

Isolation of Mycotoxigenic Fungi, Detection and Quantification of Ochratoxin A from Coffee (*Coffea arabica* L.)

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Abstract: Coffee is the most important commodity and source of export earnings in Ethiopian economy which has to fulfill the quality standards of safety up to maximum tolerable level. However, it is naturally associated with several mycoflora and some of them may produce Ochratoxin A unless careful handling measures taken place. Therefore, this research was initiated to assess the status of mycotoxigenic fungi associated with coffee and quantification of Ochratoxin A from locally consumed coffee in Ethiopia. A total of 75 coffee samples were collected from three districts namely, Haru, Homa and Nedjo of West Wollega Zone, Oromia regional state of Ethiopia. Determination of coffee associated mycoflora isolation and identification were conducted at Jimma Agricultural Research Center of plant pathology laboratory while Ochratoxin A detection and quantification were conducted at Ambo Plant Protection Research Center. Malt Extract Agar (MEA) was used for isolation and identification of mycoflora associated with coffee and ELISA kit was used to detect and quantify Ochratoxin A. The result showed that numbers of mycoflora associated with coffee were observed and five of them become the major. *Aspergillus niger* was the most dominant (73.37%) species detected from most coffee samples, followed by *Aspergillus ochraceus* (11.30%), *Fusarium* spp. (7.37%), *Penicillium* spp. (6.74%), and *Rhizopus* spp. (1.50%), respectively. Average ochratoxinA recorded was 0 (ND) ppb, 1.24 ppb and 2.02 ppb from Haru, Homa and Nedjo.

Keywords: Coffee, Ethiopia, Mycotoxigenic Fungi, Ochratoxin A

1. Introduction

Globally, coffee is the second most traded and wealth generating commodity after oil [1, 2]. Ethiopia is known for its diverse and unique Arabica coffee flavors [3]. Coffee covers 37% of agricultural exported commodities [4]. Ethiopia is the largest coffee producer in Africa and the 4th in the world next to Brazil, Colombia and Honduras by contributing 7.4% of total world coffee production [5]. Sixteen percent of the Ethiopian population depend on coffee Production, Processing and Marketing and Ethiopia exported around 180,000 metric tons of coffee and earns 800 million US\$. [6].

Several biotic and abiotic factors affect coffee production and quality in the field and after harvest. Fungal contamination and production of mycotoxins is one of the post-harvest problems that influence the quality of coffee beans. Most of the storage fungi, mainly several species of *Aspergillus* and *Penicillium* do not invade seeds to any appreciable degree before harvest. However, they can cause severe discoloration of seed in storage resulting in germination failure, due to damaged embryos or whole seeds [7]. Temperature, moisture content, storage conditions, Processing type, and durations of storage can be

important factors for mold development. Ochratoxin A frequently occurs as a contaminant in various food products and it is the main mycotoxin reported to be found in coffee in green bean, roasted coffee and instant coffee worldwide [8-10].

Ochratoxin A has carcinogenic, nephrotoxic, teratogenic, immunotoxic, and neurotoxic properties. It has been associated with nephropathy in humans. OTA is a secondary metabolites of certain fungi which is a small molecule soluble in water and it is chemically constituted by a combination of an amino acid (phenylalanine) and a polyketide to carbon 10, contains one chlorine atom necessary for its biological activity, and it is stable when exposed to heat [11]. The presence of OTA in coffee has a negative economic effect for the producing countries and consumers. Many food processors in Europe and around the world are regularly testing for the presence of OTA in commodities like coffee before destined for human consumption [12]. International Agency for Research on Cancer [13] classified OTA as group 2B (possible carcinogens to human).

Due to the economic impact, OTA takes global attention which is associated with public health. The world community is becoming sensitive for mycotoxin contamination. Many food processors in Europe and around the world are regularly testing for the presence of ochratoxin A in commodities like coffee before destined for human consumption [12]. EU has set maximum permitted level of OTA 5µg/kg for roasted and ground coffee and 10 µg/kg for soluble coffee [14]. The current study was initiated with the objective of isolation and identification of Arabica coffee bean associated fungi and detection and quantification of Ochratoxin A from locally consumed green coffee beans.

