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MYCOTOXINS AND OTHER SECONDARY METABOLITES IN SPECIES OF *FUSARIUM* ISOLATED FROM SEEDS OF CAPSICUM, CORIANDER AND FENUGREEK

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Abstract

Mycotoxins and napthaquinone pigments of seed-borne *Fusarium moniliforme*, *F. subglutinans*, *F. semitectum*, *F. oxysporum*, *F. equiseti* and *F. solani* were investigated using Thin Layer Chromatography. Equisetin, an antibiotic and a mild toxin, has been reported for the first time from several isolates of *F. semitectum*. Zearalenone, zearalenol, deoxynivalenol, moniliformin, anhydrofusarubin, fusarubin and bostrycoidin were detected from *F. moniliforme*, *F. semitectum*, *F. equiseti* and *F. solani*. A simple dichotomous key has been proposed for the identification of 6 seed-borne *Fusarium* spp. The key is based on the TLC profiles of their secondary metabolites as well as their growth response to tannin.

Introduction

Species of the genus *Fusarium* are widespread in nature, occurring as saprophytes in soil, in decaying vegetation and as parasites of wild and cultivated plants in which they cause a variety of diseases such as wilt, blight and rotting of tissues (Trenholm *et al.*, 1981). Besides, grains invaded by some species of *Fusarium* have been known to be toxic (Diener *et al.*, 1981). With the discovery of naturally occurring *Fusarium* mycotoxins viz., zearalenone, moniliformin and fusaric acid etc., the interest in toxigenic *Fusarium* spp., have increased since they adversely affect human and animal health. During studies on the seed-borne mycoflora of capsicum, coriander and fenugreek, effort has been made to examine the potentially toxigenic species of *Fusarium* for production of mycotoxins, pigments and other secondary metabolites. These have been used to develop a key for the identification of *Fusarium* species.

Materials and Methods

Collection of seed samples: Seed samples of capsicum, coriander and fenugreek from Asian, African and Latin American countries were obtained through the courtesy of Danish Government Institute of Seed Pathology, Copenhagen, Denmark. Standard blotter technique (Anon., 1966) was used for the isolation of seed-borne species of *Fusarium* which were identified after reference to Booth (1971) and Nelson *et al.*, (1983). Fungal isolates were grown and maintained on Spezieller nahrstoffarmer agar (SNA) (Nirenberg, 1976) using KNO_3 1.0 g, KH_2PO_4 1.0 g, $MgSO_4$ $7H_2O$ 0.5 g, KC1 0.5 g, glucose 0.2 g, sucrose 0.2 g, agar 15.0 g in 1000 ml distilled water. The cultures

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grown on this low nutrient medium were used for morphological study and for producing conidial suspension which was used as inoculum. For identification of *Fusarium* spp., the sporulating cultures were obtained by placing autoclaved filter paper strips at the periphery of actively growing colonies on SNA incubated at 22°C for 7 days.

Yeast Extract Sucrose (YES) Agar (Frisvad & Filtenborg, 1983) containing sucrose 150.0 g, yeast extract 20.0 g, agar 15 g in 1000 ml. distilled water; Corn Grit Agar (CGS) (Thrane, 1986) containing corn grits 75.0 g, trace metal sol., 1.0 ml, agar 15.0 g in 1000 ml distilled water and Rice Meal Agar (RA) (Thrane, 1986) containing rice meal 75.0 g, trace metal sol.,1.0 ml, agar 15.0 g in 1000 ml distilled water were used for toxin production. Rice meal was prepared by grinding rice for 3 minutes. Corn grits and rice meal were boiled for 2 h before autoclaving. The media were sterilized for 30 minutes at 15 psi. The trace metal solution containing $CuSO_4$. $5/H_2O$ (50 ppm) and $ZnSO_4$. $7H_2O$ (100 ppm), were added to ensure typical conidium colours and enhance production of secondary metabolites (Smith, 1949). Tannin-sucrose agar medium (TAN agar) containing sucrose 3.0 g, K_2HPO_4. $3H_2O$ 1.3 g, KCl 0.5 g, $MgSO_4$. $7H_2O$ 0.5 g, $NaNO_3$ 0.3 g, $FeSO_4$. $7H_2O$ 0.01 g, agar 20.0 g, tannin solution 150 ml in 850 ml distilled water was used. After autoclaving, the medium was cooled down to 50°C and before pouring intro Petri dishes a tannin solution was added to the basal medium. The tannin solution was prepared by dissolving 10 g tannin in 150 ml of distilled water. The solution was boiled for 10 minutes and carefully mixed with the basal medium to bring it to 1 liter. Isolates of *Fusarium* spp., were incubated for 7 days at 20°C.

