

Maize Milling and Modifying Atmospheric Conditions Limit Formation of Aflatoxin B₁ by *Aspergillus Flavus*

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Abstract

Occurrence of mycotoxins in foods poses a serious health concern all over the world. Aflatoxin B₁ (AFB₁) is the most toxic, with widest occurrence in various foods, but mainly in cereals and nuts and its accumulation depends on substrate and environmental factors. This study investigated the how physical status (milling) of maize kernels and atmospheric conditions (aeration, moisture and temperature) affect production of aflatoxin B₁ by *Aspergillus flavus* (ATCC 28862). Intact kernels and flour were incubated for up to 20 days in open and partially sealed petri dishes under controlled temperatures of 25 °C, 30 °C and 37 °C and initial moisture contents of 27%, 22%, 18%, 15% and 12%. It was found that on average, significantly higher ($p < 0.05$) aflatoxin B₁ level was accumulated in intact kernels (145.7 µg/kg) as compared to milled kernels (2.2 µg/kg). Also, none of the samples incubated under

partially sealed conditions, compared to up to 100% of the samples incubated in open atmosphere had detectable levels of aflatoxin B₁ after 20 days. Fungal growth was not affected by milling or aeration, but sporulation was low at 37 °C and high at 25 °C and 30 °C. The findings of this study provide baseline information on how conditions can be modified to control postharvest accumulation of aflatoxin B₁ in cereals.

Key words; aflatoxin, *Aspergillus flavus*, maize, milling, air-tight, air-open

1. Introduction

Storage environment such as humidity, aeration, and ambient temperature are among factors which influence fungal infection and mycotoxin accumulation in stored food products (Holmquist *et al.*, 1983, Mutegi *et al.*, 2013). For most of food crops especially grains, physical features like intactness of kernels are also important for their quality and stability (Kumar and Kalita 2017, Wilkin *et al.*, 2014). In Tanzania, as in most sub-Saharan Africa, maize is the leading food crop. Maize meals are prepared from maize kernels processed in different forms including intact kernels, dehulled, crushed, milled, fermented and the methods vary between communities (Gwartz and Garcia-Casal, 2014). The crop is known to be highly vulnerable to fungal infection and mycotoxin contamination and among the leading sources of exposure of the toxins to humans and animals (Darwish *et al.*, 2014).

Mycotoxins are secondary metabolites produced by some fungi that may cause disease and death in humans and animals (Bennett and Klich, 2003). The levels of exposure to the toxins and the associated effects in tropical developing countries are enormous both in humans and animals (Shephard 2008). Effects of mycotoxin in foods and feeds include illnesses due to exposure, stunted growth, frequent infections due to immunosuppression, and many other chronic health conditions to the survivors. The toxins also lead to massive economic losses due to business barriers (Milićević *et al.*, 2010). In developing countries government and other different organizations have engaged many efforts in addressing the problem (Tola and Kebede 2016).

Despite the various efforts including research and legislations that have been taken in most countries in the region, significant relief is yet to be achieved (Kimanya *et al.*, 2016). However, good findings from different studies are emerging time after time, and prospects of having total control of mycotoxin exposure are promising (Udomkun *et al.*, 2017). Methods for controlling mycotoxins that have been successfully adopted range from good agronomic farming, good harvesting and proper storage. Most of the actions are ideally achievable anywhere, but in developing countries difficulty is due to associated costs and informal food markets (Adegoke and Letuma 2013).

One area that can have impact in controlling mycotoxins is proper affordable storage (Chulze 2010). Recently, hermetic storage bags have emerged as an easier and affordable method mostly campaigned for controlling crop storage pests (Amadou *et al.*, 2016). The method, in addition of controlling pests is reported to have positive impacts on mycotoxins formation by limiting oxygen availability to the contaminating fungi (Tubbs *et al.*, 2016). Another important factor in achieving proper storage is the form in which the crops, especially grains

are stored. Maize, in most African societies is stored either as intact kernels, dehulled, crushed or milled to flour. The effects of these different physical forms on mycotoxin formation are not well established (Gwirtz and Garcia-Casal 2014). Milling maize have been mentioned as a factor contributing quality deterioration (Suri and Tanumihardjo 2016), but the way it affects mycotoxin accumulation is less reported.

