

## MYCOFLORA OF COFFEE BEANS IN THE PHILIPPINES

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### ABSTRACT

The mycoflora of coffee beans in the Philippines were determined after harvest, after drying, and roasted coffee from retail markets. Twenty-six species from 14 genera were recovered. *Aspergillus* was prevalent with eight species such as *Aspergillus chevalieri*, *A. flavus*, *A. fumigatus*, *A. japonicus*, *A. niger*, *A. ochraceus*, *A. restrictus*, and *A. terreus*. *Aspergillus niger* dominated coffee at 52.31% followed by *A. flavus* (12.95%) and *A. fumigatus* (4.61%). Other *Aspergillus* species have less than 1% prevalence. The species of *Penicillium* were *P. janczewskii* (6.12%), *P. coriophyllum* (4.67%), *P. citrinum* (2.14%) and *P. oxalicum* (1.34%). *Eupenicillium ochrosalmoneum* and *P. variable* have less than 1% incidence. Other filamentous fungi were *Cylindrocarpon didymum* (5.96%), *Cladosporium cladosporioides* (3.56%), *Rhizopus oryzae* (1.44%), *Leptosphaerulina chartarum* (1.43%) and *Fusarium verticillioides* (1.38%). Finally, *Acremonium implicatum*, *Cryosporium* spp., *Microascus* spp., *Microdiplodia hawaiiensis*, *Mucor racemosus*, *Nigrospora oryzae*, and *Pestalotiopsis* spp. were also recovered at very low frequencies. The mycoflora and incidence after harvest, after drying, and roasted beans varied according to location where the coffee beans originated. The processing methods such as drying and roasting substantially affected the degree of fungal contamination in coffee beans. The total fungal load in coffee beans increased after drying but was reduced significantly by 93 to 97% after roasting.

**Key words:** Filamentous fungi, ochratoxin A, postharvest, toxigenic fungi

### INTRODUCTION

Coffee is grown in the wide tropical countries surrounding the equator between the tropics of Cancer and Capricorn (Martins et al., 2003). In the Philippines, the production of coffee was once a major industry, which 200 years ago was the fourth largest coffee producing nation. Today, the country produces only 0.012% of the world's coffee supply with an average production of 97,428 metric tons in 2008 (BAS, 2010). Majority of Philippine coffee are produced in the mountain areas of Apayao, Batangas, Benguet, Bukidnon, Cavite, Claveria, Kalinga, and Davao.

Like other crops, coffee beans are subjected to contamination and consequent colonisation by fungi during production and postharvest stages. No coffee producing country is free from fungal contamination (Taniwaki, 2006). Extensive studies have been carried out on the mycobiota of coffee in African, Latin American, Middle East, and Asian countries (Abdel-Hafez and El-Maghraby, 1992; Bokhari, 2007; Ilic et al., 2007; Noonim et al., 2008; Pardo et al., 2004; Taniwaki et al., 2003; Taniwaki, 2006; Téren et al., 1997; Visotto et al., 2008). It is not currently known however, at which point during coffee growth, harvest and processing most fungal contamination occurred and more likely that levels increase when drying and storage are inadequate (Bucheli et al., 2000 and 2001; Bucheli and Taniwaki, 2002; Taniwaki et al. 2003; Taniwaki, 2006). Fungal contamination in coffee and an associated ochratoxin A (OTA) problem was due to faults in harvesting and storage practices (Urbano et al., 2001). Ochratoxin A is an important hepatotoxic, nephrotoxic, teratogenic and

