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Research Article
Influence of Water Activity (a_w) on Mould Infestation and Mycotoxin Contamination of Rice Fodder

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Abstract

Background and Objective: Mycotoxigenic fungi infest stored foods and feeds depending on environmental conditions prevailing in storage specially water activity (a_w) prevailing. Besides, physical conditions, the relationship of storage moulds which may act antagonistically or synergistically on individual surface moulds of fodders and feeds which in turn may influence the elaboration of mycotoxins by moulds will determine the degree of mycotoxin contamination. Therefore, it was considered worthwhile to determine the influence of water activity (a_w) on mycotoxins elaboration both qualitatively and quantitatively. Varieties of moulds are reported to infest paddy straw and elaborate mycotoxins and cause health hazards to cattle which consume it. Interaction of infesting moulds and water activity (a_w) are reported to be complex and determine mycotoxin elaboration. **Materials and Methods:** The stored paddy straw with different a_w activity was inoculated by spore suspension of individual mycotoxigenic fungi and incubated at 30°C for 15 days. Mould infested straw was analyzed for the presence of different mycotoxins. The water activity (a_w) of straw was maintained by standard methods. **Results:** The analysis of mould infested rice fodder revealed the presence of mycotoxin which differed quantitatively depending on the water activity (a_w). Moulds present in the rice fodder are likely to interact and act either synergistically or antagonistically and contribute to the management of most mycotoxigenic fungi through biologically and ecofriendly. **Conclusion:** Management of mycotoxins can be accomplished by maintaining most adverse environmental conditions and promoting antagonistic moulds. As temperature, water activity and pH have a great impact on mycotoxin production by fungi, these factors have to be considered for developing management practices.

Key words: Rice fodder, water activity (a_w), interaction, mycotoxigenic fungi, mycotoxins, synergism, antagonism, management

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In nature wide variety of fungi on the substrate interacts with each other either for space or nutrients. A variety of interactions such as antagonism, mutualism, synergism and metabiosis leading to either favourable or adverse conditions for the activity of the target fungus. The outcome of these associations in turn is influenced by variety of environmental conditions including water activity (a_w). The problem of mycotoxins is more acute under cultural conditions than what is envisaged in nature. This is probably the reason for comparatively more incidence of mycotoxins under laboratory conditions in comparison to what is experienced in nature[1]. Kumar *et al.*[2] and Palumbo *et al.*[3] elegantly discussed different aspects of interaction of fungi and components of environment on mycotoxin contamination of agricultural commodities, the key factor in preventing mycotoxigenic fungi in foods and feeds. Akbar and Magan[4] feet that water activity (a_w) influence and determine the colonization of foods and feeds by both mycotoxigenic and non-toxigenic spoilage fungi. Combined effect of high temperature and water stress in some regions, the contamination of mycotoxigenic fungi of food commodities becomes a serious problem in preventing food spoilage and degeneration of nutrients[5]. Therefore, it is important to understand how these fungi enter food chain in relation to environmental factors. Destroy or removal of mycotoxins from food or feed biologically is beset with number of problems such as economics, nutritive value and its biological safety. Prevention of mycotoxigenic mould infestation of food or feed is the best option but requires a thorough understanding of the conditions under which mycotoxigenic fungi are favoured. The information gathered from one fungal species cannot be transferred to another species[6]. Therefore, in the present investigations influence of water activity (a_w) of paddy straw on the proliferation of different mycotoxigenic fungi and mycotoxin elaboration was assessed. The water activity (a_w) of paddy straw was maintained by standard methods and its affect on mould infestation and mycotoxin elaboration was assessed and the results are discussed in this communication.

MATERIALS AND METHODS

Fungal culture: Isolates of *A. flavus, A. nidulans, A. terreus, A. ochraceus*, *P. griseofulvum* and *P. aurantiogriseum* isolated from paddy straw (*Oryza sativa* L.) and maintained on Asthana and Hawkers medium A (Glucose 10 g, KNO_3 3.5 g, KH_2PO_4 1.75 g, $MgSO_4.7H_2O$ 0.75 g, agar 16 g and distilled water 1000 mL) were employed for these studies. Seven days old cultures were employed for inoculum preparation. Inoculum was prepared in 10 mL of sterile water containing 0.05% so as to get final spore concentration to 1×10^6 conidia per mL^{-1}.