Detection of Mycotoxins and Pigments

Thin Layer Chromatography (TLC): Four mm diameter discs from the centre of 7-day old culture were removed and a drop of extraction liquid (chloroform:methanol, 2:1, v/v) was placed directly on the disc. While still wet, the mycelium side of the disc was gently pressed against the application line on a precoated TLC plate (Silica gel 60G, Merck Art. 5721; thickness of silica gel 0.25 mm on glass plate 20x20 cm). The disc was removed from the TLC plate after few seconds when a liquid front appeared. The spots were allowed to dry and the TLC plate developed in the systems described below:

System I: (Kamimura *et al.*, 1981): developing solvent, toluene/acetone/methanol (TAM) (5/3/2, v/v), spray 1, 20% (w/v) $AlCl_3$ in 60% ethanol; spray 2, 20% H_2SO_4 in water; spray 3, 0.32% (w/v) 2, 4 dinitrophenylhydrazine (2, 4-DNPH) in 2N HCl.

System II: (Filtenborg *et al.*, 1983) developing solvent, toluene/ethyl acetate/90% formic acid (TEF) 5/4/1, v/v), spray 1, 20% H_2SO_4; spray 2, 0.5% (v/v) p-anisaldehyde (ANIS) in methanol/acetic acid/conc. H_2SO_4(17/2/1) (Burmeister *et al.*, 1974).

The TLC plates were examined before and after the spray treatments in visible light as well as under shortwave UV light (254 nm) and longwave UV light (366 nm). Griseofulvin was used as an external standard in all analyses (Frisvad & Filtenborg, 1983). All retardation factors (R_f) were recorded relative to griseofulvin (relative R_f = 1.00).

Results

From 222 samples of capsicum, 88 samples of coriander and 23 samples of fenugreek a total of 1004 isolates of *Fusarium* comprising of 6 species viz., *F. moniliforme*, *F. subglutinans*. *F. semitectum*, *F. solani*, *F. equiseti* and *F. oxysporum* were obtained. Using TLC technique the mycotoxins and other secondary metabolites of species of *Fusarium* were detected.

***Fusarium equiseti*:** The TLC profiles of pigments and toxins of 200 isolates of *F. equiseti* were discernible in 4 patterns under longwave UV light (Table 1).

Pigments in all four patterns could not be identified, however, zearalenone and zearalenol were detected in 3.0% of the isolates in pattern IV at R_f 1.71 and 1.20 respectively. Another blue pigment was detected at R_f 0.81 which did not seem to be related to zearalenone family.

***Fusarium oxysporum*:** Fifty-two isolates of *F. oxysporum* showed 1 pattern of an unidentified pigment fluorescing red under longwave UV light (Table 1). The red pigment of this fungus differs at R_f value from that of *F. moniliforme* when developed in the same system. Toxins were not detected in *F. oxysporum*.

