

Isolation and Morphological Characterization of *Aspergillus flavus* and *Fusarium moniliforme* in Groundnut: Implications for Aflatoxin Contamination in Pakistan

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ABSTRACT

Groundnut (*Arachis hypogea* L), also known as peanut, is a significant oilseed crop in the world that has some important nutrients such as carbohydrates, fats, proteins, vitamins, and minerals. Nonetheless, post-harvesting in tropical areas suffers a massive setback due to the presence of mycotoxins, mainly aflatoxins, which are toxic and are produced by fungi, including *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp. Such toxins are dangerous to the health of human beings and animals, and have significant market access consequences. This paper examines the occurrence of mycotoxins in groundnut crops in two areas of Pakistan: Koont farm, Chakwal, and Attock, during the 2018-2019 growing season. The study aimed to

isolate, identify, and characterize fungal pathogens associated with groundnut seeds. Four groundnut varieties (BARI-2000, BARI-2011, BARI-2016 and Mirza Sanjwal Bora 334) were used in collecting 400 samples of seed (40 samples each) randomly and systematically. The Moisture Content was also determined to have a considerable variation in different varieties. BARI-2016 had only 21 per cent Moisture Content, but after one-month air-drying, it had dropped to 10 per cent in Mirza Sanjwal Bora 334. Fungal growth was identified on BARI-2000, BARI-2011, and BARI-2016 through a seed-borne mycoflora study. The fungi found to enter are *Aspergillus flavus* and *Fusarium moniliforme*, which are the primary contaminants. Silica gel was used in the purification and preservation of fungal isolates, and morphologic analysis was done using lactophenol blue stains on cotton. The findings showed fungal contamination in the BARI-2000 and BARI-2011 varieties to be more than that of the other two varieties. This paper highlights the need for improved practices in handling and storing crops after harvest to reduce the risk of aflatoxin contamination in groundnut crops, particularly in areas prone to fungal infestation.

Keywords: Aflatoxins analysis, *Fusarium* isolation, Peanut microbiology, Mycotoxins effects, Food Contamination

Introduction

Groundnut (*Arachis hypogea* L), which is also known as earthnut, goobers, peanut and monkey-nut is a yearly pulse. Groundnut is significant oilseed crop worldwide (Dwivedi, Crouch, Nigam, Ferguson, & Paterson, 2003). It is the 13th most nutritious crop and recorded as 4th significant oilseed crop (Reddy, Sudhakar, & Reddy, 2011), containing 10-20% carbohydrate, 40-50% fat and 20-50% protein and are rich in vitamin E, magnesium, zinc, iron, potassium, niacin, riboflavin, thiamine, folic acid, calcium and phosphorus. (Welsh et al., 2010). Its Cultivation is limited to warm temperate (zones), sub-tropical and tropical countries among 40°S and 40°N latitude. Worldwide it is presently cultivated, with whole produce of 35.9 million metric tons and on 25.2 mha, with emerging states as Asia and Africa 66%, 25% respectively (Carvalho, 2006). Crops of tropical areas frequently become polluted with aflatoxins (Shepherd, 2008). Other mycotoxins are neurotoxins which produce effects extending from necrosis to dangerous immunodeficiency or skin sensitivity and interfere with protein synthesis. *Fusarium* species are damaging plant pathogens

produce mycotoxins earlier, or nearly at post-harvest stages (Sweeney & Dobson, 1998). In emerging countries more than five billion people estimated are at risk of long-lasting exposure to aflatoxins through contaminated foods (Strosnider et al., 2006).

Penicillium spp., *Fusarium* spp. and *Aspergillus* spp. are the major species of fungus that produce mycotoxins in food commodities. Aflatoxins are acute toxic compounds and have shown to be immunosuppressive mutagen, teratogen and carcinogenic. Main mycotoxins that are recorded normally in relation to peanuts are aflatoxins. Aflatoxin trash of peanut keep away peanut growers from opening larger western markets, restricts financial occasions, increases reliance on external food assistance and badly disturbs user health. Aflatoxin, fungal growth in peanut and other products remains a challenging task in emerging countries where diet handling out and agriculture schemes are not correctly planned to handle food safety threats. This problem is increased because of sub-optimal post-harvest conditions i.e., processing, storage and handling (Wu & Khlangwiset, 2010). Deficiency of water and increased soil temperature favor aflatoxin impurity of peanut while the crop is ripening in the field and throughout marketing (Buzby & Unneveh, 2003). Related fungi and aflatoxin contamination in peanut have attracted international concerns and have been reported from various countries. Occurrences of critical aflatoxicosis from contaminated peanut in humans have been identified in India, Kenya, Thailand and Malaysia (Science, 2003). One of the early chiefs known infection of aflatoxins in 1 human being occurred in 150 villages of western India in 1974 in which 108 peoples died and 397 peoples were affected (Krishnamachari, Nagarajan, Bhat, & Tilak, 1975).