Effect of water activity: The water activity (a_w) of rice fodder was maintained as suggested by Loncin *et al.*[7]. Hundred grams of rice fodder was taken in 250 mL Erlenmeyer conical flask and different fungi were inoculated at the rate of 10 mL spore suspension. Rice fodder thus inoculated was mixed thoroughly to ensure uniform spreading of the inoculum in rice fodder. The rice fodder thus inoculated was incubated at $27\pm2^\circ$C for 15 days at different water activity (0.95, 0.92, 0.87, 0.85, 0.80 and 0.75). Five replicates were maintained and the experiment was repeated three times.

Biochemical changes: The chemical changes in rice fodder due to the inoculation of different mycotoxigenic fungi was analyzed at the end of 15 days incubation. The infested rice fodder was homogenized in distilled water and centrifuged at x1800 for 30 min to get a clear supernatant. The supernatant was taken for determining protein, phenols, free amino acids, reducing sugars, free fatty acids, total nitrogen and crude fiber as described earlier by Kiran *et al.*[8]. Ash content and loss of weight of rice fodder was determined by Ward and Johnston[9].

Mycotoxin analysis: The infested rice fodder by different fungi for 15 days was analyzed for mycotoxins as described by Surekha *et al.*[10]. One gram of fungal infested rice fodder was homogenized in methanol and shaken thoroughly for 5 min in Vertex mixer. The suspension was left for 60 min without shaking. The extracts were shaken again and filtered through Whatman no 1 filter paper. The clear extracts were employed for estimation of different mycotoxins quantitatively or semi-quantitatively as described for aflatoxins[11], sterigmatocystin and ochratoxin A[12], terreic acid[13], patulin[14], penitrem B[15] and cyclopiazonic acid[16]. The details of detection of different mycotoxins are precised in Table 1.

Statistical analysis: The results obtained in the present investigations were subjected to statistical analysis using SPSS package 12.0 version. It computed statistical parameters mean, S.D, S.E and t-value are interpreted at $\alpha = 0.05$ level of significance.

Table 1: Solvent system spray reagents used in the detection of different mycotoxins in rice fodder

Name of the toxin	Solvent system	Spray reagent	Detection	
			UV	Visible
Aflatoxins	C:A (95:5)	-	blue and green	-
Sterigmatocystin	C:M:A (1:1:1)	20% $AlCl_2$	yellow	-
Ochratoxin A	T:Ea:F (6:3:1)	20% $AlCl_3$	Bright blue	-
Patulin	T:Ea:F (6:3:1)	2% phenylhydrazine hydrochloride	-	yellow
Terreic acid	T:Ea:F (6:3:1)	Quantitative estimation	-	-
Penitrem B	H:Ea (6:4)	$Ce(SO_4)_2$ 1% in 6N H_2SO_4, $FeCl_3$	Pale green	-
Cyclopiazonic acid	T:Ea:F (6:3:1)	Ce $(SO_4)_2$ 1% in 6N H_2SO_4 2,4- DNP, $FeCl_3$ 3% in ethanol, $AlCl_3$	yellow	blue, red brown, brown

C: Chloroform, A: Acetone, M: Methanol, T: Toluene, Ea: Ethyl acetate, F: Formic acid, H: Hexane

RESULTS

The influence of water activity (a_w) on mould infestation of rice fodder and mycotoxin elaboration by different mycotoxigenic fungi was analyzed and the results are precised in Table 2.

Fungi under investigation failed to grow and elaborate mycotoxins at a_w 0.75 (Table 2). However, *A. flavus, A. terreus* and *A. ochraceus* could record poor growth and elaborated trace amount of aflatoxins, terreic acid and ochratoxin A respectively at this water activity (a_w). Interestingly, *P. aurantiogriseum* and *P. griseofulvum* failed to elaborate penitrem B and cyclopiazonic acid, respectively even at a_w 0.80. This may be attributed to hostile conditions of rice fodder or these fungi require more water activity (a_w).

