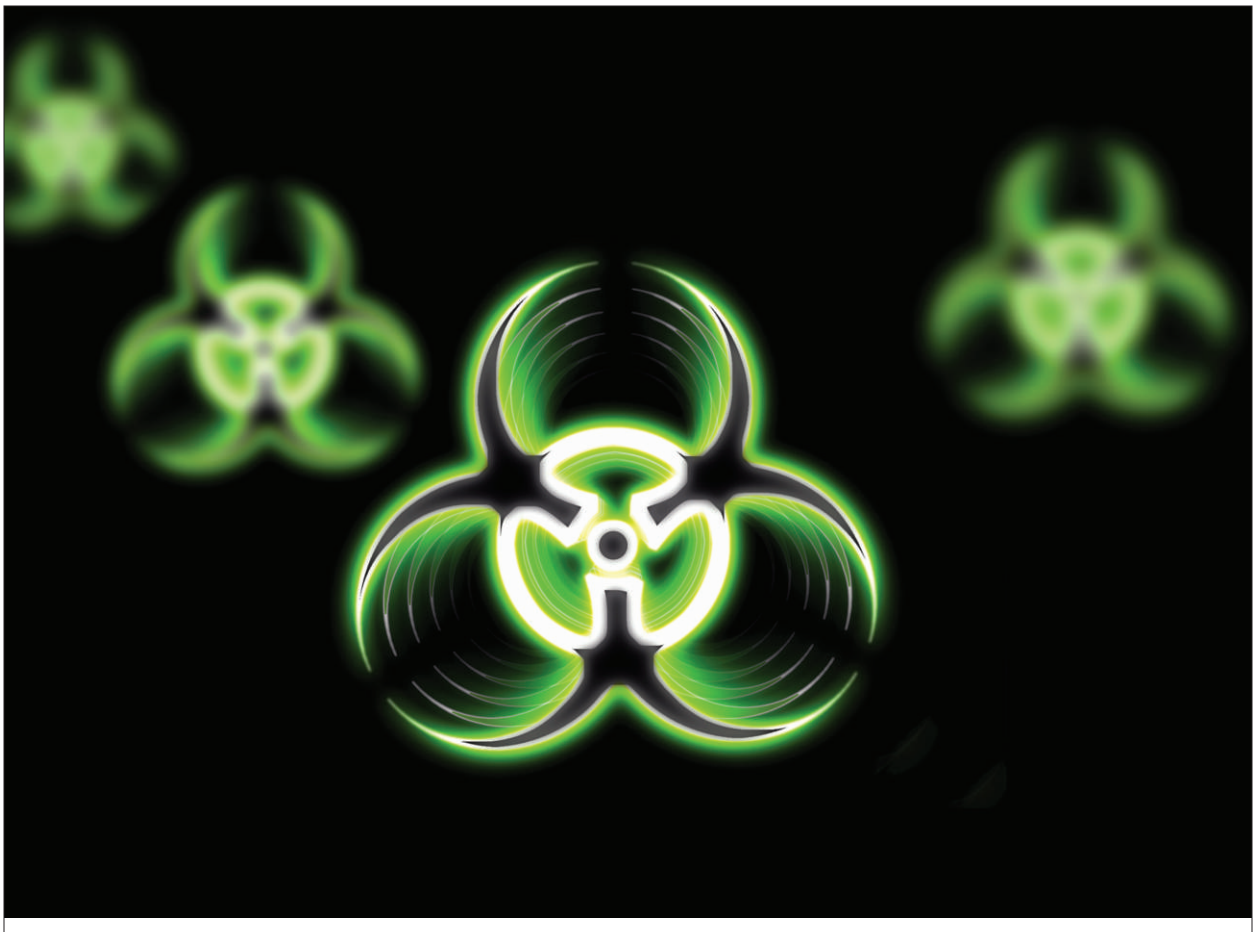

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Incidence of *Fusarium* Toxins in Rice from Karnataka, India

