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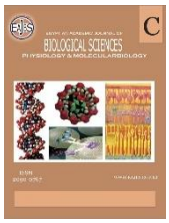

Determination of Fungal Contamination at Roasted and Unroasted Coffee Beans

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ABSTRACT

Fungal infections were observed on the coffee plant, this study aims to determine the types of fungi causing this pollution and the possibility of producing mycotoxins or not. so we make this study to isolate and identify the contaminating fungi that find in three types of Arabica coffee plants , which are common in the city of Jazan in the Kingdom of Saudi Arabia, namely (Khawlani, Luqmati and Harari) by using the dilution method on Potato Dextrose Agar media (PDA).

The results showed that some coffee samples were contaminated with fungi, which belong to 5 fungal genera, most of which belong to the Ascomycetes fungi. The species *Aspergillus niger* had the highest percentage of appearance in the coffee samples, followed *by Aspergillus flavus* and then *Penicillium sp*. The ammonia detection results showed that most *Aspergillus flavus* and *A. ochrous* isolates produced mycotoxins, while all isolates of *Aspergillus niger* and *Penicillium sp.* do not produce aflatoxins.

INTRODUCTION

Arabic coffee is one of the important tropical crops, and Brazil alone produces about 8-25% of the global coffee crop, then Vietnam and Colombia come in the next rank in terms of production, and the African continent ranks second in the production of coffee seeds. Today, coffee is one of the most popular drinks all over the world and is consumed in many countries because of its good taste, in addition to containing a high percentage of caffeine, which is the stimulant in the coffee drink. This drink also has other benefits related to facilitating the digestion process, as it contains antioxidants. Due to the increase in sales and high consumption of coffee and the increasing demand for it globally, we found coffee-producing countries seeking to implement health safety standards and control the product during production and sale to obtain a safe and high-quality product. Therefore, ancient and modern studies were conducted on this plant to obtain the finest and best ways to preserve it without any damage through harvesting or storing the crop. Arabic coffee has its main origin in Ethiopia, which is considered one of the most marketed types in the world. It is characterized by a sweet taste and strong aroma and it is consumed strongly in its pure form or mixed with some flavorings (Barrios-Rodríguez *et al.* 2022).However, some factors cannot be controlled, for example, the environmental conditions surrounding the product may cause fundamental, undesirable changes in the qualities and characteristics of the coffee plant which leads to fungal contamination of coffee beans.

According to Urbano *et al.* (2001), contamination by fungi occurs in different stages of growth, harvesting, packaging, transportation, and storage, and the high water content inside the seed, temperature, and humidity create suitable conditions for fungus growth and excretion of toxins. According to Zhang *et al.* (2013), among the factors that increase the number of *A. flavus* spores are low soil moisture and high temperature, which leads to an increase in the fungal susceptibility of the coffee plant and thus the increase of mycotoxins in the product. Mycotoxins are secondary metabolites produced by filamentous fungi that contaminate many agricultural products such as coffee beans (El-Taher *et al.,* 2012, Culliao *et al.,* 2015, Abdelghany *et al.,* 2017, Al-Rajhi *et al.,* 2023). Fungal contamination and toxin production are some of the post-harvest problems that affect the quality of coffee beans and are likely to reduce the quality of the coffee drink (Bokhari and Aly 2009 and Barcelo *et al.* 2017).

Several studies have shown the presence of mycotoxins in coffee beans (Bokhari *et al.* 2009, Urbano *et al.* 2001, and Bokhari *et al.* 2007) and they include aflatoxins AFG2, AFG1and AFB1, AFB2, and ochratoxin.

Aflatoxins are secreted by many species of the genus *Aspergillus* such as *A. flavus* (Urbano *et al.*, 2001). It is highly toxic and highly carcinogenic. It is likely to cause immunodeficiency, growth retardation, and death in humans and animals (Malaker *et al.*, 2008). Storage fungi mainly include several species of the genus *Penicillium* and *Aspergillus*, which do not invade seeds at harvest but cause contamination during storage (Barcello *et al.*,2017).

Relative humidity measurements in containers during shipment showed that an increase in humidity beyond the permissible limit causes grain rot (Bastia *et al.*, 2003). The absorption of moisture from the environment can reach levels that may allow the growth of fungi. A study on seeds stored in degrees showed low temperature and with a moisture content of more than 16% the emergence of many types of genus *Penicillium*.

