EFFECT OF WATER ACTIVITY AND TEMPERATURE ON THE GROWTH AND AFB_1 PRODUCTION OF *Aspergillus flavus* ISOLATED FROM TURMERIC

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SUMMARY

An *in vitro* experiment was conducted to determine the effect of water activity (a_w) and temperature on lag phase prior to growth, growth rate and AFB1 production by two isolates of *Aspergillus flavus* (AFg and AFt). A 6 x 5 factorial experiment replicated three times where turmeric-based media (15 ml/plate) at six a_w levels (0.85, 0.90, 0.93, 0.95, 0.982 and 0.995) was inoculated with a 6-day old *A. flavus* culture and incubated at five temperature regimes (20, 25, 30, 35 and 37° C) was carried out. Assessment of fungal growth was done daily for 10 days and lag phase prior to growth and growth rate was derived from the data. Aflatoxin B_1 (AFB_1) production was analysed after 10 days of incubation. Data showed that in all cases, no growth was observed at 0.85 a_w at all temperatures. Optimum growth rate was at 0.982 a_w in both isolates regardless of the temperature. AFB_1 production profile between the two aflatoxin-producing isolates were quite different and isolate AFt produced higher concentration of toxin than AFg. The obtained data could aid in the selection of appropriate management measures against AFB_1 contamination in commonly consumed turmeric and turmeric-based products.

Key words: Aflatoxin, *Aspergillus flavus*, lag phase, temperature, turmeric, water activity.

Spices are widely used as ingredient in food preparation and provides distinctive colour, flavour and aromas in most parts of Asia and Africa. They are rich in essential nutrients and have some medicinal properties (Egbuchua and Enujeke, 2013). They are increasingly being used by both locals and foreigners in a wide range of meals to enhance quality (Riordan and Wilkinson, 2008). In Nigeria for instance, ginger, garlic and turmeric are common spices used in most cuisine. Nigeria ranks the fourth world producer of turmeric with the northern parts such as; Gombe, Bauchi, Benue, Nassarawa and Kaduna States being the highest producers. Recent studies have focused on the efficacy of turmeric (*Curcuma longa*) on the treatment of health issues such as

diabetics, nausea, vomiting, high cholesterol, high blood pressure and inflammation (Chen *et al.*, 2007; Ernest and Pittler, 2008; Kim *et al*., 2008). Many substances have also been isolated from the rhizome of *C. longa* attributing a wide range of biological activities (Kumar *et al*., 2006) such as anti-inflammatory (Sandur *et al*., 2007; Aggarwal and Harikumar, 2009); wound healing (Mahesshwari *et al*., 2006); anticancer (Kim et al., 2012) and antibacterial activity (Gupta and Sadhuna, 2000; Naz *et al*., 2010) and this has stimulated farmers' interest in the production of this crop in Nigeria. Poor handling, transportation, processing and storage system and the way the product is displayed for sales in some Nigerian markets encourage quality deterioration of this food product. With the rain forest ecological zone of Nigeria characterized by high temperature and humidity levels, the stimulation of growth of mycotoxigenic fungi especially *Aspergillus flavus* will be encouraged. Furthermore, owing to the fact that this zone also accounts for large consumption of these products and the combined effects of high temperature and water condition, the contamination by *A. flavus* in major food products becomes a serious problem in food security and nutritional quality of food in the zone. This therefore has necessitated the need to examine the interacting effects of temperature and water activity on the level of colonization by *A. flavus* and aflatoxin B_1 ($AFB_{1)}$ production. The objective of this study was to evaluate the interacting environmental effects of water activity ($a_{w)}$ and temperature on lag phase prior to growth, growth rate and aflatoxin production of two toxigenic isolates of *A flavus* on turmeric commonly consumed in southern Nigeria. The findings of this study would aid further decisions on the best and most appropriate management measures to use in order to minimise the potential hazards occasioned by aflatoxin contamination in turmeric.

MATERIALS AND METHODS

Fungal isolate

Two aflatoxin producing fungal isolates of *A. flavus* AFg and AFt, previously isolated from ginger and turmeric respectively, were used in this study. Ginger and turmeric were collected from randomly-selected retail outlets including supermarkets, grocery stores, open market stalls and wholesale outlets in the Niger Delta Region of Nigeria in 2017. The two isolates were previously

found to produce AFB_1 by screening with Coconut Agar Media (CAM) (Lin and Dianese, 1976) and confirmation done on a mycotoxin conducive Yeast Extract Sucrose agar medium (YES, Oxoid Ltd., UK) and quantification done using HPLC.

