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ARTICLE

Determination of Fungal Species to Investigate the Aflatoxin Contamination in Rice (*Oryza sativa* L.)

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ABSTRACT: *Aspergillus* species produce aflatoxins and raise concerns about food safety in departmental stores and manufacturing mills. To address the risks posed by aflatoxins, and to advise the public on the highest quality rice that serves as a nutritious food source, an inquiry following the guidelines outlined in both local and international standards of food safety for the presence of aflatoxins is an essential requirement. Therefore, 16 white rice samples were selected randomly from low/high socio-economic departmental stores from 16 different localities. Grind powdered rice filtrate was extracted using chloroform. The filtrate applied on TLC plates and the amount of aflatoxin and moisture contents were determined. In the non-infected rice, moisture content was low (9.08%) whereas high [13.65% > 12% (standard value)] in infected ones. Four out of 8 samples of low-quality rice were contaminated with AFB_1 and AFB_2 (ranging from 22.2 to 29.3 μg/kg). All the samples except one (22.3 μg/kg) from high-quality rice were certified fit despite the contamination with AFB_1. Furthermore, phylogenetic analysis showed *Aspergillus flavus* from unfit low (Long grain brown and Brown basmati) and high-quality (Basmati-198) rice whereas *A. parasiticus* from unfit low-quality Medium-grain brown rice. The presented research proves that the detection of fungi and aflatoxins in rice grains poses a huge risk to the health of consumers. Therefore, it is necessary to check the rice grains before distribution.

KEYWORDS: Mycotoxin; aflatoxin; rice; contamination; TLC; principal component analysis

1 Introduction

Rice (*Oryza sativa* L.) belongs to the family *Poaceae*. It is an annual and perennial staple crop of tropical areas [1–3]. The Asian countries are not only the largest producers but also the exporters around the globe [4]. There are two types of rice such as; fine rice called "White Basmati Rice" which is globally renowned due to its strong aroma and grain extension during cooking [5] and the other one is "Brown Basmati Rice"

in the form of whole grain without the inedible outer husk. Other rice varieties grown in worldwide are typically limited to specific geographic areas such as; basmati rice is grown in Pakistan and India, while jasmine rice is cultivated in Thailand [6]. The characteristic taste of basmati rice, reminiscent of pandan (*Pandanus fascicularis* leaf), is due to the aromatic compound 2-acetyl-1-pyrroline [7]. Basmati rice is a favorite foodstuff in the international community. Basmati rice is mainly exported to Qatar, the United Arab Emirates, Bahrain, Kuwait, Yemen, Malaysia, Saudi Arabia, the UK and USA [8]. The rice industry contributes 21.75% to the national economy through the production of 29.5% of the rice. However, post-harvest operations impact the overall quantity of rice produced and, consequently, the economy. If the seed quality does not meet international standards it affects the rice market and can reduce yield per acre due to outdated technologies [9].

The cultivation of Basmati rice takes place during the kharif season [10]. The damp conditions favor the plant fungal pathogens and the production of aflatoxins as well [11,12]. Therefore, environmental conditions of locations between 40° N and 40° S of the equator in the growing season of a crop play a vital role in the production of aflatoxins [13,14]. Adverse drought strongly favors the presence of aflatoxins produced by *Aspergillus* (*A. flavus* and *A. parasiticus)* in grains [15]. In aflatoxin, "A" denotes *Aspergillus* (Genus), "FLA" *flavus* (Species), and 'TOXIN' means poisons [16]. The major identified aflatoxins are B_1, B_2, G_1 and G_2. The B_1 is normally predominant in cultures and food products. The most toxic aflatoxin is B1 leads to mutagenesis [17–19]. AFB_1 has been found a serious threat in countries where food safety and hygiene were not imposed strictly [20]. Pure AFB_1 is a crystalline solid that ranges from pale white to yellow and is odorless. *A. flavus* secretes AFB_1 and AFB_2, while *A. parasiticus* produces AFG_1, AFG_2, AFB_1, and AFB_2 [21]. AFB_1 toxicity is associated with severe hepato toxigenicity and carcinogenicity. Bio-transformations of AFB_1 in the liver through cytochrome P450 enzymes cause detoxification [22]. The optimum temperature for the growth of aflatoxin is 20°C–35°C. Temperatures above 40°C and below 10°C reduce the production of these toxins. Higher temperatures outside the optimal range favor the production of aflatoxin B1, while lower temperatures favor aflatoxin G1 (AFG1) [23]. Life becomes risky due to contaminated foodstuffs [24] containing B_1, B_2, G_1, and G_2 aflatoxins, which are responsible of spoilage numerous agricultural foods and feed products [25]. Rice is susceptible to the attack of fungus during harvesting in the humid season [26]. The combination of warm and damp environments is considered very conducive condition for the proliferation of toxigenic fungi that produce mycotoxins such as aflatoxin [27]. On the other hand, the consumption of unpolished rice increases nutritional excellence and health claims. However, improper drying, handling, packaging, storage, hygroscopic conditions, and transportation assist in aflatoxins production. Delays in drying cause moisture sustaining in it that ultimately leads to postharvest contamination [27,28]. Therefore, the current study involves the extraction (Chloroform) and quantification (TLC) of aflatoxins from both low- and high-grade rice samples, which were collected randomly from various regions. Furthermore, the *Aspergillus* species were phylogenetically identified from the unfit rice samples. The impact of toxicity of aflatoxins on public health along with the scarcity of data regarding aflatoxin contents in rice provided the needed base study to measure the concentration of aflatoxins in rice at local markets. This study aimed to address the concerns regarding the aflatoxin risk of rice (food) manufacturing and provide awareness to the public on highest quality rice and health issues posed by rice aflatoxins under guidelines outlined by local and international food safety standards.