carcinogenic toxin (Pitt, 1987). OTA production was earlier believed to be restricted to *Penicillium verrucosum* (Pitt, 1987; Pitt and Hocking, 1991 and 1997) and *Aspergillus ochraceus* (Ciegler, 1972; Hesseltine et al., 1972) with *P. verrucosum* predominating in temperate regions and *A. ochraceus* producing OTA in warmer areas (Moss, 1996). However, a number of additional *Aspergillus* species can produce OTA particularly those belonging to *Aspergillus* Section Nigri: *A. awamori*, *A. foetidus*, *A. niger*, *A. carbonarius*, *A. lacticoffeatus* and *A. sclerotioniger* (Abarca et al., 1994; Heenan et al., 1998; Samson et al., 2004; Téren et al., 1997; Wicklow et al., 1996; Ueno et al., 1991; Varga et al., 1996) as well as those belonging to *Aspergillus* section Circumdati: *A. cretensis*, *A. flocculosus*, *A. pseudoelegans*, *A. roseoglobulosus*, *A. steynii*, *A. sulphureus* and *A. westerdijkiae* (Frisvad et al., 2004).

As a tropical country, it is likely that environmental conditions in the Philippines are frequently conducive to fungal development in coffee beans. Taking all this information into account, this study enumerated the mycoflora in coffee beans from major production areas in the Philippines at harvest, after drying, and at the retail market outlets.

## MATERIALS AND METHODS

A total of 85 samples during the 2008 to 2009 harvest seasons were collected after harvest, after drying, and roasted beans in retail markets of Benguet, Cavite, and Davao. Distribution of coffee samples gathered is shown in Table 1. Samples were brought to Philippine Center for Postharvest Development and Mechanization (PhilMech) (formerly BPPE), Science City of Muñoz, Nueva Ecija, Philippines. Each sample (one kg) was ground using a mill and thoroughly mixed for one hour by dough mixer.

**Table 1.** Distribution of coffee samples collected from different production areas and postharvest stages.

Production area	After harvest	After drying	Roasted beans	Total
Benguet	7	15	5	27
Cavite	6	14	5	27
Davao	6	16	11	31
Total	19	45	21	85

Fungal load in coffee beans was determined by plate count technique. A representative sample from the grounded coffee beans was drawn using sterile metal scoop and a decimal serial dilution was made under sterile conditions. From the serially diluted solution, 1 ml each was poured in dichloran rose bengal chloramphenicol agar (DRBC) or Dichloran 18% Glycerol (DG18) agar. DRBC is a general purpose counting medium but specifically used for the isolation of *A. carbonarius* and *A. niger* from coffee bean samples (Hocking and Pitt, 1980; King et al., 1979; Pitt and Hocking, 1997). Strains of *A. carbonarius* and *A. niger* were recognised by their distinct dark brown to black coloration of conidia (Pitt and Hocking, 1997; Klinch and Pitt, 1988). DG18 agar (Hocking and Pitt, 1980) was also used to determine if *A. ochraceus* was present. *Aspergillus ochraceus* colonies are not densely sporulating and grow on DG18 as pale to light yellow or amber yellow colonies (Klinch and Pitt, 1988). Fungal population was accounted after 5–7 days incubation at 25 °C. All samples were analyzed twice with five replicated plates.

Fungal colonies were isolated in pure culture. Taxonomic identification of the isolates was achieved through macroscopic and microscopic observation with the aid of guidelines published for each genus or general guidelines. *Aspergillus* and *Penicillium* isolates were purified by streaking onto malt extract agar (MEA) to check for purity and then three point inoculated onto czapek yeast

autolysate (CYA) and MEA before identification based on both macroscopic characters (colony growth, colony diameter) and microscopic characters using the identification schema of Pitt (1988), Klinch and Pitt (1988), Pitt and Hocking (1997) and Samson et al. (2004). However, for *Aspergillus* and *Penicillium* species, it has always been difficult to distinguish one taxon from another by cultural and morphological means because the differences are very subtle. Hence, selected isolates belonging to section Circumdati and Nigri were sequenced of the internal transcribed spacer (ITS) gene for confirmation of species identity as described by Noonim et al. (2008) and Houbraken et al. (2007).

