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Ochratoxin A Production by Cocoa Infested *Aspergillus* Species in Ondo State, Nigeria

Olukayode Olugbenga Orole[1*]

[1]Department of Microbiology, Federal University Lafia, Nassarawa State, Nigeria.

Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

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ABSTRACT

Aim: To determine ochratoxin A production by cocoa bean seeds infested *Aspergillus* species in Ondo State, Nigeria.
Place and Duration of Study: Cocoa bean seeds were randomly collected from six local government areas namely Idanre, Akure, Owo, Akure, Oba-Akoko, and Ile-Oluji known to be highest cocoa seed producers. Samples were collected for a period of one year duration.
Methodology: Sterilized cocoa seeds were plated on potato Dextrose Agar and Sabourard Dextrose agar after which toxigenic strains were inoculated on Czapek Yeast Extract Agar and the resulting culture homogenized and spotted on TLC plate for viewing. Suspected ochratoxin A producers were inoculated separately on sterile cocoa bean seeds for 21 days after which OTA extraction were done and quantified using HPLC.
Results: Fourteen Aspergilli were isolated and identified. Twelve in Ikpenmen, eleven in Akure while the last seven were isolated from samples collected in Idanre. *Aspergillus alutaceus* were only isolated from Ikpenmen for the 12 months. *Aspergillus ochraceus, A. niger, A. niger* aggregate, *A. carbonarius*, *A. aculeatus, A terreus, A vesicolor* all produced OTA at varying concentration. *A. ochraceus* isolated from Ikpenmen samples produced the highest amount of OTA (372 µg/kg) while samples infested with *A. niger* aggregate from Akure alone produced OTA 109 µg/kg. *A. carbonarius* from all collection venues produced ochratoxin A OTA produced in Oba-Akoko by

**Corresponding author: E-mail: orolekayode@yahoo.com;*

specie *A. carbonarius* had the highest value of 539 µg/kg culture.
Conclusion: The study showed that *Aspergillus ochraceus, A. niger, A. niger* aggregate, *A. carbonarius*, *A. aculeatus, A terreus, A vesicolor* were the major OTA producers contaminating stored cocoa produce in Ondo State, Nigeria.

Keywords: Aspergillus; species; toxigenic; TLC; HPLC; concentration; contamination.

1. INTRODUCTION

Ochratoxins are metabolic products of some *Aspergillus, Penicillium*, and *Fusarium* genera found in tropics and the temperate regions of the world [1]. Ochratoxin A (OTA), ochratoxin B (OTB), and ochratoxin C (OTC) are natural fungal products while OTα, OTβ, and their other metabolites are not. The Ochratoxins are phenylalanine derivatives of a dihydroisocumarin nucleus [2] and are weak organic acids. Ochratoxin A has two ionizable groups, the carboxyl group from phenylalanine and the 8-hydroxyl group [3].

OTA was classified in-group 2B as possibly carcinogenic to human [4,5] while, the National Toxicology Program (NTP) classified it as "reasonably anticipated to be a human carcinogen" [6]. COT, [7] declared OTA a genotoxic carcinogen. It is immunosuppressive, teratogenic and mutagenic. WHO and FAO set provisional maximum intake of 100 ng/kg body weight while Scientific Committee on food of EU set 5 ng/kg body weight/day [8].

OTA, $C_{20}H_{18}ClNO_6$ (molecular weight: 403.82 daltons) is produced during fungal growth and development. *Aspergilli* reportedly implicated are *A. alutaceus, A. ostianus, A. quercins,* and *A. sulphureus, A. ochraceus* among other. Among the *Pennicillium* species are *Penicillium verrucosum* and *P. nordicum* [9]. Three strains of *P. verrucosum* have been identified, *P. verrucosum* chemotype I, *P. verrucosum* chemotype II, and Cereal-borne *P. verrucosum* chemotype III found in Denmark, Sweden, Norway, United Kingdom, Canada, and the United States [10,3].

OTA is reportedly found in coffee bean, animal products, cereals, cocoa bean, pulses, dried fruits, wines, beans, spices, nuts, processed food, olives and figs [11-14]. *A. carbonarius* and *A. niger* aggregate have been reported to cause OTA infestation of cocoa beans in African countries and in South America [15-20].

