Available online freely at www.isisn.org

Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2023 20(4): 1136-1146. **OPEN ACCESS**

Morphological and molecular identification of black *Aspergillus* spp. causing mould disease of Onion.

Shaima M. N. Moustafa* [1], Mona S. Azab[1,2], Wiam J. Aljudaya[1] and Salam S. Alsharari[1]

[1]Biology Department, College of Science, Jouf University, P.O. Box: 2014, Sakaka, **Saudi Arabia**
[2]Department of Zoology, Faculty of Science, Benha University, Benha 13518, **Egypt**

***Correspondence:** shymnabil@ju.edu.sa **Received 20 June 2023, Revised: 20 December 2023, Accepted: 24 December 2023 e-Published: 31 December 2023**

Onion black mold disease mostly occurs post-harvest, affecting bulbs during storage. The main objective of the study is to identify the black *Aspergillus* species causing onion black mold disease and determine their mycotoxin production potential. A fungus infection was realized as a black spore mass on the outer scale leaves. Fifty samples of onions with black masses were collected to isolate the pathogenic fungi. Eighty-three isolates of black *aspergilli* species were identified using morphological and molecular characteristics. Five isolates were recorded according to microscopy of the conidial structure, comprising *A. ellipticus*, *A. carbonarius*, *A. japonicus*, *A. niger*, and *A. vadensis*. Sequence data of the nuclear Internal Transcribed Spacer (ITS) rDNA was used to build a phylogenetic tree from the 25 sequences comprising our isolates. The neighbor-joining phylogenetic tree revealed the monophylogenetic relationship between species within the same section but the polyphylogenetic relationship among species from different sections. The chloroform extracts of different samples were tested for the presence of mycotoxin using thin layer chromatographic analysis, which includes the determination of the following mycotoxins: aflatoxin B1, aflatoxin B2, aflatoxin G1, and aflatoxin G2. The results indicated that aflatoxin was present in all collected samples.

Keywords: Black *Aspergilli*, Mycotoxins, Onion, Morphological and Molecular identification, ITS r RNA, Neighbour-joining tree.

INTRODUCTION

In Saudi Arabia, 112,508 tonnes of onions were produced in 2019, and it is anticipated that this number will vary by an average of 13.49% in the next years. The nation was a net exporter of onions, with around 7,976.00 hectares dedicated to their cultivation. Between 2015 and 2019, Saudi Arabian onions saw a 5% yearly rise in value while seeing a 41% annual growth in quantity.

Onion (*Allium cepa* L.) is belonged to family Amaryllidaceae, which considered the main vegetable species used and cultivated in all over the world. Onion bulb contains 2% fiber, 4% sugar, 0.1% fat, 1% protein, 89% water, vitamins B6, C and several other nutrients including folic acid (Samuel et al.2023) this displays the significance of onion in human nutrition. Aeromycology investigates the air-borne spores of fungi and their spread affects plant, animal, and human health. The onion stores normally have hanging spores, pollens, dust, insect and, fungal particles which were found to be correlated with disease onset in the onion bulbs. Many infectious operations occur during the pre- and post-harvesting of onions, as onions make their way from the field to the market. Black mold rot is considered one of the reported dangerous diseases of onions storage caused by a fungal infection. Fungal species may infect onions through wounds or the drying neck, at the top of the bulb, or in the roots. A human illness called aspergillosis is caused by the inhalation of large amounts of *Aspergillus* spores. Cough, chest pains, difficulty breathing, and fever are the main manifestations of aspergillosis. Some patients may suffer from delirium, shock, bloody coughing, chills, and blood clots. Possibly, the fungus could find a way to invade and destroy healthy tissues, causing liver or kidney failure Seyedmojtaba et al. 215) . The immune system may respond if it comes into contact with black mold, a fungus. Sneezing, coughing, congestion, and eye irritability are common symptoms. It seldom results in fatal sickness or serious illness, although it can make asthma symptoms worse.

There are around 15 distinct fungus species and 5 different bacterial species discovered to be in charge of onion infections in transport and storage all over the world. The loss resulting from these illnesses is significant and may reach 40% [3]. Several diseases, including black mold rot (*Aspergillus niger*), basal rot (*Fusarium moniliforme*), grey neck rot (*Botrytis allii*), soft

rot (*Erwinia* spp.), dry rot (*Macrophomina phasiolina*), green mold rot (*Penicillium* spp.), and blue mold rot, cause the onions to decay while they are in storage. Aimeil et al. 2023) The black mold rot (*A. niger*) is the most severe of them while in storage. On high temperatures and relative humidity, *A. flavus* & *A. niger* infect onions. *Aspergillus* sp. was said to be the main fungi responsible for the storage illnesses of onions.

Black *Aspergillus* species can be classified into *Nigri* section and *Circumdati* subgenus, they present dark colonies, between black to brown, with uni or bi-seriate strigmata Silva et al. 2011) . A fundamental classification key of the section *Nigri*, was suggested on the basis of morphological similarity, extrolite, and β-tubulin sequence (Abarca et al. 2004 Abarca et al. 2004) . Furthermore, additional species were characterized: *A. indologenus, A. fijiensis, A. neoniger, A. eucalypticola;* two species were confirmed: *A. acidus* and *A. violaceofuscus;* and *Aspergillus foetidus* were synonymous with *Aspergillus niger* relied on physiological & molecular analysis. In addition, the formerly characterized *A. lacticoffeatus* and *A. coreanus* were described as color mutants of *A. niger* and *A. acidus,* respectively (Varga et al. 2004) .

Based on the positive roles and effects of *A. niger*, further study to accurately identify and characterize this fungus is crucial. The combined approach generally referred to as polyphasic which uses morphological, chemical, physiological and molecular methods is of great interest to many mycologists because of its high efficiency in classifying complex fungi (Varga et al. 2004,Samson et al. 2004) The molecular technique based on DNA molecular marker, which is the fastest and most accurate method for fungi identification enables the phylogenetic analysis of most fungal species in *A.* section *Nigri* George M. and Ramteke2019). ITS region is one of the most widely used phylogenetic markers for most fungi. This region consists of the highly conserved 5.8S region in addition to ITS1 and ITS2, the variable region that allows differentiation of different fungal species. This region contains many predominantly RNA tandem repeats in the haploid genome which has made it very useful for species identification and hence has been proposed as a standard marker in coding DNA strand for fungal species (Mkumbe et al. 2018 and Siddiquee et al. 2007). Mycotoxins pollute a vast array of agricultural commodities and foods produced by different fungal species. These mycotoxins pose serious challenges to food safety in different countries, in which humidity and temperature conditions are optimum for the growth of fungal species and toxins' production. Possible transmission of these toxins results in potential hazards to human health (Nielsen et al. 2009). Mycotoxins are human liver carcinogens based on enough evidence for carcinogenicity in humans (Nielsen et al. 2009). Black Aspergilli are fungal species that establish colonies in food and cause spoilage. Food and Drug Administration (FDA) reported that *A. niger* is considered safer to be employed in biotechnology, especially, fermentation under particular processing conditions (Schuster et al. 2002) On the contrary, several *Aspergillus* species were presented to produce fumonisin (FB2) and ochratoxin (OTA) mycotoxins (Qi et al. 2016). Definitely, such mycotoxins make the onion and its products unsafe for human use.