The identity of other fungal isolates was also confirmed by molecular method. Mycelial plug was grown on PDA at 25 °C and harvested after 1 week. From the fungal colony, mycelia was picked by sterile wire loop and resuspended in 1 ml sterile distilled water in a microfuge tube. The tube was centrifuged for 1 minute at 12,000 rpm and the supernatant removed. Then, 200 µl of 5% Instagene™ matrix was added to the pellet and incubated at 56 °C for 30 minute. The tube was mixed in high speed vortex for 10 seconds and placed in 100 °C heat block for 8 minutes. The tube was again mixed in high speed vortex for 10 seconds and spinned at 12,000 rpm for 3 minutes. The nuclear ribosomal ITS region was amplified with primer pairs ITS1 and ITS4 (White *et al.*, 1990). Polymerase chain reaction (PCR) amplification of ribosomal DNA (rDNA) was performed at 98 °C for 2 minutes with 30 cycles of incubation for 10 seconds at 98 °C, 30 seconds at 52 °C, and 1 minute at 72 °C. Finally, at 72 °C for 7 minutes. Gene amplification was performed with the TaKaRa ExTaq system (TaKaRa, Japan). Sequencing was conducted with the ABI-Prism 377 DNA sequencing system (Applied Biosystems, California) and DNA sequencing kit (Perkin-Elmer, USA) following the ABI protocol.

## RESULTS

Table 2 gives quantification of the total fungal load of coffee beans collected from different production areas and postharvest stages. Coffee beans from Davao have the highest average viable mould count ( $2 \times 10^3$  cfu/g), followed by Cavite ( $1 \times 10^3$  cfu/g), and Benguet ( $3.8 \times 10^2$  cfu/g). The viable mould count increased in all samples after drying. Davao coffee has the most diverse mycobiota with 20 species, Cavite has 18 species and Benguet with 9 species. The predominant fungi in coffee from Benguet were *P. corilophylum*, Cavite coffee was dominated by *A. niger*, *A. flavus* and *P. oxalicum* while. *A. niger*, *A. flavus* and *Cryosporium* spp. were the main species in coffee from Davao.

**Table 2.** Quantification of total fungal load of coffee beans from various sources and sampling stages.

Sample origin	Total Fungal Load (cfu/g)			Mean
	After harvest	After drying	Roasted beans	
Benguet	$3 \times 10^2$	$7.8 \times 10^2$	$5.3 \times 10^1$	$3.8 \times 10^2$
Cavite	$1.5 \times 10^2$	$2.9 \times 10^3$	$6 \times 10^1$	$1 \times 10^3$
Davao	$2.6 \times 10^3$	$3.5 \times 10^3$	$1.4 \times 10^2$	$2.1 \times 10^3$

Table 3 shows the mean frequency of isolation of various fungi on coffee beans from Davao, Cavite, and Benguet after harvest (AH), after drying (AD), and roasted beans (RB). Coffee beans have diverse mycobiota composed of 26 species from 14 genera namely: *Acremonium*, *Aspergillus*, *Cladosporium*, *Cylindrocarpon*, *Cryosporium*, *Fusarium*, *Leptosphaerulina*, *Microascus*, *Microdiplodia*, *Mucor*, *Nigrospora*, *Penicillium*, *Pestalotiopsis*, and *Rhizopus*.

The dominant fungi of coffee was *Aspergillus* composed of eight species such as *Aspergillus chevalieri*, *A. flavus*, *A. fumigatus*, *A. japonicus*, *A. niger*, *A. ochraceus*, *A. restrictus*, and *A. terreus*. Two black aspergilli were the most frequently found fungi, isolated in 55% of the coffee beans analyzed. Of these 2,892 isolations of black aspergilli, 2,891 were *A. niger* and only one *A. japonicus*.