***Fusarium moniliforme*:** TLC profile of pigments and toxins of 418 isolates of *F. moniliforme* were delineated in 5 patterns according to the relative R_f values as well as their colour characteristics (Table 1). Four pigments of blue, blue green, light orange and red colours were distinguished on TLC plates. Except for a small number of isolates (0.8%), the red pigment at R_f 1.19 seems specific for *F. moniliforme*. Profile of pattern I in 5% isolates of *F. moniliforme* showed six pigments. The light blue at R_f 2.14, which changed to light orange colour under longwave UV light after chemical treatment was identified as anhydrofusarubin. Four unidentified pigments fluorescing blue under longwave UV light with varying intensities were detected at R_f 2.26, 1.61, 1.50 and 0.97.

Majority of the isolates (91.4%) showed a simple profile of two pigments fluorescing intense red and faint blue green at R_f 1.19 and 0.97 respectively which have been designated here as pattern II. Naphthaquinones or toxins were not detected in these two profiles. The pattern III, found only in 0.8% isolates showed two blue fluorescing spots at R_f 1.93 and 1.50. The upper spot which fluoresced in solid blue colour under shortwave UV light (254 nm) was identified as zearalenone. In pattern IV, detected in 1.9% isolates, seven spots were observed when the two developing systems were considered together. Moniliformin was identified at R_f 0.25 using TAM system (Fig.1). The spot at R_f 2.00 showed characteristic blue colour of zearalenone family under shortwave UV light. A spot at R_f 1.54 was always found close to bostrycoidin but could not be identified.

***Fusarium semitectum*:** Four chromatographic patterns were detected in *F. semitectum* (Table 1). The toxins and pigments showed blue/whitish blue or orange colours under longwave UV light. At R_f 1.35 the toxin detected was identified as equisetin. Equisetin has previously been reported from *F. equiseti* (Burmeister *et al.*, 1974) but not from *F. semitectum*. Equisetin was observed as a rose pink spot in visible light after spraying with 0.5% p-anisaldehyde.

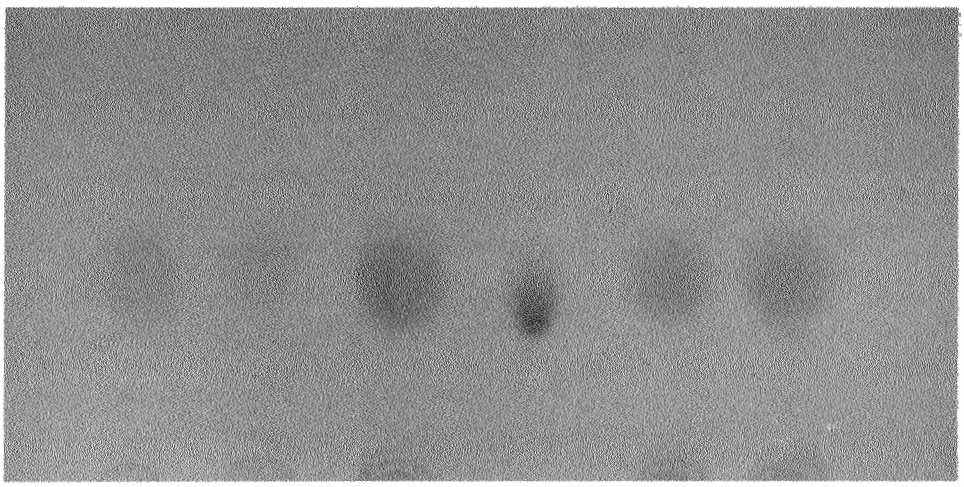

Fig. 1. Chromatographical pattern of moniliformin detected from *Fusarium moniliforme*. The TLC plate was developed in TAM, treated with 2,4-DNPH and photographed in daylight.