Critical perusal of Table 2 reveals significant changes in biochemical composition of rice fodder which varied significantly with the water activity (a_w) of fodder and the fungus involved. When free amino acids, free fatty acids, total nitrogen and ash content increased with the increase of water activity (a_w) of stored paddy straw, reducing sugars and crude fiber content decreased with the increase of water activity. The increase in ash content of fodder was significant incubated at above a_w 0.92 which may be attributed to the increased infesting fungal activity. On the other hand, marginal increase in ash content was recorded in control fodder at all a_w tried.

DISCUSSION

From the present investigations it is clear that the activity fungi in rice fodder increased with the increase of water activity and progress of incubation period. Most of the fungi under investigation failed to grow and elaborate respective mycotoxins at a_w 0.75. However, *A. flavus*, *A. terrues* and *A. ochraceus* could record poor growth and elaborate trace amount of aflatoxins, terreic acid and ochratoxin A, respectively. Interestingly *P. aurantiogriseum* and *P. griseofulvum* failed to elaborate penitrem B and cyclopiazonic acid respectively even at a_w 0.8. This may be attributed to hostile condition of rice fodder or these fungi required more water activity (a_w). Lahouar *et al.*[17] have recorded failure of *A. flavus* to produce aflatoxins at a_w 0.85. *Aspergillus nidulans* could elaborate sterigmatocystin in rice fodder maintained at a_w 0.92 and above water activity. Ochratoxin A production by *A. ochraceus* was recorded at all a_w tried but the amount of Ochratoxin A produced increased with increase of a_w above 0.87. Esteban *et al.*[18] also reported production of Ochratoxin A by *Aspergillus carbonarius* at wide range of water activity at different temperature. *Aspergillus flavus* could produce trace amount of aflatoxins when the a_w was 0.7 but increased significantly with increase of a_w 0.95. In agreement with present observations *A. flavus* and *A. ochraceus* are reported to be ubiquitous and could be active over wide range of water activity[17]. Ghali *et al.*[19] also feet that relative humidity and temperature are important critical factors in storage of food grains to keep them free from mycotoxin contamination. Belli *et al.*[20] and Kapetanakou *et al.*[21] observed production of ochratoxin A by *A. carbonarius* at higher water activity (a_w). Similarly Mitchell *et al.*[22] recorded increased growth with increase of water activity (a_w) from 0.96-0.98. Schmidt-Heydt *et al.*[23] while critically analyzing the factors contributing to mycotoxin contamination concluded that storage temperature was a key factor for aflatoxins B_1 elaboration, while water activity (a_w) influenced the aflatoxin G_1 biosynthesis. Amani *et al.*[17] and Mousa *et al.*[24] have also recorded significant amount of aflatoxin in foods with a_w 0.85-0.99 and 0.82-0.92, respectively. The differentially produced amount of either aflatoxin B_1 or G_1 may be attributed to the changing environment. It has been demonstrated that fungi produce mycotoxins to improve their competitiveness under natural conditions[25]. Rice fodder suffered significant weight loss at a_w above 0.85. Marin *et al.*[26] observed that conidia of *A. flavus* germinated freely and exhibited strong deteriorative activity at a_w 0.85 and above. On the other hand, *A. flavus* and *A. ochraceus* were active

Table 2: Influence of water activity on biodeterioration and mycotoxin production in rice fodder by some storage fungi

