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Full Length Research Paper

Fungal succession in stored rice (*Oryza sativa* Linn.) fodder and mycotoxin production

M. Surekha*, Kiran Saini, V. Krishna Reddy, A. Rajender Reddy and S. M. Reddy

Department of Botany, Kakatiya University, Warangal-506009. A. P. India.

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Succession of fungi on stored rice (*Oryza sativa* Linn.) fodder during one year (2008 to 2009) was investigated. The percentage of incidence, frequency and abundance varied with the fungi detected. *Aspergillus flavus, Aspergillus niger*, *Curvularia lunata* and *Rhizopus stolonifer* were the most common fungi and could be detected during the entire period of one year storage. Species of *Fusarium* appeared in June 2008 and persisted until January 2009. *Memmoniella echinata, Myrothecium roridum* and *Stachybotrys atra* appeared only during the latter part of the storage. Most of the fungi isolated from rice fodder were mycotoxigenic and elaborated aflatoxins, citrinin, ochratoxin A, patulin, zearalenone and satratoxin by the respective fungi.

Key words: Succession of fungi, stored rice fodder, aflatoxins, citrinin, ochratoxin A, patulin, zearalenone.

INTRODUCTION

Rice which is the major crop grown all over Andhra Pradesh is used as fodder after harvest, almost throughout the year. In villages, it is the usual practice to store fodder under open air conditions either in the form of bales or heap, and due care is not taken in protecting it from over wetting and drying. Inadequately dried hay, under faulty storage not only accelerates the deterioration but also encourages mould infestation (Lacey, 1991). Rapid drying allows few organisms to grow, but with slow drying, many saprophytic fungi are able to colonize fodder. Type of storage fungi to be colonised will vary with the storage conditions and the amount of initial water content of fodder.

Association of mycotoxigenic fungi and contamination of rice with different mycotoxins has been reported from different parts of the world (Breckenridge et al., 1986; Liu et al., 2006; Managala et al., 2006; Tanaka et al., 2007; Reddy et al., 2009). The fungi present in the fodder may cause allergy to farm workers and animals (Gotlieb, 1997; Whitlow and Hagler, 2002; Scudamore and Livesey, 1998; Seglar, 1999; Hilton, 2000; Lugauskas et al., 2002). However, not much information is available on the infestation of rice fodder by mycotoxigenic molds. Though there are several studies on succession of fungi on different food grains, practically, very limited information is available on succession of fungi on stored fodders (Surekha and Reddy, 1990; Breton et al., 1991). Further, some of the fungi colonizing fodders may elaborate mycotoxins (Cheeke, 1995; Bakutis, 2002; Naicker et al., 2007). Hence, in the present investigation, the changes in fungi on stored rice fodder and their role in mycotoxin contamination during one year storage was studied.

*Corresponding author. E-mail: magantirekha@gmail.com.

MATERIALS AND METHODS

Freshly harvested rice fodder was stored under natural conditions for 12 months. At monthly intervals, the mycoflora of fodder was analysed by employing dilution plate method (Waksman, 1922) and agar plate method (ISTA, 1985). The fungi isolated were grown in pure culture and were identified with the help of standard manuals (Ellis, 1971; Samson et al., 1984; Nelson et al., 1983). The percentage of incidence, abundance and frequency of individual fungus was calculated. Species of *Aspergillus, Penicillium, Fusarium, Myrothecium* and *Stachybortrys* which are known to be mycotoxin producers were screened for production of different mycotoxins as described in AOAC (1984). The mycotoxigenic fungi were grown in rice flour medium at 29 ± 2°C for 15 days. After the end of the incubation period, the culture filtrate was employed for isolation and characterization of different mycotoxins. Liquid-liquid extraction after clean up was employed for detection of different mycotoxins with the help of thin layer chromatography (TLC) and long wave UV light (360 nm). On the basis of fluorescence, different mycotoxins were identified and they were further confirmed with colour tests (Table 1). In screening programme, mycotoxins were determined

Table 1. Detection of different mycotoxins in rice fodder.

Mycotoxin	Solvent system	Spray reagent	Detection		References
			UV	Visible	
Aflatoxin	C : A (95:3)	----	bl, gr	--	A
Sterigmatocystin	C : M : A (1:1:1)	1	y,	--	B
Terreic acid	---	2	--	--	C
Patulin	T : Ea : F (6:3:1)	3, 4	y	y, r	C, D
Ochratoxin A	T : Ea : F (6:3:1)	5, 6, 7	bb	y, pb	E
Citrinin	T : Ea : F (6:3:1)	8	--	y, by, lo	E
Zearalenone	C : M (97:3)	8, 5, 6, 9, 10, 1	--', b, ch. bl	b, do, lp,--	E, F
Satratoxin H	C : M (97:3)	11	--	p	G
Roridin E	C : M (97:3)	11	--	p	G

