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Fungicides and the Effects of Mycotoxins on Milling Fractions of Irrigated Rice

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ABSTRACT: This study aimed to evaluate the effect of fungicides on rice cultivation, regarding the occurrence and the distribution of mycotoxins in fractions of the processed grain, by a validated chromatographic method. A method based on extraction with acetonitrile:water, determination by HPLC-DAD, and confirmation by LC-MS was validated before the mycotoxin evaluation. Control samples and samples to which triazole fungicides had been applied were collected from experimental fields for four years. Results showed that 87% of the samples were contaminated with deoxynivalenol or zearalenone, and that all samples treated with fungicide were contaminated with some of these mycotoxins. Aflatoxin B_1 and ochratoxin A were found in 37% of the samples; half of them had been treated with fungicide. Therefore, fungicides tend to be stressors for toxigenic fungi found in the fields.

KEYWORDS: *aflatoxins, deoxynivalenol, ochratoxin, zearalenone*

■ INTRODUCTION

Around 15−20% of rice grains are lost due to cultural practices and processes, variety resistance, climatic conditions, and other variables that lead to fungal contamination.[1−3] Rice crops can be damaged by fungal diseases, such as blast, brown spot, and dark spot from contamination with *Pyricularia oryzae*, *Bipolaris oryzae*, *Cercospora janseana*, and other species that attack the plant in the field and decrease its productivity. In cultivated areas, preventive measures, such as the use of fungicides, are adopted, because fungi restrict the productivity and the health of the plants.[4]

Both fungicides strobilurin and triazole are recommended for irrigated rice production to prevent loss caused by diseases, but the toxigenic fungal species might be selective and the damage they cause may not be related to productivity. Every toxigenic fungal species responds differently to fungicides, because it depends on the weather, the distribution of the active ingredient in plant tissues, the development of the plant, and the resistance of the cultivar.[4,5] These factors may be stressors in the production of mycotoxins. Fungicide formulations in emulsifiable concentrate (EC) or dispersed oil (DO) can increase penetration through the cuticle. Tebuconazole, an organic fungicide of the triazole group often applied to grain cultures, has systemic action that interrupts the functions of the cell membrane. Inhibition of sterol biosynthesis affects the synthesis of the cell membrane, hindering fungal metabolism.[6] Stressing factors trigger mycotoxin production by toxigenic fungal species, such as *Fusarium graminearum*, which contaminate crops in the field.[5−7]

Studies have shown that the mycota identified in rice has toxigenic species that can produce mycotoxins in different and complex conditions.[7,8] Researchers have found that rice bran (17.5%) and parboiled rice (15%) are the processed rice products which are more contaminated with mycotoxins that characterize contamination in the field, such as deoxynivalenol and zearalenone, and in storage, such as aflatoxin B_1 and ochratoxin A .[9−11] Rice contamination by mycotoxins might be caused by many environmental conditions, plant resistance, and toxigenic potential of the mycota, but preventive measures, such as adequate handling with the use of active ingredients which do not lead to the selection of toxigenic fungal species and promotion of their toxigenic potential, are very important to food safety.[12] The study of the effect of the fungicide on the mycotoxin occurrence is frequently carried out by isolating the toxigenic species and studying its response in vitro or in greenhouses, even though these conditions do not always reflect the conditions found in the field.[13]

Monitoring mycotoxins in rice to ensure the safety of this raw material, widely used as food, is fundamental. Determining mycotoxins requires much care regarding the physical and chemical characteristics of these compounds and their random occurrence in trace amounts. Furthermore, compounds of the same family with small structural differences, but distinct toxigenic potential, must also be determined separately.[14] Liquid chromatography is a technique applied to routine analysis in many areas, including food; the use of different detectors enables the identification and quantitation of

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compounds.[15,16] Therefore, this study aimed to evaluate the effect of fungicides on rice cultivation regarding the occurrence and distribution of mycotoxins in fractions of the processed grain by a validated chromatographic method.

