

## **Influence of Water Activity and Temperature on the Growth and Accumulation of Ochratoxin A Produced by *Aspergillus carbonarius* Isolated from Garri**

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**Abstract:** This study determined the influence of water activity, temperature and their interactions on growth, asexual sporulation and ochratoxin A accumulation in *Aspergillus carbonarius* using standard microbiological techniques. Results obtained shows that at 35°C, OTA was more produced than at other temperatures while both sporulation and mycelial growth were more enhanced at 25°C. A water activity of 0.99 produced the greatest interactions for all except for asexual sporulation that was best at 0.96<sub>a<sub>w</sub></sub>. No growth was seen at water activity range of 0.90 to 0.94 for plates incubated at 40°C. The peak time for ochratoxin A was at 56<sup>th</sup> day with rapid production within the first 28 days while the rate of production between 28 and 56<sup>th</sup> days were relatively slower. When the two factors ( water activity and temperature) were treated as combined variables for regression analysis, it was disclosed that both have a statistical significant relationship with ochratoxin A production and their regression equation were given as  $Y_1 = 31.029 - 29.14a_w - 0.052t$ ,  $Y_2 = -16.881 + 12.12a_w - 0.073t$  and  $Y_3 = -17.217 + 22.02aw - 0.082t$  for concentration of OTA in spores, biomass and medium respectively. Regression determinant confirmed that both water activity and temperature accounted for 60%, 52.6% and 42.6% of the total ochratoxin A content present respectively. This observation was also ascertained by the distribution of the bell shaped regression standardized residual histogram around zero. In conclusion, the results of this study reject the null hypothesis which states that the ability to complete wild type asexual sporulation could be a prerequisite for toxin biosynthesis. Rather, such attribute could be linked to certain extrinsic factors that includes temperature and water potential.

**Key words:** Water Activity • Temperature • OTA • Garri

### **INTRODUCTION**

Filamentous fungi are one of the three groups of fungi belonging to the Kingdom Eumycota. The other two groups are mushrooms and yeast [1,2]. Of these three groups, filamentous fungi comprise of an important class of significant commercial relevance. They are also vital for the maintenance of the ecosystems [3]. Some of these organisms act as plant pathogens causing severe crop losses and post harvest food spoilage [3,4]. Among these filamentous fungi, black *Aspergilli* are the main ochratoxin A (OTA)-producing species encountered on different food matrices in warm countries [4]. OTA has been reported on foods such as cereals, wine, grapes, cocoa, coffee, spices and dried fruits [5,6]. They are nephrotoxic, hepatotoxic, genotoxic, teratogenic and immune toxic to animals and humans and its carcinogenicity is well-established [7]. It is one

of the factors involved in causing Balkan endemic nephropathy and tumours of the human urinary tract [8]. The International Agency for Research on Cancer has classified OTA as a possible carcinogen to humans [9].

Garri is a roasted cassava food product popularly consumed in Nigeria and Africa at large [9,10]. The contamination of cassava food product (garri) [12, 13] with OTA has become a big health problem when these products are consumed by human beings [14, 15]. *Aspergillus* section Nigri and in particular *A. carbonarius*, play a central role in producing OTA in grapes [17-18]. Further reports from various parts of the world have shown that high levels of OTA are produced by different isolates of *A. carbonarius* on a variety of liquid or agar media [19, 20]. Fungal growth is strongly affected by a number of conditions, including water activity ( $a_w$ ) and temperature [21]. These factors are not only important in

fungus growth, but also in metabolite secretion. Interestingly, several researchers found a strong correlation between the growth of OTA producing *Aspergilli* (*A. ochraceus* and *A. niger*) and certain ecological conditions [22, 23]. In recent years, several documentations have been made concerning the influence of both water activity and temperature on ochratoxin A production in several foods [24,25]. However, there is paucity of information regarding the impact of ecological conditions on the growth and toxin production of Nigerian isolates of *A. carbonarius*. Information concerning the factors that cause production of OTA by *A. carbonarius* isolates is essential to develop realistic forecasting systems predicting the risk of colonization by *A. carbonarius* in garri and levels of OTA production. The main objective of the present study was to determine the effect of water activity and temperature on the growth and OTA production by *A. carbonarius* isolated from garri in Ogun State, Nigeria.