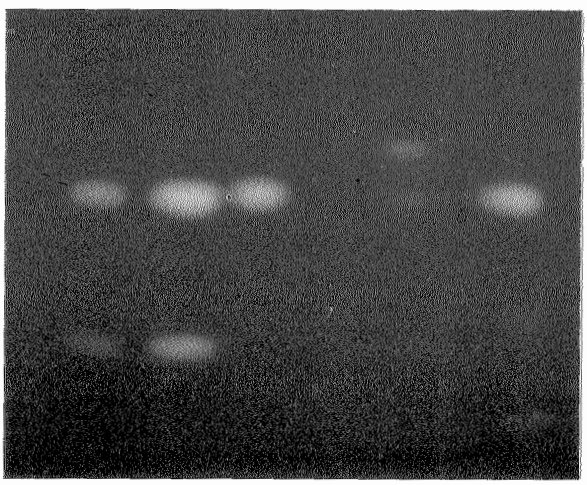

Fig. 2. Chromatographical pattern of zearalenone (upper spot) and zearalenol (lower spot) detected from *Fusarium semitectum*. The TLC plate was developed in TEF and photographed in UV_{254}.

Table 1. Chromatographical patterns of pigments and toxins of species of *Fusarium*.

Patterns of pigments and toxins/No. of Positive isolates	Relative R_f value in TEF system	Colour of pigments and toxins on TLC plates/Relative intensity of spots.		
		In visible light	Under UV light (UV_{254} UV_{366})	Under UV light (UV_{254} UV_{366}) after treating with 20% $AlCl_3$
F. equiseti				
Pattern I/58	1.28	ND	Blue green/+	Blue/+
(29.0-%)	1.10	ND	Blue green/+	Blue/+
Pattern II/45	1.42	Ros Pink/+	Light orange/+	Light orange/+
(22.5%)	1.24	ND	Blue green/+ +	Blue/+ +
	1.08	ND	Blue green/+ +	Blue/+ +
Pattern III/91	1.42	ND	Blue green/+	Blue/+
(45.5%)	1.32	Rose Pink/+	Light orange/+ +	
	1.24	ND	Blue green/+ +	Blue/+ +
	1.08	ND	Blue green/+ +	Blue/+ +
Pattern IV/6	1.71[Z]	ND	Blue green/+ +	Blue/+ +
(3.0%)	1.20[Zl]	ND	Blue green/+ +	Blue/+ +
	0.81	ND	Blue green/+	Blue/+
F. oxysporum				
Pattern I/52	1.06	Pink/+ +	Red/+ + +	Red/+ + +
(100.0%)				
F. moniliforme				
Pattern I/21	2.26	ND	Blue green/+ +	Blue/+ +
(5.0%)	2.14[A]	Light blue	Orange yellow/+	Light orange/+ +
	1.61	ND	Blue green/+ +	Blue/+ +
	1.50	ND	Blue green/+	Blue green/+
	1.19	Pink/+ +	Red/+ + +	Red/+ + +
	0.97	ND	Blue green/+ +	Blue green/+
Pattern II/382	1.19	Pink/+ +	Red/+ +	Red/+ + +
(91.4%)	0.97	ND	Blue green/+	Blue green/+
Pattern III/3	1.93[Z]	ND	Blue green/+ + +	Blue/+ + +
(0.8%)	1.50	ND	Blue green/+	Blue/+

Table 1. (Contd.)