■ MATERIALS AND METHODS

Chemical Reagents. The chemical reagents used in this study were acetonitrile, chloroform, and methanol HPLC grade, from J.T. Baker (Mallinckrodt, Phillipsburg, NJ), benzene p.a., hexane p.a., sodium chloride, and 85% phosphoric acid p.a., from Merck (Darmstadt, Germany). The water was purified by a Direct-Q UV3 system with resistivity of 18.2 MΩ·cm (Millipore, Bedford, MA). The mycotoxin analytical standards (purity >98%) were supplied by Sigma Aldrich (São Paulo, Brazil). The chemical structures of the mycotoxins are shown in Figure 1.

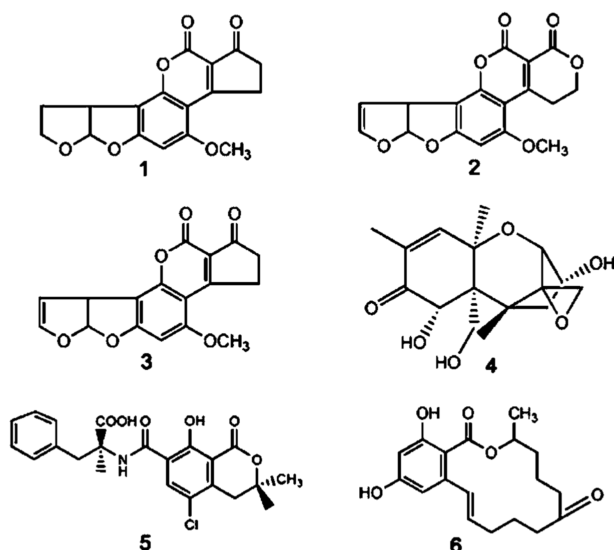

Figure 1. Chemical structures of aflatoxin B_2 (**1**), aflatoxin G_1 (**2**), aflatoxin B_1 (**3**), deoxynivalenol (**4**), ochratoxin A (**5**), and zearalenone (**6**).

Instrumentation. HPLC-DAD separation was performed by an HPLC apparatus consisting of the column 250 mm × 4.6 mm, 4 μm, Synergi Fusion-RP 80 Å (Phenomenex, Torrance, CA), a Waters 600 pump model, associated with a Waters 2996 photodiode array detector, and a Rheodyne 20 μL loop injector, connected to Empower PDA software for data acquisition. The UV spectra were recorded in the 210−400 nm range.

Liquid chromatography with mass spectrometric detection was performed by a Waters Alliance 2695 Separations Module fitted with an autosampler, a membrane degasser, and a quaternary pump. Mass spectrometry was performed by Micromass Quattro Micro API with an ESI interface. The LC separation was carried out by a column 50 mm ×3 mm i.d., 3.5 μm, XTerra (Waters, Milford, MA). Analytical instrument control, as well as data acquisition and treatment, was performed by Masslynx software version 4.1, 2005 (Waters).

Samples. The method validation for mycotoxin determination employed polished rice and rice bran which were bought in a supermarket located in southern Brazil. The rice was milled in a knife mill (Tecnal, São Paulo, Brazil), and the fractions were separated by a 32 mesh (0.5 mm) screen, for fortification with mycotoxin standards. During the method adaptation, a procedure which only used the reagents, without any mycotoxin fortification, was carried out to evaluate the initial contamination of the samples.

To measure mycotoxin production as a consequence of fungicide, an experiment was undertaken on experimental fields for four sequential crops (2006/07, 2007/08, 2008/09, and 2009/10). An experimental design of randomized blocks was used, with three repetitions and two treatments: TC, control (without fungicide application); TF, with fungicide application.

