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RESEARCH ARTICLE

Isolation and Identification of Deleterious Fungi Associated with Stored Grains and Cattle Feedstuff of Potohar Region of Pakistan

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The growth of fungus in grains and feed is favored by improper humidity and temperature during storage contributing to loss of grain quality, infections among animals and humans and production of mycotoxins. Therefore, the current study was aimed to isolate and identify fungal species among stored grains and feedstuff of Potohar region of Pakistan. For fungal screening, ten different samples were collected from storage houses situated in different cities. These samples included wheat and corn grains from Taxila, Gujar Khan and Chakwal cities, while cattle feed samples were collected from Attock city. The investigation confirmed the presence of *Rhizopus arrhizus* in wheat from two different localities in Taxila and cattle feed, respectively, *Aspergillus foetidus* and *Achaetomium globosum* in wheat from Gujar Khan and Taxila, and *Mucor indicus* in maize from Chakwal. The most predominant fungal species was *Rhizopus arrhizus.* Here we are reporting the prevalence of pathogenic and toxigenic fungal species in stored grains and cattle feed of Potohar region for the first time. Inadequate storage conditions can lead to uncontrolled multiplication of fungus, so this study will assist in optimizing the storage conditions to curb its growth for assurance of healthy food for humans and animals.

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INTRODUCTION

The grains contain microbes from field at the time of harvest (Woloshuk and Martinez, 2012). Grain is a living commodity which respires under controlled storage conditions. Upon respiration, the grains produce moisture and heat raising the humidity and temperature which accelerate the fungal growth (Mohaptra *et al*., 2016). The contaminated grains remain safe from the menace of fungal growth and production of mycotoxins if the storage conditions are well maintained. For optimization of storage conditions, it is necessary to identify the type of fungi specific to a particular area to control their growth during storage.

The cattle feed, which is composed of various cereal grains, molasses, sunflower cake, mineral mixture etc. is a high source of energy and protein with high digestibility. In Pakistan, it is composed of 15-20 % wheat and 40-50 % maize grains along with other ingredients (Iqbal *et al*., 2015). It can be contaminated with pathogenic fungi or mycotoxins if prepared with contaminated ingredients. Its aflatoxin contamination is attributed to its composition and poor storage conditions which may lead to different diseases in humans like cancer. Pathogenic fungal spores and its toxins become a part of food chain causing health hazards when contaminated meat and milk is utilized (Umer *et al*., 2017).

According to Alconada and Moure (2022), the most frequent genera causing grain infections are *Alternaria*, *Aspergillus*, *Fusarium* and *Penicillium*. Other than these genera infecting grains, *Chaetomium*, *Cladosporium* and *Rhizopus* were also reported from wheat in Pakistan (Fakhrunnisa and Ghaffar, 2006) while Niaz *et al*. (2011) isolated *Aspergillus*, *Fusarium*, *Alternaria* and *Absidia* spp. from stored maize grains which is the second major crop grown in Pakistan after wheat. *Rhizopus arrhizus* (syn. *R. oryzae*) is an opportunistic pathogen causing mucormycoses worldwide (Dolatabadi *et al*., 2014) along with gastrointestinal diseases (Ribes *et al*., 2000), and is life threatening in patients suffered from diabetic ketoacidosis. It deteriorates the plants by producing carbohydrate digestion enzymes (Ghosh and Ray, 2011) thereby reducing the grain quality in storage. In animals, it is also associated with mycotic infections in bovine abortion cases (Knudtson and Kirkbride, 1992).

Black *Aspergilli* is a group of fungi known for spoiling common foods. Few species of this group, including *Aspergillus foetidus* are ochratoxin A (OTA) producers (Téren *et al*., 1996) and typically contaminate the cereals (Cabañes and Bragulat, 2018). OTA is a stable compound which withstands ordinary food processing conditions. It is toxic and causes renal tumors in various animal species (Bui-Klimke and Wu, 2015) and in humans upon digestion and even inhalation (Hope and Hope, 2012).

