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Ability to Produce Fumonisin B_1 by *Fusarium* Species, Section *Liseola*, Isolated from Unpolished Rice in Mazandaran, Iran

[1]S.R. Aghili, [2]A.R. Khosravi, [3]T. Shokohi, [4]B. Salmanian, [5]H. Shokri and [2]O. Nikaeen

[1]Department of Veterinary Mycology, Faculty of Veterinary Medicine,
Islamic Azad University, Science and Research Branch, Tehran, Iran
[2]Mycology Research Center, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
[3]Department of Medical Mycology and Parasitology, Faculty of Medicine,
Mazandaran University of Medical Sciences Sari, Iran
[4]Education services office, Mazandaran University of Medical Sciences, Sari, Iran
[5]Department of Microbiology, Faculty of Veterinary Medicine, University of Mazandaran, Amol, Iran

Abstract: *Fusarium moniliforme* (*F. moniliforme*) is one of the most frequent fungal pathogens found in rice. Samples of rice grown in three different zones of Mazandaran Province, Iran were collected and analyzed for the presence of fumonisin B_1(FB_1). Mycological analysis revealed the presence of *F. moniliforme*-type isolates in 65.1% of the rice samples. Average levels of FB_1 per zones 1, 2 and 3 were 108-6711, 950-7711 and 98-6595 µg/g, respectively. Although Rice grown in Mazandaran province is contaminated with FB_1 and depended on geographical zone and period of collection, no significant differences were observed among samples from different zones.

Key words: *Fusarium moniliforme* · Fumonisin B_1 · Rice · Mazandaran province

INTRODUCTION

Rice (*Oryza sativa*) is considered as the most important staple food for human population worldwide, especially in the Middle East. This grain is the second highest worldwide production after maize [1]. The outer husks of the rice grain can be used to feed animals and as fertilizer or fuel [2]. Iran, as an important country in producing rice, ranks 23 in rice production and 26 in areas under cultivation and has approximate 630,000 hectares rice fields [3,4]. Rice is the main food in Iran, particularly in the northern parts which have the maximum of production too. Mazandaran is one of the provinces in northern Iran which has a favorable climate for fungal growth and mycotoxin production on foods (with average annual temperature of 17.7°C and average humidity of 75.5%). It is hot and humid for most part of the year, especially between May and October [5,6].

Rice is one of the cereals invaded by various fungal species in pre- or post-harvest. The most important fungal diseases in this product are rice blast, sheath blight, bakanae and sheath rot. Researchers have shown that *Fusarium* species chiefly caused some of these diseases [7-9].

A variety of mycotoxins can be produced by *Fusarium* species including fumonisins, moniliformin, beauvericin, fusaric acid and fusarin C [10]. Fumonisins are regarded as one of the most important groups of mycotoxins in food and feed industries and agriculture. Their contaminations in foods and feeds lead to several fatal diseases in livestocks and assumes a significant cancer risk to humans (group 2B carcinogen) [11,12]. Therefore, fumonisins have attracted a lot of attention in mycotoxin researches. The fumonisins consist of fumonisin B_1 (FB_1), FB_2, FB_3, FB_4 and other series of derivatives. Previous studies showed that FB_1 causes equine leukoencephalomalacia [13], pulmonary edema in swine [14] and has been implicated in the causation of human esophageal cancer [15]. Some researchers in Iran and in other countries have documented the fungi and mycotoxins which contaminated the cereals in Iran [16-19] and other countries [20-22] as well. As FB_1 in rice is detrimental to good health of human and animals, it sounds that sufficient researches in Mazandaran province

Corresponding Author: S.R. Aghili, Department of Veterinary Mycology, Faculty of Veterinary Medicine,
Islamic Azad University, Science and Research Branch, Tehran, Iran
P.O. Box: 14155-6453, Tel: +982161117151, Fax: 00982166933222, E-mail: aghili70@yahoo.com

have not been performed. This study tried to determine whether *Fusarium* species isolated from unpolished rice have the ability to produce FB_1 or not.