2 Materials & Methods

2.1 Collection of Samples

Rice samples (n = 16) were selected randomly from 16 different locales of Punjab Pakistan, located at latitude of 31.17° N and longitude of 72.70° E. Eight samples of excellent quality taken from fertile locations

and eight samples of low quality were taken from less fertile areas. The districts; Sialkot, Lahore, Narowal, Sheikhupura, Gujranwala, Hafizabad, Vehari, and Nankana Sahib for high-quality rice, whereas Chiniot, Mianwali, Jhang, Bhakkar, Khushab, Muzaffargarh, Rahim Yar Khan, and Layyah for low quality rice covered the sampling sites. High (low or minimum aflatoxins) and low (high aflatoxins) quality rice grades were given due to the presence of aflatoxins.

2.2 Total Moisture Content

Pre-weighed an empty Petri dish and then weighed the petri dish with sample. Weigh the dish and sample (W1). After weighing, the Petri dish was placed in the oven at 100°C. Weigh the dish again (W2) after cooling it in a desiccator. The dish was placed again in the oven for more than 2 h and then weighed again. The procedure was repeated until constant reading was obtained [29] (Fig. 1).

$$\text{Moisture}\,(\%) = \frac{\text{Total weight}\ (\text{W1}) - \text{Dry weight}\,(\text{W2})}{\text{Weight of sample}} \times 100$$

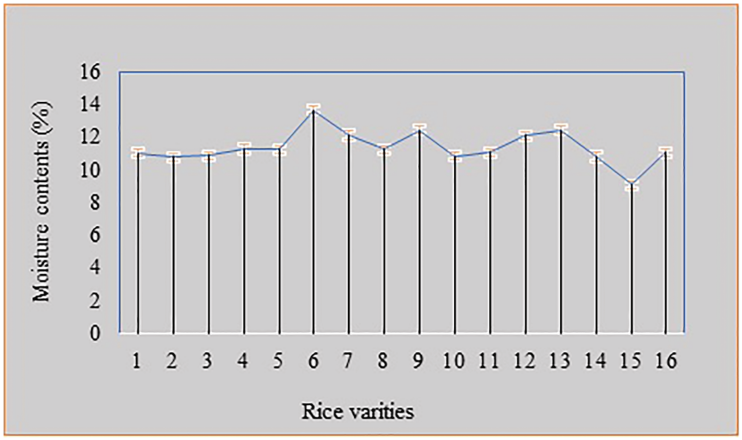

Figure 1: Moisture analysis of high white and low-quality rice

2.3 Culturing of Filamentous Species

Aspergillus species were isolated from seed samples through the agar plate method (ISTA, 1966). Nearly two hundred seeds from each sample were placed on plates with MEA (Malt extract agar) medium and incubated at room temperature or 30°C. The growth of *Aspergillus* species was observed after 6 days and further purified individually by subculturing on PDA slants. The pure culture of each fungal species was further identified by the ITS molecular method in laboratory of King Saud University with a specific voucher number (Table 1).