## MATERIALS AND METHODS

**Fungal Cultures and Selection of Media:** The strain of *A. carbonarius* used in this study was isolated from garri (a traditional fermented cassava product in Nigeria) and identified based on morphological attributes on plate and ITS sequencing [26]. The ITS sequence of the isolate was blasted for using MEGA version 4 [27] from the National Centre for Biotechnology Information (NCBI) Gen Bank, New York, NY, USA and the accession numbers of the isolate was found to shared 100% attribute with GQ468224. The strain was tested for their capacity to produce OTA on Yeast extract sucrose agar (YES agar, 30 g L<sup>-1</sup> yeast extract, 200 g L<sup>-1</sup> sucrose, 0.5 g L<sup>-1</sup> magnesium sulphate and 20 g L<sup>-1</sup> agar ) (Biotech, United Kingdom).

**Inoculum Preparation:** This was prepared as explained by Spadaro *et al.* [16]. with slight modification. Briefly, the inoculates were prepared by growing each strain on YES at 25°C for 7 days and spore suspensions were prepared by adding 10 mL of sterile distilled water containing 0.05% Tween 80 to each Petri dish and by scratching the colony surface with a sterile spatula. The conidial suspension was filtered through four layers of sterile cheese-cloth and brought to a final concentration of 1×10<sup>6</sup> conidia mL<sup>-1</sup> using a spectrophotometer. YES-agar plates were centrally point inoculated with 10 iL of the spore suspension and

incubated at 25±1°C for 7 days. The experiment was carried out in duplicate.

**Influence of the Studied Extrinsic Factors on Growth and Ochratoxin a Production:** To study the effect of temperature, petri dishes containing YES-agar were centrally inoculated with 10 iL of the spore suspension (1×10<sup>6</sup> conidia mL<sup>-1</sup>) of the strain and incubated at 20, 25, 30 35 and 40°C. The water activity of the YES-agar was modified to a<sub>w</sub> 0.90 to 0.99 by adding glycerol, as reported by Pardo *et al.* [28]. and verified by using the filter paper technique [29]. The plates were inoculated at the temperature indicated (20-40°C). Mycelial growth was determined by measuring colony diameters along two perpendicular axes, 3, 5 and 7 days after the inoculation. Two replications were done for each treatment and the experiment was carried out in duplicate. The treatments were harvested after 9 days incubation with each colony suspended in 10 ml of sterile water containing a wetting agent (Tween 80, 0.1%) to wet the spores [44]. Spores were collected by filtering through sterile glass wool and the filtrate was centrifuged to obtain a spore pellet. The number of spores was determined in defined volume relative to 0.5McFarland standard using a spectrophotometer

**Partitioning and Distribution of Ochratoxin a in Spores, Biomass and Medium:** Other preliminary experiments were made to study the effect of various a<sub>w</sub> levels and temperatures on partitioning of OTA into biomass, medium and conidia. As mentioned above, the spores were removed at the end of the incubation period. The spore pellets, mycelial biomass and medium were weighed. After separation of the spores, biomass and medium, OTA was extracted from spores and biomass by adding 1 ml of 1 mol l<sup>-1</sup> HCl and extracted three times with 3 ml chloroform and evaporated to dryness. The extracts were immediately derivatized [30]. The extracts were filtered directly through Whatman no 1 filter paper into sterile tubes while OTA was quantified by direct competitive ELISA technique using Neogen Veratox kit (Sigma, USA) according to the manufacturers instructions.

**Statistical Analysis:** Statistical tests were performed using Statistical package for social science (SPSS, Inc, 1968-2014, Delaware, Chicago) for three-way ANOVA and LSD Fisher was determined at the 95%

confidence limits for interacting factor ( $a_w \times$  temperature). Multiple regression was used to test the possibility of using water potential and temperature for predicting the level of ochratoxin A in spores, biomass and medium.

### RESULTS

Fig 1 and 2 shows the effect of water potential and temperature on the growth of *Aspergillus carbonarius* grown in YES media. At each temperature, mycelial growth increased from 0.90 to 0.99 $a_w$ . No growth was found at water activity range of 0.90 to 0.95 for the plates incubated at 40°C. Statistical analysis using ANOVA revealed that the effect of temperature, water potential and their interactions significantly influenced growth (Fvalue =7.325,  $p < 0.05$ ). Growth of the studied organism was however found to be best at room temperature followed by at 30°C after which growth rate declined. In other words, temperature of 35 to 40°C significantly slow down proliferation rate ( Fvalue = 4.803,  $p < 0.05$ ) (Table1).