Water activity (a_w)	Name of the fungus	Proteins (mg g^{-1})	Phenols (mg g^{-1})	Free amino acids (mg g^{-1})	Reducing sugars (mg g^{-1})	Free fatty acids (mg KOH/100 g)	Total nitrogen (mg g^{-1})	Ash content (mg g^{-1})	Crude fibre (mg g^{-1})	Loss of weight (%)	Mycotoxin	Amount of (intensity/ ppb/µg mL^{-1} mg g^{-1})
0.95	Control	38.5	0.60	2.76	12.00	0.30	2.50	62.0	90.0	37.0	-	-
	A. flavus	62.2	0.70	7.98	1.80	0.35	2.08	140.2	83.7	48.9	AFB_1/B_2	+5/+4
	A. nidulans	46.2	0.66	8.97	5.45	0.32	0.99	129.3	83.5	48.0	Sterigmatocystin	+2
	A. ochraceus	39.6	0.66	6.00	4.80	0.36	1.78	116.1	78.0	48.2	Ochratoxin A	+3
	A. terreus	45.5	0.90	4.86	2.85	0.33	1.25	88.7	75.4	48.5	Patulin/terreic acid	+T/35
	P. aurantiogriseum	62.5	0.72	3.96	10.00	0.37	1.87	140.0	65.0	47.8	Penitrem B	Nil
	P. griseofulvum	43.7	0.75	3.16	8.00	0.30	1.49	106.5	62.3	48.4	CPA	Nil
Statistical analysis	Mean	49.9	0.73	5.82	5.48	0.33	1.57	120.1	74.6	48.3	p<0.05 = Significant	
	SD	9.87	0.08	2.28	3.08	0.02	0.40	20.3	9.13	0.38		
	SE	4.03	0.03	0.93	1.26	0.10	0.16	7.30	3.73	0.15		
	t-value	0.03	0.01	0.02	0.004	0.01	0.003	0.001	0.009	0.00		
0.92	Control	38.5	0.54	2.76	15.00	0.30	2.50	54.6	170.0	36.0	-	-
	A. flavus	60.9	0.67	5.09	2.50	0.31	2.24	106.8	149.3	45.3	AFB_1/B_2	+3/+3
	A. nidulans	44.3	0.69	5.73	7.50	0.31	0.63	120.7	125.2	45.6	Sterigmatocystin	+T
	A. ochraceus	43.3	0.67	4.41	7.50	0.33	2.08	116.6	140.0	45.6	Ochratoxin A	+3
	A. terreus	44.0	0.92	5.16	3.75	0.34	2.08	107.9	137.8	46.5	Patulin/terreic acid	Nil/20
	P. aurantiogriseum	55.0	0.60	3.72	10.00	0.36	2.50	120.0	75.0	45.5	Penitrem B	Nil
	P. griseofulvum	46.9	0.63	2.86	8.00	0.30	1.87	77.3	100.8	46.1	CPA	Nil
Statistical analysis	Mean	49.0	0.69	4.49	6.54	0.32	1.90	108.2	121.3	45.7	p<0.05 = Significant	
	SD	7.23	0.11	1.05	2.83	0.02	0.06	16.2	8.2	0.44		
	SE	2.95	0.04	0.43	1.15	0.009	0.26	6.64	11.5	0.18		
	t-value	0.01	0.02	0.01	0.001	0.04	0.07	0.00	0.008	0.00		
0.87	Control	32.0	0.42	2.28	17.00	0.29	3.12	54.0	180.0	33.9	-	-
	A. flavus	36.6	0.63	5.51	5.31	0.37	2.77	84.6	171.5	44.4	AFB_1/B_2	+2/+1
	A. nidulans	33.0	0.46	4.75	8.50	0.33	2.49	99.8	102.3	47.1	Sterigmatocystin	Nil
	A. ochraceus	33.3	0.54	3.21	9.35	0.33	2.03	95.1	166.1	47.1	Ochratoxin A	+3
	A. terreus	37.1	0.72	4.38	3.54	0.33	1.70	95.7	141.6	46.2	Patulin/terreic acid	Nil/17
	P. aurantiogriseum	39.0	0.54	3.12	13.00	0.34	2.50	115.0	142.5	44.1	Penitrem B	Nil
	P. griseofulvum	38.7	0.56	2.50	10.50	0.29	2.34	93.7	134.3	45.8	CPA	Nil
Statistical analysis	Mean	36.2	0.57	3.91	8.36	0.33	.30	97.3	143.0	45.7	p<0.05 = Significant	
	SD	2.59	0.08	1.14	3.45	0.02	0.38	10.0	4.80	1.29		
	SE	1.05	0.03	0.46	1.41	0.01	0.15	4.08	9.01	0.52		
	t-value	0.01	0.008	0.01	0.002	0.01	0.003	0.00	0.01	0.00		
0.85	Control	32.0	0.42	2.04	20.00	0.25	3.12	52.9	192.5	33.1	-	-
	A. flavus	37.2	0.63	2.44	9.23	0.28	2.49	100.1	181.3	36.8	AFB_1/B_2	+2/+T
	A. nidulans	34.2	0.50	2.51	13.30	0.28	2.60	105.4	147.2	42.5	Sterigmatocystin	Nil
	A. ochraceus	34.8	0.46	2.91	10.90	0.26	1.87	120.0	168.0	43.5	Ochratoxin A	+T
	A. terreus	32.6	0.60	3.98	3.00	0.28	2.50	97.3	156.6	40.6	Patulin/terreic acid	Nil/12
	P. aurantiogriseum	37.5	0.51	3.00	15.00	0.29	2.50	95.0	152.5	43.5	Penitrem B	Nil
	P. griseofulvum	34.8	0.44	2.27	10.00	0.26	2.59	94.7	169.0	40.6	CPA	Nil
Statistical analysis	Mean	35.1	0.52	2.85	10.20	0.27	2.42	102.0	162.4	41.2	p<0.05 = Significant	
	SD	1.86	0.07	0.62	4.14	0.01	0.27	9.62	12.6	2.54		
	SE	0.76	0.03	0.25	1.69	0.005	0.11	3.93	5.14	1.03		
	t-value	0.009	0.02	0.02	0.002	0.004	0.002	0.00	0.002	0.001		
0.80	Control	30.0	0.42	1.80	20.00	0.23	3.75	52.0	200.0	30.9	-	-
	A. flavus	32.3	0.79	3.21	14.40	0.24	2.81	58.5	174.3	31.6	AFB_1/B_2	+2/+T
	A. nidulans	32.7	0.59	2.29	10.70	0.25	2.49	89.6	153.4	32.4	Sterigmatocystin	Nil
	A. ochraceus	31.1	0.51	2.43	9.56	0.25	1.25	74.4	146.6	32.2	Ochratoxin A	+T
	A. terreus	32.2	0.60	3.15	3.33	0.29	2.25	96.3	167.4	33.9	Patulin/Terreic acid	Nil/11
	P. aurantiogriseum	35.5	0.48	2.76	16.00	0.29	3.12	85.0	182.5	40.2	Penitrem B	Nil
	P. griseofulvum	35.5	0.52	2.03	13.00	0.25	2.50	94.8	194.4	34.3	CPA	Nil
Statistical analysis	Mean	33.2	0.58	2.64	11.10	0.26	2.40	83.1	169.7	34.1	p<0.05 = Significant	
	SD	1.84	0.11	0.47	4.50	0.02	0.64	14.4	17.8	3.16		
	SE	0.75	0.04	0.19	1.83	0.009	0.26	5.88	7.30	1.29		
	t-value	0.008	0.01	0.007	0.005	0.01	0.004	0.003	0.009	0.05		

