

Aflatoxin contamination in stored rice variety PAU 201 collected from Punjab, India

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Background & objectives: The present study was carried out on stored rice variety PAU 201 in Punjab that was not permitted for milling and public distribution due to the presence of damaged grains at levels exceeding the regulatory limits of 4.75 per cent. The aim of the study was to determine fungal and aflatoxin contamination in the rice samples to assess hazard from the presence of damaged grains. Presence of iron in discoloured rice grains was also assessed.

Methods: Stored samples of paddy of PAU 201 rice variety were collected from six districts of Punjab, milled and analysed for presence of fungal and aflatoxin contamination. Scanning electron microscopy (SEM), energy dispersive X-ray (EDX) analysis and Prussian blue staining was used to determine fungal spores and presence of iron, respectively.

Results: Aflatoxin analysis of rice samples indicated that none exceeded the Food Safety and Standards (Contaminants, Toxins and Residues) Regulations, 2011 tolerance limit of 30 µg/kg and majority of the samples had levels <15 µg/kg. The proportion of damaged grains exceeding the limit of 5 per cent was observed in 85.7 per cent of the samples. SEM and Prussian blue staining and EDX analysis of black tipped and pin point damaged rice grains did not show presence of fungal structures and presence of iron.

Interpretation & conclusions: The results of the study indicated that the stored rice samples did not pose any health concern with respect to aflatoxin contamination as per the criteria laid down by the Food Safety and Standards Authority of India.

Key words Aflatoxins - damaged grains-rice - fungal growth

Aflatoxins are secondary metabolites produced by the *Aspergillus* species namely *A. flavus*, *A. parasiticus* that contaminate a variety of agricultural and food commodities¹. These mycotoxins are recognized to be

hepatotoxins and carcinogens for humans². Because aflatoxins are unavoidable contaminants of foods, the World Health Organization (WHO) urged that their level be reduced to as low as reasonably achievable².

Regulatory authorities in different countries have set tolerance limits for aflatoxins that range from 0 to 50 µg/kg to control their levels in the food supply³. In India, a tolerance limit of 30 µg/kg has been prescribed under the Food Safety and Standards (Contaminants, Toxins and Residues) Regulations, 2011, for all foods meant for human consumption⁴.

Rice under normal conditions is not considered a commodity conducive for growth of *Aspergillus* and aflatoxin contamination but when exposed to heavy rains or high humidity could become susceptible to aflatoxin contamination^{5,6}. In India, high levels of aflatoxins have been detected in rain damaged rice grains and paddy⁷. High aflatoxin levels have also been reported in parboiled rice⁸. Various surveys conducted in different parts of the world indicated considerable levels of aflatoxins and ochratoxins in rice^{9,10}. Since rice is a staple food for majority of the worlds' population, occurrence of aflatoxins as contaminants can be a serious health concern.

A high yielding rice variety PAU201 was released in 2007 for cultivation in Punjab region by the Punjab Agricultural University (PAU), Ludhiana, India. The PAU 201 rice variety was reported to have a high percentage of black tipped/pin point damaged grains classifiable as 'damaged grains' under the Food Safety and Standards Authority of India (FSSAI) regulations, the limit of which should not be more than 5 per cent¹¹. Damaged grains have been defined as kernels or pieces of kernels that are sprouted or internally damaged as a result of heat, microbe, moisture or weather, namely, ergot affected grain and karnal bunt affected grains¹¹. Uniform quality specifications to food grains specifications are set by the Food Corporation of India (FCI)¹². The uniform specifications for presence of damaged grains in rice are 3 per cent and 1 per cent over this is permitted for presence of pin point damaged grains giving a total limit of 4 per cent¹². These specifications were relaxed for PAU 201 variety from 4 to 4.75 per cent that include pin point damaged grains¹³.