Among the eighteen dissimilar types of aflatoxins, which are known to exist, the dominant associates are aflatoxin M1, M2, G1, G2, B1 and B2, which are formed by *A. flavus* and *A. parasiticus*. *Aspergillus flavus* strains may vary between non-toxic and highly toxigenic, with a balanced chance of producing AFB1 over AFG1. Most strains of *Aspergillus parasiticus* show considerably lower variation in terms of toxigenicity and tend to produce AFB1 and variable levels of AFB2, AFG1 and AFG2 (Dors et al., 2011). Due to the extreme fear of fitness associated with the digestion of mycotoxin-contaminated diets, research worldwide aims to prove the

occurrence of aflatoxins. Many of the significant products that are prone to the fungal growth then to the toxins, include corn (popcorn, hominy and grains), dried fruits, spices, cotton seed, cashews, almonds, nut, cocoa, wheat, rice, groundnuts (uncooked, cooked and sweetened) among others which are primarily aligned as diets alignment as well as feedstuffs alignment. As such, animals are also exposed to the infection caused by aflatoxin, and when they are consumed in the form of milk and meat, infections could also occur in human beings (Kwiatkowski & de Faria Alves, 2007).

Materials and Methods

The current study involves the isolation, characterization, and morphological identification of pathogens that produce toxins in groundnuts. This experiment was carried out in the Fungal Plant Pathology Laboratory in the Department of Plant Pathology in PMAS-Arid Agriculture University, Rawalpindi, Pakistan. The information on the material utilized and the method adopted in the process of the study is presented below.

Survey and Collection of Samples

The survey of field was done from two locations (Koont farm Chakwal and Attock). The sample of groundnut varieties was collected by simple random sampling and systematic sampling. Two varieties (BARI-2000&BARI-2011) were collected from Koont farm and samples were placed in the paper bags and each bag was labeled with the variety name, date of sampling and location. After few days another survey was conducted from Attock two varieties of groundnut (Mirza sanjwal bora 334 & BARI-2016) were collected. From all two locations, 400 seeds of each variety were collected as population. After the collection of samples, immediately they were brought into the molecular mycology laboratory of Plant Pathology Department, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi for further processing.

Moisture Content Analysis

On a wet basis, the initial moisture content of groundnut seeds was 30-50% at the time of digging. Then Mirza sanjwal bora 334 variety was one-month air-dried and stored. BARI-2016 was stored without air drying. Moisture contents and seed mycoflora was assessed from seeds of each variety thrice; just after the seeds were brought in the Laboratory. Then the moisture percentage was calculated in all

available variety with the following formula.

$$\text{Moisture Content} = \frac{\text{Wet Weight} - \text{Dry Weight}}{\text{Dry Weight}} \times 100$$

Cleaning and Sterilization of Glassware

Sterilization of laboratory equipment for the research which was Petri dishes, forceps, needles, scissors, and beakers were washed with soap, and then with distilled water and then allowed to dry. All the glassware's were sterilized in an autoclave at 121°C at 15 psi (pounds per square inch) for 15 minutes.

Detection of Seed Borne Mycoflora

The sample of peanut seeds was studied regarding their correlation of seed Mycoflora through the use of a conventional blotting surface paper technique. The above-mentioned ready mixtures were tested on a random population of 400 seeds, and the samples were worked. The surface of the gathered samples was sterilized using 1% aqueous solution of Clorox after approximately 2-3 minutes. These seeds were washed with sterilized distilled water and then dried completely under two folds of sterilized filter paper to eliminate any remaining traces of chemicals. Each of the moisture-dried blotter papers was plated with a rate of 5 seeds per plate. It had grown the seedlings in a Petri dish at 25 +/- 2 °C for eight days. The diseased specimens were identified and recognized by Petri plates. This was further confirmed by placing the slides and saw under a magnifier with both low and high magnifications. Morphological characteristics of the seeds were used to study the seeds under a stereoscope. It was assumed that the incidence of disease is:

$$\text{Incidence \%} = \frac{\text{No. of Infected Seeds}}{\text{Total No. of Seeds}} \times 100$$

Isolation and Purification of Associated Fungi

Inoculation of the pathogen on available Samples was done on potato dextrose agar (PDA) and Czapek-Dox agar (CDA). The pure culture was acquired by picking the hyphal tip growing with the use of a sterilized needle, after which it was transferred to other Petri plates that were poured with PDA and Czapek media. Petri plates

were incubated at 25 °C for 57 days.