Turmeric-based media preparation, inoculation and incubation

A standard media of 5% milled turmeric agar media (50g of turmeric powder +10g of technical agar + 0.16g of chloramphenicol + 1000 ml of distilled H_2O) was used in the study. The a_w of media was modified by adding increasing amounts of glycerol to obtain the following a_w treatment levels of 0.85, 0.90, 0.93, 0.95, 0.982 and 0.995. These were checked with the a_w meter (Aqualab, Decagon devices, Inc., USA). The inoculum was prepared from a 6-days-old culture each fungal isolate grown on Malt Extract Agar (MEA) at 25° C. When agar plates were solidified, they were one-point centrally inoculated with 2 µL of the inoculum of each of the isolates of *A. flavus* (6-day old mycelia + 9 ml sterile water supplemented with 0.05% (w/v) Tween 80) for each sixty plates. After inoculation, the Petri plates were sealed with parafilm tape and kept in closed polyethylene bags at the tested incubation temperatures (20, 25, 30, 35 and 37° C). Each treatment was carried out in triplicate.

Fungal growth assessment

Assessment of fungal growth rate was done daily during the 10-day incubation period in each temperature and a_w treatment condition. The diameter of the colonies was measured daily in two directions at right angles to each other. The data was used for the determination of lag phase (days, λ) prior to growth and growth rates (mm/day, µ) for each studied factorial treatment. Data was fitted using a linear model obtained by plotting the colony diameter against time. The growth rate (mm/day) was calculated from the slope of the regression line and lag phase (days, λ) calculated by equalling the regression line formula to the original inoculum size (diameter, mm).

Aflatoxin analysis

After 10 days of incubation plugs where taken from each plates using a 4 mm cork borer into Eppendorf tubes and stored at -20^{o}C for aflatoxin analysis. Weight of Eppendorf and weight of Eppendorf + agar was taken to aid in the calculation of aflatoxin concentration in ng/g of agar. Aflatoxin was extracted with 0.75 ml of 100% methanol by shaking well for 1 h at 150 rpm at 25^o C in an orbital shaker. The extract was transferred to Eppendorf tubes and completely dried at 45^{o}C in a speed vacuum in the dark. Samples were dissolved in 1 ml of methanol: water (50:50), vortex and filtered with 0.22 µm filter (Kromega, Jaytee Biosciences Ltd., UK) into sylinazed HPLC vials using 1 ml syringes (Terumo Medical Corporation, UK). The HPLC equipment used was an Agilent 1200 Series system (Agilent, Berkshire, UK) with a fluorescence detector (FLD) (Millipore Waters, Corporation Massachusetts USA), at excitation and emission wavelength of 365 and 440 nm respectively, and flow rate of the mobile phase (methanol/water/acetonitrile, 30/60/15, v/v/v) of 1 ml/min for a running time of 12 min. Separation was achieved through the use of a C_{18} column (Poroshell 120 EC-C18 4.6 x 100 mm, 2.7 µm) preceded by a Phenomenex Gemini C_{18} 3 mm, 3 µm guard cartridge).

Data Analysis

The experiment was a 5 x 6 factorial laid out in a completely randomised design (CRD) replicated three times for each isolate. Statistical analysis was performed using Genstat 16th Edition; VSN industrial Ltd, UK for normally distributed data. Comparisons were considered significantly different at 5% probability level for all single and interacting treatments.

