



## Aflatoxin B1 (AFB1) production by *Aspergillus flavus* and *Aspergillus parasiticus* on ground Nyjer seeds: The effect of water activity and temperature

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### ARTICLE INFO

#### Keywords:

Aflatoxin  
Nyjer seed  
Oil seed  
Mycotoxin  
*Aspergillus*

### ABSTRACT

Nyjer oil seed cake supports high levels of aflatoxin B1 (AFB1) production. AFB1 is a secondary metabolite of *Aspergillus flavus* and *A. parasiticus*, classified as a Class 1A carcinogen. The aim of this study was to determine the effects of temperature (20, 27, and 35 °C) and water activity (0.82, 0.86, 0.90, 0.94, and 0.98 a<sub>w</sub>) on fungal growth and AFB1 production of *A. flavus* and *A. parasiticus* on ground Nyjer seeds over a 30-day incubation period.

Linear regression models indicated that both fungal growth and AFB1 production were significantly influenced by water activity of Nyjer seeds and incubation temperature. The two fungi did not grow on Nyjer seeds at 0.82 a<sub>w</sub> at the three incubation temperatures. The most favorable growth conditions for both fungi were 0.90–0.98 a<sub>w</sub> at 27 °C or 0.90–0.94 a<sub>w</sub> at 35 °C. The optimum temperature for AFB1 production was 27 °C for both *A. flavus* and *A. parasiticus* (with regression coefficients of 6.01 and 9.11, respectively). Both fungi were likely to produce high levels of AFB1 at 0.90 a<sub>w</sub> (with regression coefficients of 3.56 for *A. flavus* and 7.17 for *A. parasiticus*). *Aspergillus flavus* only produced AFB1 on seeds with 0.90–0.98 a<sub>w</sub> at 27 °C (in the range of 203–282 µg/kg) and on seeds with 0.90 a<sub>w</sub> at 35 °C (212 µg/kg). No detectable AFB1 was produced by this fungus in any other culture conditions that were studied.

*Aspergillus parasiticus*, in contrast, was able to produce AFB1 under all of the growth conditions. At 20 °C, this fungus produced the highest level of AFB1 (212 µg/kg) at high water activity (0.98 a<sub>w</sub>). At 27 °C, *A. parasiticus* produced high levels of AFB1 (in the range of 209–265 µg/kg) at a wide range of water activities (0.86–0.98 a<sub>w</sub>). In the entire study, the highest AFB1 concentration for *A. parasiticus* was detected on seeds incubated at high temperature (35 °C) and low water activity (0.86 a<sub>w</sub>). The findings of this study could help optimize the storage conditions of Nyjer oil seeds to reduce aflatoxin contamination.

### 1. Introduction

Mycotoxins are secondary metabolites of toxigenic species of molds, which can contaminate crops before and after harvesting. The Food and Agriculture Organization of the United Nations (FAO) estimates that approximately 25% of the cereals produced in the world are contaminated by mycotoxins (Food and Agriculture Organization, 2004; Marin et al., 2013). Mycotoxins can be found in a wide variety of food including nuts, oil seeds, spices and fruits. Mycotoxins are also found in meat, milk and other dairy products as a result of consumption of moldy feed by livestock. Aflatoxins, produced by *Aspergillus flavus*, *A.*

*parasiticus*, and rarely *A. nomius*, are the most toxic and common mycotoxins in food and feed (Abdel-Fattah et al., 1982). Aflatoxins B1, B2, B2a, G1, G2, G2a, M1 and M2 are different chemical types of aflatoxins with varying degrees of toxicity. Among these, Aflatoxin B1 (AFB1) is of particular importance, as it is highly carcinogenic (Abdel-Fattah et al., 1982) and classified by the International Agency for Research on Cancer (IARC) as class I carcinogen (International Agency for Research on Cancer, 2002). For these reasons, the United States Food and Drug Administration (USFDA) sets action level for total aflatoxin in dairy animal feed at 20 µg/kg (National Grain and Feed Association, 2011). Also, the last Commission Regulation of the European Union (EU) No

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<https://doi.org/10.1016/j.ijfoodmicro.2019.02.017>

Received 24 October 2018; Received in revised form 23 January 2019; Accepted 19 February 2019

Available online 21 February 2019

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165/2010 (Commission of the European Communities, 2006) considered stringent parameters for aflatoxin regulation. The maximum level of 8 µg/kg of AFB1 has been established in food subjected to sorting or physical treatment before human consumption, and the corresponding 2 µg/kg of AFB1 for direct human consumption.