Fig 3, 4 and 5 denotes the effect of temperature, water activity and time of incubation on the production of ochratoxin A in vitro. The maximum ochratoxin A production was obtained at 35°C followed by 25°C while relatively little or no quantity was found at 40°C. In the case of time of incubation, OTA seemed to reached its threshold limit at about 56<sup>th</sup> day of incubation. However, the rate of production between day 28 and 56 was slower compared to day 0 to 28days. For the water activity, OTA production was best at 0.99 for all temperatures. Water activity less than 0.95 produces no OTA (Table 2).

Asexual sporulation was more enhanced at 25°C and  $a_w$  of 0.95 than both 25 and 30°C for temperature and 0.99 for water activity respectively (Table 3). As shown in the Figure 6 and 7 below, the increasing order of importance of water activity to asexual sporulation was as follows; 0.96<0.97<0.98<0.99<0.95 while room temperature supported the highest sporulation density followed by 20°C and then 30°C.

Table 4 shows that both water activity and temperature have a statistical relationship with the level of ochratoxin A when both factors were treated as an independent variables. It appeared that the level of ochratoxin A could conveniently be predicted statistically from the linear relationship with water activity and temperature. The predicting equation is given by

$$Y = \alpha + \beta_1 X_1 + \beta_2 X_2$$

Table 1: Analysis of variance of the effect of water activity and temperature and their interaction on growth rate of *Aspergillus carbonarius*

Effect	DF	MS	F	P
Factor				
Temperature	4	1757	4.803	0.003*
Water activity	9	1509	7.235	0.00*
Temp $\times$ $a_w$	36	92.8	3.121	0.00*

\* $p < 0.05$  (significant)

Table 2: Analysis of variance of the effect of water activity, time and temperature and their interaction on ochratoxin a production

Effect	DF	MS	F	P
Factor				
Temperature	4	21.014	4.750	0.007*
Water activity	4	10.192	300.4	0.00*
Time	7	7.470	2.710	0.025*
Temp $\times$ $a_w \times$ time	112	5.123	54.2	0.001*

Table 3: Analysis of variance of the effect of water activity and temperature and their interaction on asexual sporulation of *Aspergillus carbonarius*

Effect	DF	MS	F	P
Factor				
Temperature	4	$1.7 \times 10^{10}$	6.084	0.002*
Water activity	4	$1.5 \times 10^{10}$	2.49	0.00*
Temp $\times$ $a_w$	16	12564	3.123	0.002*

Table 4: Regression coefficients for predicting OTA concentration in spores

Parameters	Coefficient	SE	t value	Pvalue
Constant( $\alpha$ )	.31.029	6.832	4.542	0.00*
Temperature	-0.052	0.019	2.725	0.012*
Water activity	-29.40	$1.5 \times 10^{10}$	2.49	0.00*
R <sup>2</sup>	0.60			

\* $p < 0.05$  (significant).

