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SEASONAL AND GEOGRAPHICAL ANALYSIS OF AFLATOXINS IN DIFFERENT VARIETIES OF BROWN RICE COLLECTED FROM TWO DISTRICTS OF PUNJAB-PAKISTAN

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ABSTRACT

Aflatoxins are highly toxic, carcinogenic compounds, widely prevalent worldwide, secreted by various fungal strains especially from genus *Aspergillus* including *Aspergillus parasiticus* and *Aspergillus flavus.* Brown rice having more than 17% moisture contents is considered as favorable substrates for fungal growth causing aflatoxins production. The objective of current study was to examine seasonal and geographical impact on production of aflatoxins including aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2) in different brown rice varieties procured from two districts of Punjab. High Performance Liquid Chromatography (HPLC) equipped with fluorescence detector was used to detect aflatoxins in samples including Super Kernel Basmati Rice (SK), Kainat (KN), and Supri (SP). Among aflatoxins, AFB_1 was the most common toxin detected in three varieties of brown rice especially in SK. AFG_1 and AFG_2 in the present study were not detected. A total of 200 samples were taken out of which 125 samples were positive. Among 125 samples, 100(80%) samples had greater AFB_1 content than permissible limit (2 µg/kg). In addition, 94(75%) samples had higher total aflatoxin contents than allowable limit (4 µg/kg). Aflatoxins contents in brown rice were dependent on rice varieties, regions, and months. The highest moisture and aflatoxins contents were observed in SK in the month of March collected from Gujranwala and lowest were observed in SP in the month of May collected from Multan. The seasonal comparison for aflatoxins concentrations in brown rice showed higher aflatoxins levels in the samples collected during the month of March than those procured in other months (November, January and May). Furthermore, a strong correlation between moisture contents and aflatoxins was also observed. The results of current study revealed that moisture management during storage is very significant to control aflatoxins production in brown rice varieties.

Key words: Aflatoxins, Brown rice, Seasonal variation, Punjab, Pakistan.

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INTRODUCTION

Rice (*Oryza sativa L.*) is the key staple meal consumed worldwide especially in Asian countries including China, Sri Lanka, India, Bangladesh, and Pakistan. In Pakistan, rice is amongst the most significant agronomic crop and has high demand globally, especially basmati rice due to its unique aromatic properties (Rehman *et al.,* 2017; Chandio *et al.,* 2020). According to Trade Development Authority of Pakistan (TDAP), the 9th largest rice producer of world is Pakistan with annual volumes beyond 8 million tons. In 2021/2022, the annual rice production of Pakistan was 9.3 million metric tons (TDAP, 2022). Rice contributes 0.6% of GDP (Gross Domestic Product) and 3.1% of total agricultural exports of Pakistan (GOP, 2020).

Rice is cultivated in several zones of Punjab of Pakistan which consists of about 52% of total area of rice under cultivation (Saqib *et al.,* 2018). The main rice producing areas in Punjab are Jhang, Gujranwala, Hafizabad, Sialkot, Sheikhupura, Okara, and Mandi Bahauddin (Chandio *et al.,* 2018). Most cultivated rice varieties in Punjab due to specific climate and soil characteristics are Super Kernel Basmati Rice (SK), Kainat (KN) and Supri (SP) (Gul *et al.,* 2022). The demand of brown rice (a rice without outer most layer and hull) is high in several countries due to its higher nutritional contents such as minerals, fiber, vitamins, and protein contents as compared to polished rice (Lamberts *et al.,* 2007). Similarly, other health improving components including aminobutyric acid, tocopherols, g-oryzanol, phytosterol (Gani *et al.,* 2012), proanthocyanidin, flavonoids and phenolics (Zhou *et al.,* 2014) are also present in brown rice.

However, rice exports of Pakistan have declined in last few years due to issue of mold growth and other factors such as climate change, transport costs and insecure trade routes (REAP, 2019). Brown rice is one of the favorable substrates for mold growth (Park *et al.,* 2005; Oguz, 2011). In various studies, different environmental factors such as moisture, humidity and temperature have been reported to affect mold growth and aflatoxins production (Choudhary and Kumari, 2010; Siruguri *et al*., 2012).

Aflatoxins are secondary metabolic compounds of defined species of fungi notably *Aspergillus flavus* and *Aspergillus parasiticus* (Karami-Osboo and Maham, 2018; Li *et al.,* 2018; Karami-Osboo *et al.,* 2020). Four major types of aflatoxins are aflatoxin B_1 (AFB_1), aflatoxin G_1 (AFG_1), aflatoxin B_2 (AFB_2), and aflatoxin G_2 (AFG_2), the mentioned four types are jointly recognized as total aflatoxins. These have been known to adversely affect human health especially AFB_1 that exhibits hepatotoxic, immune-suppressant, growth retardant, genotoxic and teratogenic characteristics (Bhardwaj *et al.,* 2020; Lu *et al.,* 2020; Yadav *et al.,* 2021).