Nyjer seeds (also spelled as Niger, or called Noug in Ethiopia) are oil seeds of the plant *Guizotia abyssinica* which is closely related to sunflower (University of British Columbia, 2007). Nyjer seeds are ground and pressed into cooking oil in Ethiopia, and contribute up to 50% of the Ethiopian oil seed crop. After oil extraction, dairy farmers use it to feed their cattle to increase milk production (Gizachew et al., 2016). Nyjer seeds cake used as animal feed has been found to contain high levels of aflatoxin (up to 397 µg/kg) in Ethiopia (Gizachew et al., 2016). High level of aflatoxin in milk (AFM1) from dairy cows that consumed the contaminated Nyjer seed cake was also observed (AFB1 can be metabolized to AFM1 by dairy cows, which is excreted through milk causing health hazard especially for children who consume the milk), and there was a positive correlation between AFB1 in feed and AFM1 in milk. Moreover, ground Nyjer seeds are eaten directly in Ethiopia. High levels of AFB1 contaminations have also been previously reported in staple food in Ethiopia such as Shiro (ground peas) and ground red pepper ranging from 100 to 500 µg/kg and 250 to 525 µg/kg, respectively (Fufa and Urga, 1996) suggesting that the environmental conditions in the country are suitable to support fungal growth and AFB1 production.

Numerous studies have indicated that mycotoxin such as AFB1 production is associated mainly with environmental conditions (Lin et al., 2005; Manna and Kim, 2017); therefore, it is important to determine suitable post-harvest storage conditions such as humidity and temperature in preventing the growth of fungi and the production of aflatoxin on Nyjer oil seeds. In this study, we aim to determine the conditions such as temperature, water activity and incubation period that influence fungal growth and the production of AFB1 on ground Nyjer seeds by *A. flavus* and *A. parasiticus*. The results can be used as guideline for Nyjer seed storage in order to minimize AFB1 production.

## 2. Materials and methods

### 2.1. Fungal cultures

Two AFB1 producing strains, *Aspergillus flavus* (NRRL 3357) and *A. parasiticus* (NRRL 465) were obtained from the US Department of Agriculture (USDA) culture collection. They were grown on Potato Dextrose Agar (PDA) individually at 27 °C for 5 days. The spore suspension of each strain was prepared in 5 ml aqueous solution of 0.05% Tween 80 and adjusted to 0.25 (OD<sub>540nm</sub>) which contained approximately 10<sup>6–7</sup> conidia/ml. Spore count in the inoculum was verified using an automated cell counter (TC 20, BIO-RAD, USA).

### 2.2. Inoculation and growth measurement

Nyjer seeds, which were purchased from local stores in Indiana, USA, were ground and sterilized by autoclave before use. Sterilized ground seeds (2.0 g) with approximately 0.76 water activity (a<sub>w</sub>) level were dispensed into three petri dishes (60 mm in diameter) and adjusted to different a<sub>w</sub> (0.82, 0.86, 0.90, 0.94, and 0.98) by adding sterile deionized water using a micro-pipette with membrane-tips. The following amounts of water were added per 100 g of ground seeds (1 ml, 0.82; 1.5 ml, 0.86; 2.0 ml, 0.90; 3.0 ml, 0.94; 6.0 ml, 0.98). We measured the water activity twice using a water activity meter (Aqualab, Pullman, WA, USA), and each time the seeds were shaken to make sure that the water was distributed uniformly within the seeds. Each plate was point inoculated with 10 µl of a spore suspension containing 10<sup>4–5</sup> conidia and triplicate plates were incubated at 20, 27, or 35 °C for 5, 10, 15, 20 and 30 days. The temperatures and water activities used in the study were selected by considering both the previous studies (as stated

in the discussion) that indicated a range of favorable temperatures and water activity for the growth of *A. flavus* and *A. parasiticus* (Schindler et al., 1967; Schmidt-Heydt et al., 2010) as well as the non-ideal storage environmental conditions such as in Ethiopia. To maintain the water activity of the seeds, all culture petri dishes were sealed with corning sealing tape (Sigma-Aldrich, St. Louis, MO) and incubated in mason jars in the presence of wet paper towel. The mason jars were opened every 5 days so that colony diameters on the plates could be measured as well as to keep the incubation in aerobic condition. The wet paper towel approach maintained the water activity for 30 days successfully. Water activities of plates were measured to ensure they remained the same during the 30 days incubation period. Water activity of the seeds after 5, 10, 15, 20, and 30 days were verified using the water activity meter. Fungal growth on each plate was measured every 5, 10, 15, 20 and 30 days until the colony reached the edge of the plate. The average of two diameters at right angles of fungal colony of each plate was measured and then the colony diameter of three plates of the same conditions were measured and the mean was recorded as colony diameter.