This study goals for isolation of the contaminant black molds, morphological identification of the isolated fungi and molecular characterization of *Aspergillus* species isolated from onion black molds. Determine whether sequencing analysis of (ITS) region of rDNA could be used to reveal the phylogenetic relationships of *A. Nigri* isolates from Bulb onion.

MATERIALS AND METHODS

Isolation of pathogenic fungal species

Twenty samples of onions with a black mass on the neck were collected from Sakaka City, Aljouf region, Saudi Arabia to test the presence of pathogenic fungi. Inocula of smutty black powder on the fleshy leaf of bulbs were moved to Potato Dextrose Agar (PDA) added with Rose Bengal (antibacterial). Petri-dishes were kept in dark at 25 °C for five days. Isolated fungi growing on these Petri dishes were purified using PDA media and maintained on slants having PDA media (Moustafa, 2019 and Shaima et al. 2020)

Identification of isolated fungi

Two hundred isolates of black aspergillus species were obtained and subjected to identification using morphological [(Schuster et al. 2002)], and confirmed by molecular characteristics. Isolated taxa were purified on Malt Extract Agar (agar, 15 g/L malt extract, 30 g/L mycological peptone, 5 g/L) and Czapek Yeast Agar (Sucrose 30 g/L, Yeast extract 5 g/L, Dipotassium hydrogen phosphate 1 g/L, Sodium nitrate 0.3 g/L, Potassium chloride 0.05 g/L, Magnesium sulphate 0.05 g/L, Ferrous sulphate 0.001 g/L, Zinc sulphate 0.001 g/L, Copper sulphate 0.0005 g/L, Agar 15 g/L) media. After inoculation, plates were incubated at 25°C for 6 days. The majority of the detected fungal isolates had their genomic DNA taken, amplified using PCR, and then sequenced using the ITS1 region of the rRNA gene. The majority of the time, 100 mg of mycelial powder was utilized for genomic DNA extractions; this powder was made by pulverizing a small amount of the fungal culture in liquid nitrogen with a mortar and pestle. A modified Phenol:Chloroform technique was used to isolate the genomic DNA of fungi (Samson et al. 2004) . ITS universal primer pair 1 (TCCGTAGGTGAACCTTGCGG) and ITS 4 (TCCTCCGCTTATTGATGC) were used to amplify the ITS gene, yielding amplicon products of about 500 bp. The approximately 500 bps area of the

ITS region was amplified using the PCR Master Mix (Promega ™). In every PCR experiment, a negative control (PCR mix devoid of template DNA) was conducted as well. The PCR reaction was set up as follows: a first cycle of denaturation at 95°C for 30s, annealing at 52°C for 30 minutes, and extension at 72°C for 2 minutes comprised the first 35 cycles. The last cycle was extended at 72°C for 15 minutes. The OmegaTM PCR Purification Kit was used to purify the PCR products Samson et al. 2004and,9]. Using the same reaction primers and an ABI 3730xl DNA sequencer, the purified PCR products were sequenced. The sequences pertaining to the ITS rRNA gene were submitted to the Gene Bank and subjected to the BLASTn (National Center for Biotechnology Information) search engine in order to determine a similarity index between them. MEGA (Molecular Evolutionary Genetics Analysis) software was used to alter sequences (Samson et al. 2004) . Every sequence underwent a separate BLAST search to be confirmed in Gene Bank.

Evolutionary Using the MUSCLE Multiple Sequence Alignment Program, analyses of recently acquired sequences from our isolates were aligned with extremely similar sequences from Gene Bank. ClustalX version 2.0 software was used to align the sequences and MEGA 4.0 was used for neighbor-joining and bootstrap analysis throughout the phylogenetic studies. One thousand replications were used to create the bootstrap values.

Detection of Aflatoxin by using TLC techniques:

By dissolving the solid standard in benzene:acetonitrile (98:2, v/v) and precisely quantifying the concentration using a spectrophotometer, stock standard solutions of AFB1, AFB2, AFG1, and AFG2 (10 g/mL) were created. To generate a working standard solution for measurement and spiking, aliquots of stock solutions of each toxin individually were diluted and AFB1 (0.12µg/mL), AFB2 (0.04µg/mL), AFG1 (0.07µg/mL), and AFG2 (0.07µg/mL) were added to benzene:acetonitrile (98:2, v/v). A 50 g sample of onions was weighed and extracted for 5 minutes using 300 mL of chloroform and 10 mL of distilled water [12]. Following the extraction process, 20 g of Na_2SO_4.10 H_2O was combined with the filtrate. 100 milliliters of the filtrate were evaporated in a rotary evaporator (Buchi B-481, Labortechnik AG, Switzerland) at 40 to 50 degrees Celsius. Under UV illumination at 366 nm (Chromato-Vue C-70G, Ultra-violet Products, USA), the TLC plate was visually inspected. Densitometric analysis was used to compare the regions of the samples' chromatographic peaks to those of the standard calibration curve, which allowed for the determination of the aflatoxin levels in the samples*.*

RESULTS

One of the earliest cultivated plants in existence, the onion (*Allium cepa*) is a rich source of natural organosulfur compounds (OSCs) and flavonols [1,4]. The degradation of numerous food commodities, including vegetables and fruits, is caused by a variety of pathogenic and saprophytic fungi. Seven significant fungal species were discovered in the current analysis to be present year-round in association with onion bulbs. Onion bulbs were gathered from several markets in various months during the experiment period, and Table 1 lists the prevalent fungi that were associated with them. Two hundred fungal spp. belonging to 4 genera were isolated from 20 supermarkets located in Sakaka city, Aljouf governorate, Saudi Arabia Table (1).

Table 1: Average total counts, occurrence of fungal species isolated from 20 samples of different onion cultivars collected from different supermarkets at Sakaka, Aljouf, Saudi Arabia, on PDA media at 26°C.

Isolated fungal species	total counts	Percentage of total isolated fungi (%)
***Alernaria* sp.**	18± 0.55	9± 2.13
Aspergillus niger	60± 1.32	30± 1.88
Aspergillus carbonarius	30± 0.87	15± 2.2
Aspergillusaponicus	25± 1.88	12.5± 1.76
Aspergillus. vadensis	21± 4.5	10.5± 3.54
Aspergillus. ellipticus	19± 2.66	9.2± 2.10
Penicillium oxalicum	15± 5.43	7.5± 1.76
Rhizopus stolonifer	12± 6.22	6± 0.45
Gross total counts	200	

Red onion samples exhibited the highest infection rate with *Aspergillus* section *Nigri* (62.5%) as compared to yellow and white onion samples which showed 7.5% and 5% infection rates by the same fungus, respectively (Table 2).