RESULTS

Effect of a_w and temperature on lag phase prior to growth

No growth occurred in both isolates at 0.85 a_w regardless of the temperature. At marginal temperature for growth (20^o C) at 0.9 a_w, no growth was also observed in both isolates (Fig. 1a & b). The two isolates had the longest initial lag phase at 0.9 a_w but at different temperatures. The longest average lag phase (> 6 days) were observed at 25^o C for AFg and (> 4 days) at 30^o C for

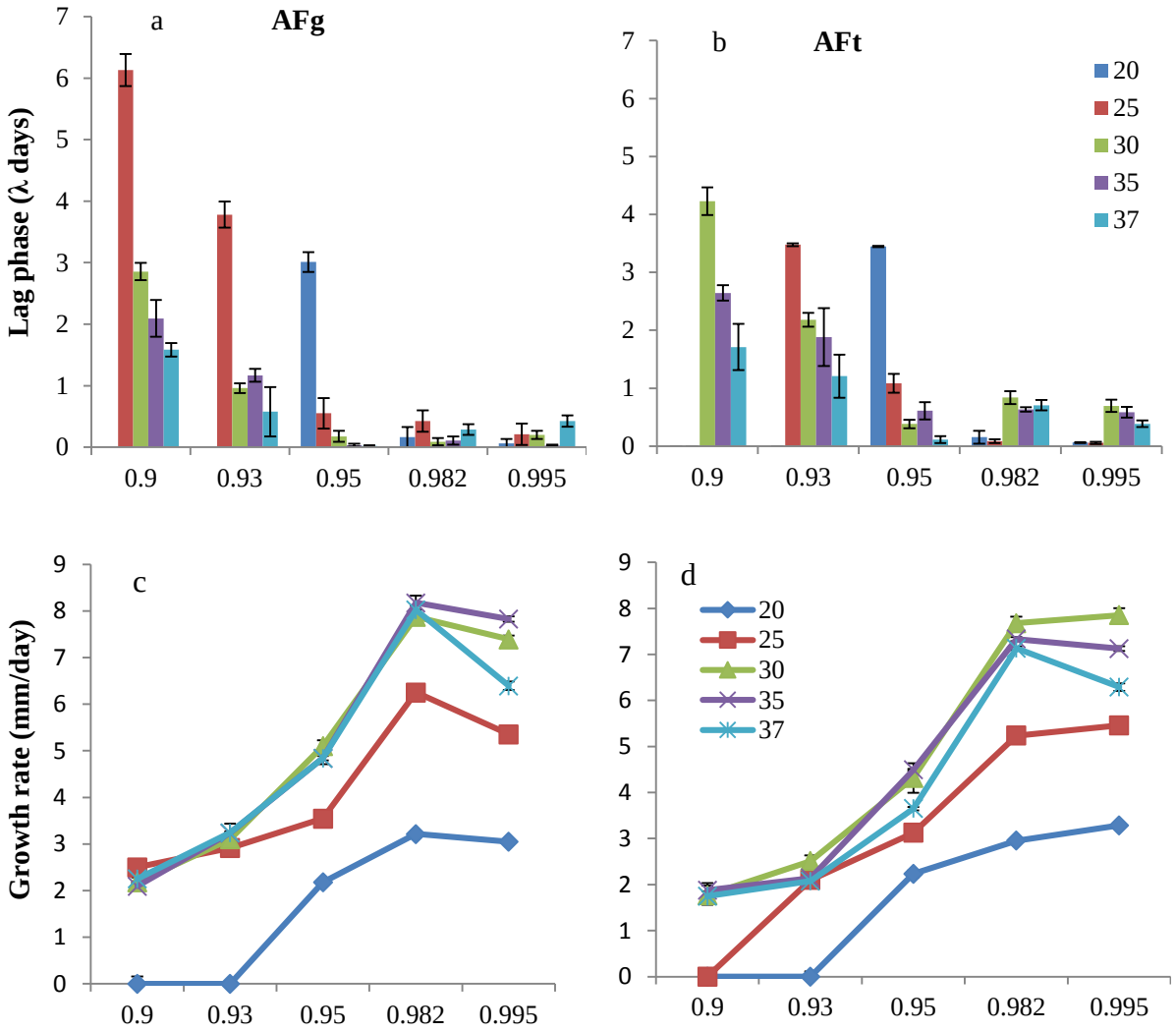
AFt. However, the shortest lag phase was at 0.982 - 0.995 a_w (<1 day) for both isolates. Analysis of variance for lag phase showed that single factors a_w and temperature and the interaction a_w x temperature were significant ($P<0.001$) (Tables 1).

