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Exploration of Fungal Infection in Agricultural Grains, Aflatoxin and Zearalenone Synthesis under pH Stress

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A B S T R A C T

Mycoflora of 25 grains samples collected from southern governorates of KSA were investigated. Fungal plates count for collected samples ranged from 0 up to 9900 CFU/g. The moisture content as an essential factor that affect fungal content of grains was also investigated and was variable among samples with range from 2.75 to 8.22%. Sixteen fungal species were identified among them Aspergilli, Penicilli and Fusaria dominate. *A. niger*, *A. flavus* and *F. oxysporium* showed high frequency among isolates, respectively. *A. flavus* and *F. oxysporium* were cultivated at different pH values and aflatoxins (B1, B2, G1 and G2) and zearalenone were detected. Maximum aflatoxins were produced at pH 5 while zearalenone was at pH 7.

Introduction

The growth of toxigenic fungi can adversely affect grain quality and even produce mycotoxins of food safety concern, which should be sensitively monitored and controlled during grain storage (Huan-Chen *et al.*, 2015). Surveillance for mycotoxins and mycotoxigenic fungi is critical for maintaining high quality grains and grain products (Sarlin *et al.*, 2006). Fungal identification is very important to provide information about which mycotoxins could be present (Maenetje and Dutton, 2007; For example, Lund and Frisvad (2003) found that samples of wheat and barley grains showing a percentage infestation of *Penicillium verrucosum* higher than that (7%) indicated by Ochratoxin A presence.

Grain spoilage and mycotoxin contamination caused by toxigenic fungi are significant food safety problems (Frisvad & Thrane, 2004; Pitt *et al.*, 2013). Grains fungal contamination have been carefully studied in several areas, and several grain types. Montes *et al.* (2009) investigated

fungal colonies on yellow and white maize grains. The major fungi encountered were *Fusarium sp*., *Penicillium sp*., and *Aspergillus sp*. When compared with the yellow hybrids, white hybrids had 34, 52 and 22% less infection by *Fusarium verticillioides*, *Aspergillus flavus*, and *Aspergillus niger*, respectively, and almost the double infection with *Penicillium sp*. Infection of maize kernels by toxigenic fungi remains a challenging problem despite the research progress (Munkvold, 2003). During grain storage, xerophilic fungi, such as *Aspergillus glaucus* and *Aspergillus restrictus*, always grow at low water activity (aw < 0.75) in grain and are always the most predominant fungal species at the beginning of storage and have relatively less effect on grain quality (Anke *et al*., 1980).

However, the growth of *Aspergillus flavus*, *Aspergillus ochraceus* and *Penicillium sp*. can seriously spoil stored grain, and even produce toxigenic or carcinogenic metabolites, which can pose a health risk to humans and animals (Nesci *et al*., 2003; Amézqueta *et al*., 2012). A total of 101 isolates of *A. flavus* and *A. parasiticus* originating from acid treated feeding grain in Sweden were identified according to conidial ornamentation. *A. flavus* was the most common (71% of all isolates) of the two species (Holmberg *et al*., 1989). *Fusarium sp*., were the most abundant species detected in maize kernels, followed by *Aspergillus sp.* of sections Flavi and Nigri and by *Penicillium sp*. Among Fusarium species, F. verticillioides was the most prevalent species, as detected by PCR directly on the kernels and on the fungi isolated from the kernels, followed by *F. proliferatum* and *F. subglutinans* (Covarelli *et al*., 2011).

Wheat were also found to be contaminated in variable amounts by potentially toxigenic fungi including *Aspergillus*, *Alternaria* and *Fusarium* (Tournas and Katsoudas, 2008). Microorganism get on grain in different ways, most often with dust from soil, from the surface of plant remnants during harvesting, transportation, storage and processing (Klich, 2002). Thirty samples of coffee beans were collected from different places of Jeddah, Saudi Arabia to determine and identify fungal population. Twenty six species belonging to 7 genera were isolated.