Solvent system: C = chloroform; A = acetone; M = methanol; T = toluene; Ea = ethyl acetate; F = formic acid. Spray reagent: 1 = 20% in $AlCl_3$; 2 = quantitative estimation; 3 = 2% phenylhydrazine hydrochloride; 4 = p-anisaldehyde; 5 = 2,4,DNP; 6 = $FeCl_3$ in ethanol; 7 = ammonia fumes; 8 = $Ce(So_4)_2$; 9 = 50% H_2So_4 in methanol; 10 = H_2So_4; 11 = phloroglucinol. Detection colours: bl = blue; gr = green; y = yellow; r = red; bb= bright blue; pb = purple brown; by = brown yellow; lo = light orange; b = brown; ch = charring; do = dark-orange; lp = light-purple. Reference: A = Stack and Pohland (1975); B = Adye and Mateles (1964); C = Subramanian et al. (1978); D = Scott et al. (1970) E = Grost-Allman and Steyn (1979); F = Mirocha et al. (1974); G = Rao et al. (1985).

only qualitatively.

RESULTS AND DISCUSSION

Mycoflora of rice fodder differed significantly both qualitatively and quantitatively with the storage time. In all, 34 fungal species representing 19 genera were detected (Table 2) during one year observation period. *Aspergillus flavus, Aspergillus niger, Curvularia lunata* and *Rhizopus stolonifer* could be detected during the entire period of observation. However, their percentage of incidence fluctuated. Species of *Fusarium* appeared in June and showed increasing trend until November and declined subsequently and finally disappeared by March. *Memnoniella echinata, Myrothecium roridum* and *Stachybotrys atra* appeared only during the latter part of the storage. When the former two disappeared by March, the latter could be spotted till the end of observation period. *Trichothecium roseum* could be spotted only at the fag end of the year, (January and February). *Sclerotium rolfsii* causal agent of foot rot of rice could be spotted during August and September. Similarly, *Syncephalastrum racemosum* was spotted during August to October, while *Phoma sorghina* could be detected during September to February with a maximum incidence in November. Species of *Penicillium* and *Paecilomyces varioti* were associated with fodder only during initial stages of storage and disappeared by October and September, respectively. There are only a few reports on occurrence of *Penicillium* species on fodder (Skaar, 1996; Brien et al., 2006). *Cladosporium cladosporioides* could be spotted after 4 months of storage but showed a continuous increase till the end of January and disappeared by March. *Chaetomium globosum* could be spotted from July with increasing population but disappeared by November. However, it was spotted again in March. *Alternaria alternata* was traced without any definite trend. Similarly, some species of *Aspergillus* occurred with varying incidence.

When agar plate method was employed (Table 3), the fungi detected were almost the same but differed in the percentage of incidence. *Chaetomium brasilense*, *Curvularia ovoides* and *Drechslera maydis* which could not be spotted in dilution plate method, could be spotted by agar plate method. On the other hand, *S. racemosum* which could be traced in dilution plate method could not be spotted in agar plate method.

The incidence of *A. flavus* followed by *A. niger* was more through out the observation period. Species of *Fusarium* showed an increase in percentage of incidence from June to January and decreased during subsequent months. *T. roseum, Aspergillus ustus* and *Aspergillus flavipes* were low in their percentage of incidence. *A. flavus, A. niger, C. lunata* and *R. stolonifer* were high in their percentage of frequency, while *A. ochraceus, S. rolfsii* and *T. roseum* were low in their percentage of frequency. The percentage of abundance of *A. flavus* was high followed by species of *Penicillium.* On the other hand, *R. stolonifer, Periconia* sp., *A. flavipes* and *M. roridum* were least in their percentage of abundance. No correlation could be observed between percentage of incidence, frequency and abundance.

Out of 24 isolates of *A. flavus* screened, 20 isolates were able to produce one or other aflatoxins (Table 4). About 50% of the isolates produced B1 and B2 toxins. Only 4 isolates out of 6 isolates of *Aspergillus nidulans* elaborated sterigmatocystin. Fifty percent *A. ochraceus* strains were able to elaborate ochratoxin A, while 25% of *A. versicolor* strains could elaborate sterigmatocystin. Out

Table 2. Succession of fungi in rice fodder (dilution plate method) in relation to mycotoxins problem.