International Agency for Research on Cancer (IARC) has ranked AFB_1 as group 1 category carcinogen. The global trade of food commodities has resulted in the worldwide contamination of aflatoxins (IARC, 2012; Althagafi *et al.,* 2021; Sun *et al.,* 2021). About 15% rice is lost every year due to inappropriate storage conditions causing fungal growth and aflatoxins production (Dors *et al.,* 2009).

Many countries have set aflatoxins permissible limits in many food stuffs due to their hazardous impacts on human health. The permissible limits established by the European Union for AFB_1 and total aflatoxins in rice are 2 µg/kg and 4 µg/kg respectively (Commission Regulation No. 1881/2006). Although Punjab Food Authority (PFA) has set maximum allowable total aflatoxins in all food substances i.e. 20 µg/kg (PPFR, 2018). However, regulatory authorities still needed work hard to enforce food regulations (Ashiq, 2015).

Various studies have reported the prevalence of aflatoxins in rice worldwide in which aflatoxins contents are higher than permissible limits (Tanaka *et al.,* 2007; Mazaheri, 2009; Elzupir, 2015; Ali, 2019). In a study conducted by Firdous *et al.* (2012) reported that 5.58% samples were contaminated with AFB_1 ranging from 2.01-16.65 µg/kg higher than the permissible limit (2 µg/kg) set by European Union.

In order to determine the extent of hazard that individuals are facing through consumption of a particular commodity in any region, it is necessary to monitor and assess the extent of contamination and adherence to limits established by regulatory authorities. Hence, the current research was planned to analyze levels of aflatoxins prevalent in different varieties of brown rice procured from two districts of Punjab. Moreover, the aim of study was to analyze the impact of seasonal and geographical variations on aflatoxins production in three varieties of brown rice. The seasonal and geographical impacts on aflatoxins production during storage will aid to develop effective strategies to control aflatoxins contamination in brown rice varieties.

MATERIALS AND METHODS

Sample Procurement: Different varieties of brown rice samples (total *n*=200) were procured from 15 warehouses from two districts of Punjab-Pakistan namely Multan (30.1575° N, 71.5249° E) and Gujranwala (32.166351° N, 74.195900° E). These two districts were selected for sampling primarily because of their different seasonal and geographical conditions. A total of 100 samples from each district were collected from warehouses with temperature range 20-25 ˚C and 45-55% relative humidity.

For seasonal analysis, brown rice samples were collected during the months of November 2019 and January, March and May of year 2020. These months were selected merely to analyze aflatoxins contents that are released in subsequent months of fungal growth. The collected brown rice varieties were corroborated in the department of Plant Protection Division, Nuclear Institute of Agriculture and Biology (NIAB) Faisalabad, Pakistan.

The rice varieties included in the study were Super Kernel Basmati Rice (*n*=88), Kainat (*n*=56) and Supri (*n*=56). In each region, total number of samples of SK, KN and SP were *n*=44, *n*=28 and *n*=28 respectively. One (6 kg) composite sample of each variety from both districts was prepared after mixing 15 sub-samples (each 400 g) drawn with the help of probe from different places (upper, middle and lower layers) of warehouse due to irregular fungal growth. Until for further inspection in laboratories of Faculty of Food Science and Nutrition, Bahauddin Zakariya University, Multan, brown rice samples were placed in plastic bags and stored at ₋4 ˚C to protect from moisture and other contaminants.

Moisture Analysis: The moisture of brown rice samples of different varieties was determined by following the AOAC methodology (AOAC, 2000).

Determination of Aflatoxins: Aflatoxins reference solution (Aflatoxin-Mix, CRM46303), trifluoroacetic acid (TFA) and Immunoaffinity columns (Eurofins, Siegen, Germany) were procured from Sigma-Aldrich (St. Louis, Mo., USA) and Romer Labs (Islamabad), respectively. Methanol and acetonitrile used during analysis were HPLC grade and purchased from Merck (Darmstadt, Germany).

Extraction and Purification: The powdered (particle size: 0.71 mm) brown rice samples (each 25 g) were added in 100 mL of methanol/water (60: 40 v/v) mixture and incubated on a rotary shaker (Thermo Scientific, Waltham, MA, USA) at ambient temperature (25 ˚C) at 200 rpm for 4-5 h. The resulting mixture were filtered using Whatman filter paper No. 42.