Table 1: Aflatoxin analysis in low quality brown Basmati Rice

Sr #	Rice samples	Aflatoxins (µg/kg)				Total	Fit/Unfit	Fungal Sp.
		B_1	B_2	G_1	G_2			
1	Kalijira rice	24.9 ± 0.01***	ND	ND	ND	24.4 ± 0.04^{ns}	Unfit	*A. parasiticus*
2	Aromatic jasmine rice	ND	ND	ND	ND	0	Fit	ND

(Continued)

Table 1 (continued)

Sr #	Rice samples	Aflatoxins (µg/kg)				Total	Fit/Unfit	Fungal Sp.
		B_1	B_2	G_1	G_2			
3	**Nutty-sweet red rice**	ND	ND	ND	ND	0	Fit	ND
4	**Haiga-mai**	ND	ND	ND	ND	0	Fit	ND
5	**Short-grain brown rice**	ND	ND	ND	ND	0	Fit	ND
6	**Long-grain brown rice**	29.3 ± 0.03***	1.52 ± 3.2	ND	ND	25.63 ± 2.1***	Unfit	*A. flavus*
7	**Brown basmati rice**	23.1 ± 0.02**	ND	ND	ND	22.2 ± 1.2 ns	Unfit	*A. flavus*
8	**Medium-grain brown rice**	25.9 ± 1.6***	ND	ND	ND	26.9 ± 1.9 ns	Unfit	*A. parasiticus*

Note: ND = Not detected (below the detection limit). Analysis was done by using one-way ANOVA and Tukey's Test. $^{**}p < 0.01$, $^{***}p < 0.001$, ns = non-significant, MTL (maximum tolerated level) of USA (FDA and FAO) and Pakistan (PSQCA) = (≤20 µg/kg).

2.4 Molecular Physiology, Extraction of DNA and Sequence Alignment

A modified version of the Doyle and Doyle (1987) CTAB technique was utilized for the extraction of the DNA genome from the samples. Nuclear ribosomal ITS regions were used to identify fungal filamentous species. According to BioEdit ver. 7.2.5 (ITSIF & ITS4) in hall 1999, consensus was obtained while homology was performed using BLAST from the NCBI (National Center of Biotechnology Information). The Genbank site was used to download the literature and initial ITS outbreak dataset (BLAST) according to a previous study [30]. However, ClustalX 2.1 was used for alignment and editing of the sequences according to Larkin et al. 2007 [31] and Bioedit [32]. The Sequences were aligned to MAFFT v. 10 using http://mafft.cbrc.jp/alignment/server/index.html [33] (accessed on 30 September 2023). The position of alignment to establish was 596. A phylogenetic tree was built by using these sequences at maximum likelihood. *Gibberella fujikuroi* was accepted as an outgroup. Phylogeny was Performed in MEGA (ver. 10.0) in accordance with the previous report [34]. Bootstrap values are adjusted based on 1000 replicas. This phylogenetic tree is helpful in finding the exact position of fungi while less than 50% of replicates branches were collapsed.

2.5 Estimation of Aflatoxins

In this study, aflatoxins in extracts of rice were estimated by comparing them to the standard official technique of AOAC method no. 977.16 [35]. Aflatoxins (B1, B2, G1, and G2) crystalline powder standards were purchased from 'Sigma–Aldrich (St. Louis, MO, USA)'. Standards were prepared by dissolving 1 mg powder in 100 mL benzene:acetonitrile (98:2; v/v). Aflatoxins (B1, G1) working standard (1 µg/mL) was individually prepared by mixing 100 µL of individual stock solution (10 µg/mL) and 900 µL of 'benzene: acetonitrile (98:2; v/v)', while B2 and G2 standards prepared by mixing '0.5 µg/mL' separately by adding 50 µL of stock solution (10 µg/mL) in 950 µL of benzene:acetonitrile (98:2; v/v).

2.6 Thin Layer Chromatography (TLC)

Pre–coated TLC plates of silica gel 60 (layer thickness 0.25 mm, 20 cm × 20 cm) were purchased from E. Merck (Germany). These plates were pre-washed for a surety that no additional compounds were present in them. Each rice sample was thoroughly mixed and ground into fine powder for experimental work via a Romer grinding mill. The 50 g of grounded sample was taken into a 500 mL conical flask, and 25 mL of water was added followed by adding the 25 g diatomaceous earth and 200 mL of chloroform. The flask was shaken for 30 min. The flask was removed from the shaker and allowed the sample to settle down. This agitated mixture was filtered and 50 mL $CHCl_3$ was added and placed in a steam bath for evaporation. Dried extracts were solubilized in 100 µL benzene:acetonitrile (98:2; v/v) and vortexed. TLC plates were spotted immediately by this sample and standardized above from the baseline (1.5 cm above the base). The spot of 5 µL was applied on the TLC plate. Two TLC tanks were taken and 50 mL ether was added in one tank, while the second tank acted as a mobile phase filled up to 50 mL with acetone:chloroform (1:9) (v/v). The TLC plate was placed on one side of the hot plate and the solution was placed on TLC by the micro-syringe. The process was carried out on the hot plate to prevent the drop from spreading on a plate. In developing first tank, plates (up till half or 3/4th solvent run) were developed with anhydrous ether for removal of impurities followed by removal of the plate for air drying. The plate was placed in the second tank upon drying. This was known as mobile phase. After the completion of this phase, the plate was taken out and again dried. Re-develop the plates in the same direction through an acetone-chloroform mixture in a TLC tank to modify R_f value of aflatoxins. The developed plates were observed under UV (λ = 254 and 366 nm) light for the presence or absence of spots of the test solution and authentic aflatoxin [29].