The most prevalent genera were *Aspergillus* and *Penicillium, while Fusarium, Mucor, Rhizopus* and *Alterneria* were recovered in moderate incidences (Bokhari and Aly 2009). Mycotoxigenic fungi including *Aspergillus*, *Fusarium*, and *Penicillium* genera are responsible for the majority of agricultural mycotoxin contamination. These fungi are common components of the microbial flora associated with many agronomic crops, including corn and sorghum (Palumbo *et al*., 2008). Aflatoxins occur in temperate and tropical regions around the world, depending on which fungal species are present. They can affect many food supplies including cereals, nuts, dried fruit, coffee, cocoa, spices, oil seeds, dried peas, beans, fruits, and, in particular, apples (Abdel-Azeem *et al*., 2015).

More than 20 different aflatoxins were identified, those belonging to the two major groups B and G are frequently found in contaminated food, especially peanuts and maize (Ehrlich *et al*., 2007; Sherif *et al*., 2009; Velazhahan *et al*., 2010;). However, aflatoxins are genotypically specific, they could also be produced by one or more fungal species (Ioannou-Kakouri *et al*., 2004), and in some cases, one species can form more than one aflatoxins (Frisvad, 1994). Zearalenone (ZEA) is produced by a variety of *Fusarium* species, including *F.*

graminearum, F. culmorum, F. cerealis, F. equiseti, F. crookwellense and F. semitectum, which are common soil fungi, in temperate and warm countries, and are regular contaminants of cereal crops worldwide (Bennett and Klich, 2003). Wheat contamination with ZEA is a worldwide problem where several reports documented the presence of ZEA in wheat samples of Brazil, Switzerland, Netherlands, Bulgaria and Germany in a level ranged from 2 to 2000 µg.kg-1 (Furlong *et al*., 1995; Bucheli *et al*., 1996; Vrabcheva *et al*., 1996; Schollenberger *et al*., 2005). This study was undertaken to recongnize the frequency and a relative percentage of fungal species in grains produced and stored in Jazan rejoin, Saudi Arabia, in order to use this information to consider possible mycotoxin occurrence in this area. Also, the objective of the current study was to explore the production aflatoxins and Zearalenone under pH stress.

Material and Methods

Samples collection

A total of 25 samples of different grains were collected from markets, local stores, agricultural cooperatives and farm fields from different districts of Jazan governorate, Saudi Arabia Samples were brought to the laboratory in sterile plastic bags and kept at 5°C. All samples were subjected to mycological analysis.

Isolation and identification of fungi

Fungal isolates were purified and transferred onto Potato Dextrose Agar (PDA) to study the macro- and micro-morphological characteristics. All the isolates of fungal species were identified up to the species using keys and manuals (Raper and Fennell 1965; Booth 1977; Samson *et al*., 1981; Barnett and Hunter 1998; Leslie and Summerell 2006). The isolation frequency (Fr) and relative density (RD) of species were calculated as follows:

Fr (%) = No. of samples of occurrence of a species/ Total No. of samples x 100

RD (%) = No. of isolated genus or species/ Total No. of isolated fungi x 100

Mycotoxins production conditions

Aspergillus flavus and Fusarium oxysporium were inoculated separately in 250ml Erlynmer conical flasks containing sterile 2% wheat natural medium. Flasks were adjusted at different pH values prior sterilization by autoclave at 121°C for 15 min. Inoculated flasks were incubated at 30°C for 10 days in dark.

Mycotoxins assay

Microtitre plate enzyme-linked immunosorbent assay (ELISA) reader (automated Chem-well) Aspergillus and Fusarium mycotoxins test kit were used to ELISA analyses. The samples were analyzed using the Aflatoxin B1, B2, G1, G2 and Zearalenone test procedure which was described by company(r-biopharm) producer (Enzyme Immunoassay for the quantitative analysis of aflatoxins, 1999 and Leszczynska et al., 2001). Ten ml of blended fungal broth has been sub-sampled with 20ml of 70% methanol and vortex for 10 min by magnetic stirrer. The extract was filtrated by Whatman no.1 filter paper and then diluted as 5ml filtered solution, 15ml distilled water and 0.25ml Tween 20. The solution was mixed by magnetic stirrer for 2min. 50 µl toxins (5, 10, 20, 45 ppb) standard solutions and 50 µl prepared test samples were added into separate wells of micro-titer plate. Plates were incubated at room temperature. The liquid was then

removed completely from the wells, the each well was washed with 250 µl washing PBS-Tween-Buffer (pH 7.2) and this was repeated two times. Subsequently, enzyme substrate (50 µl) and Chromogen (tetramethyl-benzidine, 50 µl) were added to each well and incubated for 30min at room temperature in the dark. 100 µl of the stop reagent (1M H_2SO_4) was added and the absorbance was measured at 450nm in ELISA reader.