The experiment was conducted in the Experimental Station at the Instituto Riograndense de Arroz (IRGA) (29°57′3″ S, 51°53′8″ W). The cultivar BR-IRGA 417 was sown in the minimum tillage system, with a seeding rate of 100 kg/ha. The fertilizing base was 400 kg/ha (N:P:K = 05:20:30). The nitrogen topdressing was 80 kg/ha of N when plants had three leaves, before irrigation; it was 40 kg/ha of N for plants with eight leaves before the beginning of the differentiation of the panicle primordium (DPP). The following herbicides were applied: clomazone (600 mL/ha), before the first topdressing, and bispyribac-sodium (150 mL/ha), after the second topdressing. The insecticide carbofuran (4 kg/ha) was also applied after the third topdressing (50 kg/ha urea and 50 kg/ha KCl). The fungicide tebuconazole (EC) was applied (0.75 L/ha) when panicles started to appear. These recommendations have been given by the Brazilian Rice Association regarding crop management.

After harvest, samples collected on each experimental field were dried to 13% moisture in a dryer with air injection, milled in the form of polished rice (white) and parboiled polished rice, and finally polished by a laboratory mill (Zacarria, São Paulo, Brazil). The mill was adjusted so that polished rice with ash percentage around 0.5% could be obtained. The parboiling process was conducted in the laboratory in a mass with a grain:water ratio of 1:1.5 at 65 ± 2 °C for 5 h. It was autoclaved at 116 °C ± 1 °C at pressure 0.6 ± 0.05 kPa for 10 min, dried at 50 ± 2 °C up to 18% moisture, and then dried at 40 ± 1 °C up to 13% moisture. The grains were tempered for 48 h to reduce the thermohydro imbalance before dehulling, similar to that for the polished rice. During the milling process, portions of husk, bran, and starchy endosperm were separated in the following proportions: 9%, 8%, and 80%, respectively. Afterward, the fractions were sent to the Laboratory of Mycotoxins at the Universidade Federal do Rio Grande (FURG), where they were milled by a knife mill and sieved (between 32 and 65 mesh), coded, and stored at −18 °C until extraction, which was performed by using the Tanaka Method[17] in triplicate.

Preparation of Analytical Solutions. The standard solutions were prepared by following Scott's methodology.[18] In commercial standards containing about 5 mg of mycotoxins, each solution was dissolved with 100 mL of benzene:acetonitrile (98:2 v/v) and diluted in the form of standard solutions with concentrations that were spectrophotometrically determined. To estimate the solution concentrations, the following values were used for aflatoxins B_1, B_2, and G_1, deoxynivalenol, ochratoxin A, and zearalenone: (a) molar absorptivity (ε): 19800, 20900, 17100, 5913, 5550, and 6060 mol/cm; (b) maximum absorbance wavelength: 348, 350, 355, 219, 321, and 317 nm, respectively.[19,20] The standard solutions were scanned by a Cary 100 UV/vis (Varian, Columbia, MD) spectrophotometer to verify their purity. All solutions were stored in amber bottles at −18 °C and homogenized in ultrasonic bath before use.

Determination of Mycotoxins by HPLC-DAD. Different mobile phases comprising several combinations of methanol, acetonitrile, purified water, and purified water at pH 3 were tested to provide an adequate resolution. The mobile phases were filtered through a 0.45 μm nylon membrane and degassed in an ultrasonic bath for 30 min at room temperature before use. The choice of the flow rate was based on experimental tests ranging between 0.8 and 1.0 mL/min. The column was conditioned by the mobile phase elution at the flow rate for 1 h. In the mobile phase, 20 μL of standard solution or diluted samples was injected; total separation took 20 min.

The maximum absorption spectra in the ultraviolet region and the retention times of each mycotoxin were obtained by separate individual injections. Subsequently, the retention times were confirmed by the addition of separate standards to the mixture of the seven compounds. The increase in each mycotoxin signal confirms the retention times. Injecting the mixture of six mycotoxins allowed choosing a wavelength that, in turn, enabled viewing all the others, simultaneously. The record of the signal response of each analyte was traced with the help of the data acquisition system Empower Software (Waters), which also provided the coefficient of determination (r^2), the equation of the concentration versus signal, and the linearity.