This study was therefore conducted to test in the laboratory, how different environmental and physical state factors affect mycotoxin accumulation in maize. Tested parameters included aeration and milling. Additionally, laboratory settings for moisture level and temperature were tested in order to understand how the factors should be maneuvered when setting similar or related experiments.

2. Materials and Methods

The study was conducted as a controlled experiment, in which maize kernels, inoculated with *A. flavus* conidia were kept under different conditions of moisture and aeration in laboratory and the accumulated aflatoxin B₁ quantified after specified times. The study also monitored growth and sporulation of the fungus in the kernels.

2.1 Fungal Materials

A. flavus reference strain (ATCC 28862) was used as source of spores. Growing and harvesting spores was conducted according to the method, previously described (Temba et al., 2016). In short, the stock spores were grown in malt extract agar for 5 days, and working spores were harvested and washed in 0.1% Tween 80 solution. Spores were suspended in distilled water and after quantification by Colony Forming Units count that was conducted using a Colony Counter they were stored at 4 °C and used for up to 30 days.

2.2 Maize Grains

The study was done using a white kernel variety of maize (Hybrid 33V62, Pioneer® Australia). The maize were stored at room temperature (about 22 °C) in the laboratory and used for up to 6 months. The aflatoxin contamination status of the maize was monitored during experiments by including a negative control.

2.3 Fungal Growth and Accumulation of Aflatoxin B₁ in Maize Kernels

Three sets of experiments were carried out; first to determine the effects of temperature and milling, the effect of limiting air to the experiment, and the effect of initial moisture content of the kernels.

2.3.1 Determining Effect of Temperature and Kernel Milling

About 500 g sterilized maize kernels were incubated overnight under Phosphate Buffer solution (PBS) with *A. flavus* spores suspended at a concentration of approximately 4×10^3 spores per millilitre. The fluid was then decanted and kernels dried at 40 °C for varied duration, to attain moisture contents of approximately 27%, 22%, 18%, 15% and 12%. Half of the kernels were milled and the other half left intact. For each the intact and milled kernels, 24 replicates, approximately 5 g each were taken in three groups, each with eight replicates.

Each group was incubated at specific temperature, either 25 °C, 30 °C or 37 °C for up to 20 days. The experiment set-up is illustrated in Figure 1.