It has been found that the Robusta type of coffee gives a strong yield after the roasting process. In addition, it is essential in instant coffee because it contains higher amounts of fast-soluble substances than Arabica coffee (Barrios-Rodríguez *et al.*, 2022).It has been found that consuming green coffee treatment with asparaginase and ultrasound treatment without significant sensory effects (Patil *et al.*, 2022).

The European Commission has set reference levels for the content of acrylamide, which is found in coffee, which is 400 micrograms/kg in roasted coffee and 400 micrograms/kg in soluble coffee (Pham and Hoang 2022).

The results of several studies on coffee beans stored under different storage conditions have shown that they were infected with fungi mainly from the genera *Aspergillus*, *Penicillium*, *Fusarium* and *Mucor* (Bokhari *et al.*, 2009 and Bastia *et al.*, 2003). There are also other studies on roasted coffee beans collected from the ground, coffee trees, and samples from coffee production areas, which have proven that fungi are found as natural contaminants on the surface of coffee beans, and they include two genera, *Aspergillus* and *Penicillium* (Taniwaki *et al.*, 2003).

The results of other studies also showed that *Penicillium, Fusarium* and *Aspergillus*, present as natural contaminant from the field to warehouse in coffee fruits and seeds (Bokhari 2007 and Girma *et al.*, 2008). Although the conversion of raw coffee beans into powder can eliminate fungi through the degree of effective roasting, the mycotoxins produced are not eliminated, and it has been shown in reference (Taniwaki *et al.,* 2003) One of the factors that increase mycotoxin contamination of roasted coffee beans is storage in undesirable conditions and also the low roasting temperature of coffee beans., while other studies have shown that roasting for a long time may reduce the concentration of aflatoxins (Taniwaki *et al.*,

2003) and therefore improving the quality and safety of coffee beans is necessary to reduce the problem of mycotoxins, which pose a threat to public health (Lemessa *et al.*, 2015). What affects the liver, kidneys, heart, or nerves, including what is carcinogenic.

The research aims to isolate and identify fungi contaminating commercial coffee plants available in the city of Jazan in the Kingdom of Saudi Arabia, and also determine the ability of these isolates to produce mycotoxins.

MATERIALS AND METHODS

1. Collection and Preparation of Coffee Plant:

Three types of Arabic coffee were used in the study, which are the most common in the Jazan region at Saudi Arabia these types called (Khawlani, Luqmaty, and Harari), where these samples were collected in several forms, as green beans, roasted beans, and they were also studied in the form of green coffee powder and Roasted coffee powder. roasted, where 100 gm of each type was weighed separately and kept in sterile bags, and then the samples were transferred to the laboratory to perform the required analyses.

2. Peptone Salt Solution:

This solution is used in the laboratory to recover microorganisms from multiple sources and is composed of 1g Peptone, Sodium chloride 8.5 g, 7 ± 0.2 pH at a temperature of 25 ° C, was prepared in the laboratory by dissolving 9.5 g of the medium in a liter of distilled water, mixed well and sterilized in the sterilizer at a temperature of 121 ° C for 15 minutes. Use it to revitalize the coffee plant (Lemessa *et al.,* 2015).

3. Culture Media For Isolation of Fungi:

3.1. Potato Dextrose Agar (PDA) Medium:

It was prepared according to the method mentioned in the research of Graziani *et al.* 2012), by taking 200 grams of potato extract after washing it, cutting it into small pieces, placing it in a glass beaker, and then adding distilled water to complete one liter, after that we boiled for 15-20 minutes, after which the potatoes were mashed, then filtered with a piece of clean gauze, then added to the filtrate is 20 g of dextrose and 15 g of agar. The medium was completed to 1 liter by adding distilled water and sterilized with an autoclave at a temperature of 121 °C for 15-20 minutes. Then it was cooled to 45 °C and the antibiotic L (Amoxicillin) 500 mg was added to it. Use this medium for isolation and identification of fungi.

3.2. Coconut Extract Agar:

To detect mycotoxin, the coconut medium is prepared in this way: Weigh 100 grams of grated coconut and add 300 ml of sterile water, then heat it over medium heat for 15 minutes. We filter it using sterile gauze and extract the coconut milk, to which 15 grams of agar is added. Volume to 1000 ml with distilled water, sterilize the medium at 121°C for 15-20 minutes using an autoclave (Abdul Rahim *et al.,* 2011).