During 2009-2010 the paddy of PAU 201 variety rice was not permitted for milling and subsequent public distribution due to the presence of damaged grains at levels exceeding the regulatory limits. As a result, 40 lakh tonnes of the paddy were retained in storage in rice mills and FCI godowns. The Indian Council of Medical Research (ICMR) of the Department of Health Research at the request of the Ministry of Health, Government of

India, undertook a survey with the objective to assess the extent of fungal and aflatoxin contamination in the stored paddy samples of PAU 201 collected from different districts of Punjab, and to assess whether black tipped or pinpoint damaged grains contain iron.

Material & Methods

The study was carried out in samples collected from six districts of Punjab namely Barnala, Bathinda, Faridkot, Mansa, Moga and Muktsar on the basis of availability of PAU 201 stocks in mills of these districts. Sample collection was done from August 21-23, 2010.

Collection of samples: Samples were collected from 35 rice mills in 23 locations in six districts of Punjab (Table I). A total of 35 paddy samples of PAU 201 variety were collected. In addition, 11 damaged rice samples containing discoloured, mould and insect damaged grains that were segregated by a sortex machine in the mills were also collected. Sorted damaged samples could not be collected from all the mills as only a few were functional at the time of collection of samples.

Sampling procedure: Samples were collected as per procedures recommended by the Directorate General of Health Services (DHGS)¹⁴. The quantity of paddy stored at each location ranged from 27,000 to 1,60,000 bags with 35 kg paddy per bag. At each location 2 to 10 stacks were randomly selected. An aggregate of 15-20 kg paddy sample was collected from each of the 35 mills. After thoroughly mixing about 7-10 kg per sample was separated for analysis of aflatoxin. Damaged rice sample weighing 1 kg each were collected from sortex machines in 11 rice mills.

Sub-sampling for analytical work: From each sample collected around 1 kg sample was separated using the method of quartering. The paddy samples were milled and polished in a laboratory mill resulting in an average yield of 34±7.4 per cent of head rice (range 23 to 52%) and average brokens of 35.96±7.57 (range 23-64%). A total of 150 g of the milled rice was homogenized in a laboratory mill for aflatoxin analysis. Damaged rice samples obtained from the sortex machine at the rice mills were similarly sampled and homogenized.

Estimation of percentage of damaged grains: Damaged grains were segregated into discoloured, black tipped and pin point damaged grains from a sub-sample of 20 g of milled and polished rice obtained from the paddy samples, weighed and the percentage of damaged grains in each sample was calculated as per Bureau of Indian Standards (BIS) protocols¹⁵.

Table I. Details of samples collected

Sl. no.	District	Location	Sample collected	
			Sample	Number
1	Barnala	Thuliwal	Paddy	1
			Sorted damaged rice*	1
2	Bathinda	Rampura	Paddy	8
		Rampura	Sorted damaged rice	2
		Rampura Phul Road, Rampura		
		Rampura Phul Road, Rampura		
		Bhucho		
		Bhucho		
		Bathinda		
		Gubnianana Mandi dist Bathinda		
3	Mansa	Bhikhi	Paddy	13
		Bhikhi Mansa	Sorted damaged rice	8
		Budlada, Bikhi Road Borawale		
		Biro ke kala		
		Mansa Khurd Road		
		Barnala Road Mansa		
		Barnal Road Mansa		
		Talbandi Road Mansa		
		Talbandi Road Mansa		
		FCI Depot Mansa		
		Ubhah Mansa		
		Burj Chhabar Road Vill Akalia Mansa		
		Akalia Mansa		
4	Muktsar	Muktasar Godown, Giderwaha	Paddy	6
		Giderwaha		
		Madhir Road Giderwaha		
		Butter Bakhua Giderwaha		
		Butter Bakhua Road Giderwaha		
		Butter Bakhua Road Giderwaha		
5	Faridkot	Gomti Khurd Bahanda Road Jaitu Dist Faridkot	Paddy	5
		Jaitu Bhagatuana Road Dist Faridkot		
		Bachnindi Road Jaitu Faridkot		
		Bachnindi Road Jaitu Faridkot		
		Faridpur Road Hasipul Open Faridkot		
6	Moga	Kot Ise Khan Dist Moga	Paddy	2
		Kot Ise Khan Dist Moga		
			Paddy	35
		Total	Sorted damaged rice	11