Effect of a_w and temperature on mycelial growth rate

Growth rate of *A. flavus* increased with increasing a_w with maximum mycelial growth at 0.982 – 0.995 and 30° C - 35° C (Fig. 1c & d). Optimum growth rate was recorded at 0.982 a_w in both isolates regardless of the temperature. Isolate AFg was more tolerant of drier conditions than isolate AFt. Specifically, no growth was observed at 0.9 a_w and 20° C and at 0.9 – 0.93 a_w and 20-25° C for AFg and AFt, respectively. ANOVA revealed a significant ($P<0.001$) influence of all single variables assayed (a_w and temperature) as well as their interactions on mycelial growth of *A. flavus* (Table 1).

Effect of a_w and temperature on aflatoxin B_1 (AFB_1) production

AFB_1 production profile between the two aflatoxin-producing isolates were quite different and isolate AFt produced higher concentration of toxin than AFg (Fig. 1e & f). The highest AFB_1 production of 24 - 28 ng/g was obtained at 0.982 and 0.995 a_w at 20° C for AFg and 217 ng/g at 0.95 a_w and 30° C for AFt. In isolate AFg, AFB_1 decreased with increasing temperature only at 0.995 a_w, while AFt produced trace quantities of AFB_1 at 35 - 37° C at all tested a_w levels. Generally, 25° C and 30° C was the optimum temperature AFB_1 production for AFg and AFt, respectively, as AFB_1 was detected at all tested a_w levels at these temperatures. The effect of water activity, temperature and a_w x temperature significantly ($P<0.001$) influenced AFB_1 in both isolates after 10 day of incubation (Table 1).


a
AFg
Lag phase (λ days)
b
AFt
20
25
30
35
37
0.9
0.93
0.95
0.982
0.995
c
d
Growth rate (mm/day)

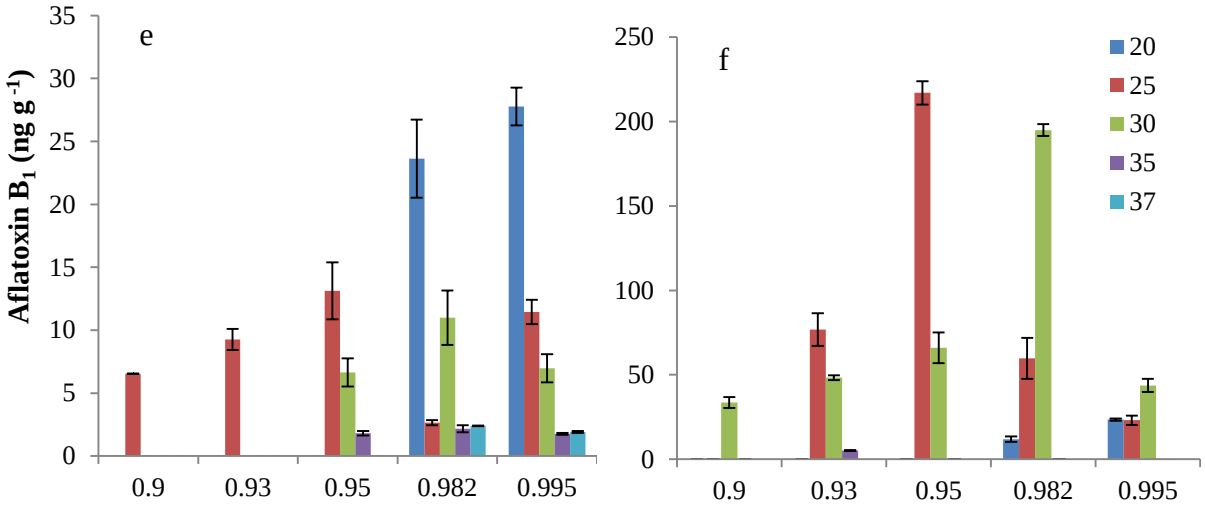


Figure 1: Effect of different temperature and water activities (a_w) on the lag phases prior to growth (a & b), relative growth rates (c & d) and aflatoxin B_1 production (e & f) of two isolates of *A. flavus* (AFg & AFt) on turmeric-based media after 10 days of incubation.

Table 1: P values for the Lag phase (λ, days), growth rate (mm/day) and aflatoxin B_1 (ng/g of agar) production of 2 isolates of *A. flavus* on different water activities and temperature on turmeric-based medium using ANOVA (normality).