Result and Discussion

Saudi Arabia climate is quite harsh, dry desert with extreme temperatures and climatically it is not very ideal for farming. In the past many agricultural programs were initiated to gain food security, ensure self-sufficiency and improve rural livelihoods. Saudia Arabia has been successful in raising yields of several important crops (Al-Shayaa *et al*., 2012). Poor storage experiences and unfavorable environmental conditions during pre- and postharvest handling of these crops were responsible for the contamination and colonization by mycotoxin producing and non producing fungi. Therefore studies on identification, frequency and their relative percentage are very important and required for further studies on fungi particularly mycotoxin producing fungi and their epidemiological significance in cereal crops cultivated and stored in Saudi Arabia.

Mould growth is determined by several environmental factors (temperature, composition of the gas atmosphere, substrate properties, including moisture content and water activity and pH) that markedly affect the composition of the mycoflora in feeds (Marin *et al*., 2000). In the current study fungal flora of 25 grains samples collected from Saudia Arabia southern governorate were investigated. Fungal count and moisture content of each sample in terms of colony forming units (CFU) per gram dry weight and percentage, respectively, were recorded (Table 1, Figure 1). Grain moisture content was a critical factor that influence establishment of the fungi. Mycological examination revealed the occurrence of 16 species of fungi belonging to 8 different genera. Identified fungal species included five Aspergilli (*A. flavus, A. fumigates, A. niger, A. ochraceous, A. tamari*), three Penicilli (*P. chrysogenum, P. notatum, P. oxalicum*), two Fusaria (*F. moniliforme, F. oxysporum*), and two Mucor (*Mucor racemosus and unidentified species)* in addition to *Chaetomium thermophilum, Rhizopus stolonifer, Syncephalastrum racemosum and Ulocladium chartarum.* Hashem (1990) reported 29 species of fungi were isolated from normal and discolored grains of barley in Saudi Arabia. The most common genera were *Ulocladium* (four species); *Alternaria, Aspergillus* and *Drechslera* (three species); *Curvularia, Fusarium, Mucor, Penicillium* and *Syncephalastrum* (two species). Mycological studies conducted to detect the colony forming unit (CUF) of fungi in 25 samples of grains revealed that some samples of different grains exhibited no clear relationship between number of CFU and diversity in fungal species detected, where some grains containing highest CFU but containing lowest fungal species. For example, sample 22 containing 98 x10^2 CUF/g grains but containing two fungal species *A. flavus and F. moniliforme*. On the other hand sample 11 containing 34.1 x10^2 CUF/g but containing 7 fungal species. In the present study, different samples of grains containing the same fungal species, A. niger, *Fusarium oxysporum* were detected in sample 3 and 20. While *A. niger, F. oxysporum*, *A. flavus* were detected in sample 7, 10, 16 and 20. Also samples (12 and 15) (21 and 23) containing the same

fungal species (Table 2). Sample 24 contains no fungal species. Wheat and other grain samples in Saudi Arabia were separately analyzed to identify the presence of fungi and their mycotoxins (Muneera, 2014), it was found that samples containing common *Alternaria*, *Aspergillus* and *Fusari um*. Another study which correlates with the data obtained in the present work is that *Aspergillus* and *Fusarium* were isolated from grain samples (barley, rice, wheat, white corn and yellow corn),collected from different localities of Riyadh region, Kingdom of Saudi Arabia, and were screened for their toxigenic potential (Amira *et al*., 2012).