2. Materials and Methods

2.1. Description of the Study Area

The studies were conducted both in the field and in the laboratory. Field study was conducted at Haru, Homa and Nedjo districts of West Wollega Zone, Western Ethiopia. The laboratory works were conducted at Jimma Agricultural Research Center Pathology Laboratory and Ambo plant protection research center.

2.2. Sampling Technique and Study Population

Three sample districts (Haru, Homa and Nedjo) from West Wollega were selected purposively due to their coffee production potential. Three peasant associations (kebeles) per district and five coffee producer farmers per associations (kebeles) were randomly selected. A total of 75 coffee samples weighing 1kg each sample were randomly collected from dry processed coffee type at farmer's storage. Simple random sampling technique was conducted to collect samples from coffee producing farmers from each sampling site of study area.

2.3. Isolation and Identification of Fungi

The coffee bean samples were surface sterilized in 5% sodium hypochlorite and was rinsed three times using sterilized distilled water. Then, 10 coffee beans were placed into a triplicate sterilized Malt Extract Agar Media amended with 1g per 1000ml streptomycin. The plates were incubated at 25°C for up to 14 days and the number of samples showing fungal growth in each Petri dish were counted and their frequency was computed using the following formula;

$$\text{Percent of Fungal infection(\%)} = \frac{\text{number of beans from which fungus isolated}}{\text{Total number of beans analyzed}} * 100$$

The growing fungi were sub cultured on Malt Extract Agar for 7 days and identified to species level based on cultural and morphological characteristics, including colony color, conidiophores, phialids, presence and size of vesicles, surface texture, color and the reverse side of mould on agar plate, mycelium, type of spore, pigmentation, Vesicle, Hyphae, Metulae, Arrangement of conidia [15-18]. The pure cultures of each isolate were preserved at 4°C on MEA slant during the study.

2.4. Detection and Quantification of Ochratoxin A by ELISA Kits

ELISA kits were used as a quick diagnostic method and most commonly used for detection of mycotoxins. The full kits containing Ochratoxin Low Matrix Microplate - 9610CH01COFN: 96 wells specific antibody, positive and negative control were used for detection of ochratoxin A. Samples extraction and processing were done as per the company procedure or order. The detection reagent is a covalent complex of these Ochratoxin A and enzyme,

alkaline phosphates. The reagent was mixed with a sample extract and the mixture was placed in the well. In the control well in mycotoxin free sample, the mycotoxins-enzyme conjugate has been saturated the bound antibody, after additions of a chromogenic substrate results in the development of color and has been quantified by the use of ELISA reader.

3. Results

3.1. Coffee Bean Associated Fungi

The result of this study revealed that total of four fungal genera were recorded from Haru, Homa and Nedjo districts of West Wollega. According to the study, the percentages of fungal genera observed 84.67%, 7.37%, 6.74% and 1.5% of *Aspergillus* spp, *Fusarium* spp, *Penicillium* spp, and *Rhizopus* spp, respectively (Figure 1). Accordingly, *Aspergillus* spp. were the most dominant fungal genera and relatively *Rhizopus* was the least. The fungal genera of *Aspergillus* found to be the most frequently isolated from

coffee bean followed by *Fusarium* and *Penicillium*, respectively where as *Rhizopus* was relatively the least one.