Isolate	Water activity (a_w)	Temperature	a_w x tempt	
AFg	P<0.001*	P<0.001*	P<0.001*	Lag phase (λ, days)
	P<0.001*	P<0.001*	P<0.001*	Growth rate(mm/day)
	P<0.001*	P<0.001*	P<0.001*	AFB_1 (ng/g of agar)
AFt	P<0.001*	P<0.001*	P<0.001*	Lag phase(λ, days)
	P<0.001*	P<0.001*	P<0.001*	Growth rate(mm/day)
	P<0.001*	P<0.001*	P<0.001*	AFB_1 (ng/g of agar)

*Significance

DISCUSSION

The current study is one of the pioneer studies to evaluate the influence of water activity and temperature on the growth and AFB_1 production by isolates of *A. flavus* on turmeric-based media at incubation conditions usually found in stores (1). These isolates may grow on turmeric and other spices produced in Africa and can cause AFB_1 accumulation in these food products (Omolola *et al.,* 2018; Uteile *et al.,* 2018). Interestingly, it is very useful to determine the optimal and marginal environmental conditions for growth and AFB_1 production by these isolates in order to effectively propose management strategies to reduce aflatoxin contamination in turmeric and turmeric-based products. The results from the current study showed that in general the isolates of *A. flavus* had similar mycelia growth rate but the amount of AFB_1 production varied significantly with AFt having produced higher toxins than AFg. The difference may not be due to the fact that AFt was originally isolated from infected turmeric, as isolate AFt also produced higher toxins than AFg when inoculated in ginger-based medium (data not shown). Rather it could be attributed to the toxin-producing ability of the isolates. Other workers had reported variation in mycotoxin production between isolates of mycotoxigenic fungi on food products and most have attributed this fact to the nature of the isolates and the ecology (Medina and Magan, 2011; Kokkonen *et al*., 2012). The mycelia growth rate profile for both isolates of *A. flavus* were similar, although AFt had shorter lag phase than AFg, but once growth was initiated, the both isolates grew equally in all growth conditions. Both isolates exhibited similar boundaries for growth in relation to a_w and temperature and range is slightly narrow. Overall, both isolates were not able to grow at 0.85 a_w at all tested temperature over the 10-day incubation period. Both isolates growth rate was optimum at 30-35^o C and 0.982-0.995 a_w. Previous works on the growth of *A. flavus* on ginger and turmeric-based media suggested a_w optimal growth of 0.98 a_w at 28±2^o C (22;26). According to Abdel-Hadi *et al*. (2012), the optimum growth of *A. flavus* was observed at 0.99 a_w and 25-30^o C on maize and conductive YES Medium, respectively. Lahouar *et al*. (2016) obtained maximum growth rate for three *A. flavus* isolates at 25 and 37^o C at 0.97 and 0.99 a_w on sorghum seeds. The optimum water activity for growth in some studies was partly different from the results obtained from the current study. For instance, (24) and (10) found that the optimum growth rate for *A. flavus* was at 0.94 and

0.96 a_w, respectively. This difference has been attributed to regional variability among the isolates. The obtained results add new information about the optimal conditions necessary for growth and inhibition of *A. flavus* on turmeric and turmeric-based products. With regards to AFB_1 production, conditions for AFB_1 by the two isolates of *A. flavus* on turmeric-based medium were optimum at 20-30^o C regardless of tested a_w and minima at 35-37^o C, although the amount of toxin produced varied considerably among isolates. Several workers have shown that the optimal conditions for AFB_1 production by *A. flavus* ranged from 25-30^{o}C and 0.96-99 a_w (8;1;20). However, the difference in the optimal conditions on aflatoxin production by *A. flavus* found in literature has been attributed to the different food products used and the studied fungal isolates (Klich, 2007; Gallo *et al.*, 2016).

CONCLUSION

The profound interacting effects of water activity and temperature on the lag phase prior to growth, growth rate and aflatoxin B_1 (AFB_1) production by the two *A. flavus* isolates on turmeric and turmeric-based media was established in the current study. The obtained optimal conditions for growth (0.982 a_w at 30 – 35^o C) and AFB_1 production (0.95 – 0.982 at 20 – 30^o C) could aid informed decision-making process in relation to temperature and a_w levels in order to minimise AFB_1 contamination during storage. This will aid in the selection of appropriate management measures against AFB_1 contamination in commonly consumed turmeric and turmeric-based products in the future.

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