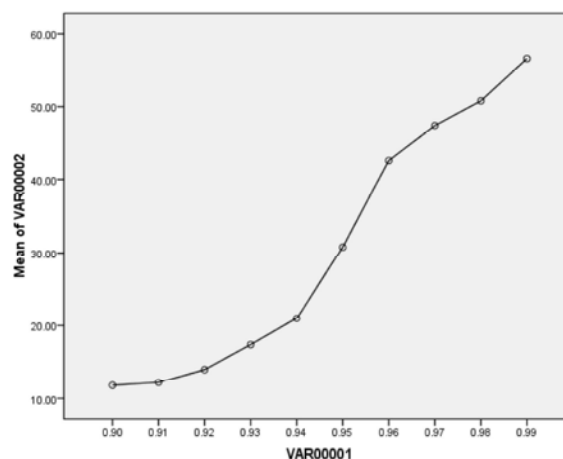


Fig. 1: Effect of water activity on mycelia growth in *Aspergillus carbonarius*

Keys

VAR00001 =  $a_w$

VAR00002 = Growth rate in cm

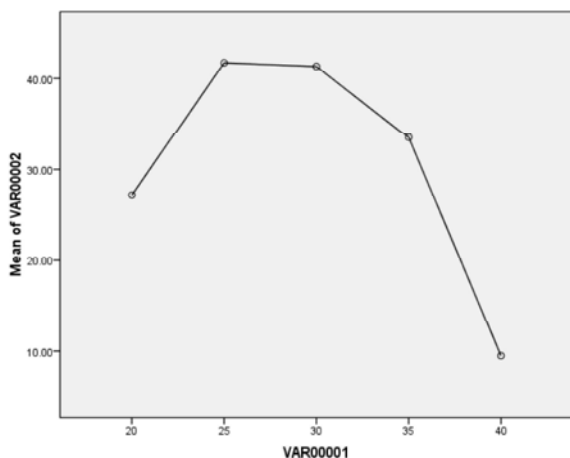


Fig. 2: Effect of temperature on mycelia growth in *Aspergillus carbonarius*

Keys  
 VAR00001 = Temperature in °C  
 VAR000002 = Growth rate in cm

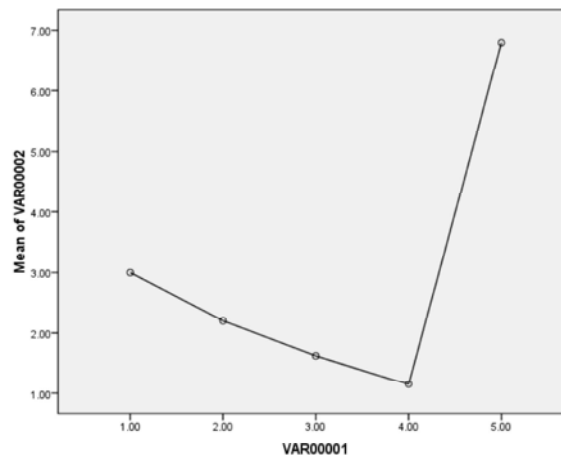


Fig. 3: Effect of  $a_w$  on ochratoxin A production by *Aspergillus carbonarius*

Keys  
 VAR00001 =  $a_w$   
 VAR000002 = Concentration of ochratoxin A in ppb  
 1.00, 2.00, 3.00, 4.00 and 5.00 represents 0.90, 0.91, 0.92, 0.93, 0.94 and 0.95  $a_w$  respectively

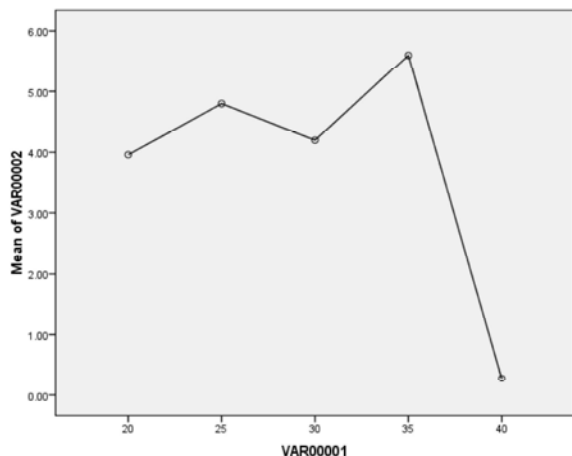


Fig. 3: Effect of temperature on ochratoxin A production by *Aspergillus carbonarius*

Keys  
 VAR00001 = Temperature in °C  
 VAR000002 = Concentration of ochratoxin A in ppb (part per billion)

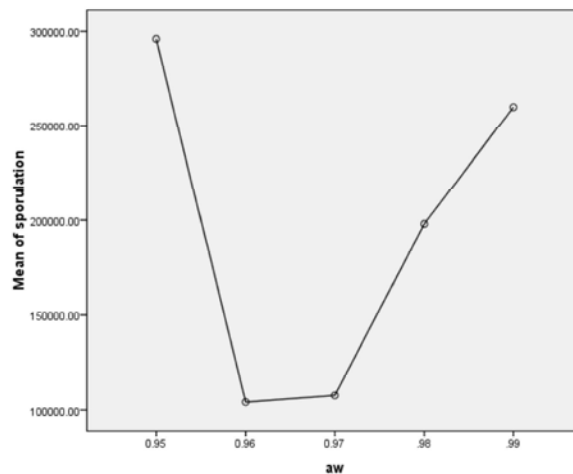


Fig. 3: Effect of time on ochratoxin A production by *Aspergillus carbonarius*

Keys  
 VAR00001 = time (days)  
 VAR000002 = Concentration of ochratoxin A in ppb

where

$\alpha$  = constant,  $\beta_{1=}$  regression coefficient of water potential  $\beta_{2=}$  regression coefficient of temperature,  $X_1 =$  water potential,  $X_2 =$  temperature therefore, level of ochratoxin A in spores can be predicted using the formula

$$Y = 31.029 - 29.14 a_w - 0.052 t \quad (1)$$

If  $a_w = 0.93$  and temperature = 250c, by substituting the values  $Y = 2.43$ . Also regression determinant ( $R^2$ ) shows that the above two factors (water activity and temperature) accounted for 60% of ochratoxin A presence in the spores.

