*Aspergillus ochraceus*
Cnk3/b	B (+++)	B (+)	-	4202.32	*Aspergillus ochraceus*
Cnk3/c	B (+++)	B (+)	-	6900.90	*Aspergillus ostianus*
Cnk3/d	+	-	-	269.28	*Aspergillus sp. (yellow)*
Cnk3/e	B (++)	-	-	583.12	*Aspergillus sp. (yellow)*
Cnk3/f	G (+)	-	-	nd	*Aspergillus parasiticus*
Cnk3/g	B (++)	B (+)	-	2627.02	*Aspergillus candidus*
Cnk3/h	B (+++)	B (++)	B (+)	12,937.5	*Aspergillus ochraceus*
Cnk3/i	B (+++)	B (+)	-	8,972.16	*Aspergillus candidus*
(Negative control)	-	-	-	nd	*Aspergillus terreus*
(Negative control)	-	-	-	nd	*Aspergillus* sp. (yellow)
D/2 (green *Aspergillus*)	B (+)	-	-	0.014	*Aspergillus* sp. (green)
N/2 (black *Aspergillus*)	B (+)	-	-	0.004	*Aspergillus awamori*

[1]Coconut agar [2]Potato dextrose agar [3]Yeast extract sucrose
B (+++): Create a fluorescence blue strong B (++): Create a fluorescence blue medium
B (+): Create a fluorescence blue weak (-): do not fluorescence.
G (+): Create fluorescence yellow-green.
nd: not detected

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