2.7 Quantitative Determination of Aflatoxin

Approximately, all spots of the same sizes of test and authentic standard aflatoxin corresponding to aflatoxins observed on preliminary plates were noticed.

The concentration of aflatoxins was determined by following common formula:

$$\textbf{B1, B2, G1, G2}\,(\mu g/\text{kg}) = \frac{\textbf{Matching of standard spot} \times \textbf{intensity} \times \textbf{dilution}}{\textbf{vol.of sample} \times \textbf{effective weight}}$$

2.8 Statistical Analysis

All the experiments were performed in triplicates. The mean square was used to determine the significant difference ($p < 0.05$) among samples and presented as mean ± standard deviation (GraphPad Prism5 software Version 5.03). PCA (principal component analysis) was used to analyse the effect of low- and high-quality rice on aflatoxins content. PCA matrices were divided into 16 rows and 3 columns for the statistical analysis of data. An optimum number of components was determined using Cattel's criterion. The input matrices were automatically scaled and measured at significant levels of a = 0.05% (correlation and PCA).

3 Results

Rice is a staple food in most of the Asian countries. The requirement depends on quality, vendors and price. The consumer preferred contaminant-free rice. However, random purchase of rice showed contamination of aflatoxins in this present study.

3.1 Moisture Analysis

Moisture plays an important role in the contamination of rice and stimulates AFs. The moisture level was determined by the AOAC method [35] (Fig. 1). The healthy rice contained less moisture. It was considered

fit because it did not support the growth of *Aspergillus flavus* and *A. parasiticus*. Infected rice demonstrated a high value of moisture that favored the fungal growth. The results of our study were in accordance with Oyedele and Adeoti [36].

3.2 Identification of Aspergillus Species

Aspergillus species were identified by using ITS regions. The phylogenetic tree determined the exact position of these filamentous fungi (Fig. 2).