For the first time, a genus *Achaetomium* and a species *Achaetomium globosum* were described by Rai *et al*. (1964). The genus is usually isolated from soil (Pote *et al*., 2018). Its closest genus is *Chaetomium* (Rodríguez *et al*., 2004). The difference between the two genera is based on the production of ascospores of dark chocolate brown and pale to mid brown color in *Achaetomium* and *Chaetomium*, respectively (Cannon, 1986). *Chaemotium globosum* is not only involved in developing human infections (Serena *et al*., 2003) but also produce five different types of chaetoglobosin, a mycotoxin (Li *et al*., 2014) but data lacks the infection causing ability and production of any type of mycotoxin by *Achaemotium globosum*. Few Mucor species are known to cause mycosis by invading animal and human tissues specifically among immunocompromised patients (Morin-Sardin *et al*., 2017) such as *Mucor indicus* (Chayakulkeeree *et al*., 2006) which also causes gastrointestinal mycosis (Deja *et al*., 2006).

Potohar plateau is recognized by highly variable rainfall frequency and its distribution pattern (Rashid and Rasool, 2011) which is not yet explored for the pathogenic and toxigenic fungal diversity among the stored grains and feed stuff. Therefore, the present study was aimed to investigate the occurrence of pathogenic and toxigenic fungi in the wheat and maize grains, and cattle feed under the storage facilities of Potohar region.

MATERIALS AND METHODS

Study area: The Pothohar Plateau, also known as the Potwar Plateau, is in the northern part of Pakistan. The approximate coordinates of the central part of the Pothohar Plateau are latitude approximately 32.5° to 34.0° north and Longitude approximately 72.5° to 74.5° east. This region includes cities like Rawalpindi, Islamabad (partly), Jhelum, and Chakwal. The region experiences a climate that varies significantly between seasons due to its subtropical location. The temperature ranges between 4°C to 40°C with an annual rainfall average between 500 mm to 1000 mm, varying across different parts of the region.

Sample collection: Ten different samples were collected from different storage facilities located in different regions of Potohar area. Six wheat grain samples were collected from Gujar Khan, Chakwal, Attock and three different localities of Taxila, two samples of maize grains from Chakwal and Attock, and two samples of cattle feed from Attock and Chakwal. Each sample was collected in a sterile bag and kept in laboratory at room temperature till fungal isolation.

Isolation of fungus: The fugus was isolated by agar plate method (Panchal and Dhale, 2011). Four grains from each sample were picked aspetically, plated on Sabouraud Dextrose Agar (SDA). Similarly, the cattle feed was placed on SDA plate in four portions and the plates were incubated at room temperature untill the fungal hyphae started to develop. Each fungal colony obtained was further purified by subculturing on the separate SDA plates and incubated at room temperature for 5 days. The fungal plates were preserved at 4°C till further characterization. Six different types of fungus were randomly selected for further characterization.

DNA extraction: Fungal genomic DNA was extracted by modified CTAB method (Zhang *et al*., 2010). Six purified fungi were randomly selected for identification, so fresh culture of fungus was harvested from SDA plate and was ground in 500 µL lysis buffer (1 M Tris-HCl, pH 8.0; 0.5 M EDTA; 6 M NaCl; 2% CTAB). The suspension was incubated at 95°C for the first day after adding 2-3µL marcaptoethanol, 20µL proteinase K and 40 µL of 10% SDS. On day 2, DNA was purified by adding chloroform: isoamyl alcohol (24: 1) into suspension and centrifuged at 1000 rpm for 10 minutes. The upper aqueous layer was transferred into a new Eppendorf tube followed by DNA precipitation with the addition of 500µL ice chilled isopropanol. The DNA pellet was washed with 70% ethanol, air dried and re-suspended in 50µL low TE buffer (10 mM Tris-HCl; 0.1 mM EDTA, pH 8.0) and stored at -20°C. The presence of DNA was visualized on 1% agarose gel containing ethidium bromide under trans UV.

