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Deteriorative changes in maize kernels due to *Aspergillus flavus* Link. and *Fusarium verticillioides* (Sacc.) Nirenberg

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Abstract: The study aimed at measuring changes in chemical composition of maize kernels due to *Aspergillus flavus* Link. and *Fusarium verticillioides* (Sacc.) Nirenberg infection. The samples of maize kernels were incubated at 28 °C for 7, 14, 21, and 28 days. The samples were analysed for mycotoxin, moisture, crude fat, crude protein, crude ash, and crude fibre. Maize kernels inoculated with *A. flavus* and *F. verticillioides* exhibited a significant decrease in crude fat. Aflatoxin B_1 (AFB_1) contamination increased in maize kernels inoculated with *A. flavus*, and fumonisin B_1 (FB_1) in kernels inoculated with *F. verticillioides*. Crude ash and crude fibre content showed no changes. Incubation time significantly affected AFB_1 and FB_1 contamination levels, moisture, crude fat, and crude protein contents. AFB_1 and FB_1 contamination were significantly correlated with crude fat degradation. The tested strains had similar deteriorative effects on maize kernels. The significant changes in the proximate composition were only observed in maize kernels with mycotoxin contamination above the regulatory limit of 10 μg kg^{-1}, thus not fit for human consumption.

Keywords: aflatoxin; fumonisin; maize kernel; mycotoxin; proximate components; fungal species

Kvarjenje koruznih zrn zaradi okužb z glivama *Aspergillus flavus* Link. in *Fusarium verticillioides* (Sacc.) Nirenberg

Izvleček: V raziskavi so bile merjenje spremembe v kemični sestavi koruznih zrn zaradi okužbe z glivama *Aspergillus flavus* in *Fusarium verticillioides*. Vzorci koruznih zrn so bili inkubirani pri temperature 28 °C za 7, 14, 21, in 28 dni. V vzorcih je bila analizirana vsebnost mikotoksinov, vode, celukopnih beljakovin, maščob, vlaknin in pepela. Koruzna zrna, okužena z glivama *A. flavus* in *F. verticillioides,* so imela značilen upad celokupnih maščob. Kontaminacija z aflatoksinom B_1 (AFB_1) se je v koruznih zrnih povečala po inokulaciji z glivo *A. flavus*, s fumonizinom B_1 (FB_1) pa po inokulaciji z glivo *F. verticillioides*. Pri vsebnostih celokupnega pepela in vlaknin ni bilo nobenih sprememb. Čas inkubacije je značilno vplival na vsebnost AFB_1 in FB_1, vsebnost vode, celokupnih maščob in beljakovin. Kontaminacija z AFB_1 in FB_1 je bila značilno povezana z degradacijo celokupnih maščob. Testirani sevi so imeli podoben kvaren učinek na koruzna zrna. Značilne spremembe v zgradbi koruznih zrn so bile ugotovljene pri njihovi kontaminaciji z mikotoksini nad predpisano vrednostjo 10 μg kg^{-1}, kar ni primerno za prehrano ljudi.

Ključne besede: aflatoksin; fumonizin; koruzna zrna; kemijska sestava; mikotoksin; vrste gliv

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1 INTRODUCTION

Maize is highly susceptible to fungal infection. Consequently, the quality of the maize kernels deteriorates (Begum et al., 2013). Fungal development can cause a considerable modification in the chemical composition of stored grains (Kakde and Chavan, 2011). Fungal infection in grains is associated with losses in carbohydrates, proteins and lipids while moisture content and free fatty acid increase. Fungi produce hydrolytic enzymes including peroxidase, amylase, pectinases, proteases and lipases. These enzymes degrade biochemical components such as fats, protein, and carbohydrates leading to the loss of dry matter (Begum et al., 2013). Bhattacharya and Raha (2002) reported a decrease in carbohydrates and fat content in maize kernels and soya beans due to post-harvest fungal infection. Jain (2008) reported a rapid increase in free fatty acids in damaged grains due to fungal infestation. Embaby and Abdel-Galil (2006) observed a reduction in carbohydrates, sugars and crude fat due to *Fusarium* in legume grains. Kakde and Chavan (2011) concluded that *Aspergillus flavus* was responsible for the maximum depletion of fat content and reducing sugars in safflower, soya bean and sesame.