### 2.3. Aflatoxin B1 extraction

Aflatoxin B1 was extracted after 5, 10, 15, 20 and 30 days of incubation from the same triplicate samples used for fungal growth measurements by transferring all the ground seeds from a plate into a tube containing 5 ml of aqueous solution of 80% methanol. The mixture was then shaken at 150 rounds per minute for 90 min and filtered through a 0.22 µm sterile syringe filter. Though fungal diameter had low variance among the triplicate plates, in order to take into account the effect of slight fungal growth variations on the AFB1 production, a 1 ml composite sample containing equal proportions of extracts from three plates incubated in the same conditions was used for AFB1 analysis. The extracts were stored at 4 °C until purification and analysis for AFB1 was performed.

### 2.4. High performance liquid chromatography (HPLC) analysis

Samples were analyzed using Thermo Scientific Ultimate 3000 HPLC equipped with fluorescence detector. The chromatogram was recorded at 365 nm excitation and 455 nm emission wavelength using a reverse phase column C18, 4.6 mm × 150 mm, 3.5 mm and isocratic mobile phase with a flow rate of 1 ml/min, consisting of the following gradient: 50% water, 40% methanol and 10% acetonitrile (HPLC grade solvents from Fisher Scientific). Before analysis, each sample was purified by an immunoaffinity column purchased from VICAM (Massachusetts, USA) based on the procedure as outlined in the protocol prior to injection into HPLC. First, 1 ml sample diluted with 4 ml of phosphate buffered saline (PBS) was loaded on the immunoaffinity column and then washed with 5 ml of PBS followed by another wash with 5 ml DI water at a flow rate of 1 ml/min. The AFB1 was eluted with 1 ml of methanol at a rate of 1 ml/min. The AFB1 standard was from *Aspergillus flavus* (Sigma-Aldrich, Milwaukee, WI, USA). The standard solution was made in methanol. There were eight standard solutions in the concentration range of 4 to 1000 µg/kg of AFB1. The calibration curve obtained proved linear with r<sup>2</sup> = 0.9991. The retention time of AFB1 was approximately 5.15 min, and the detection limit was 1.5 µg/kg under the conditions described. The validation of the HPLC method was carried out with the determination of the coefficient of variation (%CV) and the recoveries of three samples spiked with 15 µg/kg, 50 µg/kg and 100 µg/kg. The validation experiments were performed using triplicate measurements as described for the other samples. The range of %CV and recoveries were 0.8–3.8% and 99.6–109.6% respectively, which is consistent with the guidelines of Joint FAO/WHO Expert Committee on Food Additives for analysis of aflatoxin (JECFA, 2016; Kim et al., 2017).

2.5. Statistical analysis

Linear regression models were fitted to the data to assess the association between potential predictors (fungal species, water activity, temperature, and incubation time) and the outcomes (AFB1 production and colony diameter). When a predictor's relationship with the outcome of interest was not linear, then the predictor was fitted as a categorical variable in the model. All the predictors that were significant at the initial univariable analysis were considered in the multivariable regression models. Predictors were retained in the multivariable models if they remained statistically significant. Two-way interactions were also assessed. A p-value of 0.05 or smaller was considered statistically significant. The original AFB1 values were transformed to natural logarithm of AFB1 to improve the fit of the models. The model coefficients were back-transformed (exponentiated) to revert to the original scale. All statistical analyses were performed using STATA IC 15 (College Station, TX, USA).

3. Results

3.1. Fungal growth on Nyjer seeds

As shown in Fig. 1, growth of both fungi was influenced by both incubation temperature and water activity. Both grew on Nyjer seeds with  $a_w$  ranging from 0.86 to 0.98 at 20, 27, and 35 °C. No growth was detected on Nyjer seeds with 0.82  $a_w$  throughout the 30 days of

incubation at the three temperatures.