The normality of the model was also buttressed with a regression standardised residual coefficient represented in the histogram below

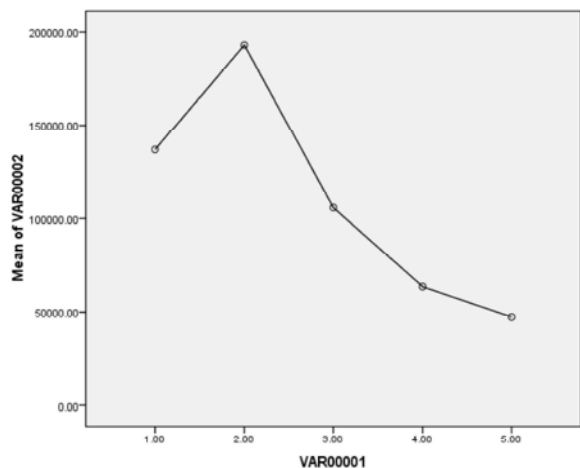


Fig. 3: Effect of temperature on asexual sporulation in *Aspergillus carbonarius*

Keys

VAR00001 = Temperature

VAR00002 = Sporulation density

1.00, 2.00, 3.00, 4.00 and 5.00 represents 20, 25, 30, 35 and 40°C respectively

Table 5: Regression coefficients for predicting OTA concentration in biomass

Parameters	Coefficient	SE	t value	Pvalue
Constant( $\alpha$ )	-16.881	8.311	2.031	0.05*
Temperature	-0.073	0.578	4.144	0.00*
Water activity	21.12	0.018	2.49	0.00*
R <sup>2</sup>	0.526			

Table 6: Regression coefficients for predicting OTA concentration in a medium

Parameters	Coefficient	SE	t value	Pvalue
Constant( $\alpha$ )	-17.217	10.759	1.060	0.024*
Temperature	-0.082	0.022	3.708	0.05*
Water activity	22.04	11.069	1.991	0.001*
R <sup>2</sup>	0.446			

Table 5 and 6 represents the relationship between water activity and temperature in relation to level of ochratoxin A in biomass and medium respectively. The regression equation for both are represented as follows as determined by the regression analysis.

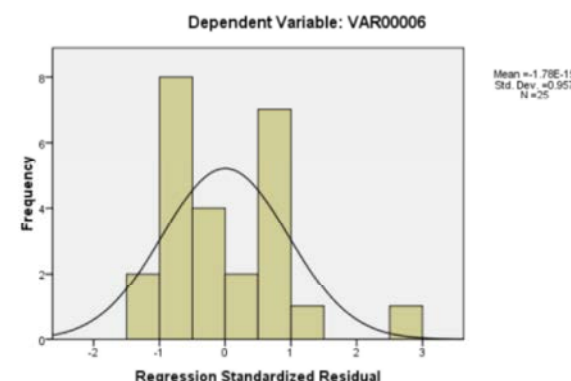
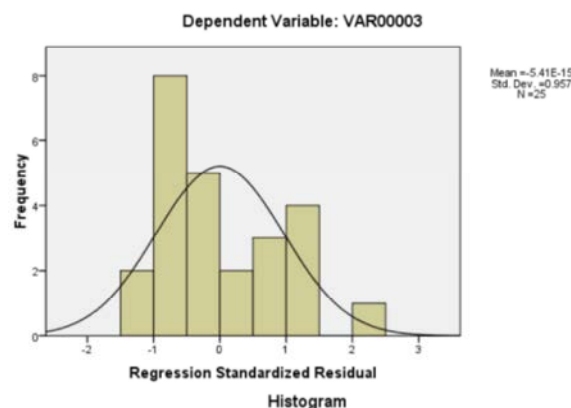
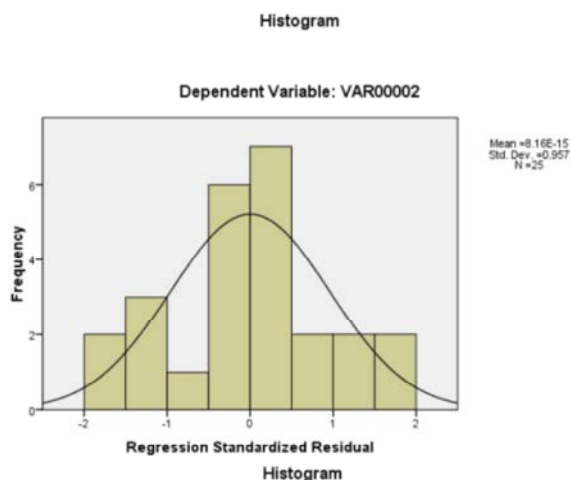
$$\text{Level of ochratoxin a in biomass} = -16.881 + 21.21a_w - 0.073t \quad (2)$$