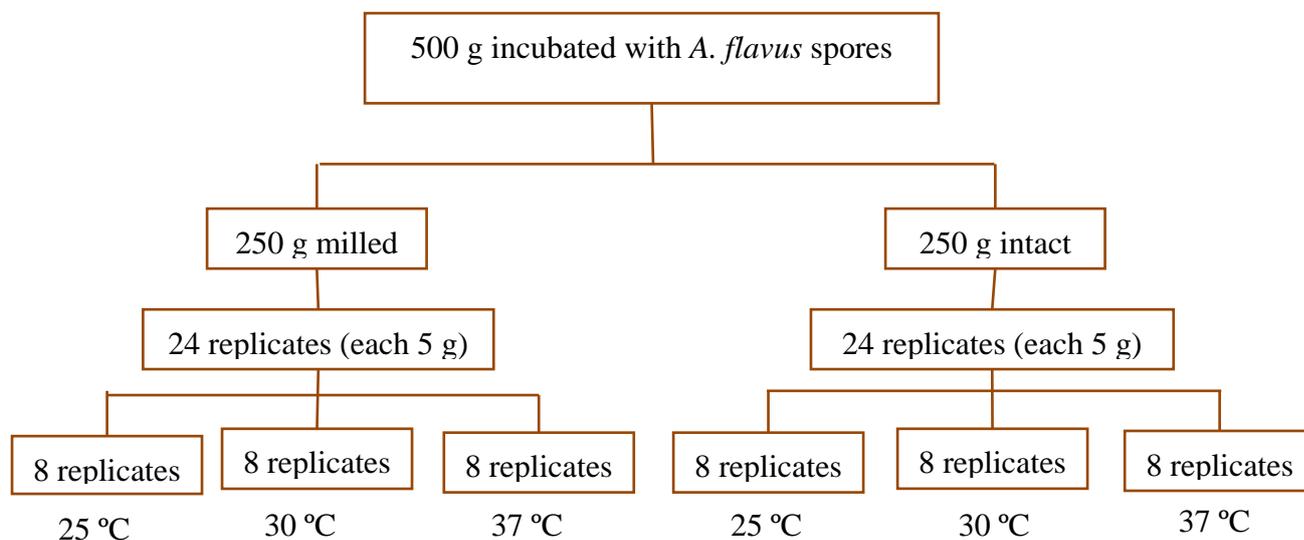


Figure 1. Illustration on replication and experiment blocks

For both milled and intact samples, half were incubated under maintained-moist condition and the rest under dry condition. Maintained-moist condition was achieved by a setting where a sample was placed in a small (3 cm) lidded petri dish, which was also placed inside a bigger petri dish (6 cm), half filled with sterile water. The large dish was also lidded to allow a constant moist atmosphere. There was no direct contact between the sample and the water. For dry condition, the maize/flour samples were placed in the small lidded dishes which were directly placed in the incubator. An additional set of 18 replicates of intact kernel in air-tight dishes were incubated at 25 °C to test aflatoxin accumulation in limited air environment. The growth characteristics of the fungus on the maize were observed at day 20 of incubation and aflatoxin B₁ was quantified by LC-MS/MS at day 0, 5, 10, and 20.

2.4 Quantification of Aflatoxin B₁ by LC-MS/MS

Sample extraction for aflatoxin B₁ analysis was done using a mixture of acetonitrile/Milli Q® water/formic acid at a ratio of 790/200/10 according to a method by Sulyok *et al.* (2006) and aflatoxin B₁ level was analysed by LC-MS/MS (Shimadzu® UHPLC Nexera, and Shimadzu® 8050) (Shar *et al.*, 2016). The analysis was conducted by using aflatoxin B₁ standards obtained from Sigma–Aldrich (Sigma-Aldrich Chemie B.V., Zwijndrecht, Netherlands). The limit of quantification for the method was 0.2 µg/kg. Data analysis was conducted by LabSolutions LCMS Ver 5.6 computer program which was used to generate raw data for mycotoxins concentrations. The data were then transferred to Excel® 2010 for formatting and cleaning, then analysed for descriptive and inferential statistics using Excel® 2010 and IBM SPSS Statistic® 2.3 computer programs.

3. Results

3.1 Fungal Growth Under Different Growth Conditions

The growth of *A. flavus* mycelia was characterised as mild, medium or massive depending on size of fungal mass (Figure 2). The growth was also on basis of by sporulation level, indicated by green colouration of the fungal mass.