Aspergillus niger (WJ-1) was the highest frequent (20 supermarkets out of 20) contributing 30% of the total count. *A. carbonarius* (WJ-2) came second, which was recovered, from 18 samples matching 15% of the total count of fungal isolates. *A. japonicus* (WJ-3) gave 12.5% of the total count but with low occurrence. *A. vadensis* (WJ-4) and *A. ellipticus* (WJ-5) were less frequently isolated representing 10.5% and 12.5% of the total

count, respectively.

Morphological and Molecular identification:

Results in Table 3 show that some species were capable to form sclerotia, comprising *A. ellipticus, A. carbonarius* and *A. japonicus*, while other species had not produced sclerotia, including *A. niger* and *A. vadensis*. Photos of compound microscopy of the conidial structure of the type strain and some illustrative species of *Aspergillus* are demonstrated in Figs (1-5).

Polymerase chain reaction (PCR) and sequencing were used to differentiate the various black *Aspergilli* isolated from onion. Genomic DNA isolation of onions fungal isolates represented with a length of ~1800 bp. The primers were designed to amplify about 600 bp of the (ITS) rRNA gene of all *Aspergillus* species (Figure 6). The size of ITS rRNA amplicons ranged between 555 - 605 bp. All sequences were deposited on GenBank under the accession numbers indicated in Table (4).

Table 2: Total numbers of black *Aspergilli* on three different kinds of twenty onion samples that were taken at 26°C on a PDA from Sakaka city.

	Red onion	Yellow onion	White onion
(CFU/g)	155± 3.76	30± 4.21	15± 1.77
Black Aspergillus occurrence (%)	125 (62.5%)	15 (7.5%)	10 (5%)

CFU: colony-forming units

Table 3: Morphological features of isolates after their incubation at 25°C for 6 days.

Species	Colony color	Vesicle serration	Vesicle shape	Conidial size (mm)	Vesicle size (µm)	Color and size of sclerotia (mm)
A.carbonarius	Brownish black	Biseriate	Globose to radiate	7-9	40-80	Pink to yellow, 1.2–1.8
A. ellipticus	Grayish brown	Biseriate	Globose to Radiate	3.5-5.5	75-100	Dull yellow to brown, 0.5–1.5
A. japonicas	Brownish black	Uniseriate	Globose to Radiate	3-5	20-35	white to cream, up to 0.5
A. niger	Dark brown to black	Biseriate	Globose to Radiate	3-5	45-80	-
A. vadensis	Brownish black	Biseriate	Globose to Radiate	3-4	25-35	-

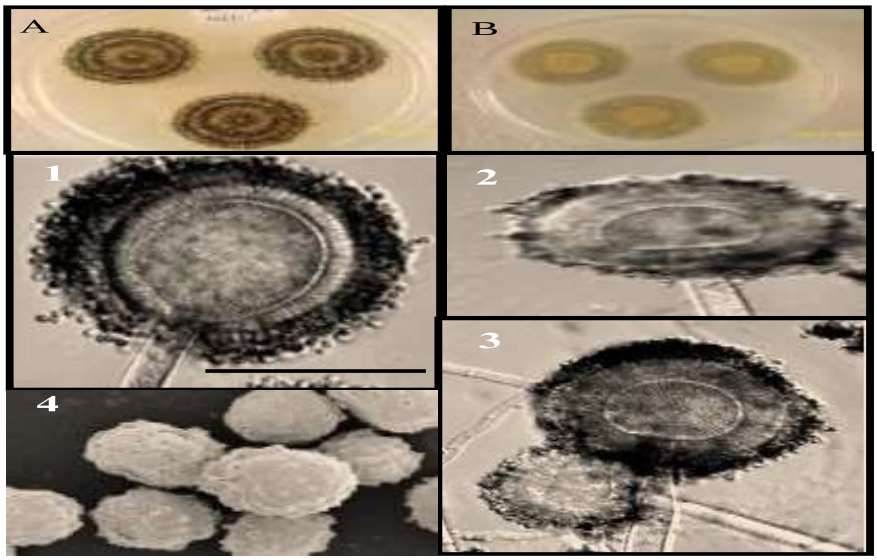

Figure 1: (A-B) Colony morphologies of *A. niger* growth on PDA media. (1-5) Mycelia growth under microscope. 1-4. Thick long conidiophore carrying biseriate sterigmata. 5. Conidia under SEM Electron Microscopy. Bar 10 µm in images (1) is the same in images 2-4, and in electron microscope images, each magnification is showed on each image, separately.

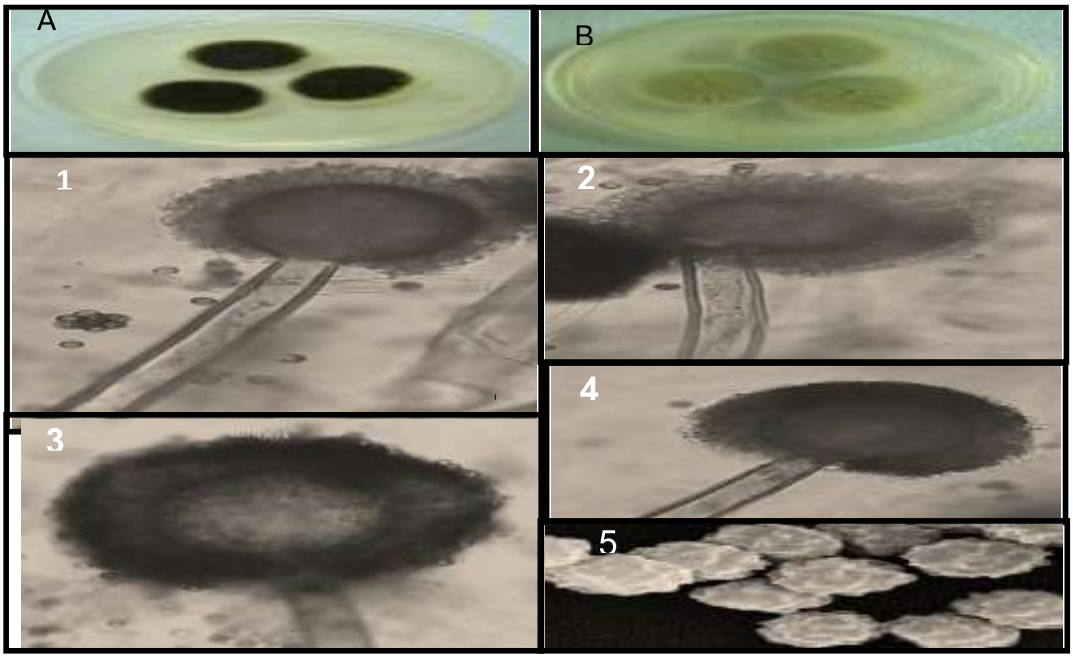

Figure 2: (A-B) Colony morphologies of *A. carbonarius* growth on PDA media. (1-3) Mycelial growth under microscope. (1-3) Globose to radiate vesicle carrying biseriate strerigmata. (4). Conidia under SEM Electron Microscopy. Bar 10 µm in images (1) is the same in images 2-3, and in electron microscope images, each magnification is showed on each image, separately.

