REDUCTION OF AFLATOXIN – B1 IN COCOA BEANS CONTAMINATED WITH ASPERGILLUS FLAVUS BY THE ESSENTIAL OIL OF AFRAMOMUM. DANIELLI USING RESPONSE SURFACE METHODOLOGY

Aroyeun Shamsideen Olusegun[1], Cocoa Research Institute of Nigeria, Nigeria

Adegoke Gabriel Olanrewaju[2], University of Ibadan, Nigeria

Varga Janos[3], University of Szeged, Hungary

Abstract: The factors and each levels used in this experiment included water activity aw (0.94 – 0.98), pH (5-9), Temperature T^{O}C (15- 35^{O}C) and essential oil of *A.danielli* (500ppm – 2500ppm). The effects of each environmental factor on reduction of A *flavus* growth and aflatoxin Bi production were determined by using a 4-factor, 5-level, Central Composite Rotatable Design (CCRD)..All experimental test runs were done in duplicate.The measure of fit of the data (R^2) was quite high for all the dependent variables: . 0.90 for *Aspergillis flavus* growth and 0.83 for aflatoxin B1 production. pH water activity, temperature and essential oil of *Aframomum danielli* affected growth of *Aspergillus flavus* and aflatoxin B1 production. The response surface methodology (RSM) plots had saddle points as stationary points which indicated the absence of unique maximum or minimum. The quadratic effects of temperature and *A.danielli* were highly significant ($p< 0.01$) with minimum *A.flavus* growth between T.^{o}C of 20 – 25^{O}C and *A.danielli* of 1500 ppm. In conclusion, the use of *A.danielli* in this study can form a synergy of barriers with other two or more environmental factors against production of aflatoxin B1 by *Aspergillus flavus.* At every combination of abiotic factors and *A.danielli*, where growth occurred, the level of aflatoxin B1 detected in contaminated cocoa bean. was less than the current regulatory standard of 20ug/kg for aflatoxin Bi in foods meant for human consumption.

Keywords: *Cocoa beans, Response Surface Methodology, A.danielli. A.flavus Reduction, Essential Oil*.

[1]Aroyeun Shamsideen Olusegun, Cocoa Research Institue of Nigeria, PMB, 5244, Ibdan.
Email: aroyeun2000@yahoo.co.uk
[2]Adegoke Gabriel Olanrewaju, Department of Food Technology , University of Ibdan, 200001, Ibadan, Nigeria.
[3]Varga Janos, Department of Microbiology, Faculty of Science,University of Szeged.P.O.Box533, H- 6701, Hungary.

INTRODUCTION

Aspergillus section Flavi has been associated with production of toxic metabolite called aflatoxin B1. Aflatoxin B1 has been classified as one of the most potent carcinogenic toxin of the funigal species *Aspergillus flavus*. Among the aflatoxins , aflatoxin B1 is more prevalent than any other analogue in toxicity and carcinogenicity (IARC, 1993). Recently,, aflatoxin B1 was discovered by Aroyeun *et al,* 2007 in cocoa beans during fermentation process.. He reported that the growth of *Asperillus flavus* in cocoa beans could be attributed to poor fermentation and other factors like pH, temperature and water activities favourable of its growth could have played a significant role. Other *Aspergillus* species identified by Aroyeun *et al*, 2007 are, *Aspergillus tamarii*, and *Aspergillus niger* Since the incidence of aflatoxin B1 in cocoa beans is a threat to lives and business, Aroyeun *et al*, 2009 reported an experiment involving the reduction of Aflatoxin B1 in cocoa beans infected with *Aspergillus flavus* using the spice *Aframomum danielli*. Several other physico chemical methods have been reported to reduce, remove or degrade aflatoxin B1 in contaminated grains. Physical methods include segregation of the contaminated seeds from good seeds (Bailey *et al*, 1998) which is effective but tedious if carried out manually. Efforts are ongoing to produce more effective method to detoxify aflatoxin contaminated food and feeds. Several workers have reported a wide range of chemical, physical and biological routes which had been taken in the attempts to reduce the toxicity of mycotoxins. Although some chemical detoxification methods (i.e ammoniation, sodium bisulphate, and calcium hydroxide treatments are effective, they do not fulfill all the requirements especially those concerning the safety of reaction products and the safeguarding of the nutritional characteristics of the treated foods and feeds (Piva *et al*, 1995). For these reasons, nutritional approach such as supplementation of nutrients or additives with protective properties against toxicity is also assuming increasing interest. Much research has been conducted in the attempt to salvage aflatoxin contaminated food commodities and to avert health risks associated with the toxin (Arpad and Radomir, 1999). Whichever decontamination process to be adopted should meet some basic criteria:

Inactivation or destruction of the toxin by transformation to non-toxic compound

Fungal spores and mycelia should be destroyed so that new toxins are not formed

The food/feed material should remain as nutritive and remain palatable

The physical properties of the food should not change significantly and must be economically feasible.. The use of *A.danielli* in reducing ochratoxin A contaminated cocoa beans has been reported (Aroyeun, 2008). . Apart from the effective OTA reduction by the spice, all the conditions listed above were met. Response surface methodology is a statistical tool involving combination of variables to achieve a desirable objectives called optimization.. Optimization is the method of choice when seeking for best alternatives from a specified sets of alternatives and modern statistical experimental designs are viewed in a way to achieve this purpose at the lowest possible overall cost (Arteaga *et al.*, 1994). In this method, a predictive plot called Response surface plots are drawn to establish the best combinations of factors for the identified desirable output.. Since several factors like pH, water activity and temperature affects the growth of *A.flavus* and production of aflatoxin B1, using the spice *A.danielli* in aflatoxin B1 reduction need to be studied to establish at which combinations of the variable factors and the *A.danielli* will optimize reduction of *Aspergillus* flavu growth and production of aflatoxin B1 in cocoa beans using Response surface methodology (RSM) Montegomary, 1997

MATERIALS AND METHODS

The factors and each level used in this experiment included aw ((0.94-0.98), pH(, 5-9), Temperature (15-35^{O}C) and *Aframomum danielli* concentrations of 500ppm,1000ppm, 1500ppm, 2000ppm and 2500ppm. The values of each environmental factor selected were based on previous studies with *A .flavus* (Ellis et al, 1993). To determine the effects of each factor simultaneously on the growth of *A.flavus* and aflatoxin B1 production. a 4 factor , 5 level central composite rotatable design (CCRD) of Zhou and Regenstein 2004 was used (Table 1). In the CCRD, variable levels were coded -2, -1, 0, +1, +2, as described by Box et al, 1978. The coded and actual values of the levels used in the CCRD are shown in table 1. All experimental tests were run in duplicates

Table I **Values of coded levels used in the Central Composite Rotatable Design for *A. flavus***

Variables		-2	-1	0	1	2
Aw	X_1	0.94	0.95	0.96	0.97	0.98
pH	X_2	5	6	7	8	9
T^{O}C	X_3	15	20	25	30	35
A.danielli (ppm)	X_4	500	1000	1500	2000	2500

Table 2: level combinations for a 4 variable Central Composite Rotatable Design CCRD for *A.flavus* growth and aflatoxin production

	Variables			
Samples	X_1	X_2	X_3	X_4
1	-2	-2	-2	-2
2	-2	-1	-1	-1
3	-2	0	0	0
4	-2	1	1	1
5	-2	2	2	2
6	-1	-2	-2	-2
7	-1	-1	-1	-1
8	-1	0	0	0
9	-1	1	1	1
10	-1	2	2	2
11	0	-2	-2	-2
12	0	-1	-1	-1
13	0	0	0	0
14	0	1	1	1
15	0	2	2	2
16	+1	-2	-2	-2
17	+1	-1	-1	-1
18	+1	0	0	0
19	+1	1	1	1
20	+1	2	2	2
21	+2	-2	-2	-2
22	+2	-1	-1	-1
23	+2	0	0	0
24	+2	1	1	1
25	+2	2	2	2