Aspergillus flavus and *Fusarium verticillioides* are commonly occurring maize pathogens that can easily survive on dead plant materials as saprotrophs. They also cause aflatoxin and fumonisin contamination (Probst et al., 2014), especially in maize kernels that provide a good natural substrate for the fungi (Perrone et al., 2014). Nutrient composition is a key factor affecting mycotoxin production in maize kernels (Ma et al., 2015). Inherent materials in maize kernels such as starch, proteins and lipids represent significant carbon and nitrogen sources potentially available during seed infection by fungi (Mellon et al., 2002). Saccharides provide the primary carbon source for mycelial growth and mycotoxin production (Mellon et al., 2005). Fanelli and Fabbri (1989), Wilson et al. (2004), and Mellon et al. (2005) reported a relationship between lipid degradation and AFB_1 production. Glucose, ribose, xylose, and glycerol are also good substrates for growth and aflatoxin production by *A. flavus* (Liu et al., 2016).

Maize serves as an important dietary staple in Sub Saharan Africa. Consequently, the nutritive value of maize is of importance. Maize is vulnerable to infection by toxigenic fungi (Abbas et al., 2006). The high temperature and high relative humidity experienced in most parts of Sub Saharan Africa, coupled with poor grain storage conditions predispose maize to toxigenic fungal attack (Oyekale et al., 2012). Consequently, it is necessary to investigate its nutritive integrity and the subsequent mycotoxin contamination during fungal infection. The objective of this study was to evaluate the effect of *A. flavus* and *F. verticillioides* infection on the proximate composition of maize kernels.

2 MATERIALS AND METHODS

2.1 INOCULUM PREPARATION

Aspergillus flavus Link. (strain PPRI1314-UKZN) and *F. verticillioides* (Sacc.) Nirenberg (strain MRC826) were obtained from the Department of Plant Pathology, School of Agriculture, Earth and Environmental Sciences, University of KwaZulu-Natal, South Africa. The fungi were plated on potato dextrose agar (Merck, Darmstadt, Germany) at 25 °C for five days, after which conidia were harvested by flooding a single culture with either Triton X-100 solution (*A. flavus*) or distilled water (*F. verticillioides*) and scraping the surface mycelia with a sterile scraper. The resulting suspensions were filtered through cheesecloth. The spore concentration was counted using a Neubauer hemocytometer, and diluted using distilled water to obtain a spore concentration of 4×10^6 cells ml^{-1} (Hruska et al., 2014).

2.2 PREPARATION OF MAIZE SAMPLES

The maize kernels were surface sterilised by immersing the kernels in a 5 % (v/v) sodium hypochlorite (NaClO) solution and stirring for one minute. The maize kernels were thereafter rinsed twice with distilled water. The moisture content (MC) of the maize kernels was then adjusted to 205 g kg^{-1} dry matter (DM) by soaking samples in distilled water for 2 hours. The samples were thereafter put in sealed plastic bags and refrigerated at a temperature of 4 °C for 72 hours to ensure uniform moisture distribution.

2.3 INOCULATION AND INCUBATION OF MAIZE

Maize was retrieved from cold storage and allowed to equilibrate to room temperature. A total of 45 samples of maize kernels each of mass 3 kg was weighed into sterilised plastic bags. Five ml of spore suspension from *A. flavus or F. verticillioides* were sprinkled on the samples and mixed manually before being transferred to the incubator. Five ml of distilled water was sprinkled on control samples. All samples were incubated at a temperature of 28 °C and sampling was done after 0, 7, 14, 21, and 28 days, respectively. The incubated samples were analysed

for aflatoxin and fumonisin content, and proximate composition.

2.4 ANALYSIS OF THE CHEMICAL COMPOSITION OF MAIZE KERNELS

Aflatoxin and fumonisin analysis were done using a liquid chromatography-tandem mass spectroscopy (LC-MS/MS) as outlined by de Kok et al. (2007). Two hundred and fifty grams of each sample was ground using a Retsch Rotor Mill (SK 1, Germany). Twenty five grams of the ground maize sample was mixed with 80 ml of acetonitrile and 20 ml of water and left to stand for 2 hours. The extract was filtered and diluted four-times with distilled water. Twenty µl of the diluted extract was injected into the LC-MS/MS for analysis.

