

CHARACTERIZATION OF FUNGAL MICROBIOTA ON RICE GRAINS FROM LOCAL MARKETS OF LAHORE

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Abstract

Pakistan is among the major rice producing countries, and more than half of the Pakistani rice produce is exported to different parts of the world. The pre and post-harvest environmental conditions of rice crop in Pakistan promote the growth of fungi in field as well as in stored rice grains. The end products have always been the main focus of food safety and quality. This study deals with the characterization of fungal microbiota on rice grains being sold in local markets of Lahore city in Pakistan.

Rice grain samples were collected from local markets, and fungal species were isolated and purified through continuous enrichment culture. Conventional morphological and physiological tests were performed to identify the mycoflora on rice grains. Phylogenetic analysis of the isolates was performed amplifying Internal Transcribed Spacer (ITS) regions.

Analysis of total mycoflora revealed the heavy load (4.33×10^5) of fungal contamination on rice grains. Most of the species were characterized as *Aspergillus flavus*, *Penicillium* spp., *Aspergillus fumigates*, *Alternaria* spp., *Aspergillus niger* and *Fusarium* spp. in this study.

Improper packaging, poor ventilation and increased humidity in the ware houses showed considerable effect on the presence of contaminating fungi in stored rice grains. Prevalence of mycotoxins producing species indicates the serious food safety threat to the consumers. This study will help to devise the strategies to control the fungal contamination in rice grains, ensuring food safety.

Key words: Rice grains, Food safety, Mycotoxins, Pakistan.

1. Introduction

Mycotoxin is derived from two words, mykes (greek word for fungus) and toxicum (latin word for poison). Mycotoxins are secondary fungal metabolites that

cause contamination in several food crops and have significant impact on human and animal health. Mycotoxicoses are the diseases caused by mycotoxins and these are associated with acute and chronic toxicity depending on the kind and dose of toxins. Currently 400 different mycotoxins have been identified from 200 different species of fungi. Nevertheless, out of these 400 mycotoxins, there are only 20 that can accumulate in concentrations posing potential health hazards to humans and animals [1].

Among several mycotoxins, aflatoxins are extremely carcinogenic, mutagenic and toxic compounds usually secreted by *Aspergillus flavus* and *Aspergillus parasiticus* species as secondary metabolites ([2], and [3]). Some other important mycotoxin producing fungi belongs to *Fusarium* and *Penicillium* species. *Aspergillus* and *Penicillium* species are the major contributor of food contamination [4]. Many factors at field conditions and during storage act together for toxin production by fungal species [5].

A number of different toxic fungi are present in food supply chain throughout the world and pose a potential threat to food security and food safety. Recently, the presence of mycotoxins in foodstuff has been paid much attention as a potential health hazard for consumers. Almost, a quarter of world's food crops is being contaminated by mycotoxins. Huge quantities of different foodstuffs including crops such as wheat, rice, maize and animal feed get contaminated by the invasion of fungal metabolites ([6], and [7]). Storage of crops without following the good storage practices (GSPs) provides an ideal substrate for the mould growth and production of mycotoxins subsequently. Tropical and temperate regions of the world are more prone towards the contamination of fungi or mycotoxins depending on the species of fungi. Dried fruits, cereals, nuts cocoa, oil seeds, coffee, beans, spices and dry beans are potentially contaminated by mycotoxigenic fungi. Ultimately, the wine and beer produced from the contaminated raw material will also carry these

potential toxins in food chain. Similarly, the livestock feeding on contaminated feed will carry these toxins in their milk and meat providing major source of entry of these toxins in human beings. The contamination of mycotoxins producing fungi is most prevalent during some stressful conditions in growing season like heavy rainfall before harvesting, drought and insect infestation. Fungal contamination usually occurs before the harvesting of crop in the field, while post-harvest contamination may be the result of delayed drying during storage or if the moisture content is exceeding the limits critical for the growth of fungus. Contamination is more prevalent on the grains sprouting at the panicle of rice if the harvesting is delayed in rainy season. It promotes the growth of *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus parasiticus*, that are major quality deterrent in grains during storage [8].