Pattern IV/8	2.00[U]	ND	Blue green/+ +	Blue/+ +
(1.9%)	1.93[Z]	ND	Blue green/+ + +	Blue/+ +
	1.61[Z1]	ND	Blue green/+ +	Blue/+ +
	1.50	ND	Blue green/+ +	Blue/+ +
	1.19	Pink/+ +	Red/+ + +	Red/+ + +
	0.40[V]	ND	ND	Blue/+
	0.25[M]			
	(in TAM System)	Pale brown/+ + +	ND	ND
Pattern V/4	0.26	ND	Blue green/+ +	Blue/+ +
(0.9%)	2.14[A]	Light blue/+	Orange yellow/+ +	Light orange/+ +
	1.61[B]	Pink/+ +	Orange yellow/+	Light orange/+ +
	1.54	Pink/+	Orange yellow/+	Light orange/+
	1.19	Pink/+ +	Red/+ + +	Red/+ + +
F. semitectum				
Pattern I/89	1.40	ND	Blue green/+	Blue/+ +
(44.5%)	1.26	ND	Light blue/+	Blue/+ +
	1.14	Pink/+ +	Orange yellow/+ +	Orange/+ +
	1.07	ND	Blue green/+	Blue/+
	0.90	ND	Blue green/+	Blue/+
	0.74	ND	Blue green/+	Blue/+
Pattern II/23	1.35[E]	Pink/+ +	ND	ND
(11.5%)	1.26	ND	Light blue/+ +	Whitish blue/+ +
	1.14	Pink/+ +	Pink/+ +	Orange yellow/+
Pattern III/80	1.26	ND	Blue green/+	Blue/+
(40.0%)	1.14	ND	Blue green/+	Blue/+
	1.04	ND	Blue green/+	Blue/+
Pattern IV/8	1.92[Z]	ND	Blue green/+ +	Blue/+ + +
(4.0%)	1.54[Z1]	ND	Blue green/+ +	Blue/+ + +
F. subglutinans				
Pattern I/6	1.04	Pink/+ +	Red/+ + +	Red/+ + +
(100.0%)				
Fusarium solani				
Pattern I/20	1.61[B]	Pink/+ +	Orange Yellow/+ +	Orange/+ +
(15.6%)	1.48	Pink/+	Light orange/+ +	Orange/+ +
	1.42	Pink/+	Light orange/+ +	Orange/+ +
	1.10	ND	Blue green/+	Blue/+
	1.10	ND	Blue green/+	Blue/+
	1.00	ND	Blue green/+	Blue/+
	0.84	ND	Blue green/+	Blue/+

Table 1. (Contd.)

Pattern II/14	1.61[B]	Pink/+ +	Orange yellow/+ +	Orange/+ + +
(11.0%)	1.42[F]	Rose Pink/+	Light orange/+	Light orange/+ +
	1.10	ND	Blue green/+	Blue/+
	1.00	ND	Blue green/+	Blue/+
	0.84	ND	Blue green/+	Blue/+
Pattern III/4	1.61	ND	Blue green/+	Blue/+
(3.0%)	1.48	Pink/+ +	Orange/+ +	Orange/+ + +
	1.42	Rose Pink/+ +	Light orange/+	Light orange/+ +
	1.10	ND	Blue green/+	Blue/+
	1.00	ND	Blue green/+	Blue/+
	0.84	ND	Blue green/+	Blue/+
Pattern IV/60	1.48	Pink/+ +	Orange yellow/+ +	Orange/+ + +
	1.10	ND	Orange yellow/+ +	Orange/+ + +
	1.48	Pink/+	Orange/+	Orange/+ + +
	1.42	ND	Blue green/+ +	Blue/+ +
	1.10	ND	Blue green/+ +	Blue/+
Pattern V/30	1.48	Pink/+	Orange/+	Orange/+ + +
(23.5%)	1.42	ND	Blue green/+ +	Blue/+ +
	1.10	ND	Blue green/+ +	Blue/+ +

Symbols +, + + and + + + indicate relative intensity of spots.

Data based on 200 isolates of *F. equiseti*, 52 isolates of *F. oxysporum*, 418 isolates of *F. moniliforme*, 200 isolates of *F. semitectum*, 6 isolates of *F. subglutinans* and 128 isolates of *F. solani*.

A: Anhydrofusarubin B: Bostrycoidin C: Equisetin M: Moniliformin ND: not detected
U: Unknown mycotoxin of zearalenone family V: Vomitoxin Zl: Zearalenol Z: Zearalenone

Eight isolates (4.0%) of *F. semitectum* showing pattern IV gave positive reaction for zearalenone and zearalenol (Fig. 2). Their identity was confirmed by comparing with their standard samples. Ninety six of the isolates of *F. semitectum* showed blue fluorescing pigments in longwave UV light in profiles of patterns I,II and III. These as well as the orange pigment could not be identified although they were checked against several known standards of pigments.