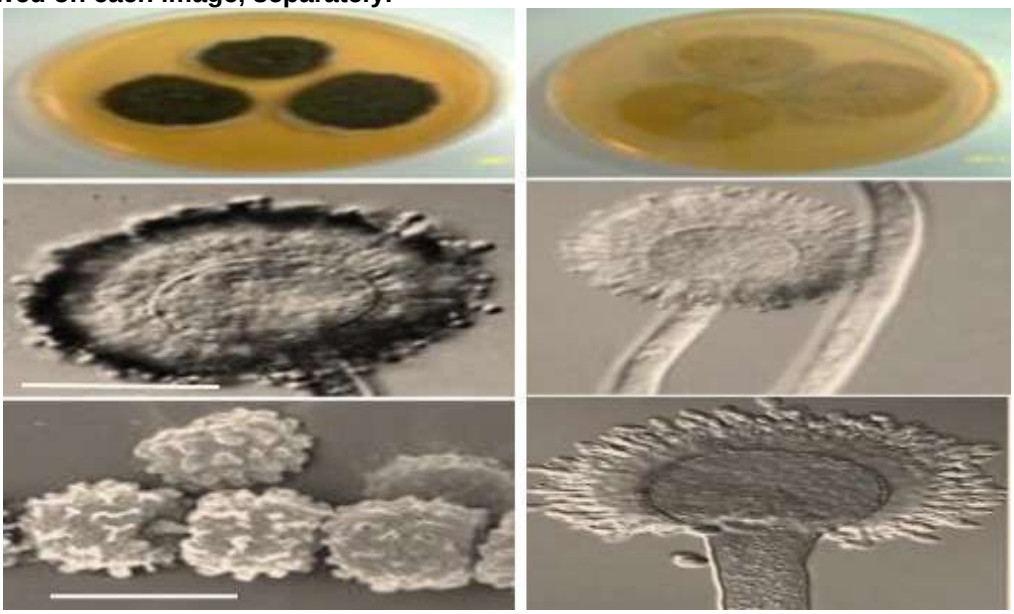

Figure 3: (A-B) Colony morphologies of *A. japonicas* growth on PDA media. (1-4) Mycelial growth under compound microscope. 1-4 Globose to radiate vesicle, uniseriate strerigmata. 5. Conidia under SEM Electron Microscopy. Bar 10 µm in images (1) is the same in images 2-4, and in electron microscope images, each magnification is showed on each image, separately

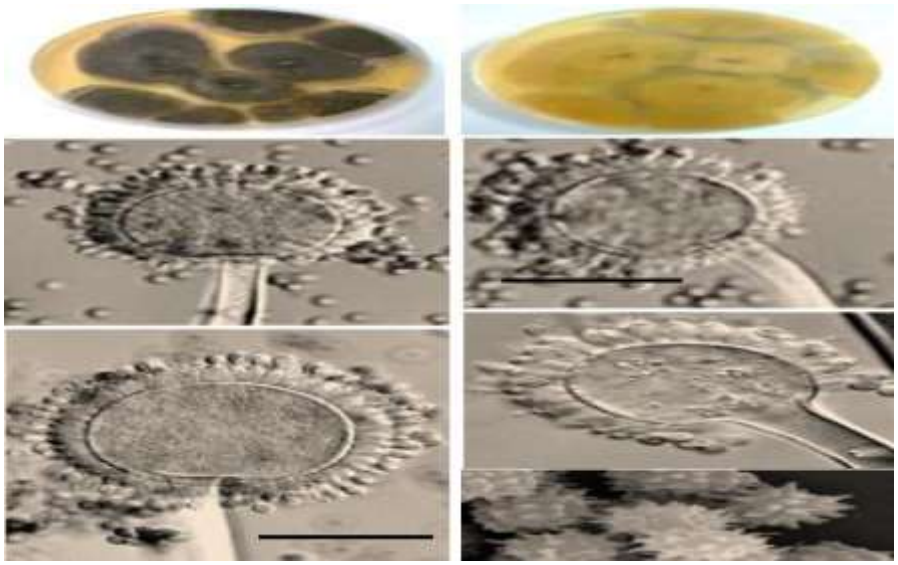

Figure 4: (A-B) Colony morphologies of *Aspergillus vadensis* growth on PDA media. (1-3) Mycelia growth under compound microscope. 1-3 Globose to radiate vesicle, Biseriate strerigmata. 4. Conidia under SEM Electron Microscopy. Bar 10 µm in images (1) is the same in images 2-3, and in electron microscope images, each owed on each image, separately.

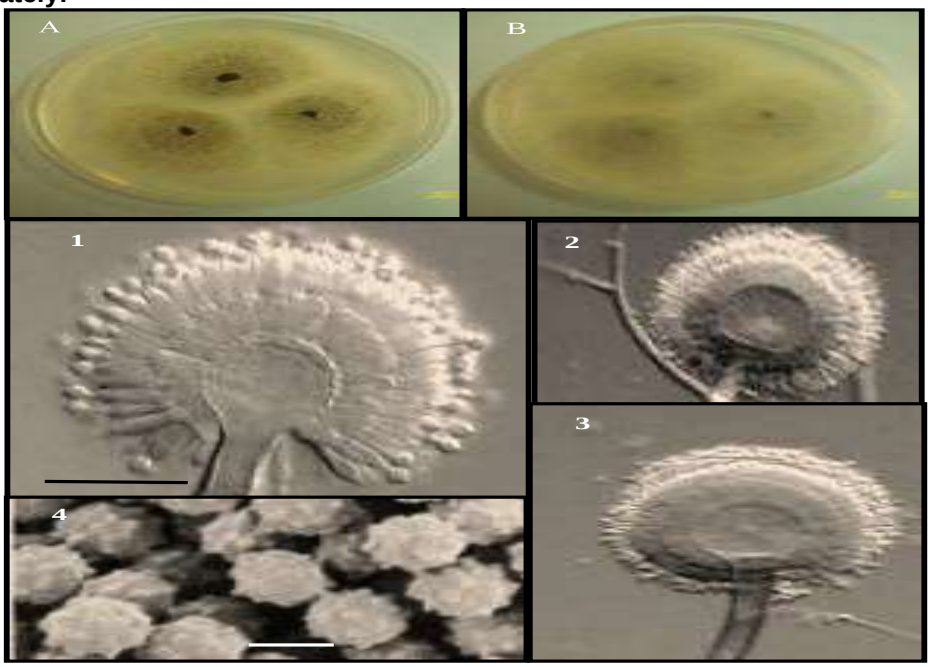

Figure 5: (A-B) Colony morphologies of *Aspergillus ellipticus* growth on PDA media. (1-3) Mycelial growth of *A.ellipticus* on PDA, under compound microscope. 1-3 Globose to radiate vesicle, Biseriate strerigmata. 4. Conidia under SEM Electron Microscopy. Bar 10 µm in images (1) is the same in images 2-3, and in electron microscope images, each magnification is showed on each image, separately.