Pakistan is an under developing country, while its climatic conditions naturally support the growth of mycotoxigenic fungi. A strong synergistic relationship has been observed between chronic exposure to mycotoxins and liver cancer in Pakistan [9]. A number of reports reveal the contamination of mycotoxins in different crops of Pakistan ([10], and [11]). Surveys and different studies conducted in Pakistan for the analysis of aflatoxins in different are summarized in Table 1.

Rice represents an ideal substrate for mould growth. The major mycotoxins reported in rice are the aflatoxins, ochratoxins A, patulin, cyclopiazonic acid, trichothecenes, deoxynivalenol and zearalenone [12]. Aflatoxins contamination is becoming such a severe problem in Pakistan especially in Basmati rice, which has affected the export of rice to much extent. To increase our global trade, it is necessary to adopt some strategies to minimize the mycotoxins problems. These strategies should include a safe pathway even from field growing to transportation and shipment. Keeping in view the above mentioned facts, there is a dire need to analyse the fungal microbiota contaminating the rice grains in our local conditions. It will give a picture about the level of contamination of different species, especially the mycotoxin producing fungi, on rice grains.

2. Materials and Methods

2.1 Sample collection

Samples of stored rice grains were collected from local markets of Lahore, a major city in Pakistan. Five towns were randomly selected in Lahore and from each selected place/town samples were collected from shops and wholesale markets of stored rice. The samples were not necessarily of the same variety and polished as well as unpolished rice were evaluated. These were not found to be stored under same environmental conditions. Samples from random places within one town were thoroughly mixed and reduced to subsample of 50 g by complete mixing and quartering of the composite sample for further studies.

2.2 Moisture content analysis

Each sample of rice grains was analysed for its moisture content following the method of Silva [13]. From each sample, 25 g of rice grains were taken and placed in previously weighed crucibles and continuously dried at 105 °C in a hot air oven until the constant weight was attained. Moisture content was determined by calculating the difference between initial weight and dry weight of the sample.

2.3 Colony counting

One gram of sampled rice grains was grounded and thoroughly mixed in 10 mL of sterile distilled water; further, 1 mL from this suspension was taken and added in 9 mL of distilled water, followed by tenfold serial dilutions up to 10^{-4} . 0.5 mL of volume from each dilution was spread on Petri plates containing 15 - 20 mL of Potato Dextrose Agar (PDA) in replicates of three. These plates were incubated at 28 °C in a static incubator for 5 days and observed daily for fungal growth. Plates showing growth more than 15 colony forming units (CFU) were selected for counting. Results were reported as CFU per gram of rice grains and colonies were enumerated following the methods of Pitt and Hocking [14].

Table 1. Occurrence of aflatoxins in Pakistani crops

Sample Category	Number of samples	Positive Samples	%age Contamination	Mean AFs ($\mu\text{g Kg}^{-1}$)	Permissible AFs Value ($\mu\text{g Kg}^{-1}$)
Wheat	20	04	20	6.6	3.6
Rice	40	28	70	4.9	4
Maize	40	34	85	53.6	20
Whole Chillies	78	26	33.3	19.4	10
Grounded Chillies	78	31	39.7	21.1	10

2.4 Isolation and preservation of mycoflora

Different species of fungi representing different genera were isolated and purified by using agar plate and blotter method [15]. Each sample was replicated thrice for each method. The plates were incubated at 28 °C. Fungi growing on different seeds were isolated from emerging colonies and pure cultures were obtained for subsequent studies. Percentage occurrence of each species was also calculated by following formula.

$$\% \text{ occurrence of species} = \frac{\text{No. of colonies of a species}}{\text{Total no. of colonies}} \times 100$$

Pure cultures of different fungi were maintained on PDA slants by inoculating the spores of actively growing pure colonies of fungal species and preserved at 4 °C in refrigerator for further studies.

2.5 Morphological characterization

The fungal morphological studies consisted of mycelium growth, colour, cellular contents and characters of fruiting bodies of fungi. Macroscopic characters like fungal colony growth, size, colour, texture, shape, reverse colour, exudates, and margin of the colony were noted for fungal identification following the Barnett & Hunter [16]. Microscopic characterization of all the fungal isolates was done by making the slides of different fungal species. The documentation of isolates i.e. size of head, vesicle shape, phalides, matulae, conidiophores and conidia characters like: conidial diameter, wall, shape, surface and conidia attachment with conidiophore was done microscopically. Identification was done by comparing the data with the synoptic keys published by Barnett & Hunter [16].