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Abstract: Contamination by molds is a severe problem in fields as well as storage conditions causing significant loss in yield and quality of several commodities. Many of these molds produce mycotoxins, which are toxic to animals and are responsible for many acute and chronic human diseases. Rice, an important food commodity is susceptible to fungal infection in field as well as storage, hence, rice samples grown under different agro-climatic conditions were screened for molds and toxins produced by *Fusarium* sp., an important toxigenic storage fungi. High incidence of storage molds in all the samples was observed. Twenty two different species belonging to 16 genera were recorded. *Aspergillus flavus*, *Fusarium moniliforme* and *Penicillium* sp. were the predominant ones. Among the 64 *Fusarium* isolates, 17 were found to be toxigenic. High performance thin layer chromatography and HPLC analyses of commodities revealed the presence of *Fusarium* toxins deoxynivalenol, nivalenol, diacetoxyscirpenol, T-2, HT-2 and zearalenone. Among these toxins, deoxynivalenol and zearalenone were found in high levels ranging from 20 to 500 μg kg^{-1}, with samples from Western Ghats showing the maximum level of 500 μg kg^{-1}.

Key words: Molds, *Fusarium* toxins, rice, Karnataka

INTRODUCTION

Molds occur widely in nature on variety of food and agricultural products. Mold contamination not only causes deterioration of foods and feeds, but also can cause food and feedborne intoxications in man and farm animals, since they may produce toxic secondary metabolites called mycotoxins (Betina, 1984). Exposure to mycotoxins in food is a widely recognized health risk as they are known to be potent toxins, carcinogens, mutagens and teratogens (Kurata and Ueno, 1984). *Fusarium* is one of the few important toxigenic genera, which can cause food spoilage and biodeterioration. Interest in *Fusarium* sp. is increasing world wide due to the discovery of a growing number of naturally occurring *Fusarium* toxins that have proved to be of threat to the human and animal health (Bhat, 1991; Bhavanishankar and Shantha, 1987; Creppy, 2002; Eriksen and Pettersson, 2004; Placinta *et al*., 1999). The involvement of this fungus in feedborne toxicity like hemorrhagic symptoms, esophageal cancer in humans etc. have focused the attention of scientific community on *Fusarium* toxins (Angsubhakorn, 1987; Calvert *et al*., 2005; Kurata and Ueno, 1984; Ueno, 1983).

The most common and important *Fusarium* toxins include deoxynivalenol (DON/vomitoxin), diacetoxyscirpenol (DAS), HT-2, T-2 and nivalenol, which are classified under trichothecenes and zearalenone (Ueno, 1983). There were frequent reports of outbreaks of *Fusarium* toxins in several parts of the world. In India, a well-known outbreak involving *Fusarium* species among humans is the scabby grain intoxication reported from Kashmir (Bhat *et al*., 1989). But hitherto not much importance

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was given to *Fusarium* toxins by researchers, especially in India, where rice is the most important food commodity and is highly susceptible to *Fusarium*, both on and off the field. Hence, an attempt has been made here to gain more information on the kind of fungi and their frequency of occurrence and on the incidence of *Fusarium* toxins in rice grown in Karnataka.

MATERIALS AND METHODS

Mold Analysis

A survey on prevalence of fungal species and on the status of *Fusarium* toxins in rice (*Oryza sativa*) from different parts of Karnataka state, India, was conducted. As first part, a total of 50 samples were procured from local markets that include government controlled APMC yards and private dealers. The samples also include the rice from various rice mills in western Ghat region of Karnataka, which reported occasional discoloration and deterioration. Sample size ranges from 300 g to 1 kg and these samples were screened for presence of storage molds by blotter test (ISTA, 1993) and also by spread plate method using PDA media amended with streptomycin sulphate (King *et al*., 1986). Mold flora were identified and enumerated by traditional procedures (Jarvis *et al*., 1983; Joffe Abraham, 1986; Lacey *et al*., 1980). Samples were analyzed for *Fusarium* sp. using banana leaf agar and isolates were identified to species level using conventional protocols (Joffe Abraham, 1986). The percent incidence was determined for both the tests and tabulated.

Toxigenic *Fusarium* isolates

Isolates of *Fusarium* sp. obtained from rice samples were screened for their toxigenic nature by inoculating them on to rice medium with 40% moisture and incubated at 28±0.5°C for 4 week for toxin production (Lee *et al*., 1986; Steyn and Vleggar, 1986).