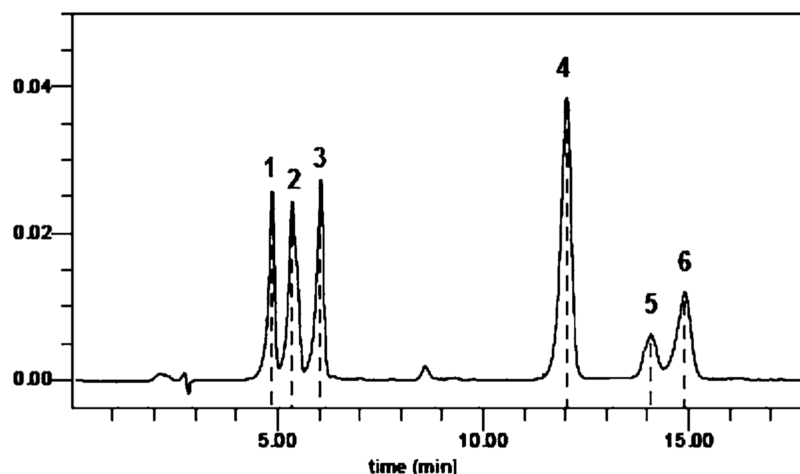

Figure 2. Chromatogram of separation of the mycotoxins in concentration 15 LOQ: aflatoxin B_2 (**1**), aflatoxin G_1 (**2**), aflatoxin B_1 (**3**), deoxynivalenol (**4**), ocratoxin A (**5**), and zearalenone (**6**).

Table 1. Recovery, RSD_r, and RSD_{pi} of the HPLC-DAD Method for Mycotoxins[a]

mycotoxins	fortification level	rice recovery (%)[b]	RB recovery (%)[b]	rice RSD_r (%)[c]	RB RSD_r (%)[c]	rice RSD_{pi} (%)[c]	RB RSD_{pi} (%)[c]
aflatoxin B_1	2LOQ	71	73	7	12	3	6
	3LOQ	72	72	5	9	7	9
	4LOQ	92	73	5	10	2	6
aflatoxin B_2	2LOQ	85	64	6	23	2	24
	3LOQ	86	71	3	25	3	20
	4LOQ	99	77	5	21	2	18
aflatoxin G_1	2LOQ	91	71	10	23	7	17
	3LOQ	92	73	8	24	5	14
	4LOQ	81	76	11	21	4	16
deoxynivalenol	2LOQ	69	71	11	24	15	22
	3LOQ	78	76	14	21	13	19
	4LOQ	86	75	15	23	12	20
ochratoxin	2LOQ	85	72	8	11	4	7
	3LOQ	87	75	5	9	3	4
	4LOQ	81	76	7	7	5	3
zearalenone	2LOQ	70	71	4	25	2	24
	3LOQ	81	69	6	24	4	25
	4LOQ	96	72	3	24	2	21

[a]RB = Rice bran [b]n = 9 (3 extractions × 3 injections) of each extract. [c]n = 3 (3 injections) of each extract.

In this study, the determination of the limit of detection (LOD) took into consideration the concentration that produced a signal which was three times the baseline noise, in a period close to the retention time of the analyte. The limit of quantitation (LOQ) was three times the LOD.

The recovery (accuracy) was evaluated by following the rice and the rice bran fortification and employing different concentration levels (2-fold, 3-fold, and 4-fold LOQ) for each standard. To evaluate precision in terms of repeatability (RSDr) and the intermediate precision (RSDpi), the spiked samples were extracted and quantitated in triplicate for each level; this resulted in n = 9 (3 extractions × 3 injections, each). RSDpi was performed on different days.

Mycotoxin Confirmation by LC-MS. The mobile phase was composed of methanol:5 mM ammonium acetate in water and 0.5% acetic acid (90:10, v/v) with flow rate of 0.2 mL/min, 10 μL injection volume, resulting in a 5-min analysis.