4. Determination of Moisture Content in Arabica Coffee Samples:

The moisture percentage of the coffee samples was estimated by drying in an oven at 105 °C for 24 hours in the laboratory of the University College in Darb.

5. Isolation and Identification of Mycotoxigenic Fungi:

The dilution method was used to isolate fungi from coffee samples according to the method recommended in the work of Lemessa *et al.*, 2015 where weigh 20 g of coffee samples , separately and placed in a sterile plastic bag. 100 ml of MRD solution was added to it and mixed for two minutes. 1 ml of the suspension was taken and transferred to the Test tube and 9 ml of MRD solution was to it to make the first concentration, then shaken by us Vortex device for 1 minute, a series of dilutions 1-10, 2-10, 3-10 were made, then transferring 1ml of each dilutions 1-10, 2-10, 3-10 to Petri dishes containing on PDA medium, three replicates for each dish, then dishes were incubated for 14 days at 28±2°C..then the total number of developing colonies was estimated.

6. Identification of Fungal Isolates:

Identifying the fungal isolates obtained from the previous steps, where the fungal colonies are purified and identified based on the macroscopic and microscopic

characteristics of the isolated fungi by using keys and manuals to complete the identification (Colli *et al.,* 1996 and Samson *et al.*, 1981)**.**

7. Detection of Mycotoxins:

Using sterile coconut medium, the sterile coconut medium was distributed in dishes with a diameter of 8 cm, then three replicates were inoculated with discs of mushroom isolates growing on the PDA medium, with a diameter of 5 mm, and at one week old in the center of the dish. The process was repeated on all the isolates, then the dishes were incubated at a temperature of 25 °C for a week. Isolates capable of producing mycotoxin were detected using a 20% ammonia solution by using filter papers saturated with the solution in the cover of the dish, then the dishes were incubated upside down for a week, at a temperature of 25 °C. The ability of isolates to produce mycotoxins is determined by changing the color of the growth on the plate to pink, red, or orange, if this change occurs, the fungal organism has the ability to produce mycotoxins (Abdul Rahim S. H., *et al.,*2011).

8. Detection of Mycotoxin Production by Using GC-MS:

Potato dextrose broth medium is used for cultivation of isolates at 28 °C for 10 days in the dark for 10 days. After the incubation period, take 10 ml of the previous broth and sub-sampled with 20 ml of 70% methanol and vortex for 10 min and make stir for all samples, then filtrated through filter paper and then diluted as 5 ml filtered solution, 0.25 ml Tween 20- and 15-ml distilled water. this extract was purified before analysis by passing through a 0.22 µm syringe filter, determination of the production of mycotoxins was used GC-MS according to the research of Leslie *et al.,* 2006. GC-MS detector 6890/5975B (Agilent Technologies) was combined with the column HP-5MS, 30 m, 0.25 mm, and 0.25 µm. The program of ChemStation was from Agilent Technologies for the system control and data processing. And used helium as the carrier gas with a column flow rate of 1 ml/min. the injection mode was the split-less injection and the volume of injection was 1 µl. The inlet temperature was 270 °C, MSD ion source temperature 170 °C, mass filter temperature 150 °C, and GC-MSD inter-face temperature 280 °C. The column temperature program was 60 °C held for 2 min, 25 °C/min to 240 °C, and 5 °C/min to 300 °C. Electron ionization (EI) was carried out at 70 eV and spectra were monitored in selected ion monitoring (SIM) mode.

RESULTS AND DISCUSSION

The obtained results in this work are summarized in the following Tables (1-7) and Diagrams (1-4).