Afterwards, the extract was diluted by adding 16 ml sodium Phosphate Buffer Saline (50 mM) having pH 7.4 in 4 mL filtered extract. The diluted extracts were further purified by passing through immunoaffinity columns. The flow rate was 1-3 ml per minute under gravity. The columns were washed with 5 ml of 10 mM Phosphate Buffer Saline/methanol (90/10 v/v). The attached toxins were separated with methanol (2 ml) by elution.

Sample derivatization and HPLC analysis: Sample derivatization for subsequent AFs analysis was completed by following the official AOAC method 2005.08 (AOAC, 2005). In screw cap vial, both samples and standards were dried under nitrogen stream and were mixed with hexane (200 µl) to dissolve aflatoxins again. Then mixed it for 30 seconds after addition of 50 µl trifluoroacetic acid. The tightly closed vials having samples were placed in dark area for 5-6 min. Then, 1.95 ml of acetonitrile/double distilled water (9:1 v/v) was added in each vial followed by shaking for 1-2 minutes using a vortex mixture. Aqueous lower layer from resulting solution containing aflatoxins was separated from vial and purified by 0.45 µm syringe filters (Millex-HV, SLHV033R) to further run on HPLC system for chromatographic analysis. HPLC (Sykam S-500 routine series) having isocratic pump (Sykam, Eresing, Germany) combined with fluorescence detector (Sykam, RF-20A) was used. An inverse phase C-18 column made up with silica gel (Welch Material, Inc., Austin, TX, USA) was used as stationary phase having 250 mm length and 4.6 mm inner diameter.

The isocratic mobile phase had 55% water, 22.5% methanol and 22.5% acetonitrile and the flow rate was set to 1ml/min. The running time of both sample and standard was 20 min. Similarly, injected volume of both standard and sample was 20 µl. The wavelengths regarding emission and excitation of fluorescence detector on which elute was analyzed were 440 nm and 365 nm respectively. The column oven temperature was adjusted at 37 ℃. The retention times for aflatoxins including AFB_1, AFG_2, AFB_2 and AFG_1 were 5.09, 6.66, 8.78 and 4.28 min, respectively.

Method Authentication: HPLC Method was authenticated by observing linearity, limit of quantification (LOQ), limit of detection (LOD) and recovery percentage. Aflatoxins working solutions (3 to 12 µg/ml in acetonitrile) were used to calibrate HPLC system (Table 1). The limit of detection (LOD) and the limit of quantification (LOQ) were computed by following the method described by Kortei *et al.* (2021). The LODs for both AFB_1 and AFG_1 was 0.05 µg/kg and for both AFB_2 and AFG_2 was 0.03 µg/kg. Similarly, the LOQs for both AFB_1, and AFG_1 was 0.15 µg/kg and for AFB_2 and AFG_2 was 0.09 µg/kg. Aflatoxins free brown rice samples were spiked with aflatoxins standard solutions of different concentrations including 3, 6, 9 and 12 µg/kg. Recovery findings were in range of 91 to 97%, 96 to 99%, 94 to 97% and 96 to 98% for AFB_1, AFB_2, AFG_1, and AFG_2 respectively (Table 2).

Statistical analysis: Data was analyzed by using software Statistix 8.1 (Informer Tech. Inc., Los Angeles, CA, USA). All the samples were analyzed in triplicates.

The data was computed using Microsoft Excel 2013 version. For statistical analysis of data, the 3-way analysis of variance (ANOVA) with interaction and 3-factor (regions, varieties and months) under completely randomized design was performed followed by Least Significance Difference (LSD) test. The probability $p \leq 0.05$ was considered as significant. The regression analysis was performed to analyze relationship between moisture content and aflatoxins contamination.

RESULTS

Moisture content

Seasonal variation: Moisture content (%) in current study was observed in three different varieties of brown rice collected during different months. The highest moisture content was observed in SK collected during the month of March (13.95±2.04%) and lowest was observed in SP (8.40±2.29%) collected during the month of May. SK had significantly different ($p \leq 0.05$) moisture content in the month of March as compared to other two brown rice varieties. The lowest moisture values in three different brown rice varieties collected from two districts of Punjab were observed in the months of January and May.

Geographical variation: Among geographical variation, the highest moisture content 13.34±1.89% was observed in SK collected from Gujranwala and lowest 8.38±1.91% was observed in SP collected from Multan. A significant difference ($p \leq 0.05$) of moisture content (%) was observed between brown rice varieties and regions of Punjab.

Furthermore, overall results of moisture contents in different varieties of brown rice collected from Multan and Gujranwala are shown in Table 3.