For mass-to-charge (m/z) ratio optimization, the individual standard of each mycotoxin was infused in the mass spectrometer (MS) at 1 μg/mL concentration. In the MS, the capillary voltage was 3.5 kV, the source temperature was 120 °C, and the desolvation temperature was 400 °C. For the linearity determination, six concentrations of standard solutions were injected. The record of the signal was traced with the help of the data acquisition system MassLynx 4, which provided the coefficient of determination and equation of concentration versus signal. The LOD and the LOQ of the instrument were also determined in the same way as those for HPLC-DAD.

RESULTS AND DISCUSSION

HPLC-DAD provided the best chromatographic separation when the following mobile phase was used: acetonitrile:water (50:50, v/v) adjusted to pH 3 with phosphoric acid (1:1, v/v) at a constant flow of 1.0 mL/min. For the quantification, the wavelength was 254 nm. The injection volume was 20 μL. The chromatogram profile of the separation of the mycotoxins is shown in Figure 2.

Linearity was studied from the LOQ to the 5-fold LOQ range. The analytical curves related to the equipment response, regarding the different concentrations of the analyte, showed that linear models were adequate for the mycotoxin concentration range, because the coefficients of determination (r^2) were higher than 0.990, as recommended by the Brazilian Agency of Health Surveillance[21] and the Brazilian Institute of Metrology.[22] The LOQ values for aflatoxin B_1 and G_1 were 0.75

μg/kg, for aflatoxin B_2, it was 0.375 μg/kg, for zearalenone, it was 0.18 μg/kg, and for deoxynivalenol and ochratoxin A, they were 1.5 μg/kg. Results showed that the method sensitivity was higher for aflatoxins because the angular coefficients of the equations had the largest variation of the concentration versus signal and that zearalenone had the lowest LOQs.

The Brazilian legislation has recommended the following maximum limits for mycotoxins: aflatoxins (B_1+B_2+G_1+G_2): 5 μg/kg in rice; ochratoxin A: 10 μg/kg in rice; deoxynivalenol: 750 μg/kg and 2000 μg/kg in processed rice and rice bran, respectively; zearalenone: 200 μg/kg in polished rice and 1000 μg/kg in rice bran.[23] The European Union legislation has set limits of 4 μg/kg for aflatoxins (B_1+B_2+G_1+G_2) and 5 μg/kg, for ochratoxin A in cereals and their milling products. Some European countries have established limits for zearalenone in cereals, such as Italy (100 μg/kg), and for deoxynivalenol in diets, such as Austria (500 μg/kg).[24] The LOQs obtained by the method are well below the limits recommended for foods, by comparison with the Brazilian and the European legislations for cereals.

Table 1 shows the recovery, repeatability (RSD_r), and intermediate precision (RSD_{pi}) values. Both precision and accuracy of analytical methods depend on the matrix, the analyte concentration, and the analysis techniques. Accuracy can vary between 2% and 20%.[25,26] In the case of accuracy, recoveries should be between 70% and 120%.[27] The RSD_r and RSD_{pi} were between 2% and 25%, and the average recovery for rice was 78%, 90%, 88%, 78%, 84%, and 82%. For rice bran, it was 73%, 71%, 73%, 74%, 74%, and 71% for aflatoxins B_1, B_2, and G_1, deoxynivalenol, ochratoxin A, and zearalenone, respectively. Therefore, in agreement with the recommended efficiency indicator, the method was suitable to be applied to this study. Low recoveries, obtained for the rice bran extractions, were expected because this is a complex matrix by comparison with white rice. The protein, lipid, and fiber averages found in bran were 13% to 15%, 15% to 17%, and 8.5% to 10%, respectively. In the case of white rice, they were 8%, 2%, and 1% to 1.8%, respectively.[28,29]

The mycotoxin confirmation was performed by a LC-MS system with the fragmentation conditions shown for each mycotoxin in Table 2.