Table 1. Moisture percentage in coffee samples**.**

Type of coffee	Moisture percentage	Storage time for old seeds
Khwlani green coffee (new)	% 2.07	
Khwlani green coffee (old)	% 1.79	2 years
Harare green coffee (new)	% 2.81	
Harare green coffee (old)	% 2.21	2 years
Luqmaty green coffee (new)	% 3.54	
Luqmaty green coffee (old)	% 2.08	2years

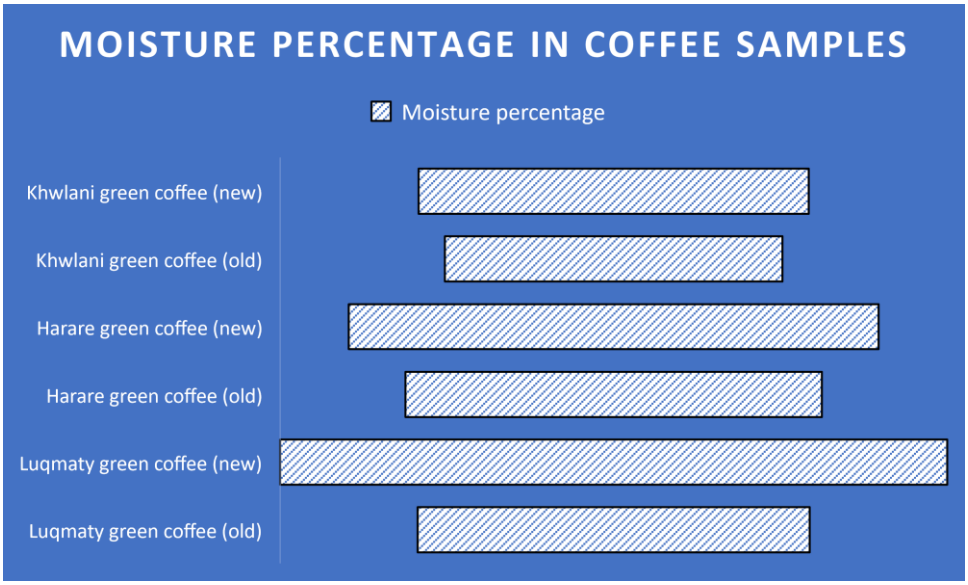

Diagram 1: Moisture percentage in coffee samples

Table 2. Percentage of fungi isolated from Khwlani coffee samples on PDA medium.

Treatment	*Asp .niger*	*Asp .flavus*	*Asp. ochraceus*	*Penicillium spp.*
Fresh new seed	22.5%	25%	0	9%
Fresh old seed	34.1%	20%	0	24%
F. ground new seed	0	12%	0	10%
F. ground old seed	20%	35%	1%	5%
Roasted new seed	0	0	0	0
Roasted old seed	8%	7%	0	0
R. ground new seed	2%	0	1%	1%
R. ground old seed	0	0	0	0

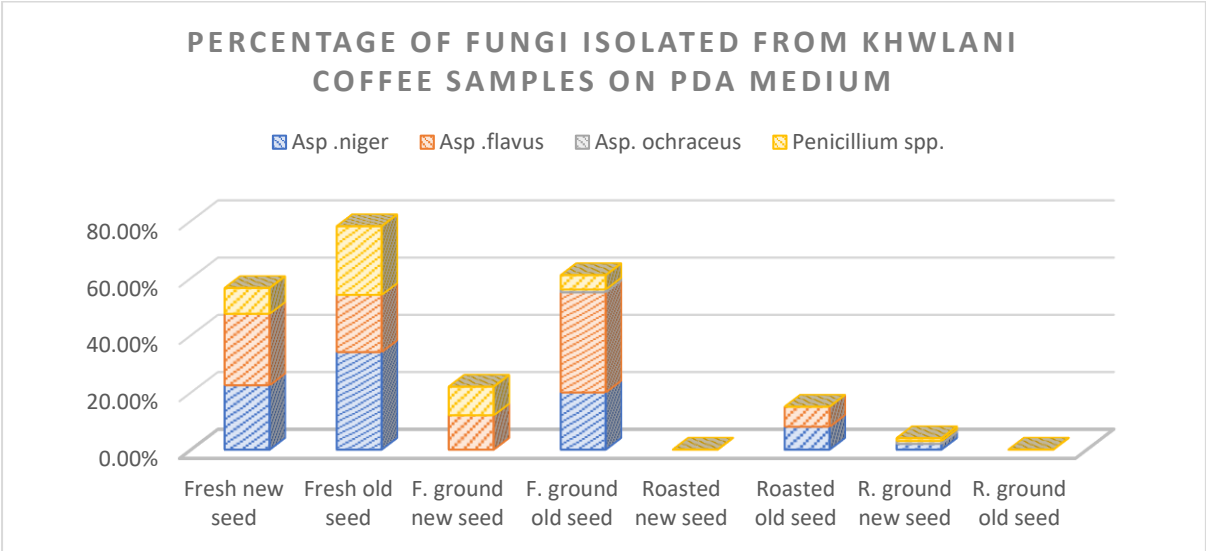

Diagram 2: Percentage of fungi isolated from Khwlani coffee samples on PDA medium**.**

Table 3. Percentage of fungi isolated from Harari coffee samples on PDA medium.