Fusarium subglutinans: Six isolates of *F. subglutinans* obtained from coriander seeds showed a simple pattern of one pigment fluorescing intense red under longwave UV light (Table 1). In a few TLC replicates traces of a blue pigment were also detected but disappeared after spraying with 20% $AlCl_3$.

Fusarium solani: In profiles of 128 isolates of *F. solani*, (Table 1), 6 pigments of pattern I either fluoresced orange or blue under longwave UV light. One of them at R_f 1.61 was identified as bostrycoidin. Pattern II consisting of 5 pigments was identical to pattern I in having bostrycoidin at R_f 1.61 but also showed a spot at R_f 1.42 fluorescing light orange under longwave UV light. This spot, by comparing with the R_f

value of the standard samples, was identified as fusarubin. The profile of pattern III showed 6 pigments under longwave UV light. It showed a consistent similarity to pattern I except for the light orange spot of fusarubin, not detected in pattern I. Majority of the isolates of *F. solani* (70.4%) showed a profile of 3 pigments discernible in patterns IV and V. A significant difference between the two was noted at R_f 1.42. This spot in pattern IV fluoresced dark orange whereas in pattern V it showed blue colour.

Growth response of *Fusarium* spp. on TAN agar

Of the *Fusarium* isolates only *F. subglutinans* was TAN^- while *F. equiseti*, *F. moniliforme*, *F. oxysporum*, *F. semitectum* and *F. solani* were TAN^+.

It was found that the inoculum should be only conidium suspension, otherwise even a small amount of substrate used as inoculum may give a false reaction in the growth test. In the present study the difference in growth response on TAN agar was used to divide the species of *Fusarium* into two groups whereas further delineation of the species was based on colour and R_f values of extracellular metabolites (e.g., pigments) excreted into the medium (Key).

Of the species of *Fusarium* only *F. oxysporum* and *F. subglutinans* showed simple TLC profiles of one pigment that fluoresced red under UV light. The two species were segregated on the basis of positive or negative growth response on TAN^+ agar as well as a different R_f value of the red pigment. Among the 4 TAN^+ species, *F. equiseti* and *F. semitectum* had a profile of 4 TLC patterns. These species were differentiated on the basis of different R_f value of pigments fluorescing blue under UV light. An orange pigment, whenever present, was also found helpful in the characterization of *F. equiseti* and *F. semitectum*. The other two TAN^+ species, *F. moniliforme* and *F. solani*, showed 5 TLC patterns of metabolites and were separated and identified on the basis of a red pigment (R_f 1.19) and a blue pigment (R_f 1.48) respectively. Occasionally an orange pigment was detected in both the species at different R_f values and proved to be of effective diagnostic value.

A key to the identification of selected species of *Fusarium* using patterns of secondary metabolites

1. Not able to grow on tannin-sucrose agar medium, one TLC pattern in TEF system, showing red pigment at R_f 1.04.
   F. subglutinans
   Able to grow on tannin-sucrose agar medium 2
2. One TLC pattern in TEF system, showing red pigment at R_f 1.06.
   F. oxysporum
   More than one TLC pattern in TEF system 3
3. Four TLC patterns in TEF system 4
   Five TLC patterns in TEF system 5
4. Blue pigments, occasionally a dark orange pigment present at R_f 1.4.
   F. semitectum

Blue pigment present, occasionally a light orange pigment present at R_f 1.32 or R_f 1.42.

F. equiseti

5. Red pigment invariably present at R_f 2.14.

F. moniliforme

Blue pigment invariably present at R_f 1.10, more often orange pigment present at R_f 1.48.