Preparation of fungal inoculum

Aflatoxigenic *A. flavus* isolates from cocoa beans isolated at some warehouses were used.. The moulds were grown on Czapeck Dox Agar (CDA) and sub cultured onto slants of malt extract agar (MEA) Difco for storage 5^{O}C. Moulds inocula were prepared by growing *A.flavus* on MEA for 7 days at 25^{O}C until sporulation. A spore suspension was prepared by washing the surface of the agar slants with sterile distilled water , followed by filtration through Whatman No 1 filter paper. Spores were concentrated using an improved Neubauer bright line hemocytometer. Appropriate serial dilutions were then made from the stock suspensions using sterile 0.1% peptone water as diluents to obtain the desired inoculum concentrations of 4 x 10^{1} .cells/ml (Teren *et al*, 1996).

Preparations and inoculation of media

Malt extract agar (MEA) was used as the basal medium. The adjustment of the water activity was made by addition of appropriate amounts of glycerol .as described. Media pH was adjusted by the addition of appropriate amount of 1M NaOH in the pre sterilized media and pH measurements was done using a previously calibrated pH meter (model, 240). All plates were (100 x 15mm inoculated with 0.5ml (2 x 10^{1} cells) using a surface plating technique based on the fact that each colony arises from a single cell .For each experimental runs, two inoculated plates and one non-inoculated (control) were examined

Examination and analysis of aflatoxin B1

After 15 days incubation at 25^{O}C, the contents of each plate showing visible mould growth were carefully transferred to 125ml flask after which 40ml of chloroform was added and the mixture taken on a precision water bath shaker (Precision Scientific, Inc., Chicago, U.S.A) for 1 hour. The mixture was then filtered twice using 24cm Whatman No 1 filter paper and the filtrate collected in a 50ml flask. The filtrate was then evaporated to dryness under a gentle streams of Nitrogen. Quantitation of the aflatoxin B1 was done using an enzyme linked immuno sorbent assay (ELISA) kit supplied by Neogen (United State of America) according to

(Teren *et* al, 1996). Calibration curve was drawn and concentrations of afb1 were measured from the curve.

RESULTS AND DISCUSSION

Interactions of temperature, pH, water activity and *Aframomum danielli* in reducing afB1 in contaminated cocoa beans.

A second-order polynomial models was used to express the responses as a function of independent variable which is given as

$$Y=B_0 + B_1X_1 + B_2X_2 + B_3X_3 + B_4X_4 + B_{11}X_{12} + B_{12}X_{12} + B_{13}X_{13} + B_{14}X_{14} B_{22}X_2^2 + B_{23}X_3 + B_{24}X_4 + B_{33}X_3^2 + B_{34}X_4 + B_{44}X_4^2$$

where Y is the dependent variable *Aspergillus flavus* growth, and , aflatoxin B_1,. Xi is the independent variable: Temperature, ^{0}C, pH, water activity and *Aframomom danielli* and B_1 is the model constant.

Model constraints and regression coefficient of the model were determined from multiple regression analysis of the experimental data and the constants and regression coefficients of the model equation are given in table. 1 The measure of fit of the data ($_r^2$) was quite high for all the dependent variable: 0.90 for *Aspergillus flavus* growth, and 0.83 for aflatoxin B_1 production,. pH, aw,, *A. danilelli* growth and temperature affected growth of *Aspergillus flavus*, and aflatoxin B_1 production. significantly at ($p< 0.05$) (table 3) .

The sign of coefficient within each equation show the direction of the effect of each independent variable, the squared products or interactions (Tab 4).

Table 3: Modeling the growth *Aspergillus flavus* in the presence of essential oil of *Aframomum danielli* aw, pH, and temperature.