The liquid chromatography (LC) had an ultra-performance liquid chromatography, ethyle bridge hybrid column (Aquity, UPLC BEH C18 1.7 µm; 2.1×100 mm column). The mobile phase A and mobile phase B were 0.1 % formic acid in water and 0.1 % formic acid in acetonitrile, respectively. The LC flow rate was 0.4 ml min^{-1}. The eluent from the LC column was directed to the mass spectrometer. The electrospray source was operated in a positive ionisation multiple reaction monitoring (MRM) mode. The MRM transitions monitored for AFB_1 were 313 $m.z^{-1}$, 241 $m.z^{-1}$, 50 V, and 47 V for parent ion, product ion, cone voltage, and collision voltage, respectively. The MRM transitions monitored for FB_1 were 722 m z^{-1}, 334 m z^{-1}, 50 V, and 40 V for parent ion, product ion, cone voltage, and collision voltage, respectively. The data acquired were analysed using Waters Masslynx™ software. The limit of detection for the LC-MS/MS was 0.5 µg kg^{-1}, whereas the quantification limit was 2 µg kg^{-1}.

The proximate components including MC, crude ash, crude fibre, crude fat, and crude protein, were anaysed using AOAC methods (AOAC, 2012).

2.5 DATA PREPARATION AND STATISTICAL ANALYSIS

A two-factor full-factorial design was used in this experiment, with the first factor at two levels and the second factor at five levels. The factors studied were fungal species (*A. flavus, F. verticillioides*) and incubation period (0, 7, 14, 21, and 28 days).

The data was subjected to analysis of variance (ANOVA) at 5 % significance level to determine the effect of *A. flavus* and *F. verticillioides* on mycotoxin contamination (aflatoxin and fumonisin), crude fat, crude fibre, crude protein and MC of grains. Where a significant result was obtained, the mean comparison was done using Duncan's Multiple Range Test. The correlation between mycotoxin contamination and proximate components was established using regression analysis. The analysis was done using GenStat® 17th Edition (VSN International Ltd, Hemel Hempstead, United Kingdom).

3 RESULTS

The proximate composition and mycotoxin concentration at the start and during the experiment are presented in Table 1. No AFB_1 and Fumonisin B_1 (FB_1) was detected in the maize kernels prior to fungal infection. In this study, crude fat and crude protein content decrease with time while AFB_1, FB_1 and MC increased. The crude ash and crude fibre was unchanged with time.

The MC was significantly ($p < 0.05$) affected by incubation period and fungal species (Table 1). The MC increased with increasing time of incubation. The highest increase in MC was observed in samples inoculated with *A. flavus* (205 to 289 g kg^{-1}, Table 1). The MC of samples inoculated with *F. verticillioides* ranged from 205 to 261 g kg^{-1}. The lowest increase in MC was observed in the control samples, ranging from 205 to 228 g kg^{-1}.

There were no mycotoxins detected in the samples before incubation. Mycotoxin contamination was significantly ($p < 0.05$) affected by fungal species and the incubation duration. The levels of both AFB_1 and FB_1 increased with the incubation duration. The control samples showed no aflatoxin contamination at the end of day seven. However, 1 µg kg^{-1} of AFB_1 was detected in the control samples on day 14, increasing to 21 µg kg^{-1} and 141 µg kg^{-1} on day 21 and day 28 respectively. FB_1 contamination was not detected in any of the control samples. The maize kernels inoculated with *A. flavus* resulted in AFB_1 contamination ranging from 409 µg kg^{-1} on day 7 to 10,508 µg kg^{-1} on day 28, while those inoculated with *F. verticillioides* resulted in FB_1 contamination ranging from 212 µg kg^{-1} on day 7 to 2,447 µg kg^{-1} on day 28 (Table 1).