Among fungal isolates *A. niger, A. flavus and F. oxysporums* showed high frequency within collected samples (84, 68 and 40 % with a relative percentage of 27.27, 22.08 and 12.99%, respectively). The other fungal genera include *A. fumigatus* (12%), A. *ochraceous* (28%), *A. tamarii* (8%), *Chaetomium thermophilum* (4%), *F. moniliforme* (8%), *P. chrysogenum* (12%) and other fungal isolates with the different level of frequency and relative percentage (Figure 2). The results of this investigation are similar to those obtained by earlier workers and show that The predominant Aspergillus species isolated were *A. flavus* (72.7%) and *A. niger* (59.1%) with the relative percentage of 51.1 and 33.3%, respectively on sorghum grains (Sreenivasa *et al*., 2010). The mycological profile of the retail wheat flour selling in different markets at Jeddah (Kingdom of Saudi Arabia) was studied by Gashgari *et al*. (2010). The most common genera were *Aspergillus* (isolated from 70% of the tested samples), *Penicillium* (30%), *Eurotium* (14%), and in a lesser extent *Fusarium* (20%) and *Alternaria* (18%). Under Jazan region conditions, four *Aspergillus* species were isolated and identified from stored wheat flour; the isolated species prevalence being *A. flavus, A. niveus, A. terreus,* and *A. niger* by rate 44.5%, 37.8%, 10.9% and 6.7%, respectively (Bosly and Kawanna, 2014).

Fig.1 Fungal Colonies Isolated from 25 Samples of Collected Grains, Control, Non Inoculated Plates Containing Growth Medium.

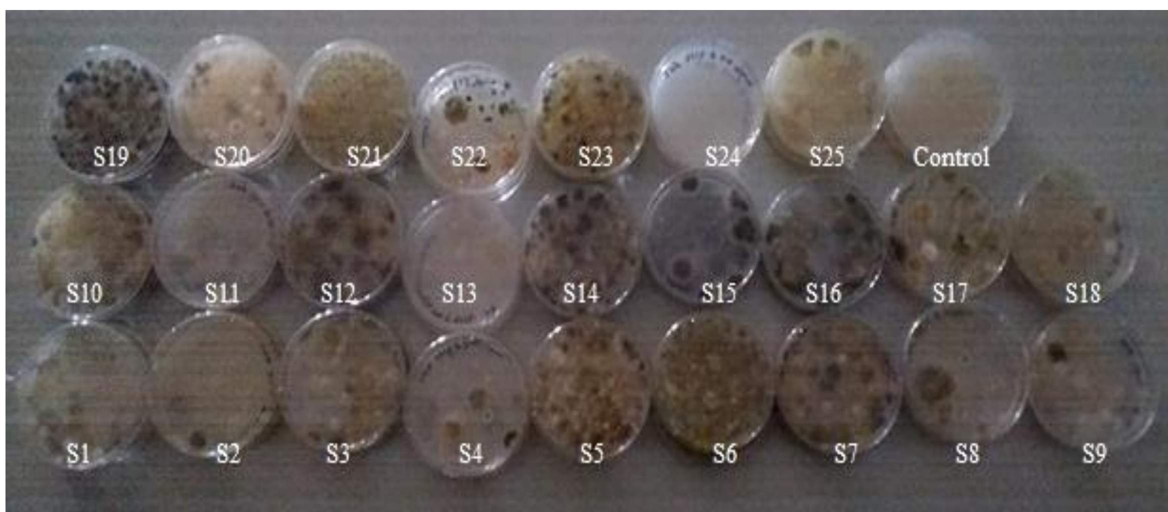

Table.1 Moisture Content and Number of Fungal Isolates (Cfu) in the Collected Grains Samples, Collected from Various Regions.