Samples of Cocoa beans	aw	pH	Tem.p ^{O}C	*A danielli* essential oil	Growth of *A.,flavus*	aflatoxin B1 (pg/g)
1	0.94	5	15	500	-	-
2	0.94	6	20	1000	-	-
3	0.94	7	25	1500	-	-
4	0.94	8	30	2000	2.00	835
5	0.94	9	35	2500	1.60	1050
6	0.95	5	15	500	-	-
7	0.95	6	20	1000	-	-
8	0.95	7	25	1500	-	-
9	0.95	8	30	2000	5.6	620
10	0.95	9	35	2500	3.2	1411
11	0.96	5	15	500	2.4	1364
12	0.96	6	20	1000	1.5	1008
13	0.96	7	25	1500	0.8	800
14	0.96	8	30	2000	0.3	422
15	0.97	9	35	2500	7.4	2023
16	0.97	5	15	500	-	-
17	0.97	6	20	1000	-	-
18	0.97	7	25	1500	-	-
19	0.97	8	30	2000	9.4	2190
20	0.97	9	35	2500	8.5	1740
21	0.98	5	15	500	-	-
22	0.98	6	20	1000	-	-
23	0.98	7	25	1500	-	-
24	0.98	8	30	2000	6.3	1212
25	0.98	9	35	2500	7.2	1452

Table 4 : Parameter estimates for growth of *A.flavus*, and production of, a.flatoxin B_1

Parameter	Growth (*A.flavus*)	AfB_1
Intercept	-138106414	61315055319
X_1	-1121536	-411992338
X_2	69612476	-30609456587
X_3	-111497	18273448
X_4	-138118	61037121
X_1^2	-2863.47	-410029
X_1X_2	282542	103733294
X_2^2	-8771438	3820139279
X_1X_3	-291.13	-205356
X_2X_3	27641	-4612084
X_3^2	59.94	18220
X_1X_4	34811	-15234666
X_3X_4	-56.49	8858.68
X_4^2	-34.53	15191

A.flavus growth) = - $138106414-1121536X_1 + 69612476 X_2 - 1114973X_3-$

$138118X_4-2863.47X_1^2-291.13X_1X_3-562.17X_4 + 34811 X_2X_4-$

$56.49X_3X_4-34.5X_4^2-87711438X_2^2+282542X_1X_2+27641X_2X_3+$

$59.9X_3^2$

$R^2 = 0.90$ p = 0.0003

.. $=61315055319-411992338X_1 30609456587X_2 + 18273448X_3-61037121X_4$

$410029X_1^2 + 103733294X_1X_2 - 205356X_1X_3-4612084X_2X_3 + 18202X_3^2$

$R^2=0.8311$ p = 0.004

Fig. 1 represented by the RSM plots had saddle points as stationary points which indicated the absence of a unique maximum or minimum. This type of response provides an advantage to food processors since a broad range of conditions could be selected to generate a desired minimum growth for *A. flavus*, and production of aflatoxin-B_1.. The quadratic effects of temperature and *A .danielli* were highly significant ($p< 0.01$) with minimum *A. flavus* growth between temperatures of 20-25 ^{0}C and *A danielli* of 1500ppm. Complete inhibition of *Aspergillus flavus* occurred at temperature range of 15 and 25^{0}C. The result obtained in this present study are in agreement with the work of Ellis *et al*, (1993). The activity of *Aframomum danielli* in inhibiting the growth of *A. flavus*, at 25^{0}C has been reported. (Adegoke and Skura, 1994). Holmquist *et al.* (1983) and Karunatne and Bullerman (1990) found that maximal growth of *A. flavus* occurred at 33-35^{0}C and decreased as storage temperature was reduced. Furthermore, *A. flavus* has been reported to grow over a temperature range of 12-43^{0}C (Ayerst 1969 and a pH range of 3.9-9.1 lie and (Lie and Marth (1968). However, it is widely accepted that microorganisms show greatest tolerance to a single environmental factor, such as temperature and pH when water activity and other factors are optimum for growth (ICMSF. 1980). Conversely, the use of *A.danielli* in this study can form a synergy of barriers with other two or more environmental factors against production of aflatoxin B_1 by *A. flavus* At every combination-of abiotic factors and *A.daniellli* where growth occurred, the level of aflatoxin B_1 detected was less than the current regulatory standard of 20ug/kg for aflatoxin B_1 in foods meant for human consumption. The low level of aflatoxin B_1 observed in cocoa beans at 0.96 water activity, pH of 8, temperature of 30^{0}C and 2000ppm essential oil of *A.danielli* can be attributed to the combined inhibitory effect of storage temperature, aw, pH and *A. danielli* on the growth of A flatoxin B1 and aflatoxin production by *A.flavus* In Table 4, only the interaction effects of *A. danielli* were significant.($p< 0.001$) with regards to *A. flavus* growth and aflatoxin-B1 production. Reduction in growth using essential oil *of Aframomum danielli* in cocoa beans contaminated with both *A. flavus* in combination with other abiotic factors as evidenced in this findings has not been reported.