MATERIALS AND METHODS

Collection of Rice Samples: One-hundred different unpolished and stored rice samples intended for human consumption were collected from 100 various rice farmers of 4 district zones of Mazandaran province, Iran, during August and November 2009 (Table 1). The locations were chosen on the basis of statistic random in each zone of province. Samples (approximately 500 g) were double packed in sterile paper bags then in appropriately labeled polythene bags. The grain samples were stored in the laboratory at room temperature in Mycology Research Center, in Mycology and Parasitology Department of Faculty of Medicine of Mazandaran University. The mycological analysis was started within a week after sampling. All the samples did not contain visible signs of mold contamination.

Isolation of *Fusarium* Species: According to International Seed Testing Association (ISTA), samples were subjected to analysis by hand halving method [23]. Briefly, 100 paddy grains were surface sterilized by %1 sodium hypochlorite for 1 min and rinsed twice in sterile distilled water for 30 seconds. Twenty-four surface sterilized grains were plated (12 per plate) on potato dextrose agar medium (PDA) containing chloramphenicol (100 mg/l). For better isolation of fungi, 4 plates were used for each sample. Two plates were incubated at 27°C and other two plates were incubated at 17°C for 7-10 days. The developing fungal colonies were counted directly after incubation. *Fusarium* species were sub-cultured on PDA medium for species identification. In this study unless otherwise indicated, all the chemicals were purchased from Merck Co., Darmastdt, Germany.

Determination of *Fusarium* Species: In this study a total of 43 *Fusarium* isolates were used. These colonies were identified on the base of morphologically and microscopically characters in different media. The isolation and identification procedures of *Fusarium* species have also been described in detail [24].

Media and Single-Spore Isolation: Different *Fusarium* species cultures were transferred to 1 ml of sterile Tween 20 in water (1:104, v/v) with a sterile wire loop. The suspensions were mixed on a vortex mixer and streaked on agar/water plates with a sterile loop. Plates were incubated at room temperature overnight. For each culture, 3 germinated spores were picked and used to inoculate PDA slants and plates. Cultures were incubated at room temperature and were grown for 1 to 2 weeks on an alternating light-dark schedule at 17 to 25°C. The slants and the plates were washed with sterile distilled water to produce conidial suspensions. Each PDA medium (12 cm diameter) was inoculated with 10^7 conidia and shaken once or twice daily for 3 days to distribute the inoculums. The cultures were incubated in the dark at 17± 2°C for 10 days.

Extraction of FB_1: Fresh fungus-infested culture material (30 g samples in triplicate) was extracted with 100 ml acetonitrile: water (80:20, v/v) by shaking on a wrist shaker for 1 h. Extracts were obtained by filtering through 12.5 cm #588 filter paper (*Schleicher and Schuel*) and stored at room temperature until used for fumonisin purification [4].

Fumonisin Sample Preparation: Samples were prepared for HPLC analysis using SPE ODS-3 columns (Whatman Solid Phase Extraction Device ODS-3, 500 mg/6 ml, cat. no. 6801-0307) on a vacuum manifold with solvent flow rate = 2 l/min. Columns were preconditioned with 5 ml methanol followed by 5 ml 1% KCL. To prepare fumonisin samples, 2 ml of sample extract was mixed with 5 ml 1% KCL and applied to a column. The column was washed with 5 ml 1% KCL followed by 2 ml of 10% acetonitrile in 1% KCL. Fumonisins were eluted from the column with 4 ml of 70% acetonitrile in water into a 10 ml silane-treated vial and dried in a heating block at 60°C under a stream of nitrogen. Samples were stored at room temperature until HPLC analysis [25].