*sortex segregated damaged rice collected from mills after milling paddy

Analysis of aflatoxins: Aflatoxins were analysed at the National Institute of Nutrition (NIN), Hyderabad, using HPLC methods as per protocol of AOAC Official Method 990.33¹⁶. The method had a Limit of Quantification (LOQ) of 5 µg/kg. Aflatoxin analysis was performed by two additional laboratories *viz.*, Vimta Labs, Hyderabad and Export Inspection Agency (EIA), Kochi for quality assurance of the data generated by the main laboratory. EIA used HPLC-MS-MS method using immunoaffinity column cleanup as per AOAC official method 991.3 with an LOQ of 2.5 µg/kg for total aflatoxins¹⁷. Vimta Labs used LC-MS-MS method with an LOQ of 1 µg/kg for total aflatoxins¹⁸.

Scanning electron microscope (SEM) study for detection of fungal growth: SEM studies using Hitachi S-3400N scanning electron microscope were performed on the rice samples milled from paddy¹⁹ in NIN, Hyderabad, to look at the surface as well as in the transverse sections of the rice grain samples that were segregated as apparently normal appearing, pin point damaged, partially damaged and fully damaged rice. As control, a local market variety was used. Before scanning grains were cleaned to remove dust and other visible particles and coated with Gold (600 A°) using E-1010 Sputter coating unit and studied at 15 KV. Pictures were captured from 50 to 7500X magnification.

Detection of iron in black tipped/pin point damaged rice grains: Prussian blue staining method: The test is based on the formation of a bright blue pigment called Prussian blue, or ferric ferrocyanide in the presence of any ferric ion⁽⁺³⁾ in the tissue/grains²⁰. Prussian

blue staining was performed on the rice grain samples segregated as normal, black tipped, pin point damaged, partially damaged and fully damaged. As controls local market rice and Ultra rice which is a reconstructed artificial rice made from iron fortified rice flour, were used.

Energy dispersive X-ray (EDX) analysis: The technique was used for identifying the elemental composition of the specimen, or an area of interest thereof. The EDX analysis system works as an integrated feature of a scanning electron microscope. For EDX analysis, the samples were placed on the microscope stub with double sided adhesive tape to study the elemental composition of the rice samples²¹.

Statistical analysis: The data were analysed using SPSS Windows Version 19.0, USA. Intra-class correlation was computed to see the agreement between the analytical laboratories. Correlation between per cent damage and aflatoxin levels was also computed.

Results

Of the 35 paddy samples analyzed after milling, none was found to be contaminated with aflatoxins at levels exceeding the prescribed regulatory limits of 30 µg/kg. Aflatoxin levels were below 15 µg/kg in 97.1 per cent of the samples. Similar results were also obtained by EIA and Vimta Labs where 94.3 and 91.4 per cent of samples had aflatoxins at less than 15 µg/kg, respectively (Table II). Results of three laboratories indicated good agreement as revealed by average measures of intra-class correlation coefficient (0.70, $P < 0.001$).

Table II. Levels of aflatoxins (µg/kg) in PAU 201 rice variety samples

Laboratory*	Aflatoxin levels (µg/kg) ¹ No. of samples							
	Milled paddy samples				Damaged rice grains ²			
	BLOQ/ND	<15	15-30	>30	BLOQ/ND	<15	15-30	>30
NIN	32 (91.4)	2 (5.7)	1 (2.8)	Nil	1 (9%)	2 (18.2%)	1 (9)	7 (63.6)
EIA	Nil	33 (94.3)	2 (5.7)	Nil	Nil	1(9%)	3 (27.3)	7 (63.6)
VIMTA	2 (5.7)	30 (85.7)	3 (8.6)	Nil	Nil	Nil	3 (27.3)	8 (72.7)
Total No. of samples		35					11	