F. solani

Discussion

The present study showed that extracellular pigments and mycotoxins produced by *F. moniliforme, F. subglutinans, F. semitectum, F. solani, F. oxysporum* and *F. equiseti*, when eluted in TEF system (Filtenborg *et al.,* 1983), showed consistent profiles that were identifiable with a particular species. These isolates in TAM system (Kamimura *et al.*, 1981), more often produced reddish-brown, pink or blue trailing streaks, sometimes in large quantities, thus interfering with the recording of R_f values of TLC spots. Since the profiles in TEF system indicated definite parameters in daylight as well as under UV light (UV_{366} and UV_{254}), coupled with the ability of *Fusarium* spp., to grow on TAN agar medium (Thrane, 1986), they were used to make a dichotomous key which could be easily used for the identification of *Fusarium* spp., together with their micro- and macromorphological characters.

Of the mycotoxins, vomitoxin was detected from a small number of *F. moniliforme* isolates. Although vomitoxin has been reported from other species of *Fusarium* growing on various media (Morooka *et al.*, 1972; Vesonder *et al.,* 1973; Mirocha *et al.*, 1976) it has not been reported from *Fusarium* isolated from seeds of capsicum, coriander and fenugreek. Vomitoxin is sometimes found concomitantly with zearalenone (Pathre & Mirocha, 1978; Vesonder *et al.*, 1981). In the present study vomitoxin has also been detected with zearalenone and thus appears to be a potential toxic contaminant of spices. It may be mentioned that trichothecenes are somehow involved in human diseases causing red-mold disease in Japan (Pathre & Mirocha, 1979) and alimentary toxic aleukia in USSR (Ueno, 1980).

Zearalenone, a well-known estrogen has been previously reported from *F. equiseti, F. moniliforme* and *F. semitectum* (Ichinoe *et al.*, 1978; Turner, 1971). Zearalenol however, has been detected for the first time from *F. moniliforme* and *F. semitectum* contaminating spices during this study. It may be mentioned that zearalenol is presumably more estrogenic than the parent zearalenone (Ueno & Tashiro, 1981). There is therefore need for more information concerning their occurrence in various parts of the world and their possible intake by man through various kinds of spices. Although no adverse effects due to zearalenone intake have been reported in man, so far, a possible health hazard connected with the daily intake of zearalenone (and zearalenol) through consumption of large quantitites of spices in third world countries should receive due attention. Studies should also be undertaken on factors affecting fungal growth and mycotoxins formation in spices, under pre- and postharvest, and/ or storage conditions.

Of the 6 species of *Fusarium* screened for mycotoxins during the present study, moniliformin was detected only in culture of *F. moniliforme* isolated from seeds of coriander whereas isolates of *F. moniliforme* from capsicum and fenugreek were not positive for this toxin. Moniliformin has been reported from *F. equiseti*, *F. oxysporum*, *F. semitectum* (Turner & Aldridge, 1983) as well as from *F. moniliforme* (Palti, 1978).

Burmeister *et al.*, (1974) reported that *F. equiseti* in cultures on corn grits produced equisetin, an antibiotic that inhibits growth of certain bacteria. In a subsequent report Vesonder *et al.*, (1979) also detected equisetin from *F. equiseti* and assigned it to be a derivative of N-methyl-2, 4-pyrollidone. It is interesting to note that equisetin is for the first time reported from *F. semitectum* during the course of this study. In view of its moderate toxicity demonstrated in mice (Burmeister *et al.*, 1974) it is unlikely that equisetin represents a mycotoxin problem in man. Probably further investigations of the antimicrobial properties of this compound may still be justified. Keeping in view the published reports and the results of the present study it would suggest that there is worldwide contamination of seed, with a variety of mycotoxin producing fungi. Since mycotoxins are a probable source of naturally occurring carcinogens in humans (Diener *et al.*, 1981), concerted effort should be made to avoid such contaminants using seed health technology.

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