After the collision cell energy optimization of the triple quadrupole, two different ion-product precursors were selected for each mycotoxin: one for quantitation and the other for qualification. These ions were monitored under time-schedule multiple reaction monitoring (MRM) conditions.

Table 2. Fragmentation Conditions in LC-MS

mycotoxin	ESI	transition (m/z) precursor Ion → product Ion	cone voltage (V)	collision energy (eV)	dwell time (s)
aflatoxin B_1	+	313 → 241[a]	45	37	0.1
		313 → 285	45	20	
aflatoxin B_2	+	315 → 259	45	30	0.1
		315 → 287[a]	45	25	
aflatoxin G_1	+	328.8 → 243.4[a]	43	20	0.1
		328.8 → 311.2	43	25	
deoxynivalenol	−	355 → 58.8[a]	17	11	0.2
		355 → 295.2	17	8	
ochratoxin	+	404 → 239[a]	30	23	0.1
		404 → 358	30	15	
zearalenone	−	317 → 131[a]	40	30	0.2
		317 → 175	40	30	

[a]Transitions used for quantitation.

The LOQ in the LC-MS was 0.0005 μg/mL for the aflatoxins B_1, B_2, and G_1, 0.001 μg/mL, for zearalenone, 0.01 μg/mL, for ochratoxin A, and 0.05 μg/mL, for deoxynivalenol. Linear calibration curves were plotted by least-squares regression of concentration versus the peak area of the calibration standards. Adequate linearity in the concentration range was obtained with correlation coefficients (r) higher than 0.97.

The validated method was applied to the samples collected in experimental fields. Results are shown in Table 3; the standard deviation of each sample triplicate complied with the method variability (it was never above 18%).

The mycotoxins deoxynivalenol and zearalenone were found in 87% of the samples. All samples treated with fungicide were contaminated with some of these mycotoxins. Deoxynivalenol was found in 67% of the samples: 55% came from treated fields, and 45% of them kept the mycotoxins even after the parboiling process. Furthermore, 40% was in rice husk and bran.

In the samples analyzed in this study, zearalenone was the second most common mycotoxin found in 47% of the samples: 64% came from treated fields and remained in 36% of these samples after parboiling. Furthermore, 93% was in rice husk and bran. Among them, 57% was also contaminated with deoxynivalenol, 21% with ochratoxin A, and 21%, with aflatoxin B_1.

These facts suggest that toxigenic *Fusarium* species contaminated the rice grain in the field, that the fungicide acted as a stressor factor, and that, during milling, the toxins were distributed among the separated fractions. The phytopathogen germination is affected by environmental variables, such as moisture, temperature, fungicides, and antagonistic microorganisms, and the rhizome depends on its own nutritional reserves. During germination, when there is water absorption, activation of hydrolytic enzymes, and germ tube development, the penetration phase enables the pathogen to penetrate by mechanical pressure or enzyme production into the surface of the host. Damage is an indirect and more efficient way for the entrance of the pathogen. Usually, penetration results in infection, a process by which the contaminant establishes contact with the host cells and gets the nutrients through them, but the defense mechanisms of the plant or adverse environmental conditions may hinder the infection.[5,12]

Resistance might be a stressful situation for the fungus, which responds with the outbreak of toxigenic potential. Because systemic fungicide is a product that can be absorbed by the leaves or roots and translocated within the plant via xylem, affecting upper and lower parts of the plant through the transpiration stream,[5,6,8] its use may have interfered in the plant metabolism and the metabolism of the fungus itself acting as a stressor. It was shown by the high occurrence of contaminated samples in the rice fractions from the fields where the fungicide was applied.