Fungus	Percentage of incidence												Percentage of frequency	Percentage of abundance
	2008									2009				
	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar		
A. alternata	2.20	3.00	-	-	-	4.50	-	-	3.20	2.10	3.90	-	50.00	7.20
A. flavipes	1.00	2.00	3.80	-	-	-	-	-	-	-	-	37.30	25.00	1.50
A. flavus	25.60	28.50	29.80	25.00	26.40	28.80	18.50	22.50	28.50	16.80	22.20	-	100.00	70.50
A. fumigatus	0.30	1.20	0.60	-	-	-	-	-	-	-	-	-	25.00	3.90
A. nidulans	7.40	8.30	6.80	-	5.30	6.00	-	6.80	-	-	-	11.00	50.00	5.20
A. niger	11.30	11.00	14.80	12.60	11.40	8.20	5.80	10.30	8.30	5.80	9.80	-	100.00	21.80
A. ochraceus	2.80	-	-	-	1.80	-	-	-	-	-	-	7.00	16.60	3.20
A. terreus	10.80	9.80	-	6.90	7.20	4.80	8.00	7.30	5.20	4.50	6.80	-	91.60	18.20
A. ustus	2.40	-	-	-	0.80	0.40	-	-	-	-	-	-	25.00	4.60
A. versicolar	-	-	-	-	-	2.60	2.20	2.30	1.50	-	-	-	33.30	9.40
Aspergillus spp	-	-	3.80	-	1.80	-	1.50	1.80	0.80	7.20	3.60	11.00	58.30	4.20
C. globosum	-	-	-	3.90	4.00	4.80	13.00	-	-	-	-	-	41.60	6.20
C. Cladosporioides	-	-	-	-	1.50	0.80	1.20	1.80	2.20	3.40	2.20	13.80	58.30	10.30
C. lunata	9.30	9.00	6.80	8.60	7.60	5.40	6.50	10.50	9.50	17.50	15.50	-	100.00	15.80
C. Pallescens	3.50	1.00	3.20	-	-	-	3.50	-	-	-	-	-	33.30	8.20
D. rostrata	-	9.80	-	2.00	1.80	-	-	1.80	-	1.50	-	8.80	41.60	9.50
D. spicifer	11.80	5.60	4.30	-	-	3.20	5.00	-	5.30	7.30	16.30	-	75.00	11.30
Fusarium spp. *(F. oxysporum, F. roseum, F. equiseti, F. moniliforme)*	-	-	6.80	9.20	9.80	8.60	17.30	19.80	15.60	10.80	5.20	-	75.00	6.80
M. echinata	-	-	-	-	-	-	0.80	1.80	2.30	6.40	2.20	-	41.60	4.90
M.roridum	-	-	-	-	-	-	-	-	0.90	3.20	1.80	-	25.00	1.80
P. varioti	1.50	2.20	6.70	7.80	0.90	-	-	-	-	-	-	-	41.60	2.70
Penicillium spp. *(P. oxalicum, P. citrinum, P. funiculosum, P. islandicum)*	1.30	3	3.80	4.00	3.60	2.60	-	-	-	-	-	-	50.00	23.90
Periconia sp	-	-	-	-	1.60	-	-	-	-	-	-	-	16.60	0.90
P. sorghina	-	-	-	-	-	3.50	2.50	3.60	3.00	2.20	1.80	-	41.60	9.30
R. stolonifer	5.50	6.3	8.80	9.00	5.40	6.50	8.80	8.20	5.20	3.00	3.10	3.50	100.00	20.80
S. rolfsii	-	-	-	-	2.50	0.90	-	-	-	-	-	-	16.60	0.80
S. atra	-	-	-	-	-	-	-	1.50	2.80	2.80	2.50	3.20	41.60	2.20
S. racemoseum	-	-	-	-	3.00	2.90	3.50	-	-	-	-	-	25.00	3.80
T. roseum	-	-	-	-	-	-	-	-	-	1.70	3.20	-	16.60	4.20
White sterile mycelium	3.40	-	-	-	3.60	5.50	2.50	-	5.70	3.80	-	5.40	50.00	3.20

Table 3. Succession of fungi in rice fodder (agar plate method) in relation to mycotoxins problem.