HPLC Analysis of Fumonisins: Samples for analysis of all fumonisins were resuspended in 1 ml methanol. The following reagents were added with mixing in order: sodium borate buffer (1 ml of 0.05 M, pH 9.5), NaCN solution (0.5 ml of 13 mg/100 ml water) and naphthalene-2,3-dicarboxaldehyde reagent (0.5 ml of 2 mg/8 ml methanol). Samples were then incubated in a heating block at 48°C for 15 min and allowed to cool at room temperature. The samples were diluted with 7 ml of mobile phase water: acetonitrile: acetic acid (40:60:1), filtered through a 0.2 mm filter (Gelman 13 mm diameter, 0.2 mm pore size, acrodisc PTFE) and an aliquot injected onto the HPLC column [26].

Statistical Analyses: Chi-square (X^2) testing was performed using SPSS software (Version 15) to assay concentrations of FB_1 relative to meteorological zone. A *P* value less than 0.05 was considered significant.

RESULTS

Twenty-three cultures were established from 100 rice samples and 43 fungal colonies were identified as *Fusarium* species. These samples obtained from 100 various rice farms of 4 districts zones. *Fusarium* colonies (69.8%) were mostly separated at 17°C maximum incubation conditions. Twenty-eight isolates of only section *Liseola (Fusarium moniliforme*) were isolated from 16 rice samples collected from 3 location zones at Mazandaran province. *F. moniliforme* (65.1%) was found to be the most dominant species isolated from all the tested samples. Variation in the ability of *F. moniliforme* to produce FB_1 was quite high and 15 of the 16 *Fusarium* colonies had ability of FB_1 production in the range of 98-7711 μg/g. Concentrations of FB_1 produced by different isolates were summarized in Table 2.

FB_1 was not detected only in one sample of *Fusarium* section *Lesiola* and 3 of them produced <110 μg/g that had a range of 98-108 μg/g. Twelve cultures produced FB_1 in amount of >900 μg/g with a range of 922-7711 μg/g. This study has also established that different *F. moniliforme* isolates were capable of producing significant quantities of FB_1 and these isolates were not limited to a given region of Mazandaran province of Iran. The culture which contained the largest number of FB_1 by HPLC analysis belonged to one rice sample from Hezar jarib neka (zone II). There was not a significant difference of FB_1 production among the *F. moniliforme* isolated from different zones ($p<0.05$).

Table 1: Meteorological data * of 4 district zones of Mazandaran province, Iran, between August and November 2009

Meteorological zone	No rice samples	Minimum temperature (°C)	Maximum temperature (°C)	Mean temperature (°C)	Mean relative humidity (%)	Mean rainfall (mm)	Meteorological stations
East moderate climate (Zone I)	55	18.1 ± 4.7	27.2±5.4	23.2± 6.0	76.0± 3.9	70.1± 56	Babolsar, Sari, Gharakheil, Dasht-e naz, Amir abad, Galogah
East mountainous climate (Zone II)	18	14.1± 5.8	23.5± 6.8	18.7± 6.3	72.8± 5.9	42.2 ± 31	Amol, Alasht,
West moderate climate (Zone III)	15	18.7± 0.5	25.2±5.0	22.0± 4.8	79.8± 2.7	147.9± 115	Noshahr, Ramsar
West mountainous climate (Zone IV)	12	9.5± 5.1	21.0± 7.0	15.2± 6.0	61.9± 7.6	23.1± 23	Siah bishe, Kojor, Baladeh

*Data supplied by the Mazandaran Meteorological Organization

Table 2: Sections of *Fusarium* species isolated from different unpolished rice samples in Mazandaran, Iran

Section	*Fusarium* species	Number of isolates	Frequency (%)
Liseola	Moniliforme	26	60.4
	Moniliforme var. Subglutians	2	4.7
Discolore	Culmorum	3	7.0
Sporotrichiella	Sporotrichoides var. tricinctum	3	7.0
	Poae	1	2.3
	Sporotrichoides var. chlamidosporum	2	4.7
Eupionnotes	Dimerum	1	2.3
Elegans	Oxysporum	1	2.3
Roseum	Arthrosporioides	3	7.0
Gibbosum	Equiseti	1	2.3
Total		43	100.0