Internal transcribed spacer (ITS) gene amplification: A set of primers ITS1 (5'-CGTCACACGTTCTT CAACC-3') and ITS4 (5'-CGTTTCACGCTTCTCCG-3') (White *et al*., 1990) were used to amplify approximately 530 bp of the ITS region from the fungal DNA extracted from six fungi. For gene amplification, 25µL of reaction mixture, containing 12.5µL of PCR master mixture (Abclonal, USA), 2µL of each primer, 5.5µL PCR water and 5µL template DNA was prepared. A PCR of 30 cycles was performed at the following conditions: initial denaturation at 95°C for 5 min, denaturation at 95°C for 30 sec, annealing at 57°C for 35 sec, extension at 72°C for 40 sec followed by final extension at 72°C for 5 min. The amplified PCR products were run on 1.5% agarose gel to

confirm the size of a product. The amplified products were sent to Macrogen, Korea for their sequencing. The sequenced ITS regions were aligned with the nucleotide sequences retrieved from the NCBI database for maximum homology by using BLAST. The ITS nucleotide sequences of each fungus were submitted to GenBank for accession numbers.

Analysis of nucleotide sequences of Pakistani fungal strains: Nucleotide sequences of Pakistani fungal strains were compared to reference strains retrieved from GenBank for each fungal species. Genbank accession numbers DQ641279, NR_163668, NR_077173 and NR_157458 were used as reference for *Rhizopus arrhizus*, *Aspergillus foetidus*, *Mucor indicus*, and *Achaetomium globosum*, respectively. The nucleotide sequences were aligned and edited by Geneious® Version 6.1.8.

Phylogenetic analysis of fungal isolates: To construct a phylogenetic tree by neighbor-joining method (Saitou and Nei, 1987), the closely related sequences to our fungal isolates were retrieved from NCBI and were aligned by Clustal W program, followed by the phylogenetic tree construction by Mega X software (Kumar *et al*., 2018).

RESULTS

Identification of fungal isolates: Six fungal species (Fig. 1) were isolated from grain and cattle feed storage facilities located in different cities i.e., Taxila, Gujar khan, Chakwal and Attock of Potohar region. Among all the fungal species, five were isolated from the stored grains while one was isolated from the cattle feed. These are likely to be field fungi which invade grains during harvesting of cereal crops. They were identified at species level by amplifying their ITS gene (Fig. 2) followed by sequencing. The genetic homology of fungal isolates TW1.1, TW15.2, TW1.2, GW3.2, CC12.1 and AW11 was found 100, 99.8, 100, 99.82, 98.70 and 100% with *Rhizopus arrhizus*, *Rhizopus arrhizus*, *Aspergillus foetidus*, *Achaetomium globosum*, *Mucor indicus* and *Rhizopus arrhizus*, respectively. The accession numbers assigned by GenBank are shown in Table 1.

Comparison of nucleotide sequences of Pakistani fungal strains with reference strains: Comparison of *Achaetomium globosum* GW 3.2 (OP948735) with the reference strain (GenBank accession no. NR_157458) revealed that there was only one difference in the nucleotide sequences which was a deletion at nucleotide position 82. *Aspergillus foetidus* TW1.2 (OP94873) comparison with reference strain (GenBank accession no. NR_163668) revealed that both are almost identical strains at least over the region sequenced in this study.

Table 1: Fungal species, their source, origin and GenBank accession numbers

Fungal Species	Source	Origin	Accession numbers
Rhizopus arrhizus TW1.1	Wheat	Taxilla	OP948736
Rhizopus arrhizus, TW15.2	Wheat	Taxilla	OP948739
Aspergillus foetidus TW1.2	Wheat	Taxilla	OP948737
Achaetomium globosum GW3.2	Wheat	Gujar Khan	OP948735
Mucor indicus CC12.1	Corn	Chakwal	OP948738
Rhizopus arrhizus AW11	Feed Concentrate	Attock	OP948734

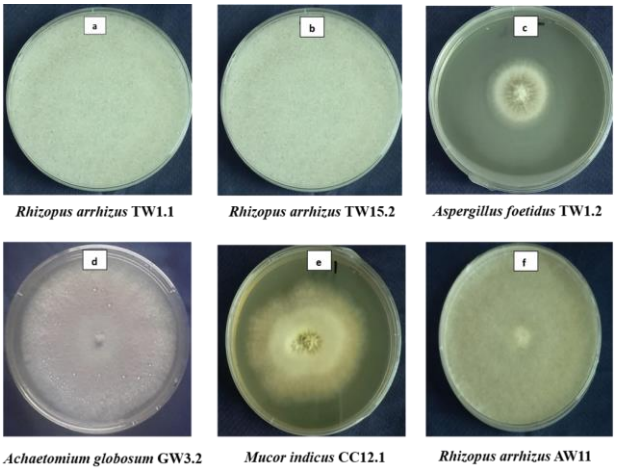

Fig. 1: Colony morphology of (a) *Rhizopus arrhizus* TW1.1, (b) *Rhizopus arrhizus,* TW15.2, (c) *Aspergillus foetidus* TW1.2, (d) *Achaetomium globosum* GW3.2, (e) *Mucor indicus* CC12.1 and (f) *Rhizopus arrhizus* AW11