Phylogenetic analysis:

Sequence data of the nuclear ITS rDNA was used to build phylogenetic trees using 25 sequences comprising our isolates. The tree indicated that all *A. japonicas* have clustered in the same division (I). Whilst, the other species have, collectively, clustered in another division (II). Each one of our species has clustered in the same sister clade with its corresponding species in the division

(II). For distance analysis, the above mentioned 25 sequences have been used to generate a distance matrix using the DNADIST topological algorithm (Table 5). Results of a distance matrix of the *Aspergillus* ITS sequences indicated that all related species of the section *Nigri* (*A*. *Nigri*) were monophylogenetic with percent identity of 100%. With the same percent identity of 100, the phylogeny of the sections: *vadensis* (*A*. *vadensis*), *japonicus* (*A. japonicus*) and *carbonarius* (*A. carbonarius*) showed that all species were closely related to each other. Otherwise, the species related to section *ellipticus* (*A. ellipticus*) were rather monophylogenetic to each other with a divergence of about 0.2 and a percent identity of 99.8. Additionally, the species related to section *vadensis* (*A*. *vadensis*) were polyphylogenetic to species of section *japonicas* (*A. japonicas*) with a percent identity of 74.8 and divergence of about 12.0. Generally, the species related to the same section were monophylogenetic, while the species from different sections were found to be polyphylogenetic.

The Neighbour-joining phylogenetic tree was plotted at scale, with branch lengths in the same units as the evolutionary distances used to infer the phylogenetic tree (Figure, 7). These data were parallel to that recorded by a distance analysis based on the alignment of ITS rRNA gene sequences among *Aspergillus* species, which revealed a great similarity in the phylogenetic relationship between species within the same section were monophylogenetic but with other sections were polyphylogenetic levels recommended by USFDA.

Recognition of Aflatoxin by using TLC techniques:

The amounts of aflatoxins (AFG1, AFG2, AFB1, and AFB2) found in the gathered onion samples are shown in Table (6). Total aflatoxins (TA) were found in red, yellow, and white onions in concentrations of 8.41, 2.4, and 1.72 ppb, respectively. Compared to yellow and white onions, red onions had the highest concentration of total aflatoxins. Additionally, a fraction of each of the four kinds of aflatoxins was present in the red onion samples. However, only two forms of aflatoxins (AFB1 and AFB2) were present in the samples of white and yellow onions. In general, it is important to note that aflatoxins' individual and cumulative concentrations are still below the FDA's suggested action thresholds.

Table 4: Identified *Aspergillus* spp., their rRNA gene, Sequence length (bp) and Genbank accession number.

	A. carbonarius	***A. ellipticus***	***A. japonicas***	***A.niger***	***A.vadensis***
rRNA gene	ITS	ITS	ITS	ITS	ITS
Sequence length (bp)	605	596	594	576	555
GenBank Accession NO.	MZ375755	MZ375762	MZ375852	MZ375759	MZ375760

Fig. 6: PCR amplification of ITS regions from genomic DNA of Aspergillus spp. A 600 bp DNA fragment amplified using ITS1 and ITS4 primer pair, L= DNA marker (1 kb), P= Positive control, N= Negative control and Lanes 1-5 represent PCR products of A. carbonarius, A. ellipticus, A. japonicas, A. niger and A. vadensis, respectively.

Table 5: Matrix of genetic distances based on alignment of ITS rRNA gene sequences of the studied isolates with highl