The crude fat content of maize kernels was 39 ± 0.5 g kg^{-1} before incubation. The crude fat content decreased with increased fungal incubation time, while the fat content for the control samples was unchanged. Both the fungal species and the length of time of incubation significantly affected the crude fat content ($p < 0.05$). The greatest reduction in crude fat content was observed in the samples inoculated with *A. flavus*. The crude fat content ranged from 39 g kg^{-1} on day zero to 19 g kg^{-1} on day 28. The crude fat content for samples inoculated with *F. verticillioides* ranged from 39 to 31 g kg^{-1}, whereas that of the control samples ranged from 39 to 37 g kg^{-1} (Table

Table 1: Variation of chemical composition of maize with A. flavus and F. verticillioides and incubated for 7, 14, 21, and 28 days (dry matter basis)

Taxon	Time (day)	MC (g kg^{-1})	Mycotoxin (µg kg^{-1})	Crude ash (g kg^{-1})	Crude fat (g kg^{-1})	Crude fibre (g kg^{-1})	Crude protein (g kg^{-1})
Control	0	205^{g}	0^{a}	11.8^{ab}	39.7^{g}	45^{a}	79.4^{f}
Control	7	209^{fg}	0^{a}	11^{a}	39^{g}	44^{a}	79^{f}
Control	14	213^{fg}	1^{b}	11^{a}	39^{g}	44^{a}	79^{f}
Control	21	218^{e}	21^{b}	11^{a}	38^{fg}	45^{a}	78^{f}
Control	28	228^{e}	141^{b}	11^{a}	37^{ef}	45^{a}	76^{df}
A. flavus	0	205^{g}	0^{a}	11^{a}	39^{g}	45^{a}	79^{f}
A. flavus	7	227^{e}	409^{b}	12^{a}	36^{e}	45^{a}	75^{cd}
A. flavus	14	243^{d}	1,259^{b}	12^{a}	33^{cd}	45^{a}	74^{bc}
A. flavus	21	274^{b}	3,032^{b}	12^{a}	31^{b}	44^{a}	73^{b}
A. flavus	28	289^{a}	10,508^{b}	11^{a}	19^{a}	45^{a}	71^{a}
F. verticillioides	0	205^{g}	0^{a}	11^{a}	39^{g}	45^{a}	79^{f}
F. verticillioides	7	217^{f}	212^{b}	11^{a}	36^{e}	45^{a}	77^{e}
F. verticillioides	14	225^{ef}	604^{b}	12^{a}	35^{d}	45^{a}	75^{c}
F. verticillioides	21	253^{cd}	1,240^{b}	11^{a}	33^{c}	45^{a}	74^{bc}
F. verticillioides	28	261^{c}	2,447^{b}	11^{a}	31^{b}	45^{a}	73^{b}
Significance Level							
Fungal taxon	<.001	<.001	0.082	<.001	0.677	<.001	
Time	<.001	<.001	0.349	<.001	0.991	<.001	
Fungal taxon × Time	<.001	<.001	0.061	<.001	0.241	<.001	
CV	0.013	0.433	0.001	0.017	0.002	0.004	
SE	0.041	994.27	0.009	0.048	0.014	0.036	
LSD $_{(p \leq 0.05)}$	0.123	2,880.3	0.026	0.139	0.04	0.104	

Means within a column followed by the same letter(s) are not significantly different according to Duncan's multiple range test ($p < 0.05$). nd = not detected

1). The mean crude fat content of maize kernels inoculated with *A. flavus* and *F. verticillioides* were significantly ($p < 0.05$) different across all treatments.

The crude protein content decreased with incubation time. Both fungal species and the length of time of incubation significantly affected ($p < 0.05$) crude protein content of samples across treatments. The crude protein content of the maize kernels inoculated with *A. flavus* decreased from 79 to 71 g kg^{-1} over the 28 days of incubation. A reduction in the crude protein content from 79 to 73 g kg^{-1} was observed in the samples inoculated with *F. verticillioides*. The crude protein content of the control samples was fairly stable, ranging from 79 to 76 g kg^{-1}.

The crude fibre content of the samples was relatively stable across all treatments. The fungal species and the incubation time had no significant effect ($p > 0.05$) on the crude fibre content of the maize kernels. The crude fibre content of the samples ranged from 45 to 44 g kg^{-1} (Table 1). Similarly, there was no significant difference in the crude ash content of the maize kernel samples across all treatments. Both the fungal species and the incubation time had no significant effect ($p > 0.05$) on the crude ash content of maize kernels. The crude ash content of the maize kernels ranged between 11 and 12 g kg^{-1} (Table 1).