Many authors have used spices to decrease growth and aflatoxin production in both aflatoxin producing fungi (Olojede *et., al.,* 1999). Inhibition of food spoilage, yeast and aflatoxigenic moulds have been achieved by monoterpenes of the spice *A. danielli* (Adegoke *et al.,* 2000). The potential of using same spice essential oil in the control of *A. parasiticus* has also been reported (Atanda *et al.,* 2007) (Basilco and Basilco, 1999) also determined the inhibitory effects of some spice essential oils on *Aspergillus ochraceus* NRRL 3174 growth and ochratoxin A production.

Cinnamon and clove oils, cinanmic aldehyde and Eugenol have also been-used in the inhibition of growth and aflatoxin production (Bullermann, 1977). Similar reports on inhibition of growth and aflatoxin production by *Aspergillus paraisticus* NRRL 2990 has been done with Garcinia kola (family Gutteferae) by Adegoke *et al.,* (1998).

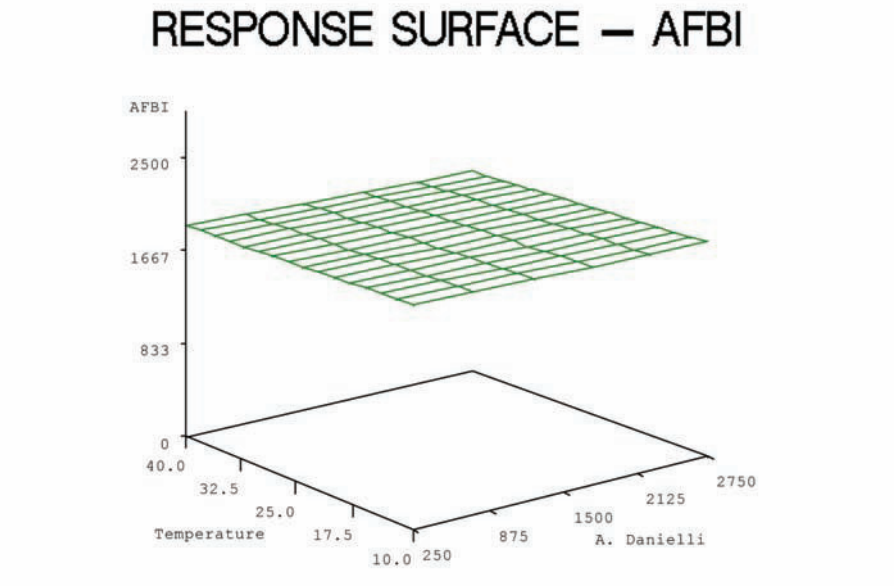


Fig 1. Response surface plots to optimize reduction of aflatoxin B1 at different *A.danielli* and temperature