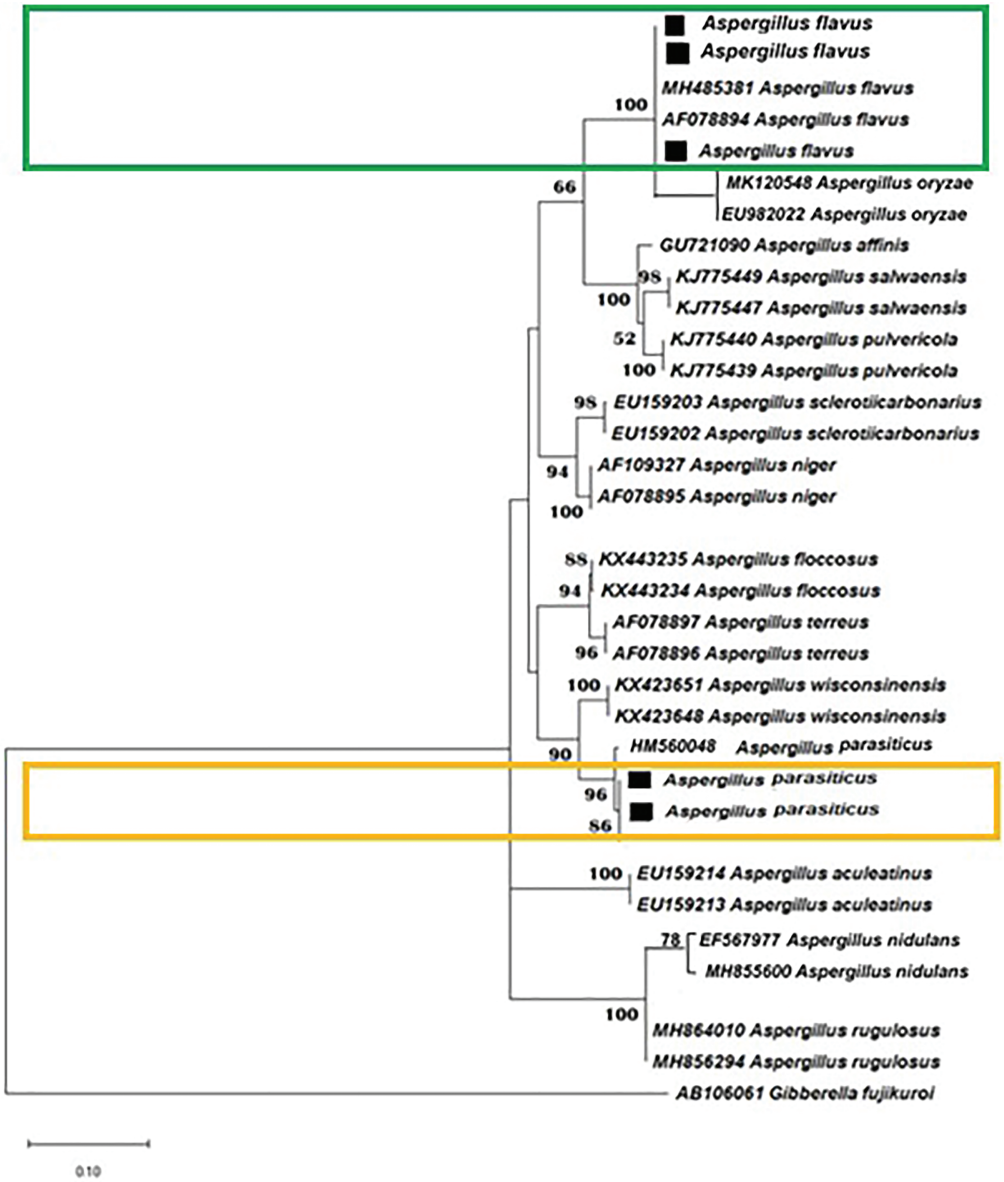

Figure 2: Phylogram of *A. flavus* and *A. parasiticus* identified from Low- and High-Quality rice contaminated with aflatoxins (R1-Long-grain brown rice, R2-Brown basmati rice, R3-Basmati-198, R4-Medium-grain brown rice, R5-Kalijira rice grains)

3.3 Determination of Aflatoxin

Aflatoxin is a major contaminant of rice due to high moisture required for its growth. The results were checked in the rice samples by the standard method of TLC by AOAC (Tables 1, 2). The results were similar to the study of Asghar et al. [27]. The detectable limit of aflatoxin is ≥1 µg/kg. Those rice samples were fit for human consumption as per MTL (<20 µg/kg) assigned by the USA (FDA and FAO) and Pakistan (PSQCA).

Table 2: Aflatoxin analysis in high quality white Basmati Rice

Sr #	Rice samples	Aflatoxins (µg/kg)				Total	Fit/Unfit	Fungal Sp.
B_1	B_2	G_1	G_2					
9	**Basmati-385**	1.94 ± 0.03^{ns}	ND	ND	ND	1.94 ± 1.01^{ns}	Fit	ND
10	**Shaheen Basmati**	1.84 ± 0.7^{ns}	ND	ND	ND	1.94 ± 0.7	Fit	ND
11	**Rahna Basmati**	Not detected	ND	ND	ND	0	Fit	ND
12	**NIAB-IR9**	1.59 ± 1.1^{ns}	ND	ND	ND	1.69 ± 0.8^{ns}	Fit	ND
13	**Super Kernal Basmati**	Not detected	ND	ND	ND	0	Fit	ND
14	**Basmati-198**	$22.3 \pm 2.4^{*}$	ND	ND	ND	$25.5 \pm 1.9^{*}$	Unfit	*A. flavus*
15	**Kashmir Nafees**	1.56 ± 1.5^{ns}	ND	ND	ND	1.59 ± 0.2^{ns}	Fit	ND
16	**Basmati-2000**	Not detected	ND	ND	ND	0	Fit	ND

Note: ND = Not detected Analysis was done by using one-way ANOVA and Tukey's Test. $^{*}p < 0.05$, ns = non-significant. MTL (maximum tolerated level) of USA (FDA and FAO) and Pakistan (PSQCA) = (≤20 µg/kg).