Treatment	*A .niger*	*A .flavus*	*A. ochraceus*	*Penicillium spp.*
Fresh new seed	52.5%	25%	11%	17%
Fresh old seed	14.1%	20%	0	0
F. ground new seed	0	12%	0	10%
F. ground old seed	68.5%	35%	5.12 %	9%
Roasted new seed	0	0	0	0
Roasted old seed	23%	7%	0	0
R. ground new seed	37.8%	10%	2%	11%
R. ground old seed	10%	17.56%	0	0

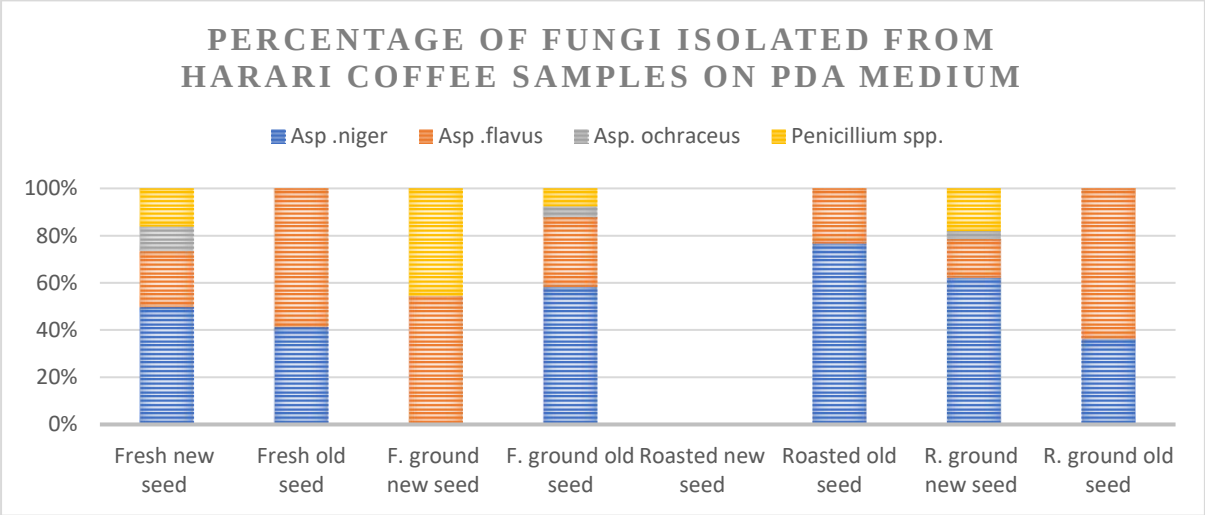

Diagram 3: Percentage of fungi isolated from Harari coffee samples on PDA medium.

Table 4. Percentage of fungi isolated from Lokmati coffee samples on PDA medium.

Treatment	*Asp .niger*	*Asp .flavus*	*Asp. Ochraceus*	*Penicillium spp.*
Fresh new seed	12.5%	15.32%	4%	16.2%
Fresh old seed	34.1%	20%	0	34.5%
F. ground new seed	10%	12%	1.2%	20%
F. ground old seed	58.7%	45.2%	1%	5%
Roasted new seed	0	0	0	0
Roasted old seed	18%	7%	0	0
R. ground new seed	17.68%	27.81%	0	1%
R. ground old seed	51.2%	10.2%	0	41.25%