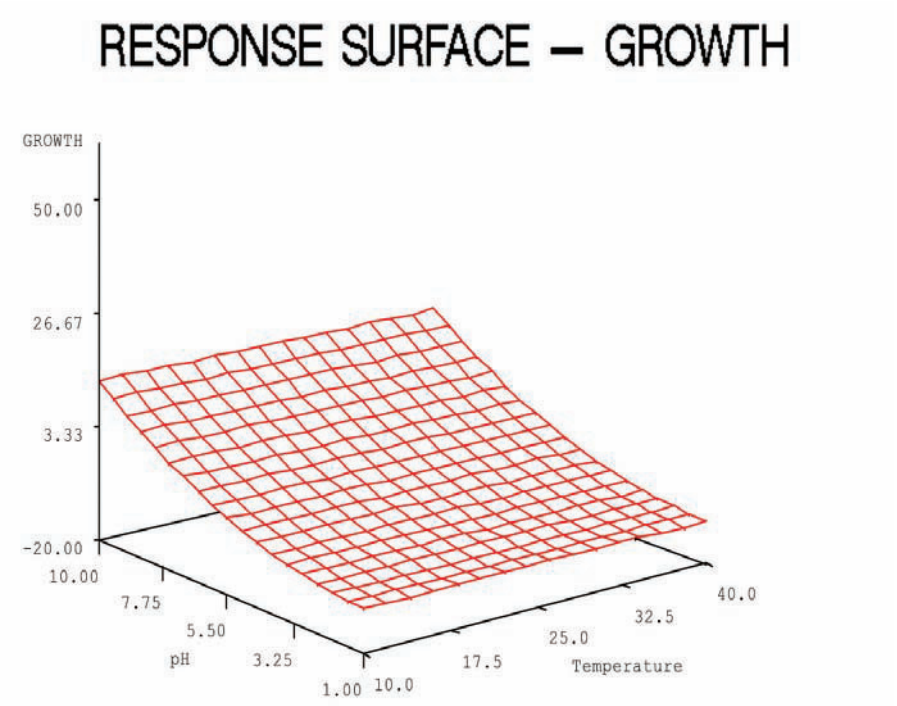
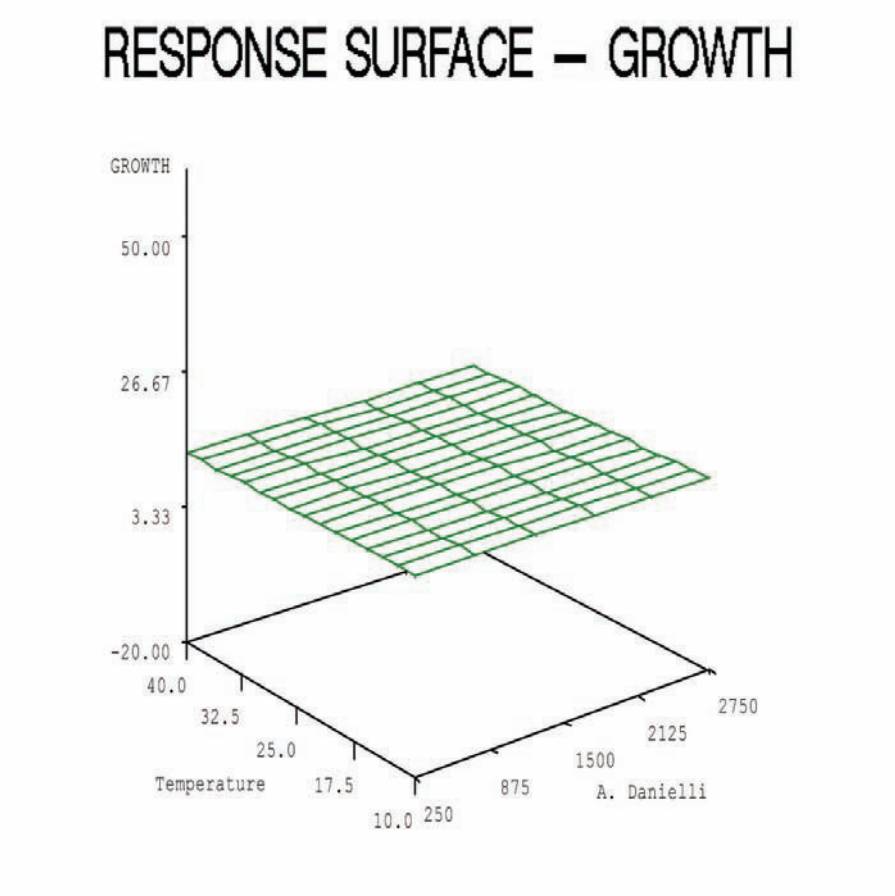
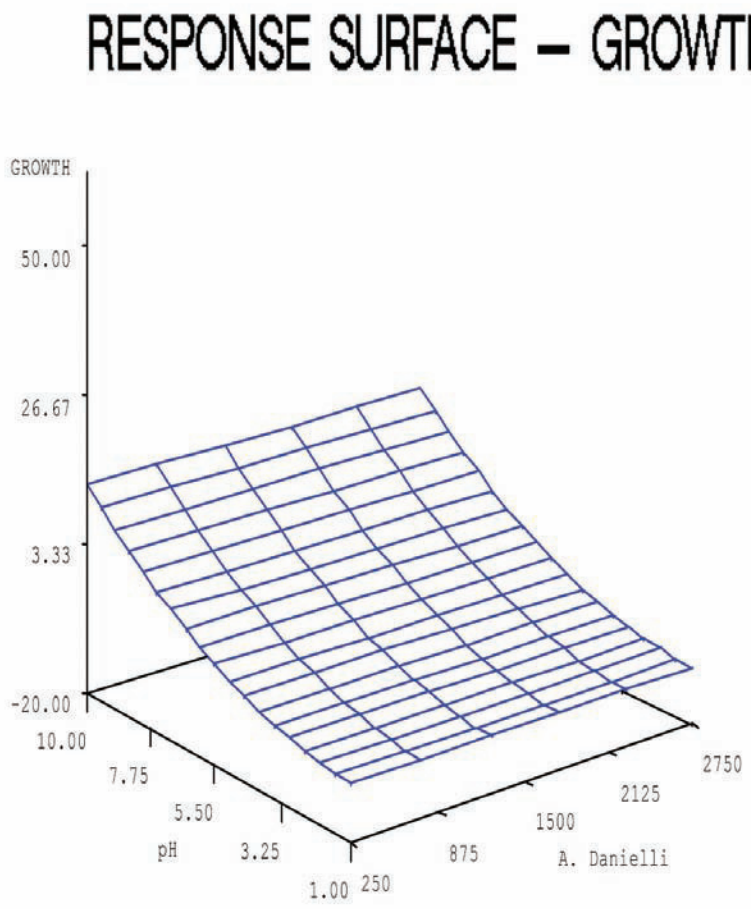