The purification of the pathogen was done in PDA and Czapek media. It can be isolated and identified as a fungal organism by the preparation of slides using a single spore.

Preservation in Silica Gel

Fungal isolates were preserved using silica gel. The Eppendorf tubes silica gel and 5% Skimmed milk solution was prepared and sterilized. After sterilization 2ml of skimmed milk was added to fully grown fresh culture plates, spores and hyphae were scrubbed with the help of a sterilized surgical blade. Skimmed milk mixed with fungal growth was added to Eppendorf tubes containing silica gel until the appearance of fungal growth.

Morphological Characterization of Associated Fungi

For the preparation of the slide, a drop of lactophenol cotton blue strain (LCBS) was placed at the center of the slide. Section of 2-3 mm fungal hyphae was picked with the help of a needle and place on the slide. Then coverslip was placed and examined under Nikon 40 X microscope for morphological characteristics.

Results and Discussion

Collection of Samples

In the current study, two fields surveyed during 2018-19 in Koont farm Chakwal and Attock fields to catch on prevailing groundnut pathogens. In Koont farm two groundnut varieties BARI-2000 and BARI-2011 and Attock BARI-2016, Mirza sanjwal bora 334 were cultivated. From all two locations, 400 seeds of each variety were collected as population. After the collection of samples, immediately the samples were taken to the Plant Pathology Department in molecular mycology laboratory, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi for further processing.

Analysis of Moisture Content

On a wet basis, the initial moisture content of groundnut seeds was 30-50% at the time of digging. Then Mirza sanjwal bora 334 variety was one-month air-dried and moisture content was reduced to 10%. BARI-2016 was stored without air drying and moisture content was reduced to 21% (Table 3.1). Moisture contents and seed mycoflora was assessed from seeds of each variety thrice; just after the seeds were

brought in the Laboratory. Then moisture percentage was calculated in all available variety thought the formula (Krzyzanowski, West, Neto, & de Barros, 2006).

Table 3.1: Analysis of Moisture Content from Collected Samples

Variety	Initial Moisture content	Sun Dry	Air Stored	Dry Moisture Content %
Mirza sanjwal Bora	30-50%	One month		10%
BARI 2016	30-50%		One month	21%

Seed Borne Mycoflora Detection

After few days fungal growth appeared on all the plates. In BARI- 2000 three seeds out of five were infected in each plate, also in BARI-2016 show two seeds out five and BARI-2011 contaminated seeds were three out of five seeds in each plate. Then a single spore was picked and slides were made to confirm the presence of *Fusarium spp.* and *Aspergillus spp.* under microscope. But one variety Mirza sanjwal bora 334 was only one seed show fungal growth. Disease Incidence is shown in the in (figure 3.1 & 3.2).