Sample No.	Grain type	Collection site	Moisture content (%)	Plate count (CFU*10^2/g)
1	*Sorghum bicolor* (Red)	Jazan	7.38	3.65
2	*Sorghum bicolor* (White)	Jazan	4.955	5.25
3	*Sorghum bicolor* (White)	Damad	5.53	15.25
4	*Sorghum bicolor* (White)	Sabya	3.18	8.1
5	*Sorghum bicolor* (White)	Abu Arish	7.5	14.45
6	*Vigna sp.*	Jazan	2.79	82
7	*Sorghum bicolor* (Red)	Abu Arish	2.75	48
8	*Helianthus annuus*	Abu Arish	7.99	3.55
9	*Triticum sp.*	Jazan	3.16	6.6
10	*Triticum sp.*	Abu Arish	3.56	4.4
11	*Zea mays*	Jazan	6.53	34.1
12	*Zea mays*	Abu Arish	4.23	3.4
13	*Arachis hypogaea*	Jazan	5.54	30
14	*Vicia faba*	Jazan	3.38	0.3
15	*Sesamum indicum*	Jazan	7.3	39.1
16	*Coffea Arabica*	Jazan	4.895	7.85
17	*Medicago sativa*	Ahad Almasarihah	6.61	13.2
18	*Hordeum vulgare*	Jazan	5.72	3.1
19	*Gossypium herbaceum*	Jazan	6.51	11.65
20	*Coffea Arabica*	Abu Arish	3.97	63
21	*Sorghum bicolor (white)*	Ahad Almasarihah	4.69	59
22	*Pennisetum glaucum*	Jazan	7.71	98
23	*Sesamum indicum*	Abu Arish	5.88	82
24	*Triticum sp.*	Sabya	4.1	0
25	*Pennisetum glaucum*	Abu Arish	8.22	99

Fig.2 Isolation Frequency and Relative Density % of Fungi Isolated form Grains Samples.

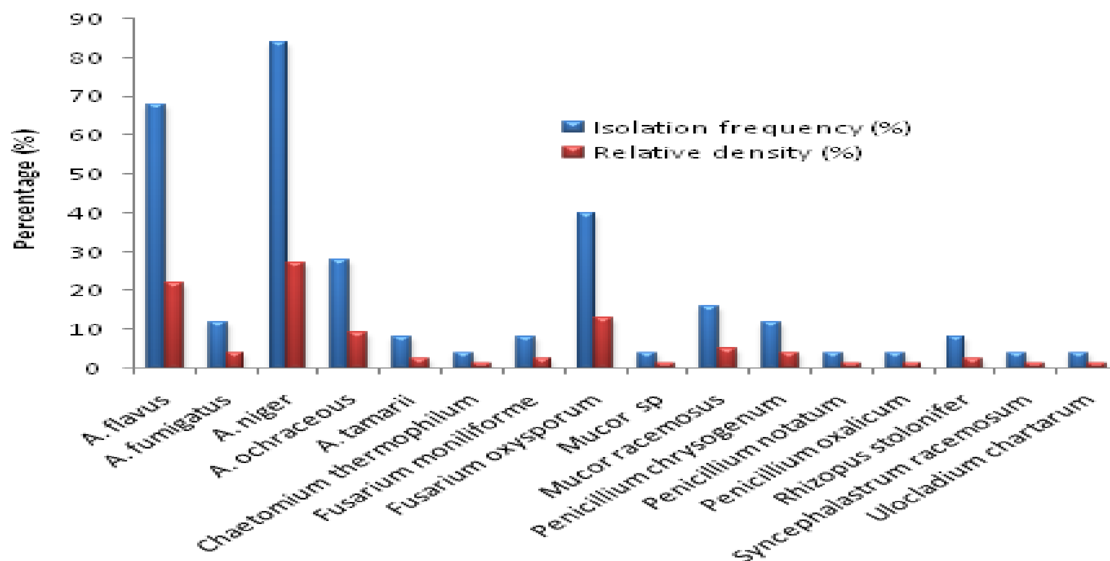

Table.2 Occurrence of Isolated Fungal Species in Collected Samples

Sample No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
A. tamari	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
A. flavus	+	-	+	-	+	+	+	-	+	+	+	+	+	-	+	+	+	-	-	-	+	+	+	-	+
A. fumigatus	+	-	+	-	-	-	-	-	-	-	-	-		-	-	-	-	+	-	-	-	-	-	-	-
A. niger	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+
A. ochraceous	-	-	-	+	+	-	-	-	-	-	+	+		-	+	-	-	+	-	-	-	-	-	-	-
C. thermophilum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
F. moniliforme	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-
F. oxysporum	+	+	-	-	-	-	+	-	-	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	-
M. racemosus	-	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Mucor sp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+-	-	-	-	-	-	-	-
P. chrysogenum	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. notatum	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P.. oxalicum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
R. stolonifer	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
S. racemosum	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
U.chartarum	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