$$\text{Level of ochratoxin a in medium} = -17.217 + 22.02a_w - 0.082t \quad (3)$$

Where

$a_w$  = water activity and  $t$  = temperature. The regression determinant for both equations shows that both water activity and temperature accounted for 52.6% and 44.6% of the level of ochratoxin A present in the biomass and medium respectively.

The histogram below represents the regression standardised residual for equation 5 and 6



## DISCUSSION

The interactions between water activity and temperature are the most critical determinant of fungal growth and mycotoxin production. Results from this study have shown that the influence of  $a_w$  and temperature on growth rate and OTA was significant. OTA production do not follow the growth rate pattern and was higher at 0.99  $a_w$  at all temperatures and the highest amount was at 35°C with production decreasing as temperature approach 40°C and no production above 40°C. This observation disclosed that the condition for mycelial growth and its asexual sporulation might be more restrictive than those for toxins production and may differ between different mycotoxins produced by the same species and between fungi producing the same mycotoxins [31-33]. O Brian *et al.* [34] reported temperature range of 25-30°C as the best favouring proliferation as well as toxin production in *Aspergillus flavus*. The little variation occurring in the two studies may however be due to differences in the species of organisms used. In another vein, this findings further enhanced that of Sanchis and Magan [33]. who reported that variation may occur in the conditions of growth and toxin production for the same organism or different organism. In this study, the influence of  $a_w$  and temperature on sporulation was shown to be significant. The highest amount of conidia produced was at 0.95  $a_w$  followed by 0.99  $a_w$  and 0.98  $a_w$  at all temperatures examined. Giorni *et al.* [35] found that maximum number of spores by Italian strains of *A. flavus* was produced at 0.96  $a_w$ . Previous studies by Gervais and Molin [36] with *Penicillium roqueforti* strains from cheese grew optimally at 0.97-0.98  $a_w$ , while maximum spore production was at 0.96  $a_w$ . Parra *et al.* [37] showed that highest amount of spores produced by a genetically engineered *Aspergillus niger* strain was at 0.95  $a_w$  when this was modified by glycerol at 35°C. The present study showed that there was no direct relationship between sporulation and OTA production in relation to  $a_w$  and temperature. The highest amount of OTA was at 0.99  $a_w$  at all temperatures and the lowest number of spores was produced at 0.96 $a_w$  at all temperatures. This may be due to the distribution of OTA between spores, biomass and ecology of *Aspergillus carbonarius* in relation to OTA gene cluster expression. Previous studies have demonstrated that *A. carbonarius* spores can contain 60-70% of the ochratoxin A content relative to the biomass [2007]. Reib [39] added different sporulation inhibitors to *A. parasiticus* NRRL 2999 and found that even without any reduction in mycelial growth,

a reduction in sporulation was correlated with AFB1 production. Other observations support the hypothesis that microbial secondary metabolite production and asexual sporulation are intimately associated [40]. Similarly, earlier observations suggested that the ability to complete wild type asexual sporulation could be a prerequisite for ST/AF biosynthesis in the genus *Aspergillus* generally [41]. For  $a_w$ , 0.99 seemed to be the best for all the examined parameters except for sporulation. This results is not surprising as many other studies have reported 0.95 to 0.99  $a_w$  as the optimum range promoting growth and toxin promotion [4,42]. Joosten *et al.* [42] and Kapetanakou *et al.* [4]. The symmetrically belled shaped of the regression standardized residual histogram that is evenly distributed around zero is an indication that the normality of the predicting equations in this study are valid. In conclusion, the outcome of this study nullified the earlier hypothesis that states that the ability to complete wild type asexual sporulation could be a prerequisite for toxin biosynthesis. Rather such attribute could be linked to certain extrinsic factors that includes temperature and water potential.

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