Table 2: Continue

Water activity (a_w)	Name of the fungus	Proteins (mg g^{-1})	Phenols (mg g^{-1})	Free amino acids (mg g^{-1})	Reducing sugars (mg g^{-1})	Free fatty acids (mg KOH/100 g)	Total nitrogen (mg g^{-1})	Ash content (mg g^{-1})	Crude fibre (mg g^{-1})	Loss of weight (%)	Mycotoxin	Amount of (intensity/ ppb/µg mL^{-1} mg g^{-1})
0.75	Control	30.0	0.36	1.56	29.00	0.20	4.37	50.8	222.5	30.5	-	-
	A. flavus	52.3	0.41	2.18	23.20	0.21	3.12	84.7	202.2	31.9	AFB_1/B_2	+1/+T
	A. nidulans	40.3	0.42	1.71	16.80	0.23	3.49	84.5	200.7	32.4	Sterigmatocystin	Nil
	A. ochraceus	32.2	0.43	2.45	12.80	0.20	1.08	84.8	180.7	32.5	Ochratoxin A	+T
	A. terreus	34.2	0.43	2.28	8.92	0.25	2.90	69.1	161.1	32.6	Patulin/terreic acid	Nil/5
	P. aurantiogriseum	33.5	0.42	2.04	17.00	0.28	3.12	75.0	187.5	40.1	Penitrem B	Nil
	P. griseofulvum	42.3	0.43	2.34	20.10	0.20	3.26	84.7	128.3	32.3	CPA	Nil
Statistical analysis	Mean	39.1	0.42	2.16	16.40	0.22	2.82	80.4	176.7	33.6	p<0.05 = Significant	
	SD	7.60	0.008	0.26	5.08	0.03	0.87	6.78	28.0	3.17		
	SE	3.10	0.003	0.10	2.07	0.01	0.35	2.76	10.4	1.29		
	t-value	0.03	0.00	0.002	0.002	0.008	0.008	0.00	0.01	0.006		