3.4 Aflatoxin in Low Quality Rice

The rice sample of low quality was highly contaminated (Table 1). In this study, four rice samples were found unfit for human consumption owing to AFs contamination in the range of 23.1 to 29.3 µg/kg including Kalijira rice, Long-grain brown rice, Brown basmati rice, and Medium-grain brown rice contaminated with aflatoxins and proved unfit for health. The highest value of AFB1 and AFB2 in a rice sample was 29.3 to 1.52 µg/kg in the case of Long-grain brown rice, respectively, higher than the standard value (>2.0). Kalijira rice (24.9 ± 0.01 µg/kg), Brown basmati rice (23.1 ± 0.02 µg/kg), and Medium-grain brown rice (25.9 ± 1.6 µg/kg) showed a significantly high amount of AFB1 with respect to Aromatic jasmine rice, Nutty-sweet red rice, Haiga-mai, and Short grain rice samples.

3.5 Aflatoxin in High Quality Rice

The high-quality rice was found to be good for health because of comparatively less contaminated (Table 2). The highest value of aflatoxins was found in Basmati-198, which was 22.3 µg/kg higher than the standard value. Five samples were contaminated with AFs but proved fit for consumption.

3.6 PCA Analysis

The results of the PCA analysis determined the relationship between low and high-quality rice and the content of aflatoxins. Three new variables were found, and the first two main components were described as much as 99.91% variability of the system. PC1 showed the largest percentage of system variability at 76.28%, while the second principal component PC2 described the system variability at 23.64%. As you can see, all parameters have a large impact on variability of the system, because these were placed in the red circle (Fig. 3A). The first principal component distinguished the fit and unfit form of rice due to the

presence or absence of fungal species. Positive PC1 values exhibited the fit rice and no fungus occurrence, while negative values of this component described the unfit rice with fungus occurrence. A strong positive correlation was found between B1 and the total, and a slightly weaker but positive correlation between them and B2. The PCA analysis explained the negative values of the first principal component (PC1) and described the occurrence of aflatoxins, while the second principal component exhibited their types (Fig. 3B). The second principal component PC2 distinguished one unfit Long-grain brown rice variety from other unfit rice varieties. The B1, B2, and Total parameters were correlated with the unfit rice type and the occurrence of fungal species. Parameter B2 characterized the unfit Long-grain brown rice, and the remaining parameters B1 and totally characterized the remaining unfit varieties (Fig. 3A and B). The effects of aflatoxin in a correlation matrix were shown in Fig. 4. Rice quality based on aflatoxins was also analyzed by PCA. The collinearity of the variables was estimated by the determinant of the correlation matrix (−1 to 0 to +1). The 0 showed a weaker correlation while 1 exhibited a strong correlation. Negative and positive signs dictated the direction of correlation.

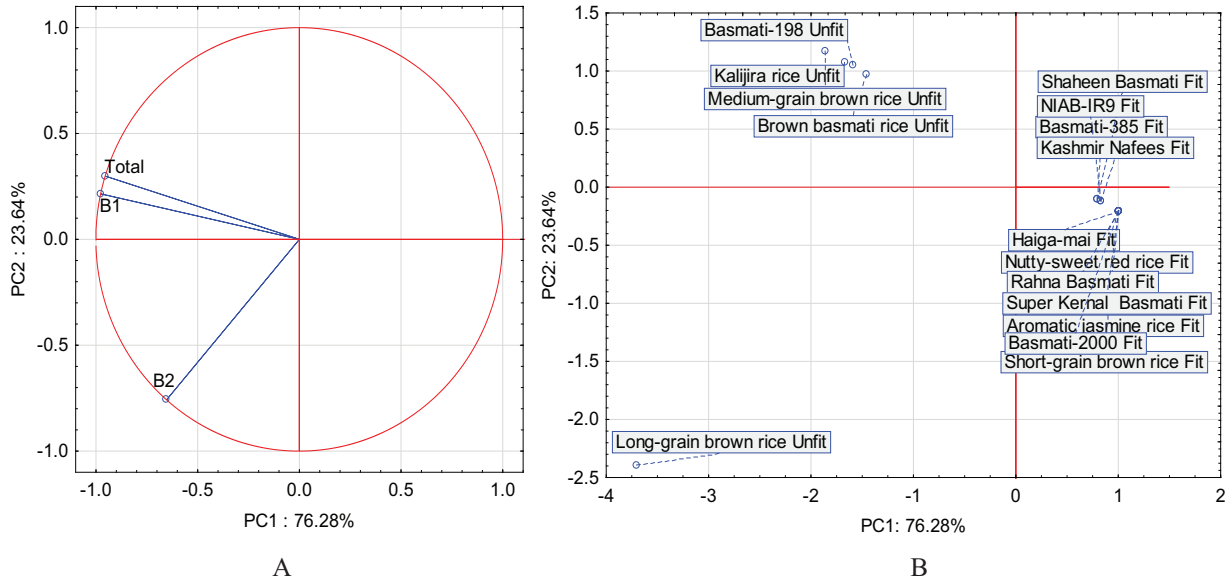

Figure 3: Loading plot (A) and score plot (B) of the principal component analysis (PC1, PC2) carried out for treatments and research parameters

r>=	-1	-0,80	-0,60	-0,40	-0,20	0	0,20	0,40	0,60	0,80

	B1	B2	Total
B1	1,000	0,474	0,994
B2	0,474	1,000	0,396
Total	0,994	0,396	1,000

Figure 4: Correlation matrix for the tested parameters of the effect of aflatoxin content