Figure 3.1: Disease Incidence

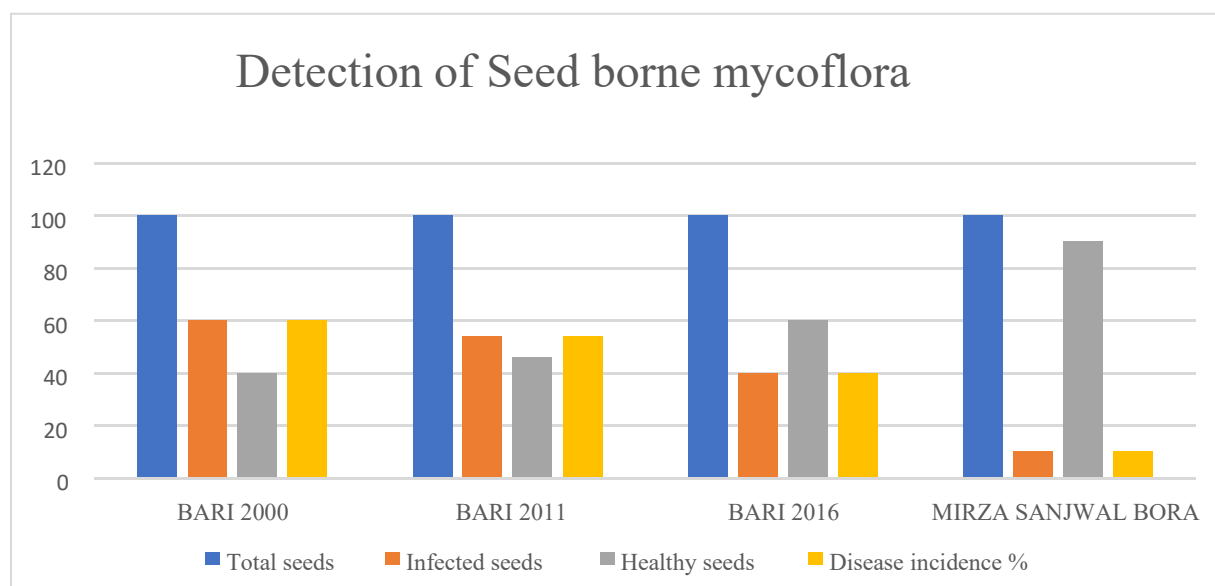


Figure 3.2: Graphical Representation of Disease Incidence % from Seeds

Isolation of Associated Fungi

Aspergillus flavus and *Fusarium spp.* were isolated from the cultural studies of media. Pure cultures were obtained through single spore/ hyphal tip method as

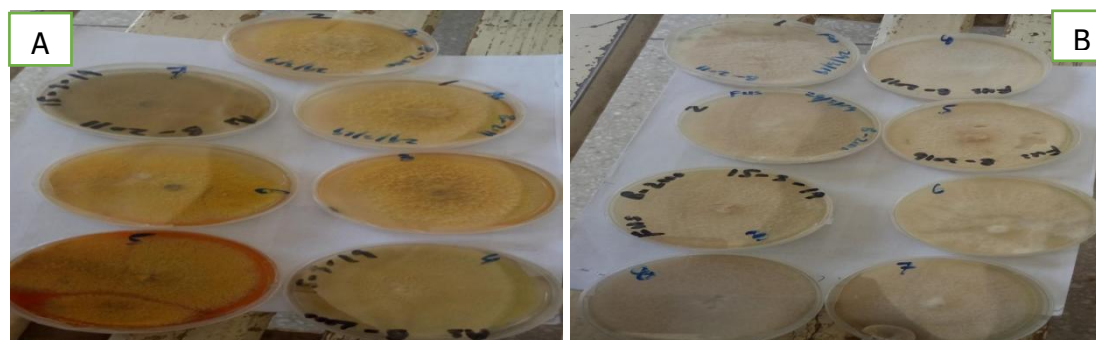


Figure 3.3: (A) Isolates of *Fusarium* spp. (B) Isolates of *Aspergillus* spp.

Morphological Characterization of *Aspergillus Flavus*

On standard media such as Czapek media and PDA morphological characterization of the *A. flavus* group were done.

Macroscopic Characteristics

Ten isolates were identified with *Aspergillus flavus*. Colonies on Czapek agar after seven days at 25°C. Different strains showed identical colony color on CDA, yellow to green or dark green, the inverse of the plate was orange (Rodrigues et al., 2007) (Figure 3.4).

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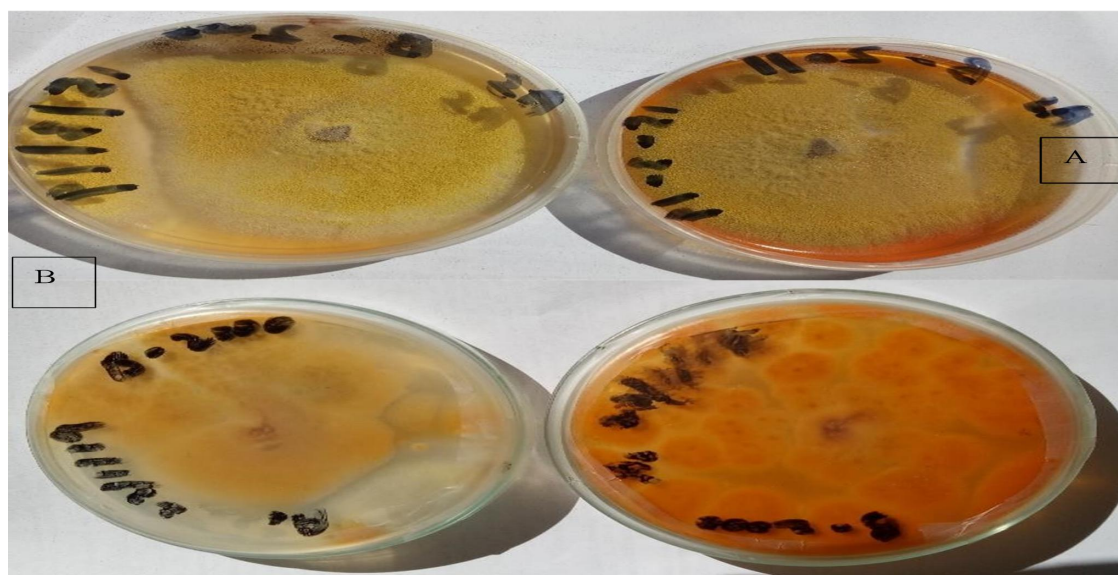