3. Results and Discussion

In present study mycoflora was isolated from stored rice grains to evaluate the contamination level in stored rice grains of Lahore, Pakistan. Rice grain samples were collected from 5 different towns of Lahore city and were further evaluated for determination of moisture content, CFU/g count, and percentage occurrence of different fungal species. Different fungal isolates from rice grain were aimed to identify and characterized on the basis of their morphological characterization.

The humidity level plays a very important role in favour of fungal growth. Moisture content of stored rice grains was calculated in order to observe a correlation between moisture level and fungal growth. The maximum moisture level observed was 14.2% with a minimum value of 7.5% in case of sample 4 (Figure 1). Substrates with high moisture contents for a length of time offer best microclimate for the growth of mycotoxigenic fungi and mycotoxins production. A positive

correlation between moisture content and CFU/g count was observed in this study, with the increase of moisture content the number of CFU/g was increased, indicating a higher level of fungal growth and contamination. *Aspergillus* and *Penicillium* species usually invade and colonize the rice grains with moisture content between 7% without ventilation and 10% with adequate ventilation.

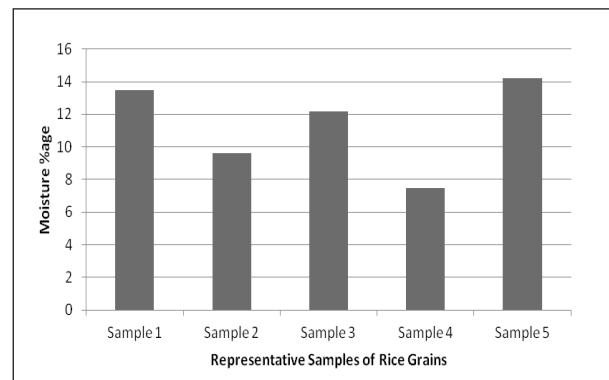


Figure 1. % of moisture content of stored rice samples

The results on CFU/g of different samples showed that mycoflora prevailed in all samples, with the maximum values of 4.33×10^5 followed by 4.1×10^5 , 3.36×10^5 , 2.5×10^5 and minimum 1.62×10^5 (Table 2). This contamination level stands above the tolerance limits, 10^2 to 10^4 CFU/g of the sample as per recommendation of Int'l Commission on Microbiological Specifications for Food.

Table 2. Total counts of colony forming units per gram of rice at dilution factor 10^{-4}

Sample No.	Average CFU / g
1	4.1×10^5
2	2.5×10^5
3	3.36×10^5
4	1.62×10^5
5	4.33×10^5

Morphological characterization of mycoflora on stored rice grain samples revealed the presence of *Aspergillus flavus* (35%) as the most frequent fungus, followed by *Penicillium* spp. (24%), *Aspergillus fumigatus* (16%), *Alternaria* spp. (12%), *Aspergillus niger* (8%) and *Fusarium* spp. (5%). Dominance of *Aspergillus* species on *Fusarium* species was analysed in all samples of rice grains under study (Figure 2). It might be due to the rice grain processing and storage conditions, that favour the growth of storage fungi i.e. *Aspergillus flavus*, instead of field fungi i.e. *Fusarium* species, more frequently found in field rather than on processed and stored grains [8].