4 Discussion

Many countries have established standard levels for aflatoxins (AFs) because of their high prevalence, risk, and toxicity to human health. For example, in the European Union (EU), the maximum level of about 4 µg/kg total has been set for aflatoxins (mainly AFB_1, AFB_2, AFG_1, and AFG_2) in cereals and AFB_1 of

about 2 µg/kg [37]. However, the maximum level of aflatoxins in rice up to standard was implied to be 20 µg/kg in USA [38] FDA, FAO, and PSQCA [38,39]. A previous study indicated the concomitant absence of AFB2, AFG1, and AFG2 [40]. Similarly, the rice contaminating *A. flavus* in our study produced one type of AF. These results were in contrast to previous studies indicating the ability of *A. flavus* strains to produce only AFB_1 and AFB_2 [40–42]. However, our results were consistent with the production of AFB1 by *A. parasiticus* strains [43]. It has also been reported that a high percentage of *A. flavus* strains synthesize AFG_1, while a minor group accumulates AFG_2 [44]. The results of this study are consistent with studies indicating that AFB1 is the most prevalent AF contaminant and has the highest concentration among rice-infecting *A. flavus* [45,46]. Firdous et al. [47] evaluated aflatoxin content in super kernel basmati rice. They determined the toxicity of AFB_1 in 13.3% of samples ranged from 1.1–32.9 µg kg^{-1}, while up to 1.0–8.1 µg kg^{-1} of 1.9% of AFB_2 toxins were found. Only one sample showed AFG_1. In basmati rice, contamination of AFB_1 was 1.0–15.4 µg kg^{-1} in 18.3% of samples while 2% of AFG_1 was also found. This study indicated the four unfit rice samples viz., Long-grain brown rice, Brown basmati, and White basmati-198 due to the presence of AFB_1 the values of 29.3, 23.1 and 22.3 µg kg^{-1} rice, respectively. Whereas, AFB2 was undetectable in these samples. The filamentous fungal species identified in these rice samples was *A. flavus*, which was also reported in similar work of Nayak et al. [48]. In their study, 50% *A. flavus* and 55% *A. flavus* were observed in raw rice samples, which revealed the higher level of contamination in rice and rice-based commodities. Similarly, Asghar et al. [27] showed 95.4% AFB_1 (1.07–24.65 µg/kg) in 250 samples and 7.6% AFB_2 (0.52–2.62 µg/kg) in 20 brown rice samples, similar trend was found in our study where aflatoxins AFG_1 and AFG_2 were unidentified. Likewise, in another study, the Tehran retail market determined AFB_1 (4.17 ng/g) in rice, and the level of contamination was >5 ng/g [49]. Asghar et al. [50] performed an experiment on export–quality basmati rice for AFs (B_1 B_2 G_1 and G_2) and detected 73.3% AFB_1 (1.17 to 6.91 µg/kg) contamination in 2047 samples with the maximum tolerated level at 2 µg/kg. The AFB_1 level in 2.3% of samples ranged from 2.05–3.36 µg/kg, 0.44% AFB_1 up to 4.07–6.91 µg/kg range AFB_1, while the remaining samples (26.7%) were within the detectable limit (>1 µg/kg). Tansakula et al. [51] also determined the aflatoxin (AF) B_1, B_2, G_1, and G_2 in 120 samples of unpolished rice and glutinous rice in Thai commodities. The total aflatoxins content determined in samples were 0.16 and 25.43 µg kg^{-1} levels of aflatoxins (particularly AFB_1 and B_2). Likewise, the district Mandi, Himachal Pradesh, India, was explored to determine the mycotoxins contamination in stored rice grains. The highest density and frequency of AFB_1 and AFB_2 was 28% and 48%, respectively, in 72% of the samples [52]. Therefore, this study aligned completely with the current study and undoubtedly witnessed the presence of Afs in the Brown basmati rice. Reddy et al. [53] performed tests on 1200 paddy rice samples. Aflatoxin (B_1) concentrations up to 0.1–308.0 µg kg^{-1} were detected in 67.8% of rice. Fredlund et al. [54] tested 99 rice samples purchased from the retail market in Swedish for determination of aflatoxins. The conducted research has shown that as much as 71% of Basmati rice contained detectable levels of AFB_1 up to 0.1 µg/kg of rice. The MTL of about 5 ng/g is assigned by the Institute of Standard and Industrial Research of Iran (ISIRI) and Mohammadi et al. [55] analyzed 4 types of AFs (B_1, B_2, G_1, G_2) in 152 samples of Iran. Their study showed contaminated levels of aflatoxin B_1 (0.09–3.3 ng/g) in 75% of rice samples that were not above the MTL. The results of this study were totally against the findings of our study. The current study also indicated that Kalijira rice, Brown basmati rice, and Long and Medium grain brown rice from brown rice samples, whereas white Basmati-198 was contaminated with AFB_1, and therefore, these rice samples were unfit for human consumption. Approximately, 200 samples of various varieties of rice were collected from Canada, Thailand, the United States, and India, which includes red, white, black, brown, jasmine, basmati and wild rice [56]. AFB_1 with the incidence of 56% and 43% was found at levels of 0.19 and 0.17 ng g^{-1}, respectively with 0.002 ng g^{-1} set as LOD (limit of detection). With a similar LOD, 23 samples found in the second year were contaminated with AFB_2. AFB_1 in both years was contaminated at levels of 1.44–7.14 ng g^{-1} and 1.45–3.48 ng g^{-1} in 5 samples, which are