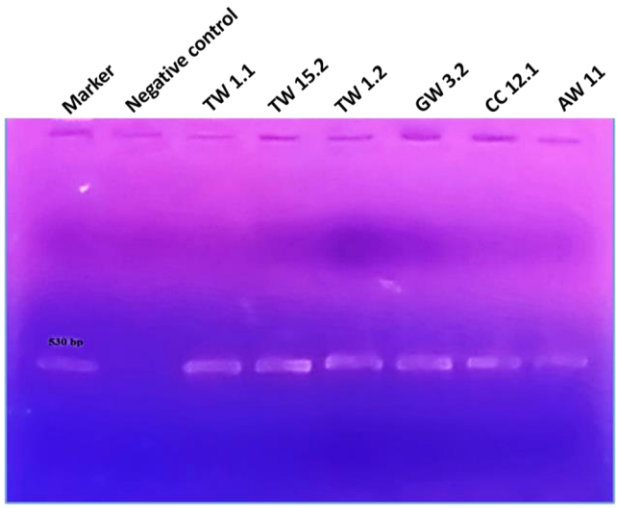

Fig. 2: PCR amplification of ITS region (~ 530 bp) of fungal species isolated in current study. The ITS of *Rhizopus arrhizus* TW1.1, *Rhizopus arrhizus* TW15.2, *Aspergillus foetidus* TW1.2, *Achaetomium globosum* GW3.2, *Mucor indicus* CC12.1 and *Rhizopus arrhizus* AW11 were amplified by PCR and visualized on 1.5 % agarose gel. Marker is the known PCR product of ~ 530 bp from a previously confirmed PCR using ITS1 and ITS4 primers.

Multiple mutations including substitution, insertion and deletion were identified when *Rhizopus arrhizus* AW11, *Rhizopus arrhizus* TW1.1 and *Rhizopus arrhizus* TW15.2 sequences were compared with the reference strain (GenBank accession no. DQ641279). A total of 6 substitutions and 2 deletions were identified in the sequence of *Mucor indicus* CC12.1 (OP948738) when compared with the reference strain (GenBank accession no. NR_077173). Nucleotide variations are shown in Fig. 3, 4, 5 and 6, respectively.

Phylogenetic analysis based on ITS region nucleotide sequences: A phylogeny was constructed to study the evolutionary relationship of isolated fungal species with each other and other fungus of the same genera (Fig. 7). *Saccharomyces cerevisiae* was used as an outgroup and the phylogenetic tree was rooted on it. The phylogenetic tree shows two distinct groups. One group is comprised of *Chaetomium*/ *Achaetomium* and *Aspergillus* species while