Aflatoxins Incidence and Contamination

Seasonal variation: The highest AFB_1, AFB_2, and total aflatoxins were 7.87±2.20 µg/kg, 2.65±1.26 µg/kg and 10.56±3.41 µg/kg respectively in the month of March and lowest AFB_1, AFB_2, and total aflatoxins were 4.82±2.17

µg/kg, 1.03±1.27 µg/kg and 5.87±3.37 µg/kg respectively in the month of May. Aflatoxins contents were significantly different ($p \leq 0.05$) in brown rice varieties in each month. It was clear from results that the highest AFB_1 content was 9.73±2.21 µg/kg in SK procured in the month of March and the lowest was 3.74±2.12 µg/kg found in the month of May in SP samples. In current study, aflatoxin G_1 and aflatoxin G_2 were not detected in any of samples of brown rice collected from two districts of Punjab. In both areas of Punjab, the aflatoxins occurrence level in three varieties of brown rice were in order: March>November>January>May.

Moreover, in both areas of Punjab, 50 (25%) samples out of 200 were taken in each month. The highest samples 41 (82%) out of 50 were contaminated with aflatoxins in the month of March and lowest 21(42 %) out of 50 were contaminated in the month of May. In the month of March, 36(72%) out of 50 and 31(62%) out of 50 had higher AFB_1 and total aflatoxins contents than permissible limits (2 µg/kg) and (4 µg/kg), respectively.
Similarly, in the month of May, 20(40%) out of 50 and 17(34%) out of 50 had higher AFB_1 and total aflatoxins than permissible limits (2 µg/kg) and (4 µg/kg), respectively.

In both regions of Punjab, the aflatoxins occurrence level in three varieties of brown rice were in order: March>November>January>May. Among month and regions wise comparison, the highest AFB_1 content 9.35±1.83 µg/kg was observed in brown rice varieties in the month of March collected from Gujranwala and lowest AFB_1 content 3.73±0.93 µg/kg was analyzed in the month of May collected from Multan.

Geographical analysis: In current study, aflatoxins contents in brown rice varieties collected from two districts of Punjab were analyzed. Aflatoxins contents differed significantly ($p \leq 0.05$) among Punjab districts. AFB_1, AFB_2 and total aflatoxins were significantly higher ($p \leq 0.05$) in brown rice procured from Gujranwala than Multan. The highest AFB_1 content 7.23±1.79 µg/kg was observed in Gujranwala and lowest 4.81±1.53 µg/kg was observed in Multan.

In addition, the highest AFB_1 content was 8.53±1.72 µg/kg in SK procured from Gujranwala and the lowest was 3.51±0.91 µg/kg found in SP samples collected from Multan. Among districts of Punjab, a total of 200 samples were taken out of which 125 samples were positive. Total 100(80%) samples out of 125 samples had higher AFB_1 content than permissible limit (2 µg/kg). Moreover, 94(75%) samples had greater total aflatoxin contents than allowable limit (4 µg/kg). The highest contamination level (71% out of 100 samples) of aflatoxins in different varieties of brown rice was observed in Gujranwala and lowest (54% out of 100 samples) was observed in Multan.

In the current study, it was examined that aflatoxins contamination level is directly correlated with moisture content in brown rice grains. A strong relationship between aflatoxins contents and moisture level in brown rice is given in Figure 1. The linear regression relationship between aflatoxins and moisture was computed by following equation:

Y = moisture coefficient(x) – intercept coefficient

Where:
Y = Aflatoxins contamination (AFB_1 or AFB_2 or AFs);
x = moisture

Among regression statistics, intercept coefficient values after regression analysis for AFB_1, AFB_2 and AFs were -3.6273 (p-value= 0.00), -3.7392 (p-value= 0.00) and -7.340 (p-value= 0.00) respectively. Similarly, moisture coefficient values with AFB_1, AFB_2 and AFs were 0.8549 (p-value= 0.00), 0.4944 (p-value= 0.00) and 1.3499 (p-value= 0.00) respectively. Coefficient of determination (R^2) value between AFB_1 and moisture was 0.7463. Similarly, coefficient of determination (R^2) of AFB_2 and AFs with moisture were 0.7773 and 0.7954 respectively. Regression analysis endorsed inference regarding contamination by aflatoxins in brown rice varieties due to moisture. The p-values for moisture coefficient showed that moisture was significant ($p \leq 0.05$) for aflatoxins contamination in brown rice varieties during storage.

Moreover, overall results regarding seasonal and geographical analysis of aflatoxins contents in three brown rice varieties are presented in Table 4. The highest AFB_1 11.37±0.46 µg/kg and total aflatoxins 16.2±0.42 µg/kg were observed in the month of March in SK collected from Gujranwala and lowest AFB_1 2.65±0.20 µg/kg and total aflatoxins 2.88±0.22 µg/kg were observed in the month of May in SP procured from Multan. SK collected from Gujranwala during month of March had significantly different ($p \leq 0.05$) AFB_1, AFB_2 and AFs contents than other brown rice varieties collected during other months (November, January and May) from two districts of Punjab.