SD: Standard deviation, SE: Standard error, Statistical inference: (i) Mean, standard deviation and standard error of different bio-deterioration of rice fodder, which revels that is fairly uniform response, (ii) t-values are significant at 0.05 level of significance to different bio-deterioration of rice fodder

even at a_w 0.75 and contributed for maximum deterioration of rice fodder and aflatoxin and ochratoxin A elaboration, which may be attributed to their xerophilic nature. Spadaro *et al.*[27] have also reported significant effect of water activity on ochratoxin A production by *A. carbonarius*. Hence, they felt that these factors should be taken into account for developing management practice for future research programs.

When protein content of rice fodder changed only fodder showed a significant increased which increased with increase in a_w activity. The increase in protein of rice fodder may be either due to infested fungal protein or it may be attributed to increased protein synthesis in metabolism. Ghorai *et al.*[28] also recorded increased in protein which was attributed to additive effect of fungal and fodder protein. On the other hand, Chattha *et al.*[29] recorded decrease in protein content of mould infested food grains.

Increase in phenols in fungal infested tissue was considered to be one of the defensive acts of host tissue. In the present investigations marginal increase was recorded in mould infestation fodder which may be due to the dead tissue of rice straw. Reducing sugars of rice straw infested by fungi decreased which may be due to their assimilation by infesting mycotoxigenic fungi. An inverse correlation was observed between water activity and free fatty acid content of rice fodder infested by fungi understudy. However, degree of changes in FAA of rice fodder varied both with the infesting fungus and water activity (a_w) of rice fodder. Increased FFA is reported to be indicative of substrate degradation. Differences in regulation of mycotoxin production may be attributed to variable conditions of the substratum in which the fungus occurs[23].

Marginal decrease in total nitrogen was recorded in *A. flavus* infested fodder at a_w 0.7 but the decrease was very significant at a_w above 0.92. Interestingly in *A. ochraceus* infested fodder total nitrogen was least at all a_w tried. However, no definite trend could be observed nitrogen content of fodder infested by different fungi. Total nitrogen content changes were minimum in *A. terreus* infested fodder at all a_w tried. Increase of ash content was maximum in *A. terreus* and *P. aurantiogriseum* infested fodder, while it was minimum in *A. terreus* and *P. griseofulvum* infested fodder.

From the present investigations it is clear that water activity has profound impact on the activity of infesting fungus resulting in increased deterioration of rice fodder and mycotoxin elaboration. Therefore, protecting the fodder from mould infestation is of paramount importance. However,

more detailed investigations are needed for keeping fodder free from mycotoxigenic fungi and mycotoxin contamination. Deeper studies involving individual factors and interaction of different factors are of paramount importance. Further, individual storage fungi may respond differently to various environmental conditions on the effect of different environmental factors on growth and mycotoxin production by fungi will increase our knowledge of the ecology of mycotoxin moulds. Such deeper studies with wide variety of fungi and complex environment may help to understand ecology mycotoxigenic fungi and help to formulate methods to protect foods and feeds from mycotoxins contamination and prevent various mycotoxigenic health hazards of farm animals.

CONCLUSION

Management of mycotoxins can be accomplished by maintaining most adverse environmental conditions for the growth of mycotoxigenic moulds and promoting the activity of antagonistic moulds. As temperature, water activity and pH are reported to influence mycotoxin producing mould growth and mycotoxin elaboration care should be taken to avoid storage of feeds under these favourable conditions.

SIGNIFICANCE STATEMENT

The present study uncovered the intrinsic role of water activity (a_w) on mycotoxin elaboration which can be beneficial to contain the growth of mycotoxigenic fungi and mycotoxin elaboration. Further, the study facilitate the researchers to uncover the critical areas of study that may researchers were not able to explain. Thus, activity mycotoxigenic fungi may be contained by creating unfavorable conditions such as low water activity (a_w) less than 0.7.

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