Out of 30 samples under analysis, 37% was contaminated with aflatoxin B_1 and ochratoxin A; 45% had been treated with fungicide during cultivation. Ochratoxin A was found in 20% of the samples: 33% was collected on treated fields, 17% was parboiled, and 83% was obtained from the external layers (rice husk and bran). Regarding aflatoxin B_1, 17% of the samples were contaminated with this mycotoxin: 80% was parboiled samples and 60% was from the external layers of the grain cultivated with fungicide. The fungal species *Aspergillus* and *Penicillium*, which produce these mycotoxins, are not

Table 3. Mycotoxins Determined in Rice Samples[a]

harvest periods	samples	mycotoxins (μg/kg)[b]			
		aflatoxin B_1	deoxynivalenol	ochratoxin	zearalenone
2006–07	1 polished rice TF		200		
	2 polished rice TC				
	3 parboiled rice TF		250		
	4 parboiled rice TC	53			
2007–08	1 polished rice TF		320		
	2 polished rice TC		120		
	3 white bran TF				350
	4 white bran TC				180
2008–09	1 natural rice in husk TF			22	11
	2 natural rice in husk TC			21	36
	3 polished rice TF		115		18
	4 polished rice TC		94		
	5 white bran TF			21	23
	6 white bran TC			21	
	7 parboiled rice in husk TF		85		
	8 parboiled rice in husk TC				
	9 parboiled rice TF	36	99		
	10 parboiled rice TC		73		
	11 parboiled bran TF				8
	12 parboiled bran TC		110	13	
2009–10	1 natural rice in husk TF		42		20
	2 natural rice in husk TC		50		
	3 polished rice TF		4		
	4 polished rice TC		29	10	
	5 white bran TF	65	60		49
	6 white bran TC		9		30
	7 parboiled rice TF		63		40
	8 parboiled rice TC		6		5
	9 parboiled bran TF	30	8		10
	10 parboiled bran TC	60	14		15

[a]TC = control (without fungicide application); TF = with fungicide application. [b]Standard deviation of triplicates below 18%

commonly found in freshly harvested product,[12]the case of this study. Again, it reinforces the hypothesis that the fungicide can select fungal species because ochratoxin A was found in the second crop, after the previous fungicide application in the field. The level of aflatoxin B_1 was higher and more frequent in the last crop under investigation.

Regarding the parboiling process, Coelho et al.[30] stated that there was migration of aflatoxins B_1, B_2, G_1, and G_2 to the starchy endosperm of the rice grain during parboiling. Regarding mycotoxin migration to the starchy endosperm during the parboiling process, Dors et al.[31] observed that the process conditions, i.e., time of soaking and autoclaving, exert influence on the migration of each mycotoxin differently. Results showed that, in harvests 2008–09 and 2009–10, the parboiled samples from treated fields were contaminated with deoxynivalenol, a fact that may have been favored by the parboiling process, thus explaining the high contamination levels in parboiled rice. In harvest 2009–10, the same response was observed for zearalenone.

The levels found in rice husk, polished rice and bran were, in general, higher in the samples collected on fields where the fungicide had been applied, but this fact does not allow the establishment of a defined quantitative behavior. Characteristic random manifestation of the fungal microbiota toxigenicity was found to be favored by the triazole application. Moreover, the mycotoxin levels found in rice samples and its products are not above the ones recommended by the legislation. However, this cereal is frequent in the human diet around the world, and risk of chronic damage may happen. It is important to choose the active principle to be used as a fungicide during crop management in the field.

The results of this study are more realistic than others found by studies conducted in vitro after microorganism isolation or in a greenhouse. Under the conditions in the fields, it is possible to make associations with other abiotic variables, such as climate, irrigation with water, and the use of other pesticides, because spores in the soil and irrigation water might intensify the stressful action of fungicides. The fact that most contaminated samples came from treated plants suggests that, although the active principle is efficient to limit plant diseases and increase productivity, mitigating mycotoxin production by *Fusarium* or other toxigenic species is not the best decision. Knowing the conditions that favor the production of mycotoxins is indispensable to avoid them during cultivation because contamination by mycotoxins has been increasingly observed in rice samples.[9,11,32−35]

Throughout four harvests, the use of fungicide in irrigated rice culture could be related to the toxigenic potential of mycota. Therefore, adopting another way to avoid plant diseases is recommended.

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Notes

The authors declare no competing financial interest.

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