Table 1. Linear regression parameters for aflatoxins

Aflatoxins	Conc. (µg/kg)	Slope	Intercept	R^2
AFB_1	3-12	1.068	0.03	0.9984
AFB_2	3-12	1.034	0.005	0.9989
AFG_1	3-12	1.074	0.135	0.9992
AFG_2	3-12	1.042	0.09	0.9998

AFB_1= Aflatoxin B_1, AFB_2=Aflatoxin B_2, AFG_1= Aflatoxin G_1, AFG_2= Aflatoxin G_2

Table 2. Recoveries (%) of aflatoxins in brown rice

Aflatoxins	Spiking conc. (µg/kg)	Measured Conc. ±SD*	Recovery (%)	(%) RSD**
AFB_1	3	2.73±0.02	91	0.73
	6	5.84±0.03	97	0.51
	9	8.34±0.04	93	0.42
	12	11.24±0.03	94	0.31
AFB_2	3	2.87±0.02	96	0.70
	6	5.76±0.03	96	0.44
	9	8.88±0.06	99	0.68
	12	11.49±0.07	96	0.62
AFG_1	3	2.85±0.02	95	0.73
	6	5.80±0.03	97	0.53
	9	8.56±0.04	95	0.52
	12	11.23±0.06	94	0.53
AFG_2	3	2.93±0.03	98	0.85
	6	5.88±0.05	98	0.93
	9	8.75±0.04	97	0.41
	12	11.56±0.05	96	0.44

*Standard Deviation; **RSD Relative Standard Deviation, AFB_1= Aflatoxin B_1, AFB_2=Aflatoxin B_2, AFG_1= Aflatoxin G_1, AFG_2= Aflatoxin G_2

Table 3. Moisture contents in three different brown rice varieties collected from two districts of Punjab

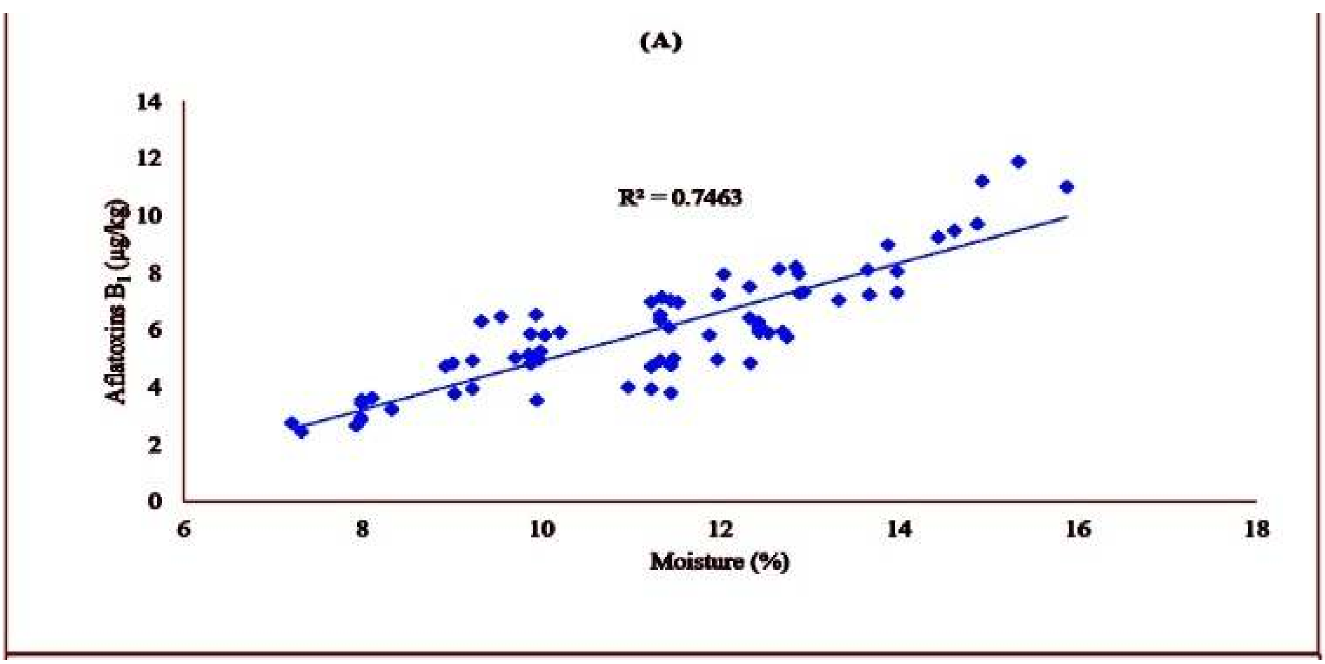
Regions	Months	Varieties**	Moisture(%)±SD*
Multan	Nov	SK	12.49±0.19^{def}
		KN	11.59±0.33^{efg}
		SP	8.03±0.07^{jk}
	Jan	SK	12.39±0.45^{efg}
		KN	11.22±0.24^{gh}
		SP	7.84±0.57^{jk}
	Mar	SK	12.52±0.42^{def}
		KN	12.07±0.64^{efg}
		SP	9.92±0.05^{i}
	May	SK	11.67±0.59^{efg}
		KN	9.40±0.48^{i}
		SP	7.74±0.37^{k}
Mean	**04**	**03**	**10.57±1.89**
Gujranwala	Nov	SK	13.84±0.17^{bc}
		KN	12.07±0.77^{efg}
		SP	9.61±0.31^{i}
	Jan	SK	12.72±0.34^{cde}
		KN	11.73±0.61^{efg}
		SP	9.85±0.14^{i}
	Mar	SK	15.38±0.47^{a}
		KN	14.65±0.22^{ab}
		SP	13.66±0.32^{bcd}
	May	SK	11.40±0.15^{fg}
		KN	10.04±0.17^{hi}
		SP	9.06±0.15^{ij}
Mean	**04**	**03**	**12.0±2.09**