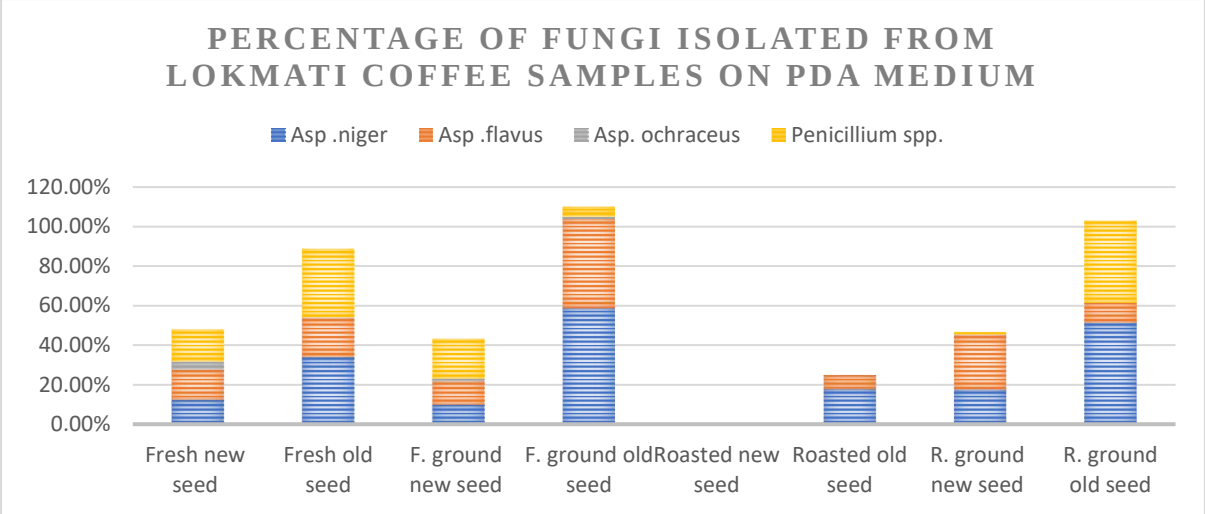

Diagram 4: Percentage of fungi isolated from Lokmati coffee samples on PDA medium.

Table 5. Percentage of fungi that preduce aflatoxin and isolated from Khwlani coffee.

Treatment	*Asp .niger*	*Asp .flavus*	*Asp. ochraceus*	*Penicillium spp.*
Fresh new seed	0	2.4 %	2.2 %	0
Fresh old seed	0	11%	1.4 %	0
F. ground new seed	0	0	0	0
F. ground old seed	0	10%	3 %	0
Roasted new seed	0	0	0	0
Roasted old seed	0	2%	17 %	0
R. ground new seed	0	0	0	0

Table 6. Percentage of fungi that preduce aflatoxin and isolated from Harari coffee.

Treatment	*Asp .niger*	*Asp .flavus*	*Asp. ochraceus*	*Penicillium spp.*
Fresh new seed	0	17%	2%	0
Fresh old seed	0	33%	0	0
F. ground new seed	0	24%	0	0
F. ground old seed	0	18%	13.2 %	0
Roasted new seed	0	0	0	0
Roasted old seed	0	21%	0	0
R. ground new seed	0	3 %	0	0
R. ground old seed	0	50 %	0	0

Table 7. Percentage of fungi that preduce aflatoxin and isolated from Lokmati coffee.

Treatment	*Asp .niger*	*Asp .flavus*	*Asp. Ochraceus*	*Penicillium spp.*
Fresh new seed	0	14.8%	2.5%	0
Fresh old seed	0	20.5%	7.2%	0
F. ground new seed	0	15%	3%	0
F. ground old seed	0	17%	2%	0
Roasted new seed	0	0	0	0
Roasted old seed	0	12.7%	0	0
R. ground new seed	0	11 %	0	0
R. ground old seed	0	21.2%	7%	0

We notice from the results in Table (1) that Luqmaty green coffee (new) is the moistest while Khwlani old coffee is the least moist.Also, we notice from our results in Tables (2),(3), and (4) that most of the isolated fungi belong to the Ascomycetes species, and the genus *A. niger* was dominant, followed by *A. flavus* and *Penicillium sp.*

These results are consistent with several studies that have shown that *Aspergillus* and *Penicillium* are natural pollutants for coffee and transmit from the field to storage (Binder *et al.,* 2007 and Suarez-Quiroz *et al.,* 2004). There were present *Penicillium* and *Aspergilli* colonies in the coffee samples, these results obtained are evidence and indicat of the pollution of air. There is a possibility that improper heat treatment during packaging, and storage transportation may lead to fungal contamination at coffee products , also due to unhealthy environmental conditions according to Graziani *et al.* (2012).

The results of detection aflatoxins from *Asp. flavus, Asp. ochraceus* and *Asp.niger* isolates using coconut medium showed that most of the *Asp.flavus* isolates were toxin-producing.