¹Aflatoxins analysed: NIN: Aflatoxin B₁+ B₂ ; EIA: B₁+ B₂+G₁+G₂; Vimta Labs: B₁+ B₂+ G₁+G₂
²Damaged grains segregated from sortex machine in rice mills
 Figures in parentheses represent % samples
 *Agreement between the results of three laboratories statistically significant at $P < 0.001$. Average measures of intra-class correlation coefficient=0.70; ND, Not detected; BLOR, below the limit of quantification.
 Limit of Quantification (µg/Kg): NIN- B₁:5; B₂:1; EIA- B₁,B₂,G₁& ,G₂: 2.5 ; VIMTA: B₁,B₂,G₁& ,G₂: 1;

Table III. Level of damaged grains in milled paddy samples

	Level of damaged grains					
	<5 (Total)*	>5 (Total)	≤1 ppd**	>1 ppd	<5 (without ppd)	>5 (without ppd)
No. (%) of samples	5(14.3)	30(85.7)	14(40)	21(60)	14(40)	21(60)
Mean % damage	4.05 ± 0.69	8.02 ± 2.03	0.8 ± 0.17	1.43 ± 0.4	4.17 ± 0.75	7.69 ± 1.59
Range % damage	3.1 - 4.65	5.15 - 12.25	0.47 - 1.0	1.02 - 2.53	2.6 - 4.9	5.2 - 10.4
Weight of damaged grains (g) (range)	0.62 - 0.93	1.03 - 2.45	0.09 - 0.2	1.20 - 0.51	0.52 - 0.98	1.04 - 2.08
Mean±SD (g)	0.81 ± 0.14	1.6 ± 0.41	0.16 ± 0.03	0.28 ± 0.08	0.83 ± 0.15	1.54 ± 0.32

*All damaged grains including pin point damage; **ppd, pin point damaged grains only
Total no. of paddy samples = 35

Aflatoxin analysis of damaged rice grains segregated by sortex machine in rice mills indicated that the regulatory limit of 30 µg/kg exceeded in 7, 7 and 8 samples of the 11 damaged rice samples analysed by NIN, EIA and Vimta Labs, respectively with levels ranging from below the limit of quantification (BLOQ) to 573 µg/kg (Table II).

The percentage of damaged grains consisting of discoloured, black tipped and pin point damage ranged from 3.1 to 12.25 per cent (Table III). Around 14 per cent of the samples had below 5 per cent damage, as per FSSAI limits. The percentage of pin point damaged grains ranged from 0.47 to 2.53 with 40 per cent of the samples having less than 1 per cent damage whereas 60 per cent of the samples exceeded the FCI specifications of 1 per cent. The percentage of damaged grains remained almost same with or without presence of pin point damaged grains. No significant correlation was observed between per cent damage and aflatoxin levels as shown by spearman's rank correlation ($r_s = -1.34$ and $P=0.44$).

No evidence of fungal contamination was seen in the rice samples collected (control) from the local market (Figs 1 and 2). SEM studies of the rice samples indicated no evidence of fungal presence on surface or at a deeper plane in the apparently normal, pin point damaged and partially damaged grains of PAU-201 rice variety (Figs 3-6). On the other hand, SEM study of the sorted, fully damaged rice grains showed fungal structures with spores both on surface and deeper regions (Fig. 7).

Prussian Blue staining to assess presence of iron in different categories of damaged/discoloured grains of the PAU 201 rice revealed that except for the Ultra rice (reconstituted artificial rice with iron fortified rice flour) none of the other samples stained blue thus indicating that the black spots of PAU-201 variety are not due to presence of iron. EDX analysis of pin point damaged grains also did not indicate presence of iron.