Figure 3.4: Showing cultural characteristics *Aspergillus spp.*(A) colony color (B) reverse of colony color

Microscopic Characteristics

Morphological characters of *Aspergillus flavus* in which Conidial heads typically radiate, 250–350µm in diameter. Conidiophore colorless, coarsely roughed, less than 1mm long by 8–12µm wide with a 1–2µm thick wall. Vesicle sub globose, 40–45µm in diameter. Sterigmata biseriata. Mutula 5–8µm long. Phialides ampulliform, 6–10µm long by 3–4µm wide. Conidia globose or sub globose, 3–4.5µm in diameter (conspicuously echinulate).

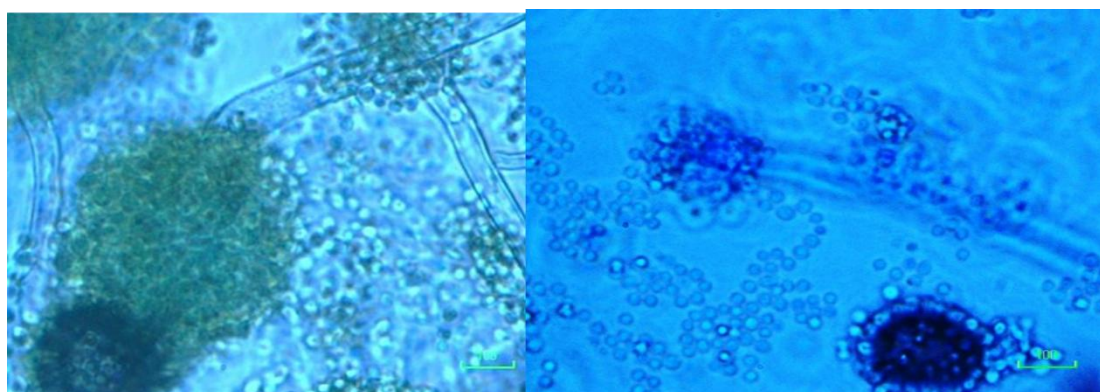


Figure 3.5: Cluster of spores and vesicle with Nikon Eclipse 2000

Conidial Head

The characteristics conidial head such as color, shape, and size are significant main investigative measures of the *Aspergillus flavus*. Conidial heads shape of differs from

globose and columnar. The phialides arrangement on the vesicle shows the shape of the conidial head. The conidial head size is determined by the length of the conidial chains and size of the vesicle. The conidial head color was determined by conidia color. *A. flavus* color was olive green or light to deep yellow-green.

Conidiophore

The conidiophore arises vertically from the foot cell which was a thick-walled branch. Conidiophores were composed of three parts: the foot cell, stipe, and vesicle. Conidiophores are frequently non-septate. *Aspergillus flavus* conidiophores were rough and hyaline.

Vesicle

The vesicle was swollen tip of the conidiophore. The shape was hemispherical, globose, clavate (club-shaped) or elliptical. Vesicles in the young *Aspergillus flavus* was elongated as the culture ages developed globose. The vesicle diameter differed from 10 to 65 μm (Afzal, Shazad, Qamar, & Nisa, 2013).

Conidia

Asexual reproductive structures were conidia also called spores. *Aspergillus* spp. conidia uni or multinucleate and one-celled structures. *A. flavus* conidia colors and shapes were roughened or smooth.