The closely related bi-seriate black aspergillus to *A. niger*, which is *A. carbonarius*, was not identified. *Aspergillus niger* and *A. carbonarius*, can easily be differentiated by conidia dimensions (3-5 µm for *A. niger* and 7-10 µm for *A. carbonarius*). The black uniseriate aspergillum isolated in this study was identified as *A. japonicus*. *Aspergillus fumigatus* was the second prevalent aspergilli at 9.5%. The green-colored aspergilla, identified as *A. flavus*, had 3.65% incidence in coffee beans. Other *Aspergillus* species have less than 1% dominance in coffee beans. The genus *Penicillium* had 16% share in the total mycobiota of coffee beans. The dominant strains were *Penicillium coriophyllum* at 9.52%, *Penicillium citrinum* (4.7%) and *Penicillium oxalicum* at 1.38%. Other *Penicillium* species have less than 1% prevalence in coffee beans and identified as *Eupenicillium ochrosalmoneum*, *Penicillium janczewskii*, and *Penicillium variabile*. Finally, other fungal species were isolated and identified as *Cylindrocarpon didymum* with 5% occurrence, *Fusarium verticillioides* at 3.75%, *Cladosporium cladosporioides* at 1.52%, and *Rhizopus oryzae* at 1.20%. Additional strains associated with coffee beans at very low frequencies (1.94%) were *Acremonium implicatum*, *Chrysosporium* spp., *Leptosphaerulina chartarum*, *Microascus* spp., *Microdiplodia hawaiiensis*, *Mucor racemosus*, *Nigrospora oryzae*, and *Pestalotiopsis* spp.

**Table 3.** Mean frequency of isolation of various fungi in coffee beans collected from Davao, Cavite, and Benguet after harvest (AH), after drying (AD), and roasted beans (RB).

Taxon	Benguet			Cavite			Davao		
	AH	AD	RB	AH	AD	RB	AH	AD	RB
<i>Acremonium implicatum</i>	20	7	0	0	0	0	0	4	0
<i>Aspergillus chevalieri</i>	9	23	0	3	63	0	0	0	2
<i>Aspergillus flavus</i>	10	89	3	0	0	10	7	53	19
<i>Aspergillus fumigatus</i>	0	21	4	0	0	2	138	328	4
<i>Aspergillus japonicus</i>	0	0	0	0	0	0	1	0	0
<i>Aspergillus niger</i>	3	29	11	25	1081	0	151	1572	19
<i>Aspergillus ochraceus</i>	0	3	0	0	0	0	0	2	0
<i>Aspergillus restrictus</i>	1	0	0	0	0	0	0	0	0
<i>Aspergillus terreus</i>	1	0	0	0	0	0	0	0	0
<i>Cladosporium cladosporioides</i>	25	30	0	0	0	0	3	22	0
<i>Chrysosporium</i> spp.	0	0	0	0	0	4	0	0	2
<i>Cylindrocarpon didymum</i>	13	5	0	0	0	0	188	50	0
<i>Fusarium verticillioides</i>	0	0	0	0	0	0	1	195	0
<i>Leptosphaerulina chartarum</i>	0	8	0	0	0	0	0	8	0
<i>Microascus</i> spp.	6	0	0	0	0	0	0	0	1
<i>Microdiplodia hawaiiensis</i>	10	5	0	0	0	0	0	0	0
<i>Mucor racemosus</i>	0	3	0	0	0	0	0	25	0
<i>Nigrospora oryzae</i>	0	0	0	0	0	0	3	0	0
<i>Eupenicillium ochrosalmoneum</i>	0	1	0	0	0	0	0	0	0
<i>Penicillium citrinum</i>	2	173	0	0	30	0	9	0	32
<i>Penicillium coriophyllum</i>	18	275	0	0	0	0	4	201	0
<i>Penicillium janczewskii</i>	0	0	3	0	0	3	0	0	14
<i>Penicillium oxalicum</i>	1	1	0	47	9	0	5	9	0
<i>Penicillium variabile</i>	0	0	0	0	0	1	0	0	0
<i>Pestalotiopsis</i> spp.	0	0	0	0	0	0	1	5	0
<i>Rhizopus oryzae</i>	1	8	1	1	30	0	0	22	1