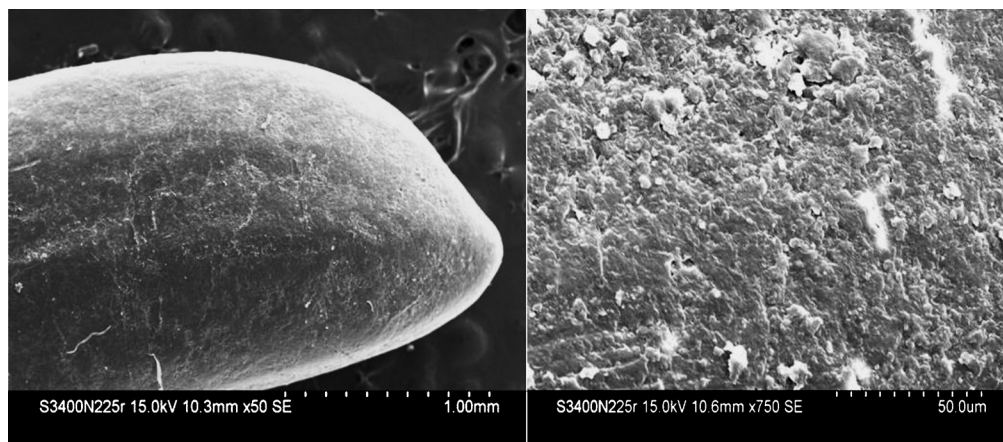


Fig. 1. SEM surface sections (at 50X & 750X magnification) of normal rice (market control) showing normal waxy and granular appearance.

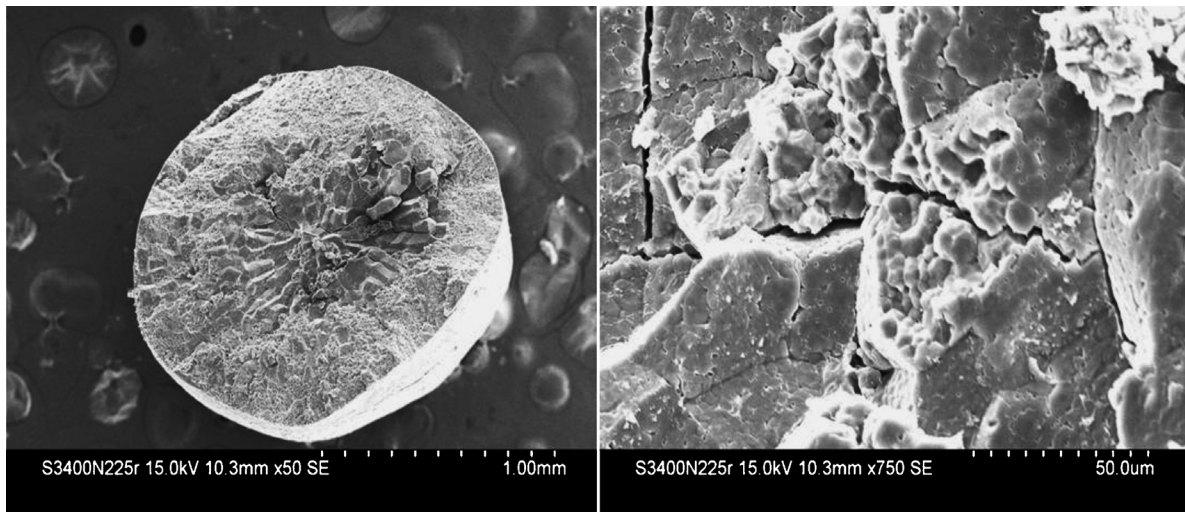


Fig. 2. SEM transverse sections (at 50X & 750 X magnification) of normal rice (market control) showing normal starch granule structure.