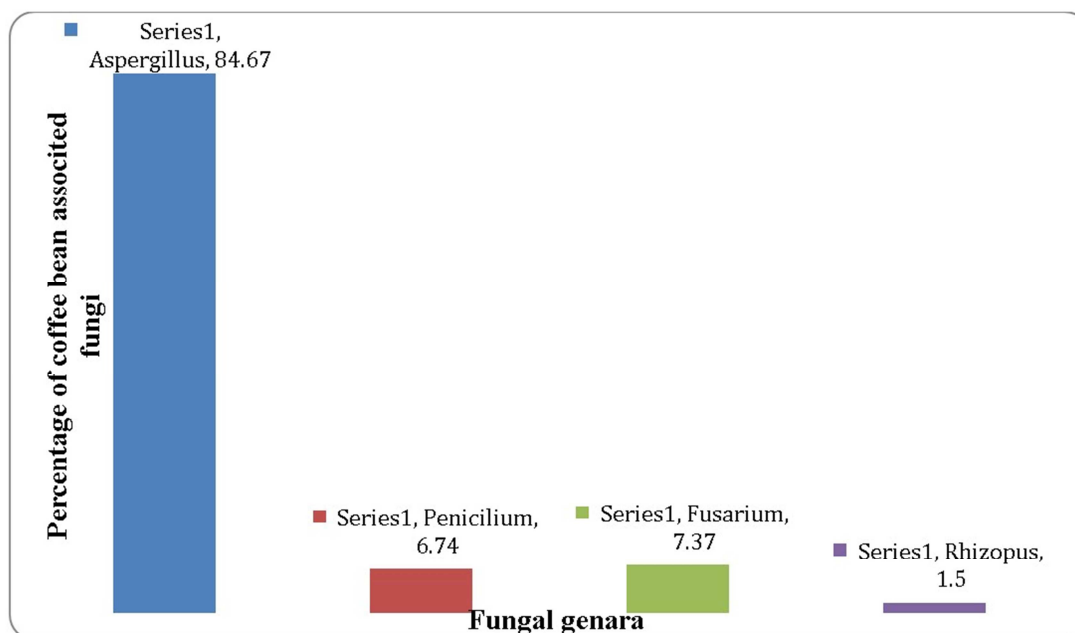


Figure 1. Percentage of total coffee bean associated fungal genera.

3.2. Mycotoxigenic Fungi Associated Coffee Bean

Aspergillus ochraceus and *Aspergillus niger* were found to be coffee bean associated mycotoxigenic fungi. *Aspergillus niger* was 73.3% while *Aspergillus Ochraceus* was 11.37%.

3.3. Coffee Bean Associated Fungi from Different Districts of West Wollega

Based on district level the composition of mycotoxigenic

fungi observed were *A. ochraceus* (3.9%, 18.1%, 5.7%), *A. niger* (64.1%, 72.3%, 91.0%), *Penicillium* (13.8%, 2.8%, 3.0%), *Fusarium* (16.8%, 4.1%, 0.4%) and *Rhizopus* (1.4%, 2.6%, 0.0%) from Haru, Homa and Nedjo, respectively (figure 2). *Aspergillus niger* was the most dominant in all districts while *Rhizopus* was the least one. *Aspergillus ochraceus* was also found in all districts with highest percentage in Homa, district followed by Nedjo and Haru.