## DISCUSSION

The fungal contamination in coffee beans in the Philippines was 97%, close to 98% contamination in Thai coffee (Noonim et al., 2008) and 93% contamination in Vietnam coffee (Ilic et al., 2007). Coffee beans in the Philippines have various mycobiota as we recovered 26 species from 14 genera. Species of *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, *Mucor* and *Rhizopus* have been found before and confirm the widespread natural contamination of coffee with these fungi (Abdel-Hafez and El-Maghraby, 1992; Batista et al., 2003; Bokhari, 2007; Daivasikamani and Kannan, 1986; Gonzalez-Salgado et al., 2005; Mislivec et al., 1983; Perrone et al., 2007; Roussos et al., 1995; Silva et al., 2000; Vissoto et al., 2008). The range of filamentous fungi recovered from coffee beans in the Philippines appears to be much greater than previously reported elsewhere. Additional species include: *Aspergillus restrictus*, *Cylindrocarpon didymum*, *Cryosporium* spp., *Fusarium verticillioides*, *Leptosphaerulina chartarum*, *Microascus* spp., *Microdiplodia hawaiiensis*, *Nigrospora oryzae*, *Eupenicillium ochrosalmoneum*, *Penicillium variabile* and *Pestalotiopsis* spp.

*Aspergillus* and *Penicillium* were the dominant and important species recovered from coffee beans in the Philippines. Many studies revealed that *Aspergillus* and *Penicillium* are natural coffee contaminants, and are present from the field to storage (Nakajima et al. 1997; Silva et al. 2000). *Aspergillus niger* and *A. ochraceus* are the two species reported to be capable of producing OTA (Abarca et al., 2001; Bayman et al., 2002; Joosten et al., 2001; Mantle and Chow, 2000; Taniwaki, 2006; Taniwaki et al., 2003; Peronne et al., 2007; Noonim et al., 2008). *Penicillium* species capable of producing OTA was not isolated from coffee beans from all sampling sites however, the presence of *P. citrinum* samples has to be considered as it is an important mycotoxin-producer (citrinin) (Pitt and Hocking, 1997). Likewise, investigation of other fungi which produce enzymes that can reduce coffee quality is also encouraged.

Black *Aspergilli* was consistent in Philippine coffee with overall average occurrence lower than reported in Brazil, Thailand and Vietnam (Leong et al., 2007; Martins et al., 2003; Noonim et al., 2008; Taniwaki et al., 2003). The black *Aspergilli* that we isolated were about 99+% *A. niger* and less 1% *A. japonicus*. *A. niger* was reported to produce OTA in coffee (Abarca et al., 1994; Heenan et al., 1998; Samson et al., 2004; Téren et al., 1997; Wicklow et al., 1996; Ueno et al., 1991; Varga et al., 1996). The closely related bi-seriate *A. carbonarius* was not identified from the representative isolates subjected to molecular technique. Whilst the incidence of *A. ochraceus* in Philippine coffee was less than 1%, this strain is a relatively important potential source of OTA in coffee products (Bayman et al., 2002; Mantle and Chow, 2000). The ochratoxigenic *Aspergillus ochraceus* has frequently been proposed as the major cause of OTA in green coffee (Frank, 1999), although a cause and effect relationship has not been demonstrated (Mantle, 1998).