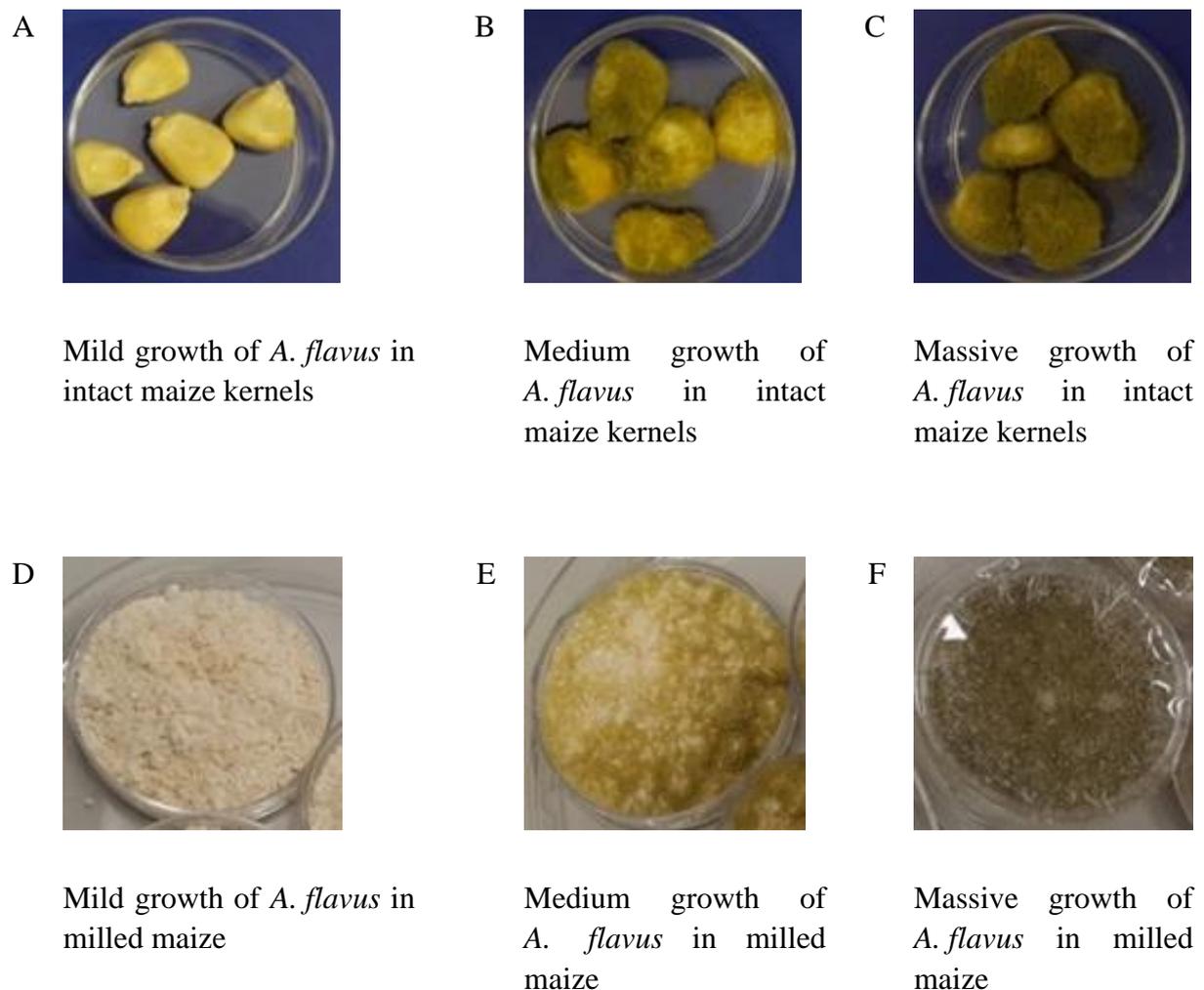


Figure 2. Pictures of samples indicating mild, medium and massive fungal growth

3.2 Effect of Kernel Milling on *A. Flavus* Growth and Aflatoxin B₁ Accumulation

After 20 day-incubation with *A. flavus* spores under moist condition, medium and massive fungal growths were observed in 18 and 9 milled kernel samples respectively, while all intact kernels demonstrated massive fungal growth. Aflatoxin B₁ was detected in 9 (33%) of the 27 milled samples and in 16 (89%) of the intact kernel samples incubated under moist condition. The difference in the frequency of accumulation of the toxin between the two groups was statistically significant ($p < 0.05$, tested by Chi-square). The average level of aflatoxin B₁ in the milled samples was 2.2 $\mu\text{g}/\text{kg}$ whereas for the intact kernels was 145.7 $\mu\text{g}/\text{kg}$ (Figure 3). The difference was statistically significant ($p < 0.05$, by t-test)

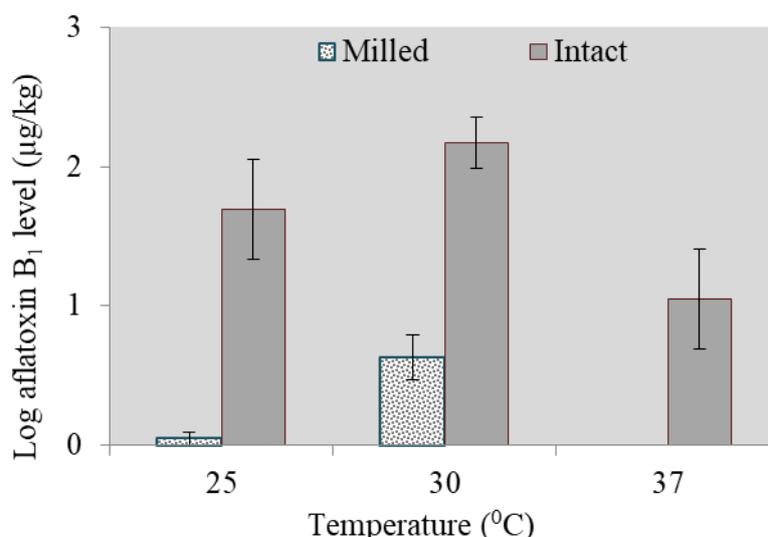


Figure 3. Aflatoxin level in maize incubated for 20 days with *A. flavus* spores, as milled and as intact kernels