The two fungi showed slower growth on Nyjer seeds at 20 °C as compared to growth at 27 and 35 °C. Neither one reached confluent growth (52 mm) during the 30-day incubation. The growth of the two fungi also increased with the water activities of the Nyjer seeds at 20 °C. Most rapid growth occurred on seeds with 0.98 followed by 0.94, 0.90 and 0.86  $a_w$ . After 30 days incubation, colony diameters of the two fungi ranged from 40 to 50 mm on seeds with 0.90 to 0.98  $a_w$ . At 0.86  $a_w$ , neither fungi showed any visible growth during the first 10 days, and then the mean diameters of *A. flavus* and *A. parasiticus* colonies grew to 29 and 22 mm, respectively at the end of 30 days incubation (Fig. 1a and b).

At 27 °C, both fungi grew rapidly on seeds with 0.90–0.98  $a_w$ . Highest growth of *A. flavus* occurred on seeds with 0.94 and 0.98  $a_w$ , on which confluent growth was detected after 10 days incubation. A slightly slower growth of this fungus was found on seeds with 0.90  $a_w$ , on which, confluent growth was observed after 15 days incubation. At 0.86  $a_w$ , *A. flavus* did not show any visible growth during the first 10 days and then the mean colony diameter grew to 43 mm at the end of 30 days (Fig. 1c). *Aspergillus parasiticus* showed slightly slower growth than *A. flavus* on seeds with 0.90 to 0.98  $a_w$ ; however, its growth at 0.86  $a_w$  was faster than *A. flavus* (Fig. 1c and d). The most rapid growth of *A. parasiticus* occurred on seeds with 0.94  $a_w$ , on which confluent growth was reached within 15 days (Fig. 1c). In seeds with 0.98  $a_w$ , *A. parasiticus* grew rapidly initially and reached its maximum mean colony diameter, 48 mm, on day 15, and then the growth stopped.