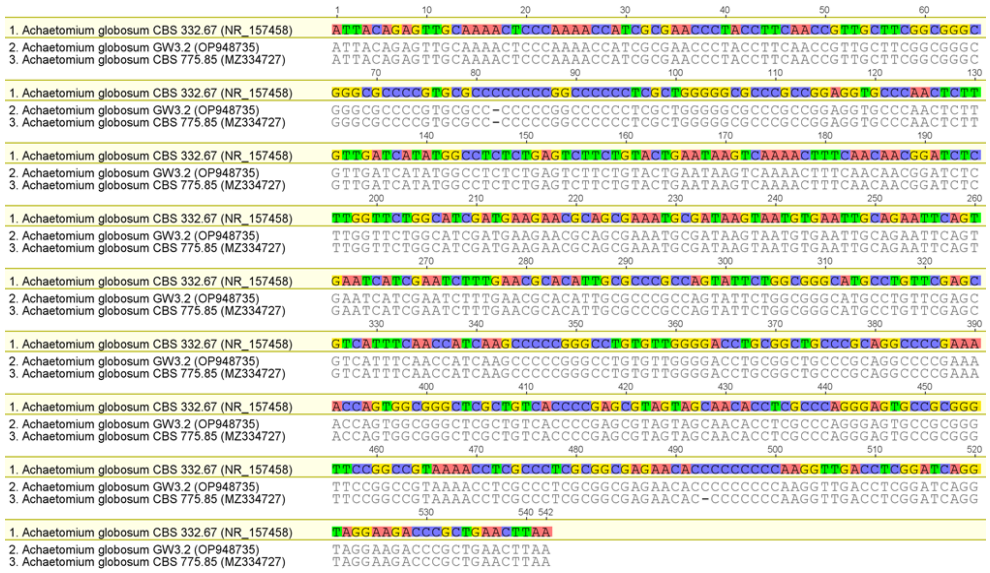

Fig. 3: *Achaetomium globosum* (OP948735) nucleotide alignment with the reference strain (NR_157458).

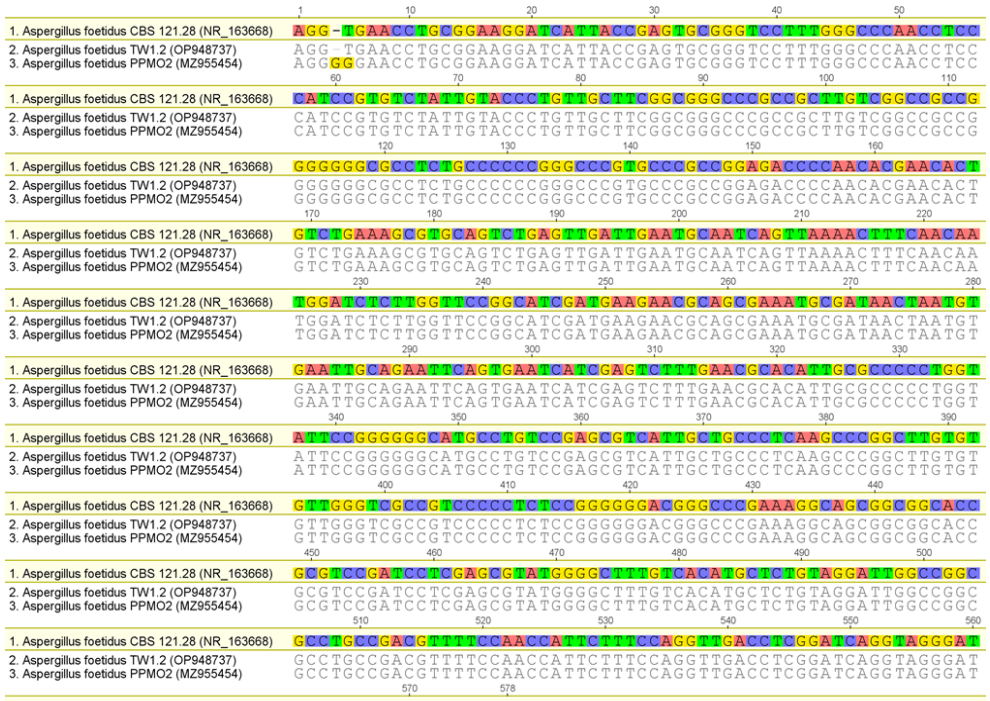

Fig. 4: *Aspergillus foetidus* (OP948737) nucleotide alignment with the reference strain (NR_163668).