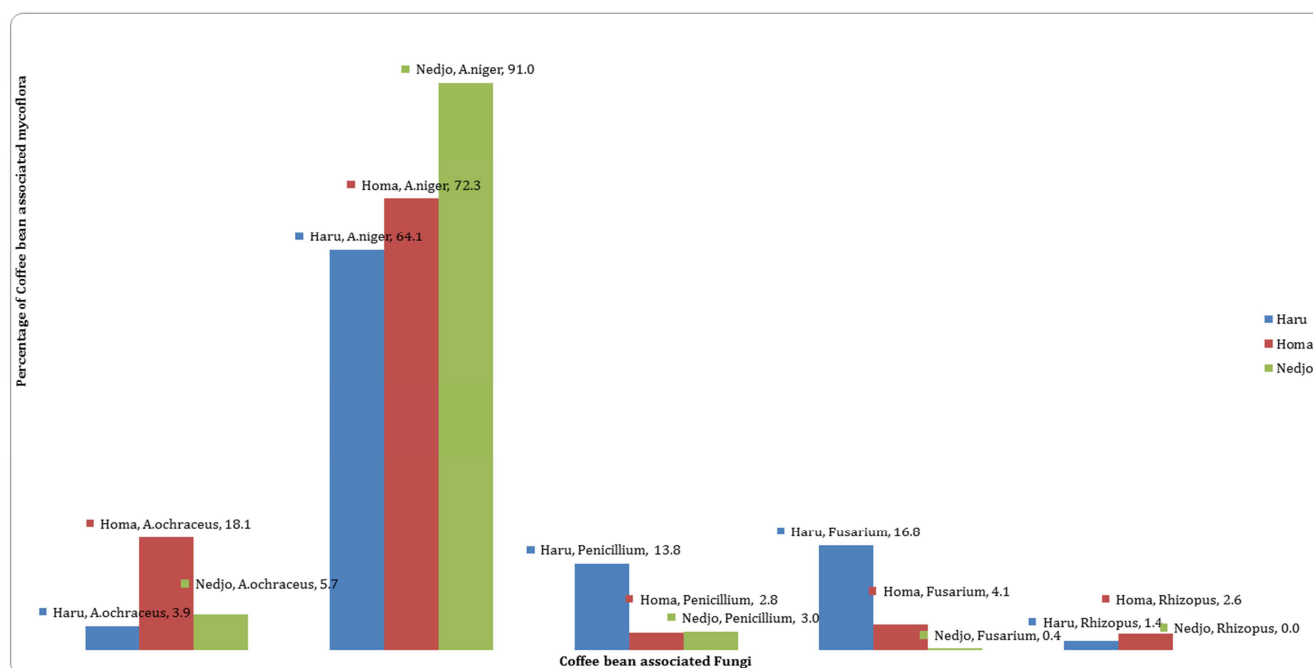


Figure 2. Percentage of coffee bean associated fungi from different district.

3.4. Correlation Matrix

3.4.1. Relationship Between Mycotoxigenic Fungi and Ochratoxin A

The result of this study revealed that there was highly significant difference positive weak correlation between *Aspergillus ochraceus* on one hand and ochratoxin A ($r=0.31$) on the other (Table 1).

Table 1. Ochratoxin A concentration using ELISA kit.

	<i>A.ochraceus</i>	<i>A.niger</i>	Ochratoxin A	Moisture content	Visible mold overgrowth
A.ochraceus		0.14	0.31**	0.25*	0.11
A.niger	0.14		0.12	0.10	0.27*
Ochratoxin A	0.31**	0.12		0.23*	0.20
Moisture content	0.25*	0.10	0.23*		0.44***
Mold condition	0.11	0.27*	0.20	0.44***	

Where: *A.niger*=*Aspergillus niger*; *A.ochraceus*=*Aspergillus ochraceus* *** Significant at $P<0.001$, ** Significant at $P<0.01$, * Significant at $P<0.05$.

3.4.2. Relationship Between Mycotoxigenic Fungi and Mold Condition

The result of this study revealed that there was significant difference positive weak correlation between *Aspergillus niger* on one hand and ochratoxin A ($r=0.27$) on the other (Table 1).

3.4.3. Relationship Between Mycotoxigenic Fungi and Moisture Content

There was also significant difference positive weak correlation between *Aspergillus ochraceus* on one hand and moisture content ($r=0.25$) on the other.

3.4.4. Relationship Between Ochratoxin A and Mold Condition

There was positive weak correlation between ochratoxin A on one hand and mold condition ($r=0.20$) on the other.

3.4.5. Relationship Between Ochratoxin A and Moisture Content

Similarly, there was significant difference positive weak correlation between ochratoxin A on one hand and moisture

content ($r=0.23$) on the other.

3.4.6. Relationship Between Mold Condition and Moisture Content

There was highly significant difference positive intermediate correlation between moisture content on one hand and mold condition ($r=0.44$) on the other (Table 1).

3.5. Level of Ochratoxin A from Green Coffee

The result revealed that the level of Ochratoxin A were different from district to district. Haru district was found to not detectable amount while Homa was found 1.23 $\mu\text{g/kg}$ (1.23 ppb) OTA level and Nedjo was relatively the highest one which was detected a mean of 2.03 $\mu\text{g/kg}$ (2.03 ppb). The occurrence of *Aspergillus ochraceus* is not necessarily mean that there is ochratoxin A but the factors like moisture content may play a role for the production of Ochratoxin A. Thus while *Aspergillus ochraceus* observed from the samples collected from Haru, but we couldn't detect any ochratoxin A from the district.