Super scripts (^{a,b,c,d,e,f,g,h,i,j,k}) in same column show significant difference ($p \leq 0.05$); *SD means Standard deviations; **SK= Super Kernel, KN= Kainat, SP= Supri

Table 4. Seasonal and geographical analysis of aflatoxins in three varieties of brown rice collected from two districts of Punjab.

Regions	Months	Varieties**	Samples (*n*)	Aflatoxins (µg/kg)±SD*		
				AFB_1	AFB_2	Afs
Multan	Nov	SK	07(11)	6.13±0.26^{ef}	1.81±0.07^{d}	7.99±0.31^{de}
		KN	05(07)	4.97±0.35^{hi}	1.56±0.04^{hi}	6.74±0.13^{hi}
		SP	04(07)	3.54±0.10^{m}	0.49±0.06^{l}	4.08±0.11^{l}
	Jan	SK	05(11)	5.83±0.08^{g}	1.68±0.03^{efg}	7.62±0.10^{ef}
		KN	03(07)	3.92±0.10^{kl}	1.53±0.07^{i}	5.49±0.09^{j}
		SP	03(07)	2.97±0.24^{n}	0.36±0.03^{no}	3.36±0.23^{m}
	Mar	SK	08(11)	8.09±0.13^{c}	2.69±0.05^{b}	10.9±0.34^{b}
		KN	05(07)	6.20±0.31^{ef}	1.78±0.03^{de}	8.02±0.31^{de}
		SP	04(07)	4.89±0.07^{i}	0.28±0.03^{op}	5.18±0.08^{j}
	May	SK	05(11)	4.78±0.06^{i}	1.56±0.04^{i}	6.39±0.13^{i}
		KN	03(07)	3.76±0.20^{lm}	1.43±0.04^{j}	5.22±0.18^{j}
		SP	02(07)	2.65±0.20^{o}	0.22±0.03^{p}	2.88±0.22^{n}
Mean	**04**	**03**	**54(100)**	**4.81±1.56**	**1.28±0.77**	**6.15±2.27**
Gujranwala	Nov	SK	09(11)	8.38±0.52^{c}	3.86±0.08^{b}	12.24±0.58^{c}
		KN	06(07)	7.46±0.45^{de}	2.73±0.13^{c}	10.19±0.34^{de}
		SP	04(07)	6.44±0.11^{fg}	1.85±0.11^{f}	8.29±0.12^{f}
	Jan	SK	09(11)	7.38±0.12^{e}	2.75±0.07^{c}	10.14±0.05^{e}
		KN	05(07)	6.24±0.13^{g}	2.11±0.10^{e}	8.35±0.23^{f}
		SP	03(07)	5.14±0.11^{hi}	1.34±0.12^{ij}	6.48±0.23^{h}
	Mar	SK	11(11)	11.37±0.46^{a}	4.83±0.04^{a}	16.2±0.42^{a}
		KN	07(07)	9.48±0.23^{b}	3.92±0.05^{b}	13.40±0.18^{b}
		SP	06(07)	7.20±0.13^{e}	2.39±0.06^{d}	9.59±0.15^{e}
	May	SK	06(11)	7.00±0.02^{ef}	1.23±0.09^{jk}	8.23±0.09^{f}
		KN	03(07)	5.88±0.05^{g}	1.05±0.06^{k}	6.92±0.05^{gh}
		SP	02(07)	4.84±0.09^{i}	0.72±0.14^{l}	5.56±0.11^{i}
Mean	**04**	**03**	**71(100)**	**7.23±1.84**	**2.39±1.28**	**9.63±3.07**