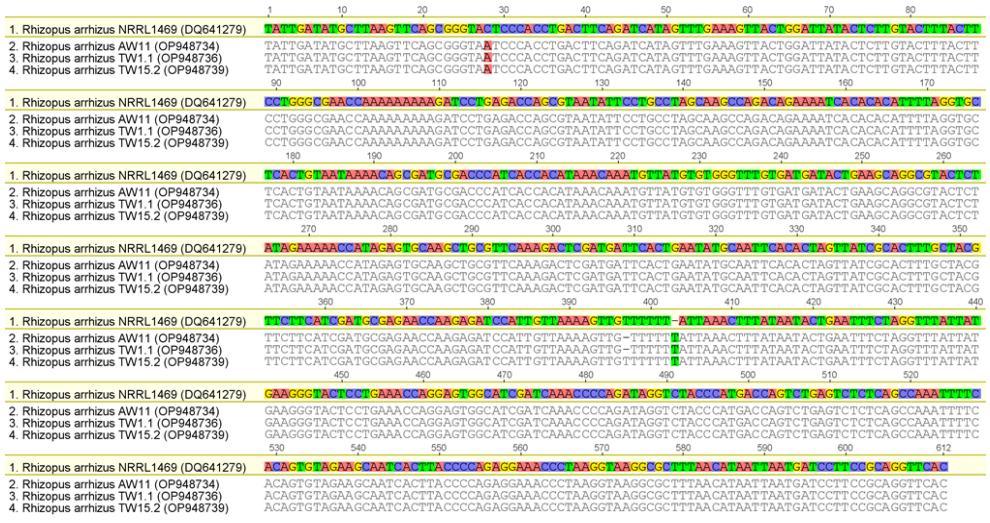

Fig. 5: *Rhizopus arrhizus* (OP948734, OP948736, OP948739) nucleotide alignment with the reference strain (DQ641279).

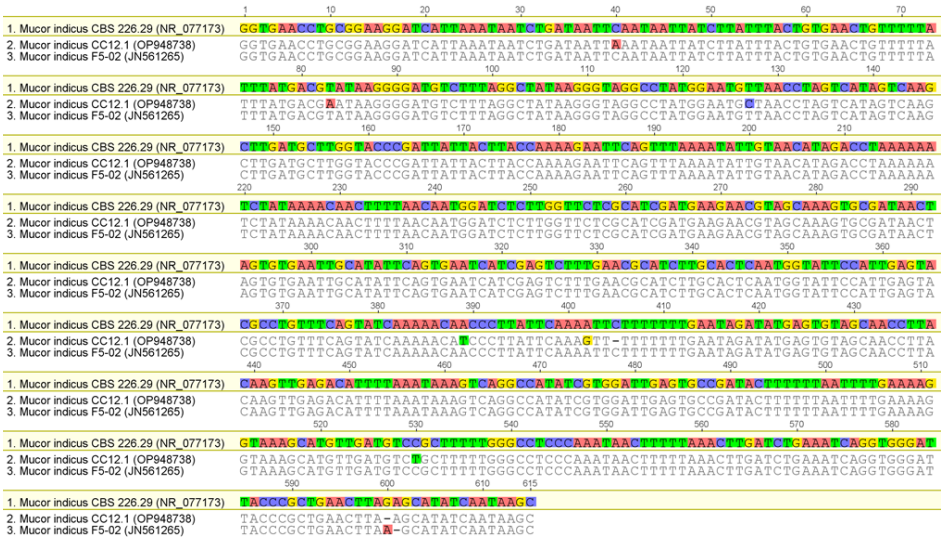

Fig. 6: *Mucor indicus* (OP948738) nucleotide alignment with the reference strain (NR_077173).