3.3 Effect of Aeration on *A. flavus* Growth and Aflatoxin B₁ Accumulation

At day zero the milled samples had a moisture content of 27% while the intact kernels had moisture of 22%. At day 5, the moisture in air-limited flour and whole kernel samples dropped to 26% and 20% respectively, whereas for air-free samples the moisture did not change for samples incubated in moist atmosphere, but dropped to 13% for milled samples and 11% for whole kernel samples incubated under dry atmosphere. Medium vegetative growth of *A. flavus* was observed in both air-tight and open-air, intact kernels whereas massive growth was observed in the milled samples.

None of the samples under air-limited condition, 33% of the milled samples and 100% of the intact kernel samples incubated under moist conditions had detectable levels of aflatoxin B₁ after 20 days incubation at 25 °C. The average levels of aflatoxin B₁ are given in Table 1.

Table 1. Growth of *A. flavus* and accumulation for milled and intact samples incubated in air-limited or air-free moist condition

Sample	Aeration	MC at day 0 (%)	MC at day 5 (%)	Fungal growth	Frequency (and average AFB ₁ level) at day 20
Milled	Air-tight	27	26	Massive	0%
	Open air	27	27	Massive	33% (0.45 µg/kg)
Intact	Air-tight	22	20	Medium	0%
	Open air	22	22	Medium	100% (173.7 µg/kg)

MC = Moisture content (%)

3.4 Effect of Temperature on *A. flavus* Growth and Aflatoxin B₁ Accumulation

Intact kernels incubated at 25 °C and 30 °C expressed medium vegetative fungal growth and massive growth at 37 °C. The milled samples expressed massive growth of the fungus at all three temperatures of incubation; 25 °C, 30 °C and 37 °C. In both intact and milled kernels, at 37 °C the fungal mass appeared white woolly (low sporulation) as compared to the green powdery (massive sporulation) at 25 °C and 30 °C. Lowest accumulation of aflatoxin B₁ was recorded at 37 °C compared to 25 °C and 30 °C (Figure 2). Whereas all intact kernel samples incubated under moist open-air conditions at 25 °C and 30 °C had detectable levels of aflatoxin B₁ (average 173.7 µg/kg and 228 µg/kg respectively), 67% (average 35.4 µg/kg) of the corresponding samples incubated at 37 °C were detected to have aflatoxin B₁. None of the milled samples (including the ones incubated under open-air, moist atmosphere) had detectable level of the toxin whereas at 25 °C and 30 °C 33% and 67% respectively the flour samples had the toxin detected.

3.5 Effect of Moisture on *A. flavus* Growth and Aflatoxin B₁ Accumulation

The effects of sample moisture level at the start of the experiment and duration of incubation on aflatoxin B₁ production are given in Figure 4. At the incubation temperature of 25 °C and under moist atmosphere, aflatoxin B₁ was detected in few samples after 5 days. Maximum levels of the toxin were detected after 10 days, where, with initial moisture contents of 22% and 27% highest average levels of 214 µg/kg were recorded. At 18% and 15% initial moistures aflatoxin B₁ was 152.5 µg/kg and 108 µg/kg respectively, whereas lowest average levels were recorded in samples with 12% initial moisture level (42 µg/kg). Aflatoxin B₁ levels recorded at days 15 and 20 were not significantly different from the levels obtained at 10 days.