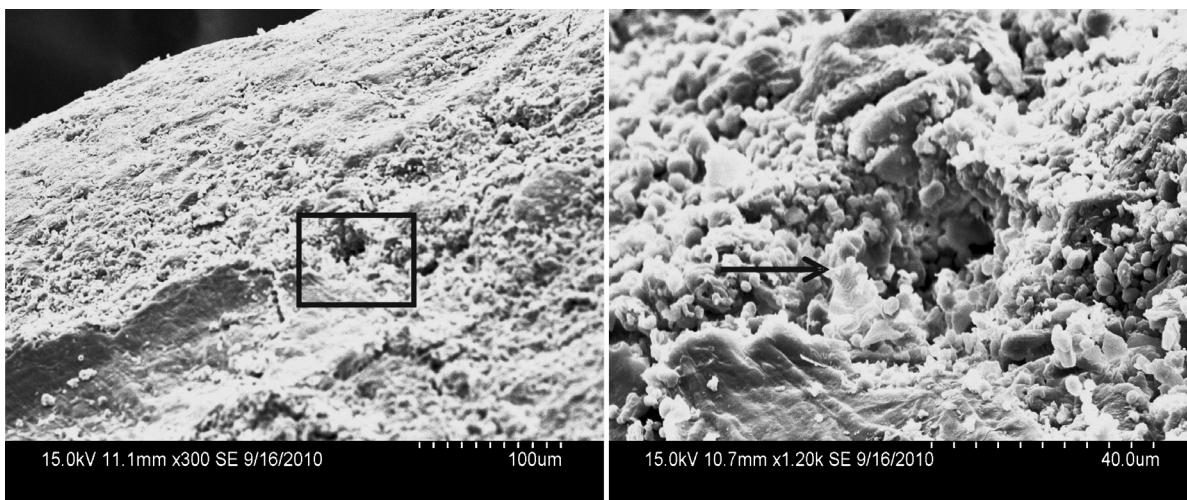


Fig. 3. SEM surface sections (at 300X & 1200X magnification) of apparently normal PAU 201 rice showing pin point hole (in box) & absence of fungal structures (arrow).

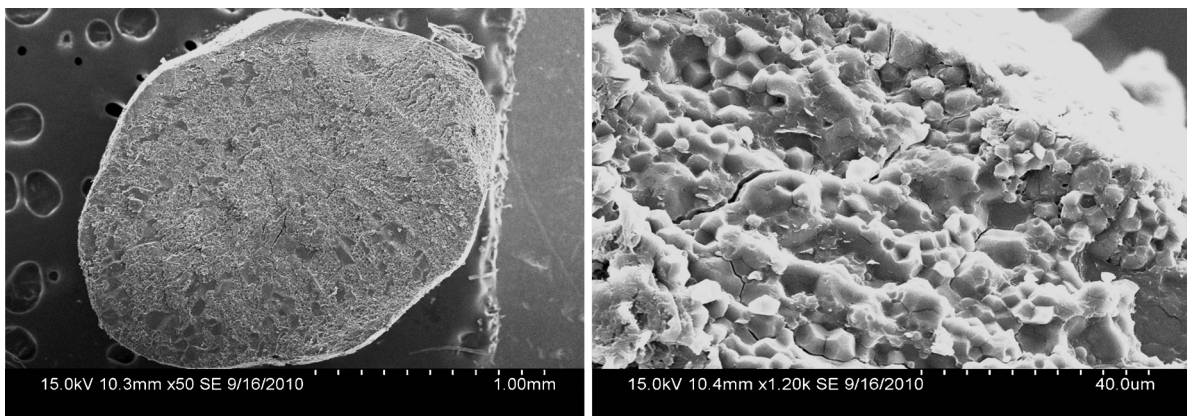


Fig. 4. SEM transverse sections (at 50X & 1200X magnifications) of apparently normal PAU 201 rice showing normal structures.