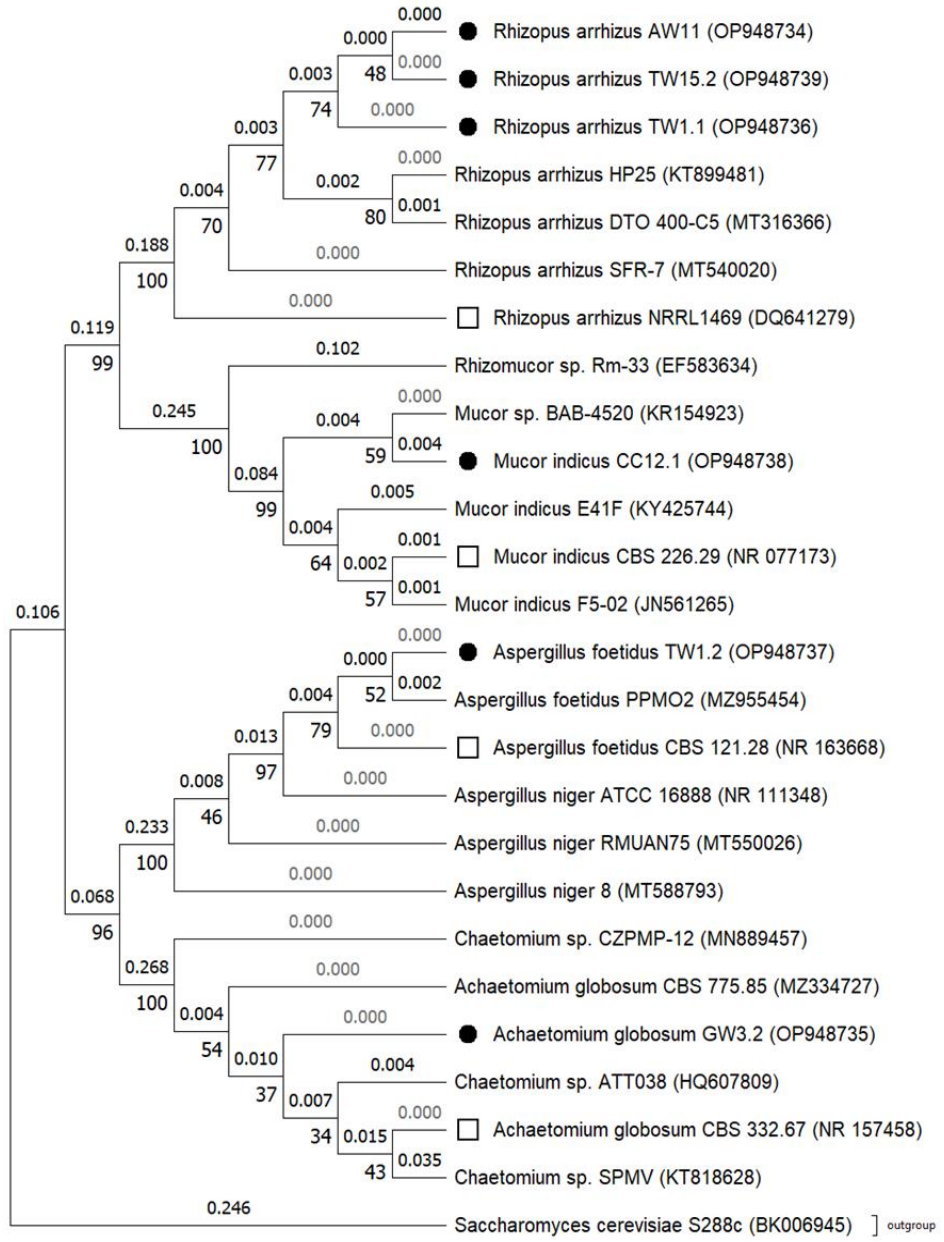

Fig. 7: A phylogenetic tree was inferred by Neighbor-joining method using combined ITS sequences of fungal species isolated in this study and related sequences retrieved from NCBI. Pakistani fungal isolates sequenced in current study are indicated as black dots and the reference sequences for each species are indicated by squares. Saccharomyces cerevisiae was used as an outgroup and the tree is rooted on Saccharomyces cerevisiae. Bootstrap percentages are indicated below the nodes. Branch lengths are highlighted above the branches. Bootstrap value of 1000 was adjusted. Evolutionary analyses were conducted in MEGA11.

the other group consists of *Rhizopus* and *Mucor* species. *Chaetomium spp.* form the basal clade including *Achaetomium globosum* GW3.2, depicting a common ancestor for other isolated fungal species. The clade of *Aspergillus* spp. is originated from the basal cluster. *Aspergillus foetidus* TW1.2 is more closely related to the

group consisting of *Rhizopus arrhizus* AW11, *Rhizopus arrhizus* TW1.1 and *Rhizopus arrhizus* TW15.2 than other *Aspergillus* species included in the current analysis. From this group, a cluster of *Rhizopus* spp. encompassing *Rhizopus arrhizus* AW11, *Rhizopus arrhizus* TW1.1 and *Rhizopus arrhizus* TW15.2 is diverged, and make another group which includes *Mucor* spp. M*ucor indicus* CC12.1, isolated in this study, also fall in this group.