Super scripts (^{a,b,c,d,e,f,g,h,i,j,k,l,m,n,}) in same column show significant difference ($p \leq 0.05$); *SD means Standard deviations; **SK= Super Kernel, KN= Kainat, SP= Supri, AFB_1= Aflatoxin B_1, AFB_2=Aflatoxin B_2, AFs= Total aflatoxins



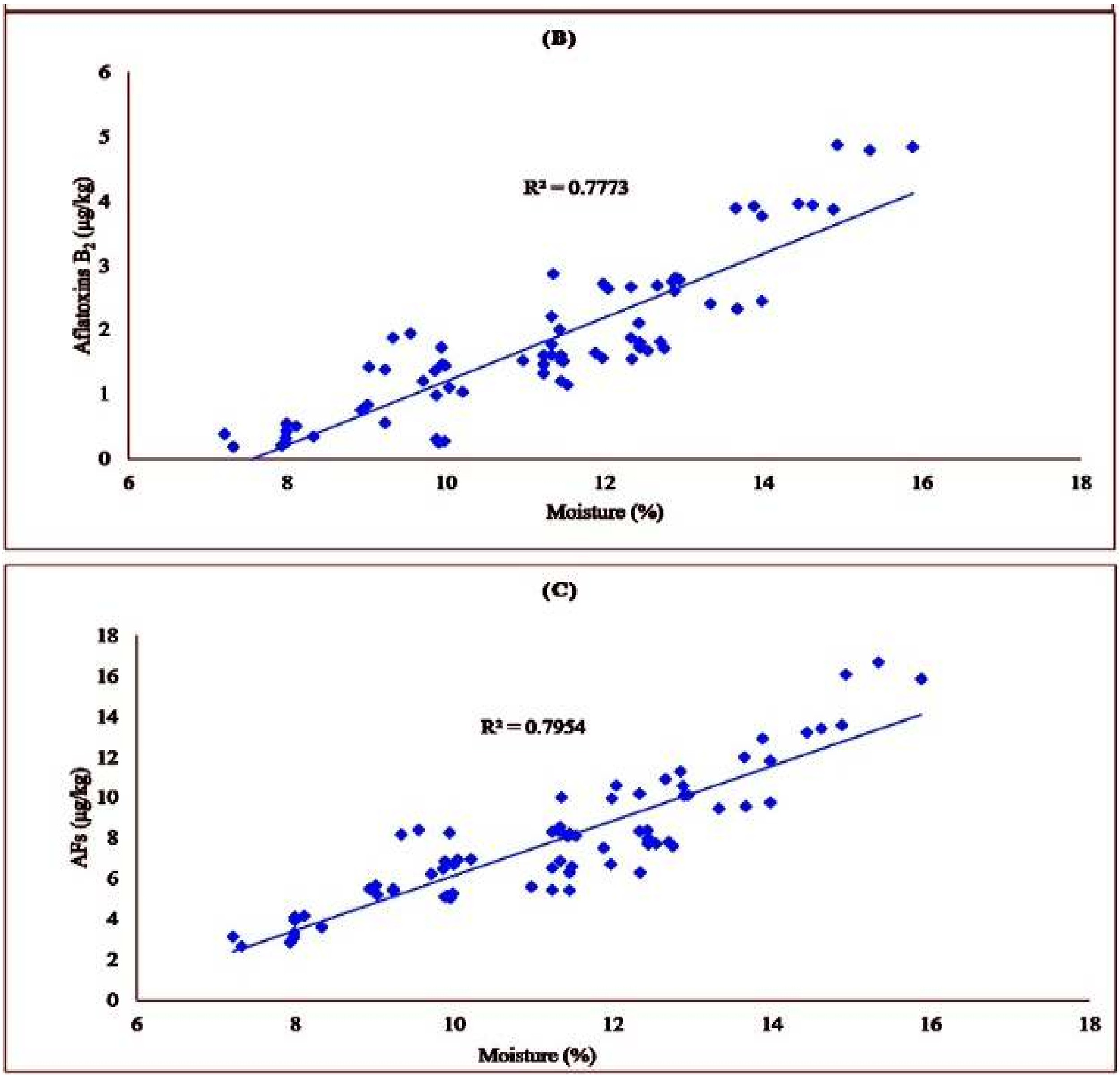


Figure 1. Scatter diagram showing correlation between moisture and aflatoxins; A (AFB_1), B (AFB_2) and C (AFs) in three brown rice varieties collected from two districts of Punjab.