the most contaminated; this includes red and black rice taken from Thailand and basmati from India [57]. Reiter et al. [58] analyzed AFs in different varieties and fractions of a total of 81 samples of rice including whole, short, long grain rice, basmati rice, and puffed rice in Vienna. Results analysed a detectable number of aflatoxins in 24 samples out of the total in which 15 samples contained AFB_1 and only one in $AFB_{2,}$ while B_1 and B_2 were found in a range of 0.45–9.86 and 1.5 μg kg^{-1}, respectively. AFG_1 and AFG_2 was not determined in the samples. These samples exceeded the limits of AFB_1 set by EU, e.g., one at a range of 2.16 μg kg^{-1}, 2nd in the range of 2.85 μg kg^{-1} while 3rd was about 9.86 μg kg^{-1}. The results of Reiter et al. [58] were contradictory to our findings as our study indicated the contamination of AFB_1 in Long-grain brown rice, while short grains of rice were contaminated free from any type of aflatoxin. The results are also in close agreement as reported by Castells et al. [59] work, which studied two artificially contaminated varieties of husked rice with AFs. AFs of about 356–818 μg/kg and 244–645 μg/kg were found in medium-grain and long-grain rice, respectively. Yazdanpanah et al. [49] recommended the regular screening of AFB_1-infected rice, because the consumption of a high quantity of AFB_1 is toxic to the liver, cyclic nucleotide phosphodiesterase activity in the brain, liver, heart, and kidney tissues can be inhibited by AFB_1. These all result in severe impairment of the metabolism of proteins, carbohydrates, and lipids. Previous studies have shown that rice grains are susceptible to AFs (AFB_1 and AFB_2) accumulation [60,61]. Both the present observations and previous results indicate that rice grains are a major source of AF contamination. These results confirm that unless appropriate steps are taken, the risk of AF poisoning in humans from rice infected by toxic *A. flavus* strains cannot be eradicated. Since rice consumption is common in many nations, it is highly advised to keep an eye on the levels of AFB_1 in rice grains that have been stored as a preventive step [62]. The presence of AFs in the majority of the samples examined here suggests that regular, national-level programs to track the levels of AFs in rice grains are necessary. By using appropriate storage procedures, the concentration of AFs should be decreased [63].

5 Conclusion

The determination of fungal species associated with aflatoxin contamination in rice (*Oryza sativa* L.) is critical for ensuring food safety and public health. This study revealed that certain fungal species, particularly *Aspergillus* spp., were the primary culprits and responsible for the production of aflatoxins in rice. Identifying these fungi through advanced molecular and microbiological techniques not only helps in understanding the contamination pathways but also assists in developing effective prevention and control strategies. Given the global consumption of rice as a staple food, the presented study strongly endorses that mitigating aflatoxin contamination is essential for reducing the risk of liver cancer, immunosuppression, and other health problems linked to aflatoxin exposure. Future efforts should focus on improving agricultural practices, post-harvest handling, and storage conditions to minimize fungal growth and aflatoxin production, ensuring rice remains a safe and nutritious food source. Further research into biocontrol methods and genetic resistance in rice varieties could also offer sustainable solutions to this critical issue.

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