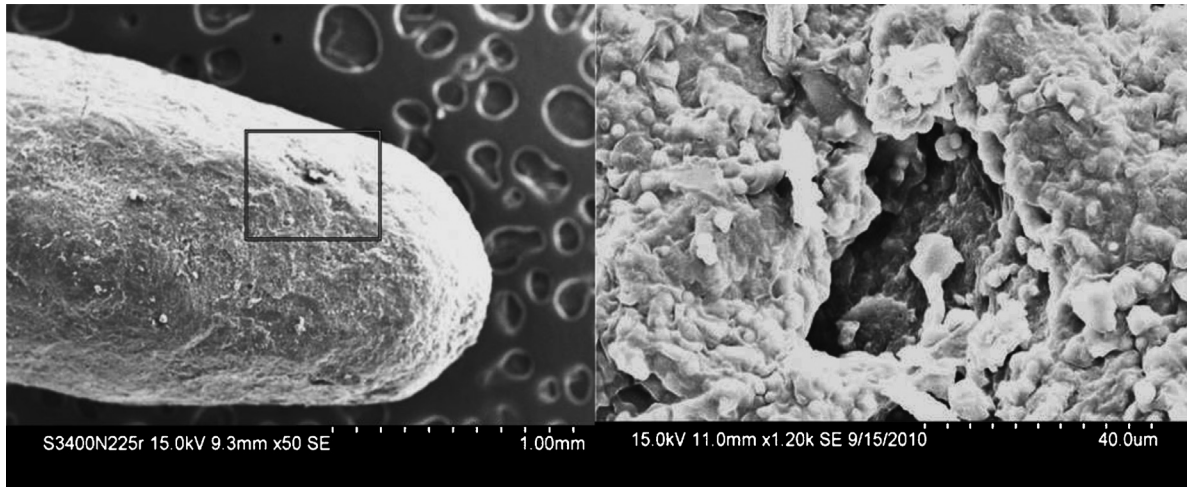


Fig. 5. SEM surface sections (at 50X & 1200X magnification) of pin point damaged PAU 201 rice showing affected areas (in box) and absence of fungal structures.

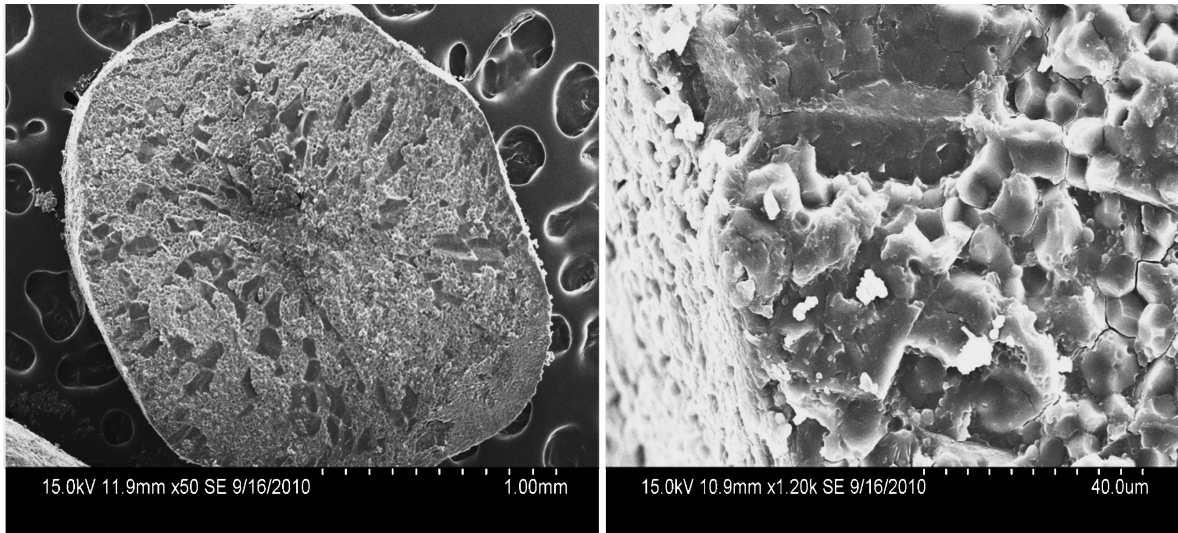


Fig. 6. SEM transverse sections (at 50X & 1200X magnifications) of partially damaged PAU 201 rice grains showing normal structure.