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	
1		100.0	100.0	100.0	100.0	85.0	85.0	85.0	85.0	85.0	96.3	96.2	96.3	96.3	96.3	96.3	96.3	96.3	96.3	96.5	88.8	88.8	86.8	87.8	88.8	1
2	0.0		100.0	100.0	100.0	85.0	85.0	85.0	85.0	85.0	96.3	96.2	96.3	96.3	96.3	96.3	96.3	96.3	96.3	96.5	88.8	88.8	86.8	87.8	88.8	2
3	0.0	0.0		100.0	100.0	85.0	85.0	85.0	85.0	85.0	96.3	96.2	96.3	96.3	96.3	96.3	96.3	96.3	96.3	96.5	88.8	88.8	86.8	87.8	88.8	3
4	0.0	0.0	0.0		100.0	85.0	85.0	85.0	85.0	85.0	96.3	96.2	96.3	96.3	96.3	96.3	96.3	96.3	96.3	96.5	88.8	88.8	86.8	87.8	88.8	4
5	0.0	0.0	0.0	0.0		85.0	85.0	85.0	85.0	85.0	96.3	96.2	96.3	96.3	96.3	96.3	96.3	96.3	96.3	96.5	88.8	88.8	86.8	87.8	88.8	5
6	11.4	11.4	11.4	11.4	11.4		100.0	100.0	100.0	99.8	85.6	85.4	85.6	85.6	85.6	84.3	84.3	84.3	84.3	84.5	76.4	76.4	74.8	75.7	76.4	6
7	11.4	11.4	11.4	11.4	11.4	0.0		100.0	100.0	99.8	85.6	85.4	85.6	85.6	85.6	84.3	84.3	84.3	84.3	84.5	76.4	76.4	74.8	75.7	76.4	7
8	11.4	11.4	11.4	11.4	11.4	0.0	0.0		100.0	99.8	85.6	85.4	85.6	85.6	85.6	84.3	84.3	84.3	84.3	84.5	76.4	76.4	74.8	75.7	76.4	8
9	11.4	11.4	11.4	11.4	11.4	0.0	0.0	0.0		99.8	85.6	85.4	85.6	85.6	85.6	84.3	84.3	84.3	84.3	84.5	76.4	76.4	74.8	75.7	76.4	9
10	11.1	11.1	11.1	11.1	11.1	0.0	0.0	0.0	0.0		85.6	85.4	85.6	85.6	85.6	84.3	84.3	84.3	84.3	84.5	76.4	76.4	74.8	75.7	76.4	10
11	3.4	3.4	3.4	3.4	3.4	10.6	10.6	10.6	10.6	10.5		99.8	100.0	100.0	100.0	94.5	94.5	94.5	94.5	94.5	85.4	85.4	83.4	84.3	85.4	11
12	3.6	3.6	3.6	3.6	3.6	10.9	10.9	10.9	10.9	10.7	0.2		99.8	99.8	99.8	94.3	94.3	94.3	94.3	94.3	85.2	85.2	83.2	84.1	85.2	12
13	3.4	3.4	3.4	3.4	3.4	10.6	10.6	10.6	10.6	10.5	0.0	0.2		100.0	100.0	94.5	94.5	94.5	94.5	94.5	85.4	85.4	83.4	84.3	85.4	13
14	3.4	3.4	3.4	3.4	3.4	10.6	10.6	10.6	10.6	10.5	0.0	0.2	0.0		100.0	94.5	94.5	94.5	94.5	94.5	85.4	85.4	83.4	84.3	85.4	14
15	3.4	3.4	3.4	3.4	3.4	10.6	10.6	10.6	10.6	10.5	0.0	0.2	0.0	0.0		94.5	94.5	94.5	94.5	94.5	85.4	85.4	83.4	84.3	85.4	15
16	3.4	3.4	3.4	3.4	3.4	11.9	11.9	11.9	11.9	11.6	5.0	5.2	5.0	5.0	5.0		100.0	100.0	100.0	99.8	86.1	86.1	84.1	85.0	86.1	16
17	3.4	3.4	3.4	3.4	3.4	11.9	11.9	11.9	11.9	11.6	5.0	5.2	5.0	5.0	5.0	0.0		100.0	100.0	99.8	86.1	86.1	84.1	85.0	86.1	17
18	3.4	3.4	3.4	3.4	3.4	11.9	11.9	11.9	11.9	11.6	5.0	5.2	5.0	5.0	5.0	0.0	0.0		100.0	99.8	86.1	86.1	84.1	85.0	86.1	18
19	3.4	3.4	3.4	3.4	3.4	11.9	11.9	11.9	11.9	11.6	5.0	5.2	5.0	5.0	5.0	0.0	0.0	0.0		99.8	86.1	86.1	84.1	85.0	86.1	19
20	3.4	3.4	3.4	3.4	3.4	11.8	11.8	11.8	11.8	11.6	5.2	5.4	5.2	5.2	5.2	0.0	0.0	0.0	0.0		86.3	86.3	84.3	85.2	86.3	20
21	0.4	0.4	0.4	0.4	0.4	11.8	11.8	11.8	11.8	11.5	4.1	4.3	4.1	4.1	4.1	3.6	3.6	3.6	3.6	3.6		100.0	98.0	98.9	100.0	21
22	0.4	0.4	0.4	0.4	0.4	11.8	11.8	11.8	11.8	11.5	4.1	4.3	4.1	4.1	4.1	3.6	3.6	3.6	3.6	3.6	0.0		98.0	98.9	100.0	22
23	0.4	0.4	0.4	0.4	0.4	12.0	12.0	12.0	12.0	11.8	4.1	4.4	4.1	4.1	4.1	3.7	3.7	3.7	3.7	3.7	0.0	0.0		99.1	98.0	23
24	0.4	0.4	0.4	0.4	0.4	11.9	11.9	11.9	11.9	11.7	4.1	4.3	4.1	4.1	4.1	3.6	3.6	3.6	3.6	3.6	0.0	0.0	0.0		98.9	24
25	0.4	0.4	0.4	0.4	0.4	11.8	11.8	11.8	11.8	11.5	4.1	4.3	4.1	4.1	4.1	3.6	3.6	3.6	3.6	3.6	0.0	0.0	0.0	0.0		25
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	

MT628904 A. niger WA-TKA
MT588793 A. niger 8
MT550026 A. niger RMUAN75
MT530272 A. niger SF_996
MZ375759 A. niger Van Tieghem WJ-1
MT529130 A. japonicus EF_481
MK311337 A. japonicus hlb15
KC128815 A. japonicus VIT-SB1
MW031913 A. japonicus P3843
MZ375852 A. japonicus WJ-3
AY656631 A. ellipticus IMI 172283
AJ876881 A. ellipticus MUCL 31303
EF661194 A. ellipticus NRRL 5120
AY585548 A. ellipticus CBS 70779
MZ375762 A. ellipticus WJ-5
MG857649 A. carbonarius Q
JF838359 A. carbonarius MUM05.18
JF838358 A. carbonarius MUM03.59
AF459731 A. carbonarius NRRL 346
MZ375755 A. carbonarius WJ-2
MH862923 A. vadensis CBS 113365
MH613142 A. vadensis IHEM 26351
MZ227347 A. vadensis INF73
AY585549 A. vadensis CBS 113365
MZ375760 A. vadensis WJ-4

Table 6: Levels of Aflatoxins in the collected onion samples.

Aflatoxins	Red onion	Yellow onion	White onion
AFG1	0.61*± 0.21	-	-
AFG2	0.35± 0.24	-	-
AFB1	4.89± 0.60	1.19±0.1	1.46±0.32
AFB2	2.56± 0.23	1.21±0.09	0.26±0.02

***PPb: Parts per billion**

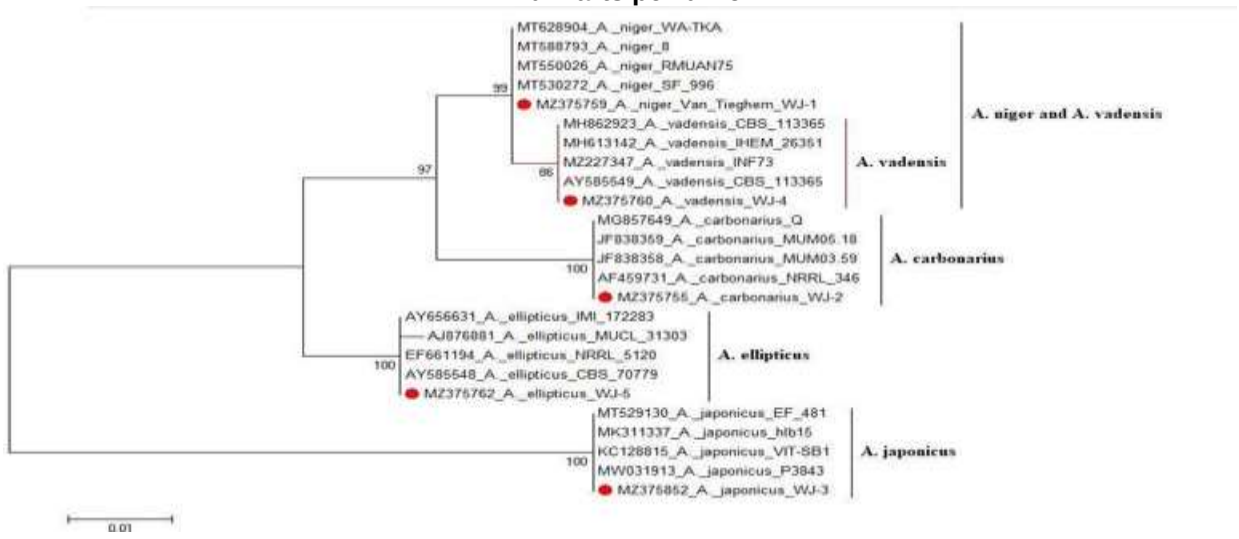

Figure 7: Phylogenetic analysis of ITS region derived from Neighbors- Joining method using MEGA 4.0 software. (Oval red shapes represent our isolates and remaining are reference sequences).