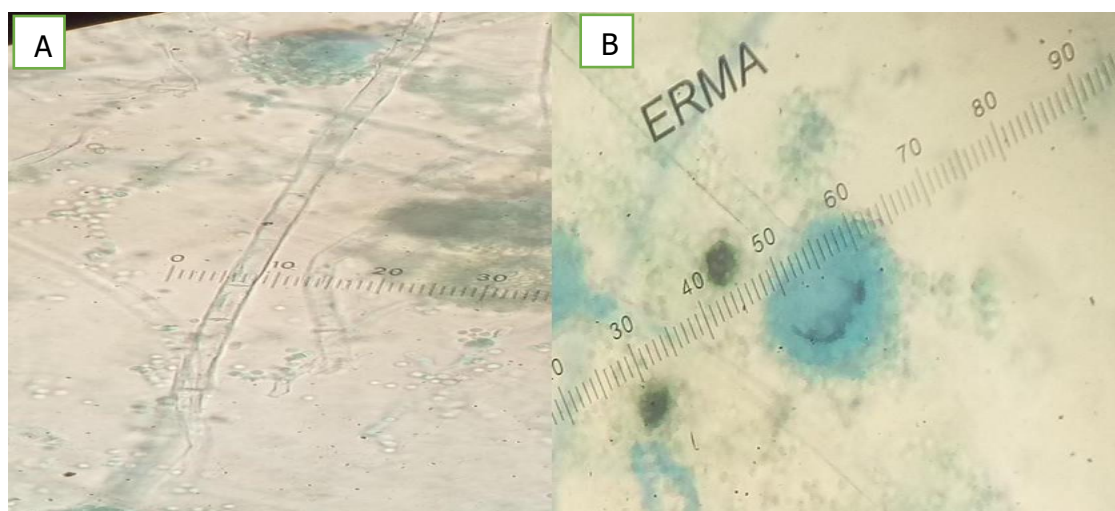


Figure 3.6: Hyphae with septation (A) Vesicle size $45\mu\text{m}$ (B)

Table 3.2: Morphological Characteristics of *Aspergillus Flavus*

Isolates	Colony color	Colony texture	Conidiophore	Conidia (Size μ m)	Shape	Vesical Size (μ m)	color	Hyphae
ASPBC2	Yellowish green	velvety to woolly	colorless, coarsely roughened	4.5 $\pm$ 1.2	Globose	40.2 $\pm$ 3.4	Yellow	Septate and hyaline
ASPBC2	Olive green	velvety to woolly	colorless, coarsely roughened	4.02 $\pm$ 1.6	Globose	44.7 $\pm$ 2.3	Yellow	Septate and hyaline
ASPBC2	Greenish yellow	Loccose,	colorless, coarsely roughened	4.4 $\pm$ 1.7	Globose	45.2 $\pm$ 1.9	Yellow	Septate and hyaline
ASPBC2	yellow to dark green	velvety to woolly	colorless, coarsely roughened	4.5 $\pm$ 1.8	Globose	45.2 $\pm$ 2.1	Yellow	Septate and hyaline
ASPBC11	yellow to dark green	velvety to woolly	colorless, coarsely roughened	4.3 $\pm$ 1.7	sub Globose	43.7 $\pm$ 4.8	Yellow	Septate and hyaline
ASPBC11	yellow to dark green	velvety to woolly	colorless, coarsely roughened	4.2 $\pm$ 1.9	Globose	44.2 $\pm$ 2.8	Yellow	Septate and hyaline
ASPBC11	yellow to dark green	velvety to woolly	colorless, coarsely roughened	4.4 $\pm$ 1.8	Globose	43.4 $\pm$ 3.8	Yellow	Septate and hyaline
ASPBA16	Yellowish green	velvety to woolly	colorless, coarsely roughened	4.3 $\pm$ 1.8	Globose	45.2 $\pm$ 2.2	Yellow	Septate and hyaline
ASPBA334	Yellowish green	velvety to woolly	colorless, coarsely roughened	4.5 $\pm$ 1.9	Globose	42.7 $\pm$ 3.8	Yellow	Septate and hyaline

Sterigmata

The fertile area of the vesicle grows particular conidiogenous cells defined as Sterigmata. The metulae was primary layer sterigmata and the phialides were the secondary layer. The phialides have risen from the metulae in biserial species while from the vesicle phialides were formed right in uniseriate spp. Conidia cells were produced directly on the phialides. Usually, phialides were non-septate. Mostly *Aspergillus flavus* species are biserial certain species are uniseriate

Morphological Characterization of *Fusarium Moniliforme*

Morphology identification of all *Fusarium moniliforme* was done based on the *Fusarium Laboratory Manuals*. The 10 *Fusarium* isolates obtained in this study, based on their morphological characters, belong to those identified in these manuals. Tentatively, it was possible to classify 10 isolates in a single species of *Fusarium* of

close morphology, viz., *Fusarium moniliforme*. The isolates were identified morphologically and did not exhibit significant differences amongst them based on the parameters such as colony color, pigmentation, days to fill 9 cm dish, growth habit, micro-conidia size, micro-conidia shape, macro-conidia size, macro-conidia shape, phialide, occurrence of chlamydospores, septation in macro-conidia, diameter and inter-septal distance (Rafique, Rauf, Naz, & Shabbir, 2015) (Table 3.2).