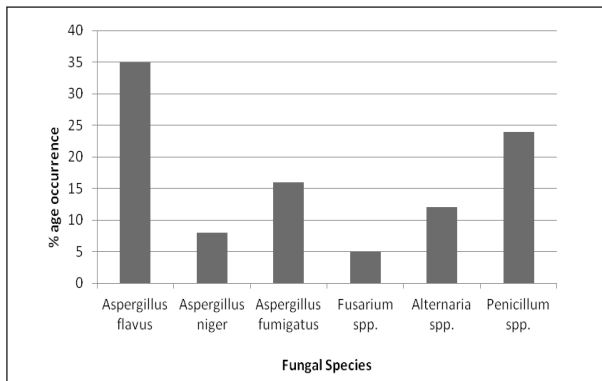


Figure 2. Occurrence of different fungal species isolated from rice grains in %

The highest *Aspergillus* spp. and *Penicillium* spp. counts were detected from different stored grain samples, which are the main contaminant of rice grains in present study. The presence of fungal growth indicates the anticipated contamination of mycotoxins, as high prevalence of *Aspergillus* spp. indicates the contamination of aflatoxins in rice grains as reported by Aydin *et al.*, [17]. The higher percentage occurrence of *Aspergillus* spp. followed by another most frequent *Penicillium* spp. (24%) was observed in present study. Ackermann [18], observed the presence of the different genera *Alternaria*, *Aspergillus*, *Penicillium*, *Mucor*, *Fusarium* and *Rhizopus* in stored grains. High frequency of *Aspergillus* and *Penicillium* were also reported when analysing stored barley grains.

Eight isolates (A to H) from stored rice grain samples, showing distinct morphological features were isolated and subjected to further studies for their identification and characterization. All of these isolates were grown on malt extract agar (MEA) plates and differentiated based upon their macroscopic and microscopic characteristics. Macroscopic study was based on: colony's color, diameter, reverse color, exudates and textures. On the basis of macroscopic study isolates can't be differentiated. Further microscopic study involved the arrangement, color, diameter, shape, size, wall characters, cellular contents, conidial heads, conidiophore, sterigmata, conidia and conidial arrangements [19].

Conidial heads of all isolates of *Aspergillus* varied from each other, some were: radiated, loosely radiated, elongate, globose (Figure 3 and 6) and clavate (Figure 4 and 5), and they also differ in their length and diameter. Colonies of *Aspergillus* exhibits different colors consisting: yellowish green (Figure 3), green (Figure 4), dark green (Figure 5), olive green (Figure 6) for isolates belonging to *Aspergillus flavus* and grey colored for *Aspergillus fumigatus* (Figure 7) and black colored for *Aspergillus niger* (Figure 8). All these characters indicate that *Aspergillus* isolates differ significantly in their morphology. Conidiophores also exhibit variable morphology i.e. thick and thin cellular contents, smooth

and roughened wall etc. Sterigmata of some isolate are biseriata (Figure 3 and 8) and most being uniseriate (Figure 4, 5, 6 and 7). Conidial diameter is also another important character and ranges from 3 to 8 μ in most of the *Aspergillus* species. Some other isolates belonging to *Fusarium* (Figure 9) and *Alternaria* (Figure 10) were also studied macroscopically and microscopically for their significant morphology. The morphological diversity in different structural features of mycelia has also been reported by Geiser *et al.*, [20], supporting our findings.

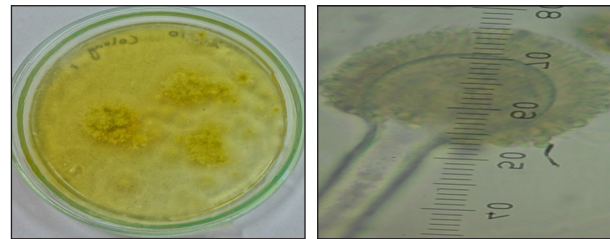


Figure 3. Conidiophore and pure culture of isolate A (*Aspergillus flavus*)

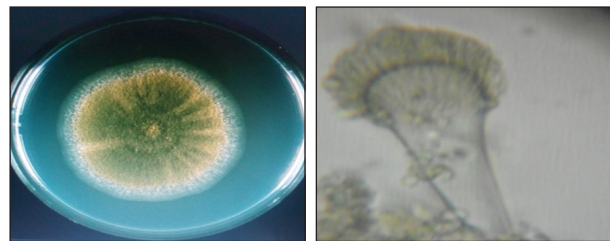


Figure 4. Conidiophore and pure culture of isolate B (*Aspergillus flavus*)

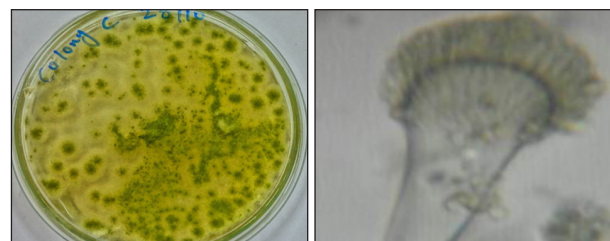


Figure 5. Conidiophore and pure culture of isolate C (*Aspergillus flavus*)