The relationship between mycotoxin contamination and both crude fat and crude protein was best described by second order polynomial equations shown in Table 2. A high coefficient of determination (R^2) was observed

Table 2: Regression equations for crude fat and crude protein of maize kernels contaminated with AFB_1 and FB_1

Mycotoxin	Equation	R^2
AFB_1 vs Crude fat	$1 \times 10^{-7}x^2 - 0.003x + 38.57$	0.986
FB_1 vs Crude fat	$1 \times 10^{-6}x^2 - 0.0061x + 38.74$	0.963
AFB_1 vs Crude protein	$1 \times 10^{-7}x^2 - 0.002x + 77.45$	0.821
FB_1 vs Crude protein	$1 \times 10^{-6}x^2 - 0.0065x + 78.84$	0.944

between mycotoxin contamination and crude fat content (R^2 = 0.986 for AFB_1 and R^2 = 0.963 for FB_1). The coefficient of determination between crude protein content and both AFB_1 and FB_1 was 0.821 and 0.944 respectively. No correlation was observed between mycotoxin contamination and either crude ash or crude fibre content.

4 DISCUSSION

The MC of the maize kernels increased with incubation time. A similar observation was made by Islam (2016) on stored black gram (*Vigna mungo* (L.) Hepper). The increase in moisture content with incubation time is attributed to respiration by maize kernels and fungi (Magan et al., 2004). The mycelial growth increased with time as evidenced by the progressive increase in AFB_1 and FB_1. The increased mycelial biomass escalated the respiration of fungi, hence, high MC on day 28 compared to the minimal change in MC at the start of the experiment. The increase in MC was higher for maize kernels inoculated with *A. flavus* compared to *F. verticillioides*. The incubation temperature of 28 ˚C was optimal for the growth of *A. flavus* (Pratiwi et al., 2015) but unfavourable for *F. verticillioides* whose optimum temperature is around 25 ˚C (Garcia et al., 2012).

The AFB_1 and FB_1 contamination of maize kernels increased over time because of the increasing mycelial biomass. There was a high AFB_1 contamination as compared to FB_1 contamination. *Aspergillus flavus* grows faster than *F. verticillioides* at the incubation temperature of 28 ˚C (Garcia et al., 2012; Pratiwi et al., 2015). Aflatoxin B_1 contamination observed in the control samples could have been caused by internal infection (Mellon et al., 2007).

The greatest depletion of crude fats occurred in maize kernels inoculated with *A. flavus*. This observation is consistent with the findings by Kakde and Chavan (2011) who reported that *A. flavus* was responsible for the maximum depletion of fat content in cereals and oilseeds. Embaby and Abdel-Galil (2006) also observed a reduction in crude fat content in legume seeds due to *Fusarium* sp. *Aspergillus flavus* and *F. verticillioides* produce lipases that hydrolyse fats into fatty acids, which are subsequently degraded to provide a carbon and energy source (Kinderlerer, 1993).

The decrease in crude protein content observed in this study agrees with the findings of Reed et al. (2007) who associated changes in the protein content of maize with fungal degradation. The depletion of protein is attributed to its utilisation during the growth and metabolism of fungi (Bhattacharya and Raha, 2002). Liu et al. (2016) reported that amino acids such as glutamate, aspartate and arginine significantly promote AFB_1 production by *A. flavus* indicating protein utilisation. Results from this study are in tandem with previous research findings (Bhattacharya and Raha, 2002; Rheeder et al., 2009; Liu et al., 2016) that associated protein depletion with fungal deterioration.

Hydrolytic enzymes produced by *A. flavus* and *F. verticillioides* break down fats and proteins for use in fungal growth and development, which in turn creates conducive conditions for the production of mycotoxins (Liu et al., 2016). Fats are preferred over proteins as carbon substrates, hence, the high correlation between fats and mycotoxin contamination (Mellon et al., 2007).

5 CONCLUSION

Aspergillus flavus and *F. verticillioides* caused significant ($p < 0.05$) changes in the levels of crude fat and crude protein content of maize kernels. Although aflatoxin contamination was highly correlated with the depletion of crude fats, such changes can also be caused by *F. verticillioides,* which produces FB_1. The proximate composition of maize samples with allowable mycotoxin contamination (< 10 μg kg^{-1}) was similar to uncontaminated maize kernels. Significant changes in proximate components were observed at mycotoxin contamination levels higher than the regulatory limit of 10 μg kg^{-1}, thus not fit for human consumption.

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