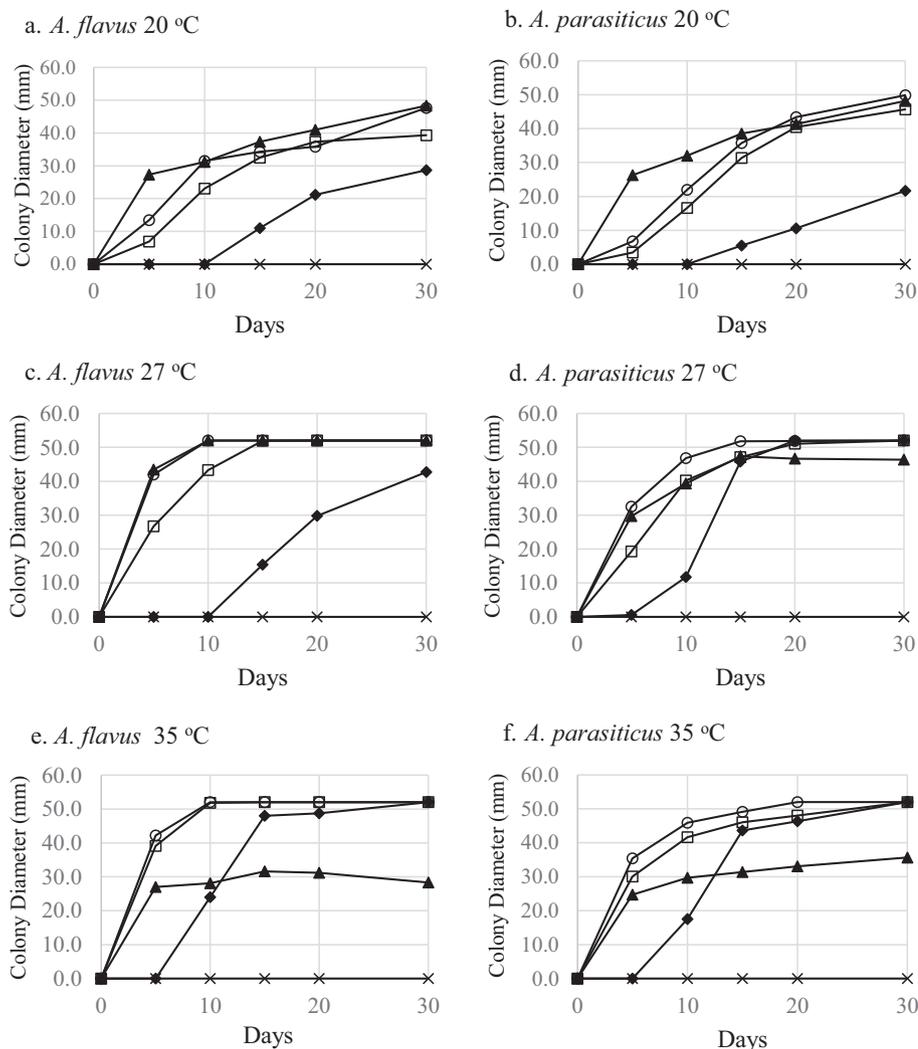


Fig. 1. Mean colony diameters of *A. flavus* (panels a, c, and e) and *A. parasiticus* (panels b, d, and f) on Nyjer seeds with 0.82 (x), 0.86 (♦), 0.90 (□), 0.94 (○), or 0.98 (▲)  $a_w$  at 20, 27, and 35 °C.

On seeds with 0.90 and 0.86  $a_w$ , near confluent growth of *A. parasiticus* was detected after 20 days. This fungus also showed a lag period with no visible growth during the first 5 days of incubation (Fig. 1d).

At 35 °C, the two fungi grew best on seeds with 0.90 and 0.94  $a_w$ . *Aspergillus flavus* reached confluent growth after 10 days incubation on seeds with 0.90 or 0.94  $a_w$  (Fig. 1e). *Aspergillus parasiticus*, which had slower growth than *A. flavus*, reached confluent growth after 20- and 30-days incubation on seeds with 0.94 and 0.90  $a_w$ , respectively (Fig. 1f). At 0.98  $a_w$ , the two fungi only grew rapidly during the first 5 days and then showed very little growth afterward (Fig. 1e and f). The maximum mean colony diameters of *A. flavus* and *A. parasiticus* were 32 and 38 mm, respectively. In contrast, at 0.86  $a_w$ , the two fungi did not grow during the first 5 days, then grew rapidly from day 5 to 15. Both reached confluent growth after 30 days incubation (Fig. 1e and f).

### 3.2. AFB1 production on Nyjer seeds

Although *A. flavus* and *A. parasiticus* showed similar growth patterns on Nyjer seeds under all incubation settings in this study, they did not produce similar levels of AFB1 under the same incubation condition. *Aspergillus flavus* produced AFB1 on seeds within a narrower range of water activity (0.90–0.98  $a_w$ ) and temperatures (27 and 35 °C), while *A. parasiticus* produced AFB1 on seeds with a wider range of water activity (0.86–0.98  $a_w$ ) at all three incubation temperatures. As shown in Fig. 2a, at 27 °C, *A. flavus* produced high levels of AFB1 (282, 261, 203  $\mu\text{g}/\text{kg}$ ) on seeds with 0.90, 0.94 and 0.98  $a_w$ , respectively. At 35 °C, it only produced AFB1 (212  $\mu\text{g}/\text{kg}$ ) on seeds with 0.90  $a_w$ . The fungus did neither produce any detectable AFB1 on seeds with 0.86  $a_w$ , nor on

seeds incubated at 20 °C. In contrast, *Aspergillus parasiticus* produced AFB1 under all culture conditions (Table 1 and Fig. 2b). The highest AFB1 concentration (267  $\mu\text{g}/\text{kg}$ ) for *A. parasiticus* was detected on seeds with low water activity (0.86  $a_w$ ) incubated at high temperature (35 °C). At 27 °C, *A. parasiticus* produced high levels of AFB1 (228, 252, 209 and 265  $\mu\text{g}/\text{kg}$ ) on seeds with 0.86, 0.90, 0.94, and 0.98  $a_w$ , respectively while at low incubation temperature (20 °C), the highest AFB1 concentration produced (212  $\mu\text{g}/\text{kg}$ ) on seeds with high water activity (0.98  $a_w$ ).

### 3.3. Regression modeling

The multivariable linear regression model indicated that fungal growth was significantly associated with water activity and temperature (Table 2). Only 18.3% of *A. flavus* produced detectable levels of AFB1, while 68.3% of *A. parasiticus* samples produced AFB1. There was no significant difference in the growth of the two fungi; therefore, the same regression model is applicable for both *A. flavus* and *A. parasiticus*. The optimal water activity level for fungal growth was in the range of 0.90–0.98 (with 0.94  $a_w$  having the highest coefficient) and the least favorable condition was at 0.86  $a_w$ . The optimum temperature for growth was at 27 °C (with a regression coefficient of 13.11), while the least amount of growth occurred at 20 °C.