DISCUSSION

Black mold disease of onion is one of the most

common and very serious disease, which were observed in many markets of Sakaka city, Aljouf region, Saudi Arabia. Most fungi that cause black mold disease are members of the genus *Aspergillus* known as the *Aspergillus* black mold, which usually grows in warm climates with high humidity and temperature. In the present investigation, all fungal species associated with onion black mold disease from the Sakaka market were isolated and identified. Two hundred fungal isolates were gathered from 20 samples of three onion cultivars in the markets of Sakaka region. These isolates have been recognized into 4 genera. Red onion samples had the highest infection with *Aspergillus* section *Nigri* when compared with white and yellow samples. *A. niger* recorded dominant species, representing 62.5% of the total obtained isolates. In consistence with our results, *Aspergillus niger* was reported as the dominant species in onion tested.

Morphological discrimination of a great number of black Aspergilli revealed 15 distinguished taxa. Most of these species could be discriminated by combining colonial and micro- morphological features. Whilst, it is still hard to discriminate using morphology. These taxa can be discriminated by sequencing ITS. Consequently, it was essential to make precise descriptions of the various species of pathogenic fungi of onion. Especially, several species of the *Nigri* fungi are well-known producers of aflatoxins: fumonisin and/or ochratoxin. Results clarified that all compiled isolates were identified as *Aspergillus Nigri* species, which were confirmed by ITS gene sequences. Sequences were accepted in the GenBank database having accession No. *A. carbonarius* (MZ375755), *A. ellipticus* (MZ375762), *A. japonicas* (MZ375852), *A. niger* (MZ375759), and *A. vadensis* (MZ375760).

Accumulative total aflatoxins in the present study were 12.53 ppb in all collected onion samples. Samples did not exceed the maximum range Maximum allowed by USFDA which is 20 ppb. Aflatoxin G1, Aflatoxin G2, Aflatoxin B2 and Aflatoxin B1 were detected in the collected samples of red onion, while Aflatoxin B1 and Aflatoxin B2 were detected in both yellow onion and white onion. (Anwar et al. 2019)was presented similar results where aflatoxin contamination of onion had values lower than 20 ppb. (Arowora et al.2012) observed that some agricultural products in Baboko Market, Nigeria demonstrated aflatoxin concentrations higher than the 20 ppb recommended by USFDA. They concluded that the commodity traders and populace have to be sensitized to the risks of aflatoxin consumption.

Aspergilli section *Nigri* is generally determined worldwide and thus has the capability to develop under a large variety of environmental conditions, like temperature and humidity. The non- growth on plates of *A. ellipticus* above 30°C. In addition, *A. carbonarius* and *A japonicus* cannot grow above 35^{o}C, so the optimum temperature is at 30 ^{o}C. Bragulat et al. 2019 reported that the best temperatures for the growth of *Aspergillus carbonarius* were in the ranges of 25–35°C. However, *A. niger* and *A. vadensis* still grew until 45 ^{o}C, while the optimum temperature was 25 ^{o}C. Results of this study are agreeable to Al-Garni et al.2007who reported that *Aspergillus niger* was considered a thermophile and unable to grow at 10°C & 55°C. However, Nawar. 2008 observed that *Aspergillus niger* failed to grow at 15 °C, which is different from our results. Many researches were supported by our results; (Nawar et al. 2008) and Alwakee et al. 2008 reported that the optimal degree of temperature to growth of *Aspergillus niger* was 30 °C. Whereas, our results disagreed the results of Palacios-Cabrera et al. 2005 who determined the optimum temperature of *A. niger* is higher than 30 °C. The optimum temperature of *A. niger* was found to be 25 °C (Al-Garni et al. 2007).

To determine the phylogenetic position of *A. niger* isolates from Indonesia, molecular identification and genetic analysis of the isolates based on the ITS region were carried out. Identification of the isolates at the genus and species level was guaranteed by comparing them to ITS reference sequences obtained from NCBI (Nielsen et al. 2009). In the present study analysis, the complete gene sequences were used including the informative sites. Distance matrix constructed for the five studied Aspergillus species ITS rRNA genes sequences and twenty reference sequences from the NCBI database by the DNADIST topological algorithm.

The phylogenetic relationships between Aspergillus species were well supported by the data obtained by the Neighbor-joining phylogenetic tree, which did not adjust for distance based on the alignment of ITS rRNA gene sequences. These findings showed that species in one area that were monophylogenetic had a large deal of similarity in their phylogenetic relationships, but species in other sections were polyphylogenetic. These findings aligned with those of (Kjaerbølling et al. 2020) who investigated the evolutionary connections within the Flavi division using a lineage deriving from 200 genes. The tree's internal branching received strong support (100 of 100 bootstraps on the majority of branches).

CONCLUSIONS

The research paper focuses on onion black mold disease caused by Aspergillus species and their mycotoxin production potential, with a specific focus on the situation in Saudi Arabia. The study isolated 83 Aspergillus species from onions with black masses in Saudi Arabia and identified them using morphological and molecular characteristics. The study found the presence of aflatoxin in all collected samples, with the highest infection rate in red onions. The genetic analysis of the isolates showed distinct phylogenetic relationships among the Aspergillus species. The study also examined the detection of aflatoxin using thin layer

chromatographic analysis and found that the cumulative total aflatoxins in the collected onion samples were below the FDA's suggested action thresholds. The paper also discusses the importance of accurate identification and characterization of these fungi, the potential hazards of mycotoxins on human health, and the significance of the study in understanding and managing black mold disease in onions. The study provides crucial insights into the prevalence of Aspergillus species, their phylogenetic relationships, and mycotoxin contamination in onion samples, with implications for food safety and public health

Supplementary materials
Not applicable.

Author contributions
All authors have read and agreed to the published version of the manuscript.

Funding statement
This study was supported by the …….

Institutional Review Board Statement
Not applicable.

Informed Consent Statement
Not applicable.

Data Availability Statement
Data are available on request to the corresponding author.

Acknowledgments
Not applicable.

Conflict of interest
The authors declare that there are no conflicts of interest

REFERENCES

Abarca ML, Accensi F, Cano J, Cabanes FJ. Taxonomy and significance of black aspergilla.

Aimei Wang, Md. Nahidul Islam, Anders Johansen, Minna Haapalainen, Satu Latvala, Merete Edelenbos, Pathogenic *Fusarium oxysporum f.* sp. *cepae* growing inside onion bulbs emits volatile organic compounds that correlate with the extent of infection, Postharvest Biology and Technology, 2019, Volume 152, Pages 19-28, https://doi.org/10.1016/j.postharvbio.2019.02.010.