Analysis of *Fusarium* Toxins

Analysis of *Fusarium* toxins was carried out by dry grinding samples to the consistency of flour in a laboratory mill to pass through 20 BSM sieve. Ground samples were extracted and analyzed for the presence of type-A trichothecenes such as diacetoxyscirpenol, T-2 and HT-2 and type-B trichothecenes such as deoxynivalenol and nivalenol as well as zearalenone according to Abbas *et al*. (1984) and Bosch and Mirocha (1992). Briefly, 100 g of sample was moistened with 25 mL distilled water and extracted with methanol-water (55:45), defatted with n-hexane and partitioned with dichloromethane. The solvent was evaporated to dryness; the residue was dissolved in 5 mL of methanol and an aliquot of each sample was resolved by TLC by comparison with standards from Sigma and quantified by using chromo-densitometric scanning of TLC plates with Camag TLC scanner 3 (HPTLC) (Kamimura *et al*., 1981).

The standard mycotoxins (Sigma) and the sample extracts were applied to TLC plates precoated with silica gel 60 (Kieselgel 60, E. Merck, Darmstadt, Germany) and the plates were developed in solvent systems of chloroform-acetone (60:40) for trichothecenes and chloroform-ethanol (93:7) for zearalenone. After development the plates were air-dried and trichothecenes were detected by spraying first with 4-(p-nitrobenzyl) pyridine solution [1% in chloroform-carbon tetrachloride (40:60)]. After drying trichothecenes appear as blue spots on white background under UV light (Bennet and Shotwell, 1990; Takitani *et al*., 1979). For zearalenone detection, plates were air dried after development and observed under UV light (both long and short wave) and sprayed with aluminum chloride (20% in ethanol) solution and reexamined under long wave UV for fluorescence spots. The identity and amount of trichothecenes were confirmed by HPLC (LC 50 Shimadzu). Separations were achieved on a C18 reverse phase column using water-acetonitrile-methanol (5:4:1, v/v/v) as an isocratic mobile phase at a flow rate of 0.5 mL min^{-1}. Diode array detector was used at 236 nm and fluorescence detector was recorded at excitation 236 nm and emission 464 nm. Quantification was by comparison with reference standards (Abbas *et al*., 1984; Bosch and Mirocha, 1992).

RESULTS AND DISCUSSION

Mold Analysis

Blotter test of rice grains revealed a total of 22 different fungal species belonging to 16 genera. The most common fungal species associated with these samples were *Rhizopus* sp., *Penicillium* sp., *Fusarium* sp. and *Aspergillus* sp. in that order. *Fusarium moniliforme* was the most predominant mycoflora (1-67 % incidence) followed by *Rhizopus* (0-61% incidence), *Penicillium* (0-60% incidence), *Fusarium solani* (0-49% incidence) and *Aspergillus flavus* (6-48 % incidence). The results were further supported by plate count studies (Table 1). A total of 19 species belonging to 13 genera were recorded with species of *Penicillium* being the most common group observed with mean $\log_{10}$ cfu 1.81 g^{-1} followed by *Fusarium moniliforme* ($\log_{10}$ 1.73 g^{-1}) and *Aspergillus flavus* ($\log_{10}$ 1.67 g^{-1}) (Table 1).

Toxigenic *Fusarium* Isolates

A total of 64 *Fusarium* isolates were isolated from rice samples and checked for their toxigenicity. Eleven isolates were found to be toxigenic (Table 2). Seven isolates produced deoxynivalenol. Whereas

Table 1: Incidence of molds on rice samples

Molds	Percent incidence* (range)	Mean mold count** ($\log_{10}$ cfu g^{-1})
Aspergillus flavus	6-48	1.67
A. niger	0-30	1.12
A. candidus	0-27	1.31
A. nidulans	0-6	0.62
A. fumigatus	0-6	1.53
Fusarium solani	0-49	1.36
Fusarium moniliforme	1-67	1.73
Fusarium oxysporium	0-13	1.45
Alternaria alternata	0-6	1.16
Botrydiplodia theobromae	0-2	0.87
Aschochyta sp.	0-2	0.40
Cephalosporium sp.	0-6	0.00
Cladosporium sp.	0-34	1.10
Curvularia lunata	0-10	0.67
Chaetomium globosum	0-6	0.45
Colletotrichum sp.	0-4	0.00
Trichoderma sp.	8-10	1.48
Mucor sp.	0-25	1.04
Nigrospora oryzae	0-15	0.80
Penicillium sp.	0-60	1.81
Rhizopus sp.	0-61	1.30
Verticillium sp.	0-2	0.00