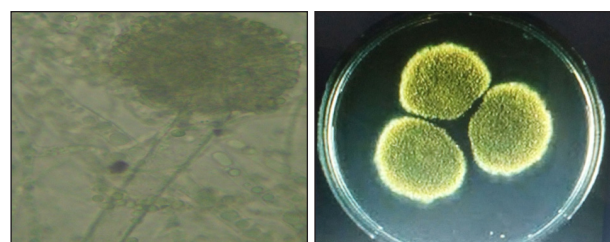


Figure 6. Conidiophore and pure culture of isolate H (*Aspergillus flavus*)

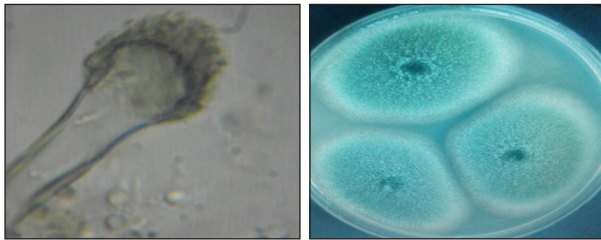


Figure 7. Conidiophore and pure culture of isolate D (*Aspergillus fumigatus*)

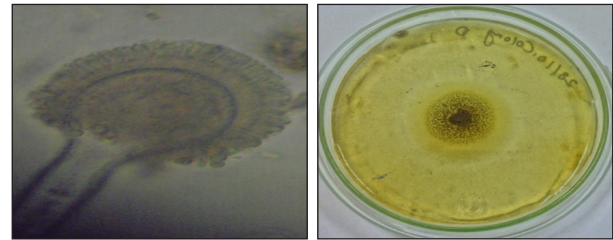


Figure 8. Conidiophore and pure culture of isolate E (*Aspergillus niger*)

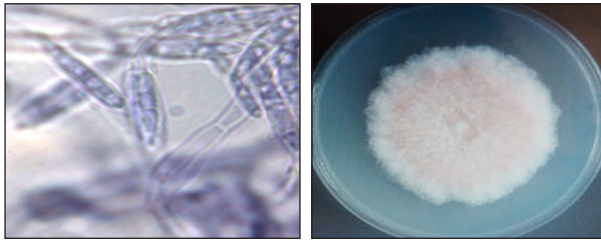


Figure 9. Conidiophore and pure culture of isolate F (*Fusarium* spp.)

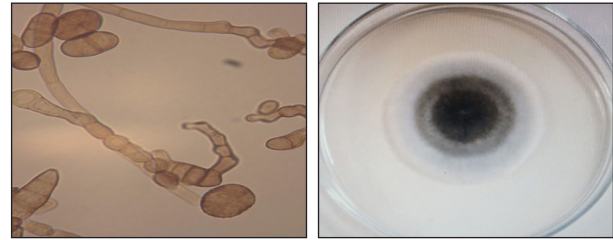


Figure 10. Conidiophore and pure culture of isolate G (*Alternaria* spp.)

Keeping in view the above results, it is obvious that the contamination level of mycotoxigenic fungi in our stored rice grains is very high. Food safety is a burning issue throughout the world. Currently the emphasis is on minimizing the risk of toxins in food commodities and utmost desire is preventing them from reaching consumers. This study suggests the adoption of pre and post-harvest practices to decrease the contamination level in rice grains. Further studies on the molecular characterization of these isolates, and quantitative determination of mycotoxins in stored rice grains are in the way.

4. Conclusions

- In the present study, mycoflora was isolated from stored rice grains and different fungal isolates were characterized on the bases of their morphological characters. There was a positive correlation in the moisture content of rice grains and presence of fungal species.

- Characterization of rice grains mycoflora revealed the contamination of *Aspergillus flavus* (35%) as most frequent spp., followed by *Penicillium* spp. (24%), *Aspergillus fumigatus* (16%), *Alternaria* spp. (12%), *Aspergillus niger* (8%) and *Fusarium* spp. (5%).

- The high level contamination of mycotoxigenic fungi indicates the potential contamination of mycotoxins to be expected in stored rice grains in Pakistan.

- This study highlights situation of rice grains safety in Pakistan, and need to educate the stake holders to devise the control / decontamination strategies of mycotoxins residues in stored rice grains.

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