Al-Garni, S.M., S. Kabli, F. Al-Shehrei and Z. AlGanawi, Mycoflora associated with some textiles in Jeddah City. JKAU, 2007. 19: 93-113.

Alwakeel, S.S., Indoor fungal and bacterial contaminations on household environment in riyadh, Saudi Arabia. Saudi J. Biol. Sci., 2008. 15: 113-119.

Antonie van Leeuwenhoek. 2004; 86: 33-49.

Anwar IAG, Sabahelkhier MK and Babiker EE, Detection of Aflatoxins in Foodstuffs (milk, egg, banana and onion) from Three Locations (Khartoum, Omdurman and Bahri) in Khartoum State, Sudan, International Journal of Biochemistry & Physiology, 2019 Volume 4 Issue 4.

Arowora. K.A., Abiodun, A.A. , Adetunji, C.O. , Sanu, F.T. , Afolayan, S.S, & Ogundele, B.A, Levels of Aflatoxins in Some Agricultural Commodities Sold at Baboko Market in Ilorin, Nigeria, Global Journal of Science Frontier Research, 2012, vol 12 Issue 10.

Bragulat, Maria & Abarca, M Lourdes & Castellá, Gemma & Cabañes, F.. Intraspecific variability of growth and ochratoxin A production by Aspergillus carbonarius from different foods and geographical areas. International Journal of Food Microbiology. (2019). 306. 108273. 10.1016/j.ijfoodmicro.2019.108273.

George M. and Ramteke P. W. Morphology, molecular identification and phylogenetic analysis based on internal transcribed spacer (ITS) of the ribosomal nuclear DNA (rDNA) sequence of a pathogenic fungal isolate Aspergillus niger LKO1. J. Soci. Trop. Plant Res., (2019), 6(2): 166–170.

Khanal, M., Bhatta, B. P., & Malla, S. Isolation and Characterization of Bacteria Associated with Onion and First Report of Onion Diseases Caused by Five Bacterial Pathogens in Texas, U.S.A. Plant

disease, (2023). 107(6), 1721–1729. https://doi.org/10.1094/PDIS-09-22-2206-SR

Kjærbølling I.; Vesth T.; Frisvad J. C. et al. comparative genomics study of 23 Aspergillus species from section Flavi. NATURE COMMUNICATIONS, (2020), 11:1106.

Mkumbe B. S.; Pangastuti S. A. and Susilowati A. Phylogenetic Analysis Based on Internal Transcribed Spacer Region (ITS1-5.8S-ITS2) of *Aspergillus niger* Producing Phytase from Indonesia. Int. Conf. Sci. Appl. Sci. (ICSAS) (2018).

Moustafa, S. Possibility of reducing presence of harmful fungi in air-conditioner windows using a transcendental anti-fungal chemical. Egypt. Acad. J. Biol. Sci. G. Microbiol. 2019, 11, 59–70. https://doi.org/10.21608/eajbsg.2019.69072

Nawar, S.L. Prevention and control of fungi contaminated stored pistachio nuts imported to Saudi Arabia. Saudi J. Biol. Sci., 2008, 15: 105-112.

Nielsen, K.F.; Mogensen, J.M.; Johansen, M.; Larsen, T.O.; Frisvad, J.C. Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. Annal. Bioanal. Chem. 2009, 395, 1225–1242.

Palacios-Cabrera, H., M.H. Taniwaki, J.M. Hashimoto and H.C. De Menezes, Growth of *Aspergillus ochraceus*, *A. carbonarius* and *A. niger* on culture media at different water activities and temperatures. Braz. J. Microbiol., 2005. 36: 1-15.

Qi, T. F., Renaud, J. B., McDowell, T., Seifert, K. A., Yeung, K. K., & Sumarah, M. W. Diversity of Mycotoxin-Producing Black Aspergilli in Canadian Vineyards. Journal of agricultural and food chemistry, (2016). 64(7), 1583–1589. https://doi.org/10.1021/acs.jafc.5b05584.

Samson RA, Houbraken J, Kuijpers A, Frank JM, Frisvad JC. New ochratoxin or sclerotium producing species in *Aspergillus* section *Nigri*. Stud Mycol. 2004; 50: 45-61.

Samson, R.A.; Visagie, C.M.; Houbraken, J.; Hong, S.B.; Hubka, V.; Klaassen, C.H.W.; Perrone, G.; Seifert, K.A.; Susca, A.; Tanney, J.B.; et al. Phylogeny, identification and nomenclature of the genus Aspergillus. Stud. Mycol. 2014, 78, 141–173.

Samuel J. Bunu, Benjamin U. Ebeshi, Deghinmotei Alred-Ugbenbo, Edebi N. Vaikosen, Kashimawo J. Adesegun, Akpevwe C. Kogoro, Ogechukwu L.Chukwuemerie "Quantification of Secondary Metabolites and Chromatographic Analysisof *Allium Cepa*, Liliaceae Ethanolic Extract". IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS), (2023), 18(2), pp. 16-22.

Schuster, E.; Dunn-Coleman, N.; Frisvad, J.C.; Van Dijck, P.W. On the safety of *Aspergillus niger*—A review. Appl. Microbiol. Biotechnol. 2002, 59, 426–435.

Seyedmojtaba, S. Jacques, G. Pascal, A. G. Sybren de Hoog, Johan W. Mouton, Willem J. G. Melchers, Paul E. Verweij, *Aspergillus* and aspergilloses in wild and domestic animals: a global health concern with parallels to human disease, *Medical Mycology*, 2015. Vol. 53(8), 765–797.

Shaima M.N. Moustafa, Rania H. Taha, Usage UV Irradiation for Reducing Fungal Contamination of Loose Nuts in Al Jouf Markets, Egyptian Academic Journal of Biological Sciences, G. Microbiology, Article 3, Volume 12, Issue 1, June 2020, Page 19-27, https://doi.org/10.21608/eajbsg.2020.78190

Siddiquee S.; Abdullah F.; Guan T. S. and Aziz E. R . Phylogenetic Relationships of Trichoderma harzianum Based on the Sequence Analysis of the Internal Transcribed Spacer Region -1 of the rDNA. J. Appl. Sci. Res., (2007), 3(9): 896-903.

Silva, D. M., Batista, L. R., Rezende, E. F., Fungaro, M. H., Sartori, D., & Alves, E. Identification of fungi of the genus *Aspergillus* section nigri using polyphasic taxonomy. Brazilian journal of microbiology : [publication of the Brazilian Society for Microbiology], (2011). 42(2), 761–773. https://doi.org/10.1590/S1517-838220110002000044.

Varga, J., Frisvad, J. C., Kocsubé, S., Brankovics, B., Tóth, B., Szigeti, G., et al. New and revisited species in Aspergillus section Nigri. Stud. Mycol. (2011). 69, 1–17. https://doi.org/10.3114/sim.2011.69.01.