DISCUSSION

The moisture content and aflatoxins contamination in brown rice varieties in the month of March collected from Gujranwala were higher than Multan (Table 3 and 4). These findings may be due to wet season and geographical location of Gujranwala which are appropriate for fungal growth and aflatoxins production. Aflatoxins contamination is mostly observed due to relatively higher humidity and temperature. Moisture contents of rice at harvesting time is mostly between 16-37% which is most suitable for fungal growth especially *Aspergillus* species including *Aspergillus parasiticus* and *Asergillus flavus* (Reiter *et al.,* 2010; Sarker *et al.,* 2015).

The recommended moisture level for brown rice storage (more than one year) in rodent proof room is ≤9% that is mostly labelled on plastic bags (Naseer *et al.,* 2014; Su´arez-Bonnet *et al.,* 2013). In Pakistan, flood irrigation is mostly adopted to cultivate rice crop resulting in humid conditions which promote fungal growth and aflatoxins production (Redfern *et al.,* 2012). The pre and post-harvest aflatoxins contamination may occur mostly if moisture contents, temperature and water activity are beyond the recommended limits and the produce is not dried on time (Asghar *et al*., 2013; Lv *et al.,* 2019; Muga *et al.,* 2019).

In current study, the average moisture contents for three brown rice varieties collected from two districts of Punjab ranged from 7.74±0.37 to 15.38±0.47% (Table 3). Similarly, average AFB_1, AFB_2 and AFs contents in three brown rice varieties collected from two districts of Punjab ranged from 2.65±0.20 to 11.37±0.46 µg/kg, 0.22±0.03 to 4.83±0.04 µg/kg and 2.88±0.22 to 16.2±0.42 µg/kg respectively (Table 4). Our results were also in line with the observations of Mukhtar *et al.* (2016) in which moisture contents and aflatoxins contamination in super kernel basmati rice samples (*n*=48) were examined. Average moisture content in super kernel rice samples collected from four districts of Punjab ranged from 9.5±0.92 to 10.7±1.16%. The total 28 out of 48 rice samples were contaminated with total aflatoxins. Among 28 contaminated rice samples, 27 samples were positive for AFB_1 and 16 were positive for AFB_2. All the 27 out of 28 contaminated rice samples had higher AFB_1 contents than permissible limit (2 µg/kg). Similarly, 26 rice samples out of 28 had higher total aflatoxins contents than allowable limit (4 µg/kg). In addition, a strong positive relationship was observed between aflatoxins (B_1+B_2) and moisture contents in which coefficient of determination value (R^2) was greater than 0.6.

In our study, total 125 out of 200 (62.5%) samples had aflatoxins contamination. Among 62.5% positive samples, 80% (100) samples had higher AFB_1 than allowable limit (2 µg/kg). Moreover, 94 out of 125 samples had greater AFs contents than permissible limit (4 µg/kg). A similar study was reported by Reddy *et al.* (2009) in which AFB_1 content was observed in total 1200 rice samples that were exposed to rain due to open storage stacked in field. Among 1200 rice samples, 67.8% rice samples were contaminated with AFB_1 ranging from 0.5 to 38.5 µg/kg. The findings reported by Asghar *et al.* (2014) were also in agreement with our study in which AFs contents were increased in brown rice due to high moisture contents (≥30%). Total 95.4% brown rice samples were contaminated with AFs and its range was 1.07 to 27.27 µg/kg. Same findings were reported by Taligoola *et al.* (2011) in Uganda where Pakistani rice samples were analyzed in which AFB_1, AFB_2 and total aflatoxins contents ranged from 16.08 to 120.06 µg/kg, 4 to 20 µg/kg and 20 to 50 µg/kg respectively.

The highest aflatoxins contamination and moisture content in current study were examined in SK. These results may be due to lowest tocopherol contents in SK as compared to other two brown rice varieties. Almost similar study was reported by Iqbal *et al.* (2014) in which tocopherol level (vitamin E) and aflatoxins contamination were examined in five Pakistani rice varieties including Super Basmati Rice, KS-282, Basmati PK-385, IRRI-6 and IRRI-9. The rice varieties that had higher tocopherol contents had lower level of aflatoxins contamination. The highest 53.2 ± 1.5 mg/100 and lowest 40.2 ± 2.5 mg/100 tocopherol levels were examined in IRRI-9 and Super Basmati Rice respectively. Similarly, highest 12.45 µg/kg and lowest 8.9 µg/kg total aflatoxins contents were observed in Super Basmati Rice and IRRI-9 respectively.

Conclusion: Aflatoxins contamination in three brown rice varieties collected from two districts of Punjab is a major point of concern. It is concluded that all three brown rice varieties (SK, KN, and SP) collected from two districts of Punjab during different months had different contents of aflatoxins and moisture values. A direct relationship was examined between aflatoxins contents and moisture. Hence, it is very important to manage moisture level in brown rice varieties during storage to inhibit fungal growth and aflatoxins production.

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