*: Tested by standard blotter method; **: Tested by plate count method

Table 2: Toxigenic *Fusarium* isolates from rice samples

	Mycotoxin produced			
Isolate*	Nivalenol	Deoxynivalenol	T-2	Zearalenone
Smg-3	-	+	-	-
Smg-4	-	+	-	+
Smg-5	-	+	-	-
Smg-6	-	+	-	-
Rcr-11	-	-	+	-
Sdr-15	-	+	-	+
Tum-18	+	-	-	-
Cta-20	-	+	-	-
Bgl-21	-	+	-	-
Bgl-22	-	-	+	-
Man-26	+	-	-	-

*: None of the isolates produced HT-2 or diacetoxyscirpenol

Table 3: Natural contamination of rice samples with *Fusarium* toxins

	Rice	
Toxin	Positive samples	Range (μg kg^{-1})
Deoxynivalenol	12/50	20-500
Diacetoxyscirpenol	4/50	100-200
HT-2	0/50	ND
Nivalenol	3/50	50-100
T-2	0/50	ND
Zearalenone	6/50	20-300

Table 4: Mycotoxicological analysis of rice samples

		Toxin (μg kg^{-1})			
Samples	Mold count ($\log_{10}$ cfu g^{-1})	Nivalenol	Deoxynivalenol	Diacetoxyscirpenol	Zearalenone
Wg-1	3.36	--	300	--	--
Wg-2	3.28	--	200	--	300
Wg-4	3.20	--	300	100	--
Wg-5	3.34	--	500	--	100
Wg-6	3.30	50	--	--	--
Wg-7	3.32	--	--	200	--
Wg-9	3.35	--	200	--	200
Wg-10	3.33	--	400	100	--
Pl-2	2.99	--	100	--	20
Pl-9	2.98	--	50	--	100
Pl-15	3.08	50	--	--	--
Pl-22	2.96	--	100	--	--
Pl-28	3.16	--	20	--	50
Pl-30	3.00	100	--	--	--
Pl-35	2.97	--	200	--	--
Pl-36	2.99	--	200	--	--
Pl-39	3.05	--	--	100	--

*: None of the samples yielded produced T-2 or HT-2 toxin

nivalenol, T-2 toxin and Zearalenone were produced by two isolates each. None of the isolates produced HT-2 and diacetoxyscirpenol. Regarding co-production of mycotoxins, only Zearalenone was produced as a co-product along with deoxynivalenol by two isolates smg-4 and sdr-15. Remaining five of the deoxynivalenol producers and two each isolates which produced Nivalenol and T-2 were able to produce single toxins only.

Analysis of *Fusarium* Toxins

The analysis of rice samples for natural occurrence of mycotoxins revealed the presence of trichothecenes as well as zearalenone (Table 3). deoxynivalenol was the most common toxin found (12/50 samples) followed by zearalenone (6/50), diacetoxyscirpenol (4/50) and nivalenol (3/50). Quantity wise also occurrence of deoxynivalenol was highest (500 μg kg^{-1}) followed by zearalenone (300 μg kg^{-1}). T-2 and HT-2 toxins were not detected in any of the samples tested.

Enumeration of *Fusarium* in Toxin Positive Samples

Among the rice samples, *Fusarium* count ranged from 0.96 $\log_{10}$ cfu g^{-1} in Pl-22 to a maximum of 1.36 $\log_{10}$ cfu g^{-1} in Wg-9. A mean count of 0.76 $\log_{10}$ cfu g^{-1} was recorded in samples which did not yield any toxins (Table 4). Three samples yielded nivalenol in the range of 50 to 100 μg kg^{-1}, 12 samples yielded deoxynivalenol, which ranged from 20 to a maximum of 500 μg kg^{-1} in Wg-5. Four samples yielded diacetoxyscirpenol in the range 100 to 200 μg kg^{-1} and six samples yielded zearalenone in the range of 20 to 300 μg kg^{-1}. T-2 and HT-2 toxins were not detected in any of the samples. Eight samples yielded more than one toxins.

A critical analysis of the results of these samples, which, when grouped under two chief agro-climatic zones to which they belong revealed that those samples from western ghat region showed

Table 5: Mycotoxicological analysis of rice samples from western ghat and Karnataka plains

Samples	No. of samples	Mold count (log_{10} cfu g^{-1})	Toxins			
			T-2	Diacetoxyscirpenol	Deoxynivalenol	Nivalenol
Western ghat	10	3.31	ND	3/10	7/10	1/10
Plains	40	3.02	ND	5/40	12/40	1/40

higher mean *Fusarium* count of 1.31 log_{10} cfu g^{-1} when compared with samples from plains, which showed 1.02 log_{10} cfu g^{-1} (Table 5). Percentage of toxin positive samples was also high in western ghat region as 70% were deoxynivalenol positive, 30% were diacetoxyscirpenol positive and 10% were nivalenol positive whereas, for samples from plains it is 30% for deoxynivalenol, 12.5% for diacetoxyscirpenol and 2.5% for nivalenol. T-2 toxin was detected in none of the samples.