Name of the fungus	Percentage of incidence												Percentage of frequency	Percentage of abundance
	2008									2009				
	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar		
A. terricola	0.30	-	-	-	-	-	-	-	-	-	-	-	8.33	6.33
A. alternata	1.20	3.00	-	-	-	2.30	-	-	-	-	-	-	25.00	1.32
A. flavus	28.30	30.30	30.20	28.40	28.80	28.60	20.30	18.30	16.20	16.20	22.30	30.30	100.00	22.28
A. niger	10.50	14.50	11.00	13.30	12.40	10.30	8.30	7.40	8.30	6.40	9.80	13.00	100.00	15.42
A. ochraceus	2.40	3.00	0.10	-	-	-	-	-	-	-	-	-	25.00	0.89
A. terreus	11.30	12.30	3.50	-	-	-	10.30	11.20	8.40	5.30	6.80	-	66.60	4.37
C. brasilense	-	-	-	0.80	-	-	-	-	-	-	-	-	8.33	3.81
C. globosum	-	-	-	10.50	11.30	14.30	15.40	10.50	-	-	-	-	41.66	6.45
C. Cladosporioides	-	-	2.40	4.30	6.30	-	-	-	3.50	2.60	3.80	2.40	58.33	4.32
C. lunata	6.30	7.00	6.80	8.50	9.50	6.70	11.50	12.30	12.30	10.50	9.30	12.40	100.00	0.71
C. ovoides	2.40	-	0.40	-	-	-	-	-	-	-	-	-	16.66	0.47
C. Pallescens	5.40	2.80	9.30	10.30	0.40	-	-	0.30	-	-	-	-	50.00	0.82
D. maydis	-	0.80	0.40	-	-	-	-	-	-	-	-	-	16.66	1.32
D. rostrata	-	-	-	-	0.40	0.70	1.30	-	-	-	-	-	25.00	0.21
D. spicifer	1.40	14.40	11.30	2.30	11.80	4.30	-	-	9.40	8.60	3.40	2.80	83.33	1.35
Fusarium spp. *(F. oxysporum, F. roseum, F. equiseti, F. moniliforme)*	-	-	3.60	5.80	8.80	9.70	10.30	11.40	12.80	12.80	9.40	-	75.00	10.24
M. echinata	-	-	-	-	-	3.20	11.80	13.80	10.80	23.40	20.20	-	50.00	8.34
M. roridum	-	-	-	-	-	-	-	4.00	5.80	5.80	1.20	-	33.33	5.21
Penicillium spp. *(P. oxalicum, P. citrinum, P. funiculosum, P. islandicum)*	11.30	4.60	6.60	7.80	0.80	12.40	-	-	-	-	-	-	50.00	2.32
Periconia sp.	0.20	-	-	-	-	-	-	-	-	-	-	4.40	16.66	0.21
P.sorghina	-	-	-	-	-	-	2.80	4.80	4.30	0.20	1.80	-	41.66	1.72
R. stolonifer	10.80	10.30	8.50	7.30	8.80	7.30	6.30	-	-	-	-	15.30	66.66	5.85
S. rolfsii	-	-	-	-	-	1.00	1.50	1.30	-	-	-	-	25.00	0.92
S.atra	-	-	-	-	-	-	-	4.30	8.20	8.20	12.30	15.20	41.66	2.21
White sterile mycelium	8.40	-	-	0.50	-	-	-	-	-	-	-	4.10	25.00	1.08

of 19 *Aspergillus terreus* strains screened, 3 and 10 were able to produce patulin and terreic acid, respectively. Species of *Fusarium* could elaborate only zearalenone. When 5 strains of *Fusarium moniliforme* were screened, 3 could elaborate zearalenone. Out of 3 strains of *Fusarium semitectum* and *Fusarium solani* each, 2 and 1 strain could elaborate zearalenone, respectively. Five strains of *Fusarium oxysporum* could elaborate zearalenone when 7 strains were screened. When 5 strains were screened, only 2 and 3 strains of *Penicillium oxalicum* and *Penicillium citrinum*, respectively, were able to produce citrinin. None of the isolates of *Penicillium islandicum* were mycotoxigenic. Out of ten strains

Table 4. Toxigenic potential of fungi colonized in stored rice fodder.

Name of the fungi	No. of strains examined	Percentage of incidence (%)	Name of the mycotoxin
A. flavus	24	83.33	Aflatoxin
A. nidulans	6	66.60	Sterigmatocystin
A. terreus	19	52.63	Terric acid
A. terreus	19	15.73	Patulin
A. ochraceus	12	50.00	Ochratoxin A
A. versicolor	4	25.00	Sterigmatocystin
P. oxalicum	5	40.00	Citrinin
P. citrinum	5	60.00	Citrinin
P. islandicum	2	0.00	Citrinin
F.moniliforme	5	66.00	Zearalenone
F. semitectum	3	66.60	Zearalenone
F. solani	3	33.30	Zearalenone
F. oxysporum	7	71.42	Zearalenone
S. atra	10	60.00	Satratoxin H
M. roridum	5	60.00	Roridin E

of *S. atra,* 6 were able to produce satratoxin H. Three strains of *M. roridum* were able to elaborate roridin E, when 5 strains were screened.

From the present investigations, it is clear that fungal colonization of rice fodder differed with time. Some of the typical trichothecenes producing fungi appeared late in fungal succession. The incidence of *A. flavus* did not differ much with the storage time. An increase in storage time was conducive for fungal proliferation. The incidence of *Fusarium* increased considerably with the storage time. It is also clear that rice fodder supported the growth of varieties of mycotoxin producing fungi and if due care is not taken during storage, they may pose a serious threat to the health of cattle and in turn, man.

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