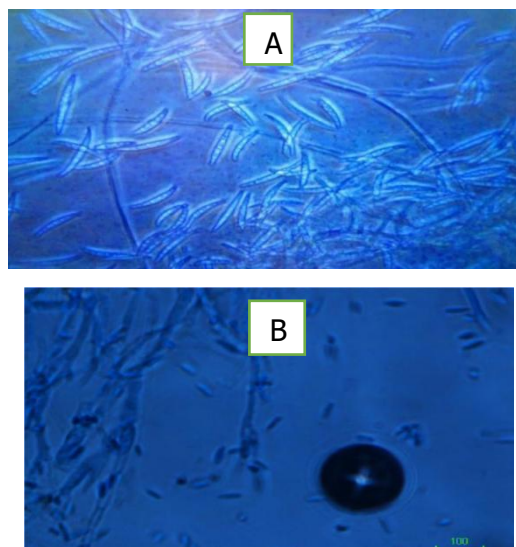


Figure 3.7: Macrospores of *Fusarium spp.* with septation (A), Microspores of *Fusarium spp.* (B)

Colony Color

In addition to the media, the aerial mycelium was also found in the same colors. Encoding the same as colony color determined the different isolates. All the isolates of *Fusarium* were white. This observation in this parameter made the identification of the cultures to be isolates of *Fusarium moniliforme*. Colony color is an auxiliary character when it comes to identifying a fungal species, and hence, it was not easy to identify the species using the colony color.

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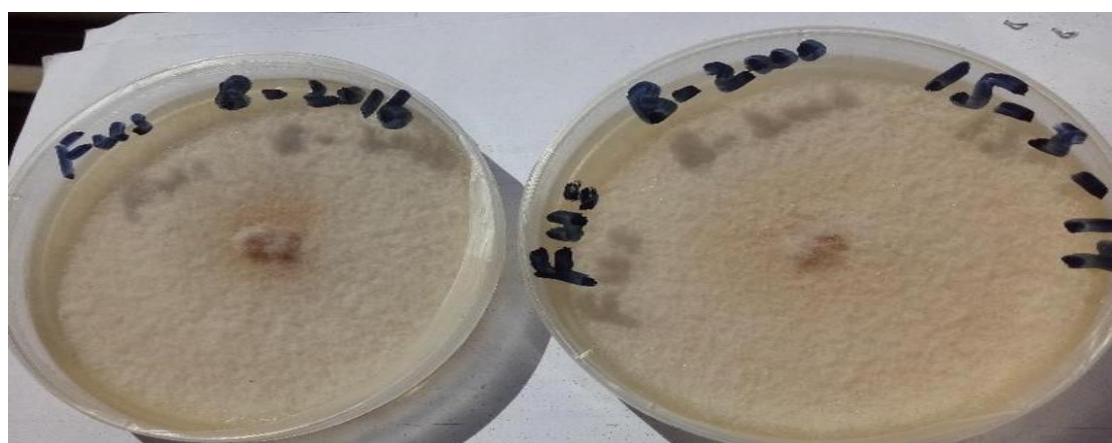


Figure 3.8: *Fusarium moniliforme* colony color and mycelial growth

Growth Habit

Growth habits of *Fusarium moniliforme* are mercifully conducted, whereby it has a mycelium at a point above the medium where the growth processes occur. The same was done in the aerial mycelium of isolates in question (Figure 3.8). The isolates showed the same fluffy mycelial growth patterns. About ten isolates viz. FBA334, FBA16, FBA16, FBA16, FBC11, FBC11, FBC11, FBC2, FBC2 and FBC2 showed fluffy growth habit.

Pigmentation

Other factors that are of secondary consideration in the identification of the species of *Fusarium* are the pigments. The isolates were observed to detect any pigmentation on plates of Czapek media after 15-20 days of incubation at 25°C. The majority of the isolates also showed no significant pigmentation.

Colony Diameter

The days required to fill 9 cm Petri plate for each isolate on Czapek and PDA media varied from 7-11 days. All the isolates were discovered to be the fastest growing on Czapek medium and required a minimum of 7 days to cover the plate. Conversely, the isolates have consumed the plates in 11 days or less, indicating they are all slow-growing. The statistics on this parameter were used in the determination of the fast and slow-growing species of *Fusarium moniliforme*.