We notice from our results in Tables (5),(6), and (7) that most of the isolated fungi isolated from Harari coffee able to produce the toxins, also all isolates of *A. flavus* able to change the color of the colonies, while the most isolates of *Aspergillus niger* and *Penicillium sp* were not mycotoxin-producing, and the studies of Bokhari and Ali (2009) have proven that the presence of mycotoxins as patulin, Aflatoxins B1, B2, G1 and G2 in coffee beans under the conditions of the treated environment and different environmental conditions.

Fungal toxins, including aflatoxin, as secondary metabolites belonging to the genus *Aspergillus*, are not formed in all cases of growth of these species, and the isolation of these fungi does not mean the presence of toxins in them except under specific environmental conditions, such as high humidity with appropriate temperatures (Graziani *et al.* 2012).

According to Nakajima *et al.* (1997), the rate of fungal contamination increased during wet processing, mechanical processing, and the drying processes of coffee beans, but not all strains produced carcinogenic toxins. The results of our research also proved that roasting for a long period reduces or prevents the growth of fungi, which reduces the concentration of aflatoxin.

The study also showed that new green coffee beans have a higher percentage of fungi than old coffee, and this may be due to the water content in green coffee being higher than that of roasted coffee.

Also. We noted that the best type of coffee is Khwlani coffee, where the least number and types of fungi were in it, also Khwlani coffee seeds are characterized by being large in size and covered with a green oily layer that characterized by distinctive smell. Perhaps this is reason help Khwlani coffee to prevent the growth of fungi.

Our study also showed that a high percentage of moisture in the coffee sample leads to an increase in the number of fungal isolates, Therefore, the study recommends working to completely reduce the humidity during harvesting and storing coffee beans in order to preserve the fruits and the product and raise the efficiency of coffee, which plays a major role in the global economy and trade.

CONCLUSION

This study proves that fungi are found as natural pollutants on the surface of coffee beans and notice that the processing of raw coffee beans into powder can eliminate the fungus through the effective roasting degree, the mycotoxins produced are not completely eliminated. Moreover, this study demonstrates the efficiency of qualitative method as coconut milk agar and ammonia vapor the detection of aflatoxigenic fungi from coffee beans.

Declarations:
Normative Consent: From January 2023 to March 2024
Ethical Approval: The University of Jazan Ethical Committee approved the study, and it was carried out under the Helsinki Declaration's ethical principles.
Competing Interest: Authors do not have any conflict of interest to declare,
Authors Contributions: The authors have equally contributed to writing , designing , compiling and final editing of the manuscript
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ARABIC SUMMARY

تلوث نبات البن العربي بالفطريات والكشف عن السموم الفطرية المنتجة في القهوة

منى سعد عاشور[1]،انتظار مصطفى عباس [1]، أميمة عبد الوهاب رضوان[2] ، سميرة أحمد عواد[1] ، عبير عبد العزيز محسن [1]،علوية حسين أحمد المشاى[1] ، مواهب وداعة الله موسى[3]

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لوحظت إصابات فطرية على نبات القهوة، وتهدف هذه الدراسة إلى تحديد أنواع الفطريات المسببة لهذا التلوث ومدى إمكانية إنتاج السموم الفطرية من عدمه، فقد قمنا بهذه الدراسة لعزل وتشخيص الفطريات الملوثة لثلاثة أنواع من نباتات القهوة العربية المنتشرة في مدينة جازان بالمملكة العربية السعودية وهده الأنواع هي (الخولاني واللقماتي والهراري) وذلك باستخدام طريقة التخفيف على دكستروز البطاطس.

وقد أظهرت النتائج أن بعض عينات القهوة ملوثة بالفطريات التي تنتمي إلى 5 أجناس فطرية ينتمي معظمها إلى فطريات Ascomycetes. كان للنوع *Aspergillus niger* أعلى نسبة ظهور في عينات القهوة، يليه *Aspergillus flavus* ثم sp *Penicillium*.

أظهرت نتائج الكشف عن الأمونيا أن معظم عزلات *Aspergillus flavus* و *Aspergillus* ochrous أنتجت سموم فطرية، في حين أن جميع عزلات *Aspergillus niger* و*Penicillium sp* لا تنتج الأفلاتوكسينات في العينات المعزولة بالبحث