Several reasons could be attributed for the variability and complexity of fungal load in coffee beans. Variation in climatic conditions, harvesting, processing method, and drying could substantially affect degree of fungal infection in coffee beans (Silva et al., 2000). The fungal load seems to be related to farmers' practice of harvesting, processing and drying. In Cavite for example, berries which fall to the ground are collected and mixed with mature and good berries for sun drying in the open ground, cemented pavement, or road for 10-29 days. Cherries fallen onto the soil are likely to have increased levels of OTA and might harbor OTA-producing molds which could rapidly be propagated in the drying yard (Bucheli et al., 2001; Bucheli and Taniwaki, 2002). The direct contact of coffee with ground soil could have increased mold count after drying. Ground patios must be avoided, since soil is the natural habitat of ochratoxigenic fungi and other microorganisms as well (Batista et al., 2009). Meanwhile, the lesser fungal load in Davao coffee could be attributed to the modified dry process that involves immediate removal of the pulp after harvest and a shorter drying time. Berries contain plenty of water (59-63%), with an easily accessible carbon source in the form of free sugars that makes them an ideal substrate for the development of molds and OTA formation

(Bucheli and Taniwaki, 2002). The wet processing and drying method in Benguet yielded lowest fungal count. The depulping process reduces significantly the fungal load and risk of OTA contamination during the subsequent fermentation and drying steps (Bucheli and Taniwaki, 2002). Wet processing appears less susceptible to infection by *Aspergillus* spp. and OTA contamination (Frank, 1999) as manifested by low incidence of this genus in the samples collected from Benguet. Since the fruit pulp is an excellent substrate for the growth of OTA-producing strains (Joosten et al., 2001), its removal eliminates a very suitable substrate. Moreover, the careful drying of depulped berries in Benguet using plastic net, laminated sacks, galvanized sheets, or bamboo mats could have reduced mould infection and subsequent OTA formation. The roasting process (218°C for 30 minutes) decreased the total fungal load in coffee beans from 93 to 97%. Fungal contamination of *A. niger* was reduced from 63 to 100% after roasting the coffee beans.

Our study provided first report on the mycoflora in coffee in the Philippines. We aimed to use these data as benchmark information for follow-up studies to improve the quality of coffee destined for local and export markets. Such study, for example, is the evaluation of the toxigenic potential of *A. niger* and *A. ochraceus*. The variability of fungal contamination from different growing areas and postharvest stages suggests follow-up investigations of the relationship between the mycobiota and presence of OTA in coffee, local climatic conditions and processing factors. Particularly, studies on the a) distribution of fungi with the potential to produce OTA, in coffee beans throughout the harvest, drying, and storage; b) investigation of the relationship between the presence of OTA in coffee and local climatic conditions; and c) the influence of processing practices on OTA production.

Meanwhile evaluation of the presence of OTA in coffee beans is very important in the context of consumer protection and food safety. Regulatory authorities in some coffee consuming countries have set maximum limits for OTA in coffee in recent years. For green coffee limits range between 5 and 20 parts per billion (ppb); between 3 and 10ppb for roasted coffee; and between 4 and 10ppb for soluble coffee (FAO, 2010). Likewise, the time of invasion of coffee by toxigenic fungi is also of great importance in understanding the problem of OTA in coffee and developing control strategies. The solution to the problem of OTA in dried coffee appears to lie in improvements in agricultural practice (Taniwaki et al., 2003).

## CONCLUSIONS

Coffee beans in the Philippines have diverse mycobiota with *Aspergillus* and *Penicillium* as prevalent and important specie recovered. The coffee beans from Davao have the highest mean viable mould count followed by Cavite and Benguet. The processing methods such as drying and roasting substantially affect degree of fungal infection in coffee beans.(What abt roasting?) The high fungal contamination was characterized by fruit contact with the soil and by inadequate postharvest handling of the product during drying in ground patios. Ground patios must be avoided, since soil is the natural habitat of ochratoxigenic fungi and other microorganisms as well. The adoption of the Good Agricultural Practices (GAP) will significantly reduce the risk of microorganism contamination under the conditions of coffee fruit and bean deterioration as well as reduce OTA production. The present study showed that there is a need for follow-up studies to improve the quality of coffee destined for local and export markets.

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