Table 3: Concentrations of fumonisin B_1 produced by different isolates of *Fusarium moniliforme* isolated from unpolished rices at mazandaran province, Iran

Local strain Number	Fumonisin B_1 concentration (µg/g)	Locality (Meteorological Zone No.)
FM_1	6711	Jouybar (I)
FM_2	6012	Bahnamir (I)
FM_3	4115	Babol (I)
FM_4	ND	Bahnamir (I)
FM_5	108	Babol (I)
FM_6	2993	Babol (I)
FM_7	950	Hezar jarib neka (II)
FM_8	7711	Hezar jarib neka (II)
FM_9	108	Galougah (I)
FM_{10}	1121	Amol (II)
FM_{11}	5795	Amol (II)
FM_{12}	922	Feridonkenar (I)
FM_{13}	1140	Ramsar (III)
FM_{14}	6596	Tonekabon (III)
FM_{15}	3098	Chalous (III)
FM_{16}	98	Royan (III)

- ND= not detected

DISCUSSION

Fumonisins are a series of structurally related sphingosine analog toxin produced by *F. moniliforme* and other fungi [10]. The most abundant and one of the most active members of this series is FB_1. We reported here the detection of FB_1 by HPLC in rice seed harvested from different farms in Mazandarn province, where rice is a major crop.

The natural occurrence of fumonisins in rice was first reported by Abbas *et al*. [27] and there are some other reports on fumonisins in rice [16]. In the first report of rice contaminated with fumonisins, it was shown by HPLC that 40% of rice was positive for FB_1 at levels of 4.3 mg/kg [27]. However, no recovery data for the analysis of fumonisins in unpolished rice are published to date. In the present study analysis of 28 strains of *F. moniliforme* isolated from rice indicated that 65.1% of the strains produced significant levels of FB_1 (98-7711 µg/g) when grown on *Fusarium* cultures. The observation indicated that *F. moniliforme* isolates from unpolished rice samples is capable of producing fumonisins in culture. This result is consistent with *F. moniliforme* being the source of fumonisins detected in naturally contaminated rice samples from fields or during stored with *Fusarium* species. This finding showed that when conditions are conducive to infection, *Fusarium* contamination of rice seeds can occur. Although only FB_1 was quantitated in this study, it is likely that many additional mycotoxins and other secondary metabolites were produces as well. Previous studies reported that the strains of *Fusarium* species isolated from maize in Canada have produced the mutagenic fusarins [28] and strains isolated from maize, wheat, asparagus and rice in Italy have been able to produce the cyclic peptide toxin beauvericin in addition to FB_1 [29]. In present study average levels of FB_1 per zones 1, 2 and 3 were 108-6711, 950-7711 and 98-6595 µg/g, respectively. FB_1 levels in samples from zone 2 were higher than those in samples from the other two zones, independent of the collection period. Chi-square test indicated that there were not significant differences in FB_1 levels as a function of geographical zone and collection period. As a result, the difference in fungal infestation and fumonisin production among the zones and periods of collection could be due to differences in harvesting techniques, collection phase, storage conditions, source of *Fusarium liseola* section isolates in soil and/or environmental conditions (temperature, relative humidity and rainfall). The effect of rainfall and relative humidity on fumonisin levels showed a drastic variation during the harvesting season, which could have produced physiological stress on the crops [30]. In addition, the results could be related to the differences in agricultural practices in each zone, where farmers used a different handling process before and during harvest.

It was concluded that *F. moniliforme* is one of the most important *Fusarium* species coexisted with contamination or disease in pre- or post- harvest of rice seeds. Thus, rice grown in Mazandaran province is contaminated with FB_1 with levels varying within geographical region and maize collection period and needed for monitoring for FB_1.

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