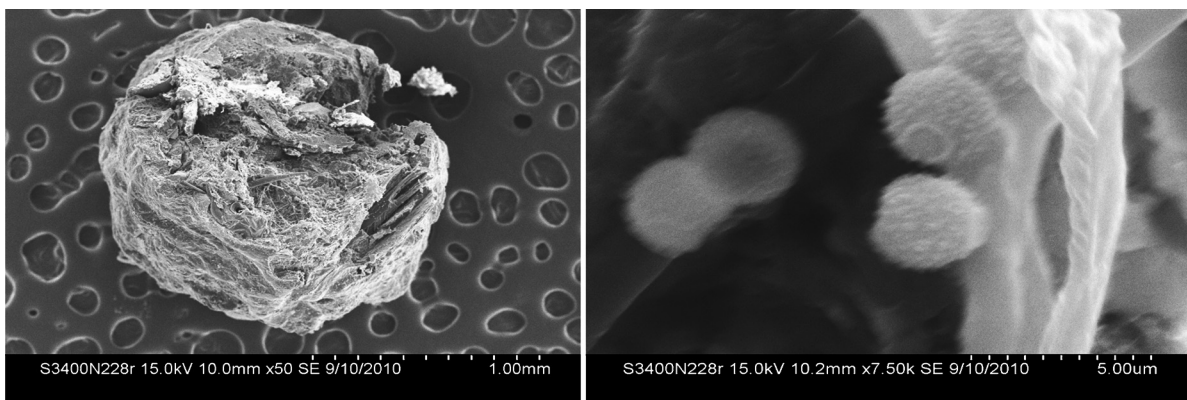


Fig. 7. SEM surface sections (at 50X & 7500X magnification) of fully damaged PAU 201 rice grain showing irregular appearance and spore morphology.

Discussion

Damaged food grains resulting from fungal invasion and subsequent spoilage may produce mycotoxins that are known to be toxic to humans and animals²². Thus the presence of damaged grains in rice is an important factor that is considered for mould contamination and the FSSAI regulations established norms to ensure that such damaged grains do not exceed 5 per cent in rice¹¹. The extent of aflatoxin contamination, found in the PAU 201 stored rice in the present study was consistent with reported studies on aflatoxin contamination in rice with majority of samples having levels below the tolerance limits for aflatoxin in India^{6,7}. On the other hand, aflatoxin levels exceeded the tolerance limits in damaged rice grains.

Although the sensitivities of the analytical methods used by the three laboratories for aflatoxin analysis differed, a reasonably good agreement in the results was observed. Variability in the results of aflatoxin analysis of contaminated commodities have been discussed earlier²³.

Rice kernels discoloured by fungi are classified as damaged grains/kernels by FSSAI and FCI^{4,12}. The black tip, pin point or peck damage is reported to be the cause of insect and fungal activity and occurs under conditions of heavy rainfall during kernel development²⁴. Such discoloured grains were observed to break easily during milling reducing the percentage of head rice and lowering quality as well as the price of rice²⁵. Earlier studies on black tipped rice did not indicate presence of aflatoxins or other mycotoxins and were not found to be toxic in experimental studies with chicks and ducklings²⁶. The SEM studies of the damaged and discoloured rice grains in the present study revealed fungal growth only in fully damaged grains as reported earlier^{27,28}.

The presence of discoloured and damaged grains is reported to be high in the PAU 201 rice²⁹. This was observed in the present study where pin point damage and other discoloured grains exceeded the regulatory limits. The FCI had relaxed these specifications up to 4.75 per cent at the request of the Government of Punjab, to expedite the milling of paddy of PAU 201 and other varieties and to maximize the procurement of rice in drought year¹³.

The black tipped discolouration in rice was earlier attributed to the presence of iron²⁶. The presence of high levels of iron in certain pigmented varieties of rice such as black rice that had high levels of anthocyanins

have been reported earlier³⁰. However, in the present study Prussian blue staining and EDX analysis of discoloured rice grains did not reveal presence of iron. These results rule out the possibility that the black tips on rice are due to presence of high iron content.

In conclusion, the results of the study indicated that the stored paddy of PAU-201 rice variety were in compliance with the food safety and standard regulations of the FSSAI with respect to aflatoxin contamination.

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