DISCUSSION

In our study, *Rhizopus arrhizus* TW1.1 and *Rhizopus arrhizus* TW15.2 were prevalent among wheat collected from two different localities of Taxila. *Rhizopus arrhizus* prevails in Potohar region as Liaquat *et al*. (2019) isolated it from tomato where it was found responsible for causing brown rot. Arif *et al*. (2017) also reported it from the same region as a causative agent of fruit rot in yellow oleander. Various cereal grains other than wheat also inhabit *Rhizopus arrhizus* as shown by the studies of Wilson *et al*. (2016) and Cara *et al*. (2018), who isolated these fungi from stored maize and barley grains.

Another fungus in this study was identified as *Aspergillus foetidus* TW1.2 which was isolated from wheat grains collected from Taxila. The *Aspergillus* spp. was found among the fresh and the stored sesame seeds of Potohar region, capable of producing aflatoxins as investigated by Ajmal *et al*. (2022). According to Al-Wadai *et al*. (2013), *Aspergillus* is the most common occurring genera among the wheat grains, and if the wheat and other grains contaminated with *Aspergillus* are used as ingredients in animal feed, they may produce toxins which is then consumed by animals. In this regard, the study by Usman *et al*. (2019) focused on isolation of aflatoxigenic *Aspergillus* spp. from animal feed.

Along with the isolation of *Rhizopus arrhizus* and *Aspergillus foetidus*, another species *Achaetomium globosum* GW3.2 was isolated from the wheat of Gujar Khan region. *Achaetomium* spp. are soil saprophytes. Rodríguez *et al*. (2004) isolated new species of *Achaetomium* from Indian soil which supports our finding that wheat attained *Achaetomium globosum* from field and retained during storage. To our knowledge its presence among cereal grains is not reported yet. It can be considered toxigenic as *Achaetomium* originated from *Chaetomium* (Rodríguez *et al*., 2004) which is also indicated by the phylogenetic tree constructed in this study.

The results showed that *Mucor indicus* CC 12.1 was associated with corn collected from Chakwal. *Mucor* spp. is also prevalent in Potohar region known for causing rot infection in fruits such as rotting of *Eriobotrya japonica* (Abbas *et al*., 2018). The presence of *Mucor* spp. was also noticed among the maize grains collected from fields of Thailand (Inyawilert *et al*., 2020) while its presence was observed among wheat grains imported from Argentina and Kazakistan stored in Iranian silos (Okhovvat and Zakeri, 2003).

To maintain the quality of feed and to preserve animal health, it is prerequisite to keep the constant check on raw materials used as ingredients (Krnjaja *et al*., 2010). Veterinary feeds are produced mainly from wheat and maize grains (Khalifa *et al*., 2022). The cattle feed collected in this study was contaminated with *Rhizopus arrhizus* AW11. It can be assumed that the fungal contamination present in cattle feed might be due to the addition of *Rhizopus oryzae* contaminated wheat grains, and its presence in wheat samples has been shown in current study. Our findings are in accordance with the results of Krnjaja *et al*. (2010) who reported *Rhizopus* as a dominant genus in animal feed. In short, the *Rhizopus arrhizus* was a dominant fungal species among all the samples collected from Potohar region.

Conclusions: Our study revealed the presence of pathogenic and toxigenic fungal species among stored wheat, maize and feed concentrate of Potohar region of Pakistan for the first time. The wheat was susceptible to pathogenic *Rhizopus arrhizus*, toxigenic *Aspergillus foetidus* and *Achaetomium globosum* while pathogenic *Mucor indicus* was isolated from maize. *Rhizopus arrhizus* was also found in cattle feed. If the growth of deleterious fungi is not controlled by maintaining storage conditions, it can deteriorate grain quality that may cause infections and produce toxins to affect the health of humans and animals. The novel information of this study, which revealed the types of fungi, can be considered for the optimization of storage conditions to protect the quality of wheat and maize grains and ensure the quality of feed concentrate made from cereal grains.

Authors contributions: MAK, IAK and AHT designed and executed the project and organized the data. MAS, NN and MAZ made a significant contribution to the idea of the study and interpreted the results and drafted the manuscript. SAB, RHP and YA reviewed the manuscript. SS and BJ facilitated in the research and collected and compiled the data.

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