Table 2. Ochratoxin A concentration using ELISA kit.

Districts	N ^o of sample analyzed	Average OTA concentration in $\mu\text{g/kg}$ (ppb)
Haru	25	0 (ND) Not detected
Homa	25	1.23 $\mu\text{g/kg}$ (1.23 ppb)
Nedjo	25	2.03 $\mu\text{g/kg}$ (2.03 ppb)
Mean		1.08 $\mu\text{g/kg}$ (1.08 ppb)

*ppb=part per billion

4. Discussion

Four major fungal genera *Aspergillus*, *Fusarium*, *Penicillium* and *Rhizopus* were detected in association with coffee beans studied in West Wollega Zone. *Aspergillus* spp was dominantly associating with coffee beans which accounts (84.67%), *Fusarium* (7.37%), *Penicillium* (6.74%) and *Rhizopus* (1.50%). The results were in line with Geremew *et al.*[19] who reported that *Aspergillus* (79%), *Fusarium* (8%), *Penicillium* (5%) found in association with coffee beans. Urbano *et al.*[20] also reported that isolated filamentous fungi in coffee beans from Brazil they found that *A. ochraceus* and *A. niger*.

The moisture content of the coffee bean plays great role for the production of OTA as well as for the growth of mold formation over the cherry of coffee. *Aspergillus niger* was also significantly correlated with mold formation of the coffee cherries. This indicates that, coffee without mold formation may develop Ochratoxin A and moisture content is the most factorsfor OTA production. OTA has been detected and quantified by ELISA. The present study indicated that the concentration of OTA from green coffee that ND (not detected), 1.23 $\mu\text{g/kg}$ and 2.03 $\mu\text{g/kg}$ from Haru, Homa and Nedjo, respectively. This result is in agreement with Geremew *et al.*[19] who reported concentration of OTA in locally consumed coffee from Ethiopia was 1.5 $\mu\text{g/kg}$.

A significant feature of ochratoxin is that it occurs in a

wide variety of commodities such as raisins, barley, soy products and coffee in varying amounts but at relatively low levels [21]. Culliao and Barcelo [22] reported that OTA was more commonly detected in Robusta coffee (37%) than in Arabica coffee (26%). The highest level of OTA found in the dried whole cherries of Arabica 97 µg/kg, while for Robusta the highest level of 120 µg/kg OTA was found. Romani, *et al.* [23] also reported that the detected OTA from green coffee beans was 18–48 µg/kg in Congo. The current study revealed that when toxigenic strains of *Aspergillus ochraceus* shown positive significant correlation with OTA. This shows that the presence of toxigenic strains involves a great risk of OTA presence when it get favorable conditions. These mycotoxigenic fungi have a potential to produce high amount of OTA.

5. Conclusion

In conclusion, 75 coffee cherry samples has been collected from three districts of West wollega from, Haru, Homa, and Nedjo. The samples has been tested for Arabica coffee been associated mycoflora and level of concentration of ochratoxin A. There was a significant difference among the treatments on *A. ocraceus*, *A. niger*, *Penicillium sp.*, *fusarium sp.* and *Rhizopus sp.* from Haru. There was also a significant difference on *A. ocraceus*, *A. niger*, *Penicillium sp.*, and *Rhizopus spp.* from Homa and there was a significant difference on *A. ocraceus*, *A. niger*, *Penicillium sp.*, *fusarium spp.* from Homa. While there was no significant difference among treatments on *fusarium spp.* from Homa and *Rhizopus spp.* from Nedjo. The result showed that numbers of mycoflora associated with coffee were observed while five of them become the major. Among these *Aspergillus niger* were the most dominant (73.37%) detected from most coffee samples, while *Aspergillus ochraceus* (11.30%), *Fusarium spp.* (7.37%), *Penicillium spp.* (6.74%), and *Rhizopus spp.* (1.50%) were identified. ELISA kit has been used to detect and quantify ochratoxin A. The average ochratoxin A concentration detected was 0 (ND) Not detected 1.23 µg/kg (1.23 ppb) and 2.03 µg/kg (2.03 ppb) from Haru, Homa and Nedjo respectively.

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