Temperature and water activity were significantly associated with AFB1 production for both species (Tables 3 and 4). The optimum temperature for AFB1 production was 27 °C for both *A. flavus* and *A. parasiticus* (with regression coefficients of 6.01 and 9.11, respectively). Both fungi were likely to produce high levels of AFB1 at 0.90  $a_w$  (with regression coefficients of 3.56 for *A. flavus* and 7.17 for *A. parasiticus*). In addition, 0.98  $a_w$  was also favorable for AFB1 production by *A. parasiticus*. Also, *A. parasiticus* produced significantly higher levels of AFB1 ( $p < 0.001$ ). Incubation time was not significantly associated with AFB1 production.

## 4. Discussion

This study has shown the influence of water activity and temperature on the growth and AFB1 production of *A. flavus* and *A. parasiticus* on Nyjer seeds. Both fungi grew on Nyjer seeds with 0.86 to 0.98  $a_w$  at 20, 27 and 35 °C. Overall, *A. flavus* grew slightly faster than *A. parasiticus* did in most growth conditions. The optimum water activity for growth depended on the incubation temperature of the seeds. At a low incubation temperature (20 °C), the two fungi grew most rapidly on seeds with high water activity (0.98  $a_w$ ); whereas at a high incubation temperature (35 °C), both showed rapid growth on seeds with 0.90 and 0.94  $a_w$ . The optimum temperature for growth also depended on the water activity of the seeds. On seeds with low water activity (0.86  $a_w$ ), these two fungi showed a lag phase with no visible growth for 5 to 10 days and the lag phase became shorter and the growth became faster when incubation temperature increased from 20 to 35 °C. On seeds with 0.90 or 0.94  $a_w$ , both fungi showed rapid growth at 27 and 35 °C; whereas, on seeds with a high water activity (0.98  $a_w$ ), the two fungi grew most rapidly at 27 °C.

A previous study showed that the maximum growth of *A. flavus* on sorghum grains was observed at 0.99  $a_w$  and at 37 °C and the minimum water activity for growth was 0.91  $a_w$  (Lahouar et al., 2016). It was also reported that *A. parasiticus* can produce aflatoxin in the range of 20–40 °C with  $a_w > 0.90$  on YES agar (Schmidt-Heydt et al., 2010). Earlier studies on artificial substrate such as wort agar showed that the most favored temperature range was between 29 and 35 °C for the growth of *A. flavus* (Schindler et al., 1967). In this study, overall the least fungal growth was observed at 0.86  $a_w$  and 20 °C for both *A. flavus* and *A. parasiticus*. The differences in optimal conditions for fungal growth suggest that not only fungal strains but also the substrates are important determining factors.