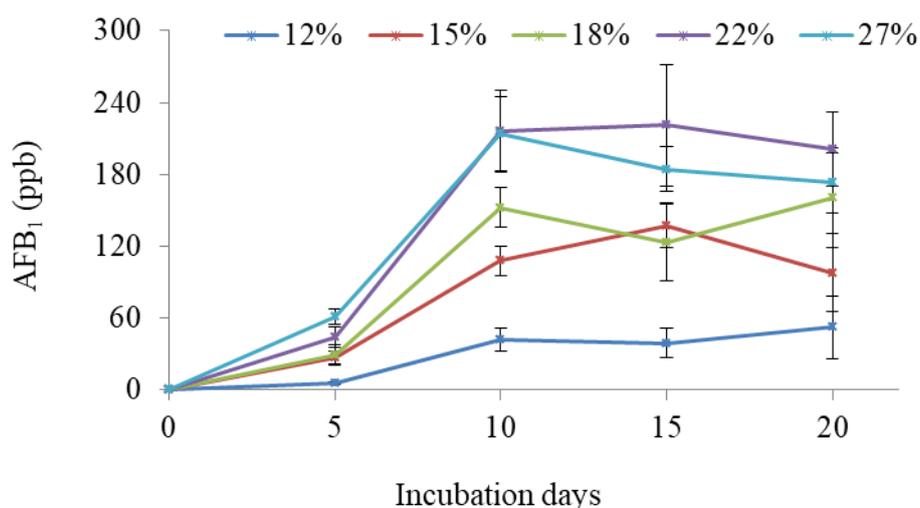


Figure 4. Aflatoxin B₁ levels with different initial sample moisture content

4. Discussion

The current study has indicated that milling maize into flour suppresses aflatoxin B₁ production by *Aspergillus flavus*, but not fungal growth. Risk that aflatoxin will be formed in maize kept intact is about three times higher as compared to milled maize. This might be explained by the fact that in milled maize nutrients are easily accessible to the fungi and hence reducing nutrient shortage stress. It has been found that upon some sorts of stress like drought, temperature or infection, maize cells release reactive oxygen species, which in turn are known to induce aflatoxin formation in the fungi through inter-kingdom communication (Fountain *et al.*, 2016).

The study has also indicated positive relationship between fungal growth and milling of the kernels. This is probably due to the soft and easy penetrable growth media supplied by the flour as compared to the hard intact kernels. Distribution and availability of different nutrients including trace elements have been indicated to influence growth of *Aspergillus flavus* in corn (Lillehoj *et al.*, 1974).

The study has demonstrated that limiting air presence in the samples reduces or prevents aflatoxin B₁ production but not fungal growth. This might be due to shortage of oxygen in or accumulation of carbon dioxide which might create an uncondusive environment for aflatoxin production. It has been indicated that hermetic storage and modified atmospheric conditions affect accumulation of aflatoxin and fungal growth in some food products (Maina *et al.*, 2016). Oxygen, or low CO₂ environment is important for aflatoxin formation but not for fungal growth. *Aspergillus* spp can grow in suitable substrates even under very limited air condition provided that adequate moisture is available (Villers 2014).

Fungal growth, sporulation and aflatoxin B₁ production were optimal at 20 °C and 30 °C as compared to at 37 °C, growth was massive but sporulation and aflatoxin production were minimal. This indicates a linkage between sporulation and aflatoxin production by *A. flavus* as previously indicated in other studies on the fungus (Giorni *et al.*, 2012). Poor sporulation at 37 °C might be attributed to non-supportive metabolic changes at the high temperature.

The study indicates that high moisture facilitates more production of aflatoxin B₁. The importance of moisture in fungal growth have been extensively studied (Mousa *et al.*, 2011), and it has been recommended to have a maximum dryness or minimal moisture level of cereals and other crops, prior to storage as a way of minimizing aflatoxin production.

The study has indicated that under the laboratory condition, the maximum aflatoxin levels were achieved after 10 days with no further increase for up to 20 days. It is understood that aflatoxin B₁ is a stable compound, which might imply that no more aflatoxin B₁ was being formed beyond the 10 days although the fungi were still alive. Comparable results have been reported in a previous study by Giorni *et al.* (2011) which indicated that production of aflatoxin in different media flattens with time under constant due to the change in ecological and nutritional substrates.

The findings of this study add up in setting postharvest measures for controlling aflatoxin accumulation in foods, especially during storage. Recently, the use of hermetic bags among

farmers has been advocated aiming at controlling insect pests during storage. The findings of this study imply that the same hermetic storage might have an effect in reducing aflatoxin accumulation. However reducing aeration means preserving moisture and hence extends favourable conditions for fungal growth. The growth of fungi will lead to rotten foods and might facilitate production of other mycotoxins such as fumonisins. Therefore in order to achieve maximum benefit of reducing aflatoxin B₁ in foods by limiting aeration, it is necessary to ensure optimal dryness of the products is reached.

5. Conclusion

From the findings of this study it can be concluded that maize milling and limiting aeration reduce aflatoxin formation by *Aspergillus flavus*. It is also conclusive that growth of the fungi and sporulation are dependent on moisture status and optimal temperature, but less affected by limiting air supply.

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