A.: *Aspergillus*, C.: *Chaetomium*, F.: *Fusarium*, M.: *Mucor*, P.: *Penicillium*, R.: *Rhizopus*, S.: *Syncephalastrum*, U.: *Ulocladium*

Table.3 Aflatoxins and Zearalenone Production by *A. flavus* and *F. oxysporium*, Respectively under Different pH

Mycotoxin (µg/kg)	pH			
	3	5	7	9
Aflatoxin B1	2.92	3.57	3.31	2.99
Aflatoxin B2	0.67	0.95	0.88	0.71
Aflatoxin G1	0.0	0.54	0.50	0.40
Aflatoxin G2	0.31	0.44	0.41	0.0
Total Aflatoxins	3.90	5.50	5.10	4.10
Zearalenone	60.5	52.9	37.5	13.9

The obtained data on the frequency and relative percentage of fungi would be of a great importance for predicting the extent of pre- and postharvest infection and colonization of grains. The high frequency and relative percentage of *A. niger* and *A. flavus* species should be of primary concern for grains experts and policy makers in this region to minimize or prevent the economic losses caused by these fungi and also to reduce the exposure of human to mycotoxins. Broggi *et al.* (2007) stated that the major fungal species isolated was *Fusarium verticillioides* in maize in Argentina. Relative density of Aspergillus isolates was maximum for *A. niger* aggregates and *A. ochraceous* (30% each) followed by *A. flavus* (26%), *A. parasiticus* (11%) and *A. carbonarius* (3%) in maize meal (Saleemi *et al.*, 2012). The fact that *Aspergillus* species was the most dominant species in the grains samples under study was reported by different investigators in different areas around the world especially in the warm and subtropical regions (Gashgari *et al.*, 2010; Saleemi *et al.*, 2012; Bosly and Kawanna , 2014).

Mould growth and mycotoxins production are determined by several environmental

factors (temperature, composition of the gas atmosphere, substrate properties, including moisture content and water activity and pH) that markedly affect the composition of mycoflora and in turn the mycotoxis in feeds (Marin *et al*., 2000). In the provided study pH among the critical factors was investigated for both aflatoxins and zearalenone as main mycotoxins produced by Aspergilli and Fusaria that were the most frequent fungal genera among all samples. Aflatoxins are known to be potent hepatocarcinogens in animals and human.

Therefore, the presence of toxigenic fungi and mycotoxins in grains stored for long periods of time presents a potential hazard to consumers health. Our result (Table 3) described that optimum pH for Aflatoxin B1, B2,G1 and G2 production was 5, then decreased. Aflatoxin G1 and G2 were inhibited at pH 3 and 9 respectively. Acidic pH 3 inhibited accumulation of AFG1 more than aflatoxins. The differential effect of pH on accumulation of aflatoxin G1 probably results from a decrease in activity of one of the biosynthetic enzymes involved in its formation. Buchanan and Ayres (1975) reported that maximal growth and aflatoxin production occurred at initial pH levels of 5.0, 6.0, and 7.0 respectively. Initial pH levels less than pH 6.0 favored production of the aflatoxin B, whereas levels greater than pH 6.0 favored production of the aflatoxin G. Yabe *et al*. (1999) report that the cytochrome P450 monooxygenase necessary for aflatoxin G1 formation is more unstable than the P450 monooxygenase required for aflatoxin B1 formation. Zearalenone production decreased with increasing pH, the optimum production pH was 9. El-Kady and El-Maraghy (1982) studied the effect of pH on zearalenone production by *F. oxysporium* in a pH range of 5 to 8 and their results suggests that zearalenone productivity reduced in alkaline pH values in agreement with the provided data;

However, they determined that optimum productivity was at pH 7 while in the provided study it was pH 5 this variation may be a result of *F. oxysporium* variety and/or variation in other cultivation conditions especially temperature as temperature directly affected the rate of zearalenone synthesis (Sherwoodand Peberdy, 1974).

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