*Aspergillus parasiticus* produced AFB1 at water activity levels ranging

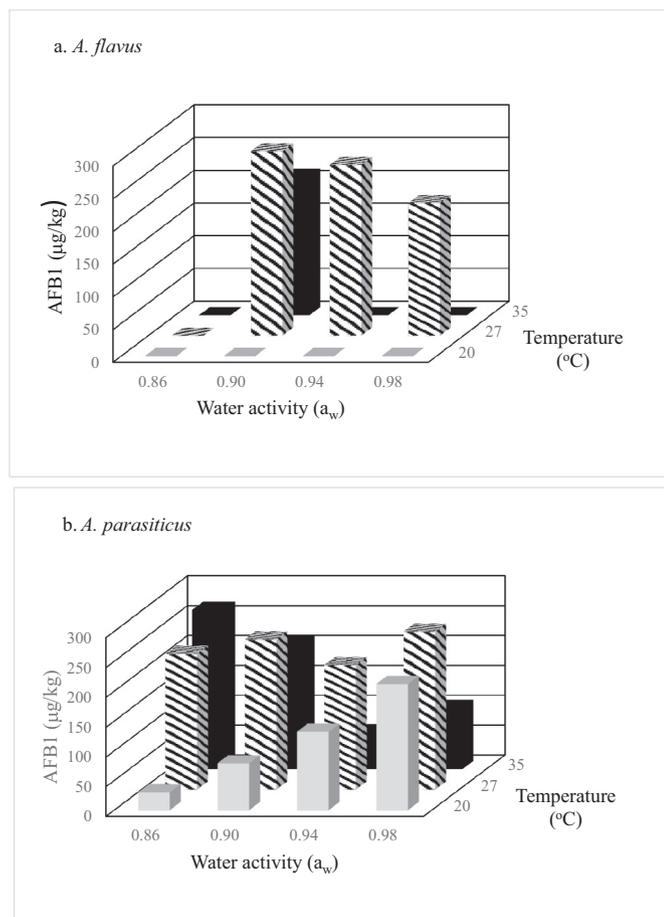


Fig. 2. The maximum AFB1 production of the five sampling times (5, 10, 15, 20 and 30 days) by *A. flavus* (panel a) and *A. parasiticus* (panel b) at 20 °C (●), 30 °C (◐), and 37 °C (■) each combination of temperature and  $a_w$  studied.

**Table 1**

Aflatoxin B1 (AFB1) concentration<sup>a,b</sup> (µg/kg) produced by *A. flavus* and *A. parasiticus* at each water activity (a<sub>w</sub>), temperature and sampling incubation time.

Species	Day	Temperature (°C)																	
		20			27			35			20			27			35		
		Water activity (a <sub>w</sub> )																	
0.86			0.90			0.94			0.98										
<i>A. flavus</i>	5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	47	ND					
	10	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	29	ND					
	15	ND	ND	ND	ND	131	ND	ND	261	ND	ND	ND	ND	ND					
	20	ND	ND	ND	ND	282	212	ND	ND	ND	ND	203	ND	ND					
	30	ND	ND	ND	ND	24	ND	ND	15	ND	ND	ND	ND	ND					
<i>A. parasiticus</i>	5	ND	ND	ND	ND	252	210	ND	206	48	41	265	ND	ND					
	10	ND	ND	7	ND	146	153	ND	158	56	134	190	103	ND					
	15	ND	228	178	35	157	ND	ND	149	62	175	176	ND	ND					
	20	ND	ND	7	23	156	196	ND	169	33	212	168	ND	ND					
	30	30	36	267	79	77	150	132	209	8	35	195	ND	ND					

ND: Not detectable.

<sup>a</sup> Limit of detection:1.5 µg/kg.

<sup>b</sup> Limit of quantitation:4 µg/kg.

**Table 2**

Multivariable linear regression model showing the effects of water activity, incubation time, and temperature on fungal colony diameter (mm).

Predictor	Category	Coefficient	p-Value	95% confidence interval
Water activity	0.86	1 (reference)		
	0.90	17.76	< 0.001	12.46–23.05
	0.94	19.08	< 0.001	13.78–24.37
	0.98	11.85	< 0.001	6.56–17.14
Incubation (days)		0.93	< 0.001	0.71–1.14
Temperature (°C)	20	1 (reference)		
	27	13.11	< 0.001	8.52–17.69
	35	9.08	< 0.001	4.5–13.67
Intercept		3.97	0.174	–1.78–9.73

**Table 3**

Multivariable linear regression model showing association of water activity and temperature with level of aflatoxin B1 for *A. flavus*.

Predictor	Category	Coefficient (exponentiated)	p-Value	95% confidence interval
Water activity	0.86	1 (reference)		
	0.90	3.56	0.018	1.25–10.09
	0.94	1.81	0.255	0.64–5.15
	0.98	2.41	0.095	0.85–6.84
Temperature (°C)	20	1 (reference)		
	27	6.01	< 0.0001	2.43–14.82
	35	1.31	0.523	0.53–3.22
Intercept		0.51	0.132	0.20–1.23

**Table 4**

Multivariable linear regression model showing association of water activity and temperature with level of aflatoxin B1 for *A. parasiticus*.

Predictor	Category	Coefficient (exponentiated)	p-Value	95% confidence interval
Water activity	0.86	1 (reference)		
	0.90	7.17	0.01	1.63–31.54
	0.94	4.11	0.061	0.93–18.09
	0.98	5.88	0.02	1.33–25.12
Temperature (°C)	20	1 (reference)		
	27	9.11	0.001	2.52–32.87
	35	2.17	0.228	0.61–7.85
Intercept		2.25	0.21	0.62–8.12

0.86–0.98 a<sub>w</sub> at varying degree at 20, 27 and 35 °C during 30 days of incubation. However, AFB1 production by *A. flavus* on Nyjer seed only occurred at 27 and 35 °C. At 27 °C, *A. flavus* produced AFB1 on Nyjer seeds with a wider range of water activity (0.90–0.98 a<sub>w</sub>); whereas at 35 °C, AFB1 was only detected on seeds with 0.90 a<sub>w</sub>. According to the regression model, the most favorable temperature for AFB1 production for both fungi was 27 °C. Other studies have reported the optimal condition for both *A. flavus* and *A. parasiticus* to produce aflatoxin in cereals was 0.99 a<sub>w</sub> and 33 °C (Milani, 2013), suggesting that the optimum incubation period and temperature required for maximum AFB1 production could vary significantly due to the nature and composition of the substrate and differences in fungal species.