Fig 2: Response surface plots to optimize reduction of A.flavus growth at different temperature and pH


RESPONSE SURFACE – GROWTH
GROWTH
50.00
26.67
3.33
-20.00
40.0
32.5
25.0
17.5
10.0
Temperature
250
875
1500
2125
2750
A. Danielli


RESPONSE SURFACE – GROWTH
GROWTH
50.00
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A. Danielli

CONCLUSION

The use of *A.danielli* in this study can form a synergy of barriers with other two or more environmental factors against production of aflatoxin B1 by *Aspergillus flavus*. At every combination of abiotic factors and *A.danielli*, where growth occurred, the level of aflatoxin B1 detected in contaminated cocoa bean. was less than the current regulatory standard of 20ug/kg for aflatoxin Bi in foods meant for human consumption.

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BIOGRAPHY

Dr Aroyeun is a Chief Research Officer in the Crop Processing and Utilization of the Cocoa Research Institute of Nigeria, Ibadan He is an erudite scholar in the area of processing,. quality control and Microbiology/safety of foods. He is a renown scholar in the fields of mycotoxins, especially in reduction of ochratoxin A in cocoa and cocoa products

Professor Adegoke is a professor of Food Microbiology/Safety of the University of Ibadan and the Head of Department of the Food Technology Department. He has worked widely in the area of inhibition of growths of pathogenic organisms (bacteria and moulds) in foods using natural means such as essential oils of spices like *Aframomum danielli*.

Varga J. is a Research fellow in the Department of Microbiology of the University of Szeged, Hungary. He is a widely traveled scientist and a prominent research fellow in the area of mycoxin research and mycology.

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