Present investigation suggests the high incidence of molds like *Penicillium* sp., *Aspergillus* sp., *Fusarium* sp. and *Rhizopus* sp. on rice samples. These fungi are most common under Indian conditions and are mostly saprophytic in nature. A few show facultative parasitism under favorable conditions (Bhat, 1988; Dakshinamurthy and Shukla, 1991; Ghosh and Nandi, 1981; Janardhana *et al.*, 1999).

Plate count studies supported the blotter studies but with the exception *Rhizopus*, whose incidence was found low under plate count studies. This is because *Rhizopus* is the predominant saprophytic fungi occur mainly as a surface contaminant (Alexopoulos *et al.*, 1996) whereas the other three fungi are known to invade and colonize the intercellular spaces (Agrios, 2005; Alexopoulos *et al.*, 1996). Presence of high percentage of mycoflora, particularly *Aspergillus* sp., *Fusarium* sp. and *Penicillium* sp. is important because these species are known to produce harmful toxins (Chelkowsky, 1991). The toxins produced by these fungi particularly from *Aspergillus* and *Fusarium* sp. are harmful to humans as well as animals. Among these, trichothecenes and zearalenone produced by *Fusarium* spp. are some of the important ones, which pose significant health risk to humans as well as animals (Creppy, 2002; Eriksen and Pettersson, 2004; Placinta *et al.*, 1999). The investigations revealed the occurrence of these toxins on Indian rice in significant levels as few of the samples showed higher levels of deoxynivalenol and zearalenone than codex limits prescribed for cereals. This shows adaptability of the fungus *Fusarium* that produces toxins under varied climatic conditions (Toffe Abraham, 1986).

The toxigenic nature of the isolates is the result of genetic constitution of the organism and its reaction with the environment (Joffe Abraham, 1986). Most of the isolates produce single toxin and among the co-producers none of the deoxynivalenol producers produced nivalenol. This agrees with the findings of Ichinoe *et al.* (1983) and others (Greenhalgh *et al.*, 1983; Tanaka *et al.*, 1988), who demonstrated that isolates of *Fusarium graminearum* were chemotaxonomically subdivided in to deoxynivalenol-adeoxynivalenol and nivalenol-fusarinon-x producers with differences in their geographical distribution. Interestingly, diacetoxyscirpenol occurred only as a co-product either with deoxynivalenol or with both deoxynivalenol and T-2.

The high incidence of molds in rice samples from western ghat region may be due to prevailing weather conditions in that region. This zone lying between the Arabian sea and the peninsular India receives high rainfall particularly during monsoon season, which corresponds with growth and harvest period of rice and sometimes proper drying of paddy was not possible due to intermittent or continuous rainfall. High moisture content in paddy and subsequent moisture in rice is congenial for *Fusarium* development and toxin production whereas in case of rice from plains, the growing conditions were semiarid type with low rainfall and low humidity which are not so ideal for mold growth.

The variation in occurrence of molds among the samples can be explained by the fact that, in India rice is grown under different agro-ecological situations and processed mostly under natural conditions and prevailing environmental conditions play an important role in natural microbial load of the commodities. As *Fusarium* is an important saprophytic fungus, which require high moisture content

(Bhat, 1988; Chelkowsky, 1991; Christensen *et al*., 1980; Dakshinamurthy and Shukla, 1991; Patkar, 1993), their incidence is low on commodities, which are grown and/or processed under dry conditions. Whereas, commodities grown either under irrigated or high rainfall conditions and processed under humid conditions are usually loaded with high fungal species.

High incidence of molds and presence of toxins in rice samples indicates bad handling of these commodities by farmers as well as millers and traders who store under poor conditions, which encourages mold growth and toxin production. Till recently mycotoxicological quality measures in India were primarily aimed at the detection and reduction of aflatoxins but the present study indicates that, adequate measures have to be taken also in respect of *Fusarium* toxins in general and deoxynivalenol and zearalenone in particular.

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