Aflatoxin biosynthesis involves the oxidative degradation of lipids (lipid peroxidation) in the presence of fatty acid hydroperoxides (Bircan, 2006). Lipid peroxidation and aflatoxin production is enhanced in the presence of polyunsaturated fatty acids (De Luca et al., 1995; Fanelli and Fabbri, 1989). For example, linoleic acid and trilinolein activate more aflatoxin production than saturated fatty acids (Fanelli and Fabbri, 1989). The oil content of Nyjer seed varies from 30 to 50% and its fatty acid composition is predominantly linoleic acid (polyunsaturated fatty acid) (University of British Columbia, 2007). The high level of AFB1 in the Nyjer seed cake observed in our previous study was probably due to the oil extraction process, storage temperature, moisture conditions and the presence of residual fatty acids.

Nyjer seed is indigenous to Ethiopia and contributes up to 50% of the Ethiopian oil seed crop (Gizachew et al., 2016; University of British Columbia, 2007). Due to the high consumption of oil seed and oil seed products, there is a global concern over the high level of aflatoxin contamination of these products. In countries in South East Asia and Africa, where the climate is hot and humid, high level of aflatoxin contamination in oil seeds and oil has been reported. Studies conducted in China (Li et al., 2009) reported up to 68.5 µg/kg of AFB1 in peanut and 20.5 µg/kg in sesame pastes. Up to 956 µg/kg of AFB1 was recorded in crude rice bran oil in India (Jayaraman and Kalyanasundaram, 2009), and up to 71.1 µg/kg of AFB1 was observed in sunflower oil in Sudan (Elzupir et al., 2010).

The oil seed shells normally protect the seed from infestation by *Aspergillus* fungi. However, the broken shells and the presence of moisture during the oil extraction process allow more fungal growth. Our experiments on the whole seed at various conditions showed either slow growth or the fungi didn't grow. Though there is evidence that aflatoxin production starts during harvest, the post-harvest moisture and temperature conditions of the seeds support further fungal growth. Thus, appropriate control of these factors at the early stages of storage

and processing of the oil seeds is recommended to minimize aflatoxin production.

Though there are a few studies on environmental factors that influence fungal growth and AFB1 production on different cereals and grains, to our knowledge, this is the first report on the effects of water activity, temperature and incubation period on fungal growth and AFB1 production on Nyjer seeds. The findings of this study could help optimize the storage conditions of the Nyjer oil seed before and after oil extraction. Also, future studies are needed on aflatoxin contamination of the oil extracted from Nyjer seeds.

In conclusion, *A. flavus* (NRRL 3357) and *A. parasiticus* (NRRL 465) were able to grow on Nyjer seeds with 0.86–0.98  $a_w$  at 20, 27 and 35 °C. The most favorable growth conditions were 0.90–0.98  $a_w$  at 27 °C or 0.90–0.94  $a_w$  at 35 °C. *Aspergillus parasiticus* was able to produce AFB1 under all studied growth conditions, whereas *A. flavus* only produced AFB1 on seeds with 0.90–0.98  $a_w$  at 27 °C and on seeds with 0.90  $a_w$  at 35 °C.

## Abbreviations

AFB1	Aflatoxin B1
AFM1	Aflatoxin M1
DI	Deionized Water
HPLC	High Performance Liquid Chromatography
EU	European Union
FAO	Food and Agriculture Organization
USFDA	Food and Drug Administration of the United States
USDA	United States Department of Agriculture

## Acknowledgements

This study was supported by Purdue University Northwest, United States, through internal research grant fund (ID: 21010000). We thank Jessica Orr and Yung-Chen Hsu for their valuable assistance in the laboratory. We also thank Susan Delatorre for ordering the various laboratory items required for the research.

## Declarations of interest

None.

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