Shape and Size of Micro-Conidia

Micro-conidia formed by almost all isolates were abundant after 7 days at 25°C+2 °C in pure cultures. They were also present in abundance at the aerial

mycelium in false heads on phialides, forming chains. The morphology of the whole micro-conidia cell is also a characteristic feature, and this parameter further assisted in the differentiation of the isolates into species. In this paper, the oval-shaped micro-conidia were seen predominantly, but globose, pyriform, and obovoid-shaped conidia were also seen. The length and width of the microconidia were measured in a light microscope at 40x magnification in 10 random determinations using an ocular micrometer (Figure). The size of micro-conidia also showed minor differences in the mean of the overall average of micro-conidia, which was 4.8 ± 0.46 in length, 2.52 ± 0.20 in width.

Shape and Size of Macro-Conidia

The macro-conidia were observed in shapes that were long and slender (Figure 3.7). Ten isolates of the fungus, viz. FBA334, FBA16, FBA16, FBA16, FBC11, FBC11, FBC11, FBC2, FBC2 and FBC2 produced the macro-conidia. Measurement of the size of macro-conidia was also done randomly 10 times by using an ocular micrometer under a light microscope at 40X magnification. The average size of macroconidia was 18.39 ± 2.35 in size.

Table 3.3: Fusarium Moniliforme Morphological Characteristics

Isolates	CC	GH	D	Macroconidia		Microconidia	
				Size μm	Shape	Size μm	Shape
FBA334	Creamy white	Fluffy	9	18.1 ± 0.97	long and slender	3.6 ± 0.7	Oval
FBA16	Creamy white	Fluffy	9	18.7 ± 2.7	long and slender	4.5 ± 1.7	Club
FBA16	white	Fluffy	9	19 ± 3.1	long and slender	4.6 ± 1.6	Oval
FBC11	white	Fluffy	9	18.7 ± 3.3	slightly straight	4.7 ± 0.9	Oval
FBC11	Creamy white	Fluffy	9	18.7 ± 2.7	slightly straight	4.8 ± 1.9	Oval
FBC11	Creamy white	Fluffy	9	18.5 ± 2.9	long and slender	4.9 ± 1.9	Club
FBC2	Creamy white	Fluffy	9	17.5 ± 2.9	long and slender	4.7 ± 0.9	Club
FBC2	Creamy white	Fluffy	9	18.2 ± 2.8	long and slender	4.5 ± 1.8	Club
FBC2	Creamy white	Fluffy	9	17.7 ± 2.9	long and slender	4.6 ± 1.7	Oval

Conclusion

Groundnut (*Arachis hypogea* L) also known as earthnut, goobers, peanut and

monkey-nut is a yearly pulse. Groundnut is significantly known oilseed crop worldwide. Groundnut is cultivated in six landmasses and more than 100 states. Peanut seeds contain 10-20% carbohydrate, 40-50% fat and 20-50% protein and are rich in vitamin E, magnesium, zinc, iron, potassium, niacin, riboflavin, thiamine, folic acid, calcium and phosphorus. *Penicillium* spp. *Fusarium* spp. and *Aspergillus* spp. are the major species of fungus that produce mycotoxins in food items. While there are numerous other mycotoxins produced by fungi, the five major classes are deoxynivalenol, ochratoxin A, fumonisins, zearalenone, and aflatoxins. In Africa, these toxins arise certainly in nutrients from different region. Symptomatic survey and sampling were done in September-October 2018 from Koont farm and BARI-2000, BARI-2011 was collected then from Attock fields BARI-2016 and Mirza sanjwal bora 334 infected and healthy seeds were collected. Each variety 400 seeds were collected as a population. Moisture content analysis was done by Mirza sanjwal bora 334 variety was one-month air-dried and moisture content was reduced to 10%. BARI-2016 was stored without air drying and moisture content was reduced to 21%. In order to detect seed-borne mycoflora, the sample was surface sterilized with a 1 per cent solution of Clorox for 2-3 minutes. These seeds were sterilized by washing in sterilized distilled water and then allowed to dry thoroughly on two folds of sterilized filter paper to remove any chemical traces. They were placed in petri plates containing sterilized blotter paper in the fungal plant pathology laboratory. After isolation 20 isolates were recovered. These fungal isolates showed similar cultural and morphological characteristics to *Aspergillus flavus* and *Fusarium moniliforme*. These isolates were preserved. Based on cultural characteristics and morphological characterization the *Aspergillus flavus* and *Fusarium moniliforme* were confirmed. The highest incidence was recorded at Koont Farm Chakwal varieties BARI-2000 and BARI-2011 followed by Attock varieties BARI -2016 and Mirza sanjwal bora 334.

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