Field and storage fungal contaminants of grains in Nigeria had previously been reported by Adejumo et al. [21]. Fungal deterioration of seeds, grains and feed stuff is a chronic problem in the developing countries field and storage system as most of them are in tropical hot and humid climate. The presence and growth of fungi may cause spoilage of food and its quality and quantity [21]. Adejumo et al. [22], Dongo et al. [23], and Makun et al. [24] all gave reports of Nigerian agricultural crops and products infestation with spoilage fungi and subsequent production and contamination with ochratoxin A and other mycotoxins such as aflatoxin, fumonisins, and zearelanone.

OTA LD_{50} varies among species, and influenced by the feeding method, the solvents, presence of other mycotoxins and the composition of the diet. Ochratoxin A binds the plasma protein making it easily absorbed by the upper GIT depending on the animal species [25,26]. The binding covalent bond between OTA and plasma proteins limits its transfer and distribution thus increasing its half life within the cells [27]. Elimination and removal of OTA and other metabolites from the body occurs slowly through the biliary and renal excretion [28-30].

2. MATERIALS AND METHODS

2.1 Isolation and Identification of Aspergilli

Isolation of fungi was done using the modified methods of Makun et al. [13]. Air dried cocoa beans were surface sterilized by immersing in 70% ethanol for a minute, and subsequently in 50% NaOCl solution for 3 minutes after which the seeds were plated on Potato Dextrose Agar (PDA) and Sabourard Dextrose Agar (SDA). The fungal species were characterized based on their morphological and cultural characters using taxonomic guides and standard procedures according to Pitt [31], Kozakiewicz [32], Klich [33], and Matasyoh et al. [34].

2.2 Identification of Suspected Ochratoxigenic Fungi

The modified methods of Adetunji et al. [35] and Chilaka et al. [36] were used to access ochratoxin A production. Plates containing prepared Czapek Yeast Extract Agar (CYA) already inoculated with 6 mm diameter of 7 day old colony of isolated fungi and incubated for 7 days at 28℃ were used. Ten (10 g) of the culture medium was gently removed from the Petri dish and added to a vial containing 20 ml of dichloromethane for ochratoxin A extraction. These were homogenized, filtered through Whatman No. 1 filter paper and spotted on a two-dimensional Thin Layer Chromatographic (TLC) plate.

2.3 Ochratoxin A Production by Toxigenic Isolates

The modified methods of Kumar et al. [37] were used in OTA production. Fifty (50 g) dried cocoa bean seeds were cleaned and washed then soaked in 50 ml distilled water and sterilized at 121℃ 15 psi for 20 min. The sterile seeds were inoculated with pure culture of toxigenic fungal spores ($1x10^6$), and allowed to go through fermentation at 27℃ for 21 days.

2.4 Extraction and Quantification of Ochratoxin A from Fungi

Extraction and subsequent quantification of toxin was done utilizing the modified methods of Yazdani et al. [38] and Adetunji et al. [35]. Ten grams (10 g) culture medium was scrapped into test tube (32 x 200 mm) containing 20 mL chloroform: acetone (85:15 v/v) for ochratoxin A extraction. The suspension was incubated at room temperature (25℃) for 15 - 20 min and agitated every 5 min using a vortex stirrer. The extract was filtered through Whatman no.1 filter paper and the filtrate evaporated to dryness under 40℃ in an air circulated oven dryer. The residues were resuspended in 500 µL of methanol and aseptically filtered using a 0.2 µm syringe filter and quantified using HPLC.

A Shimadzu HPLC set-up was used for quantification of ochratoxin A. The mobile phase is composed of acetonitrile, water, and acetic acid (48:51:1). The HPLC apparatus was fitted with a fluorescent detector, a C 18 column 3.9 x 150 mm, 5 µm. The injection system is a Rheodyne 7125 with 100 µL loop. The HPLC pump was set at 1 mLmin-1 flow rate. The excitation and emission wavelength of the scanning fluorescent detector were set at 333 and 470 nm.

2.5 Statistical Analysis

The results were expressed as means and Standard Error of Means (SEM). Analysis of Variance was obtained and the means were separated using Tukey's Kramer post hoc test at $p \leq 0.05$.

3. RESULTS

3.1 Isolated Aspergilli

Fourteen *Aspergillus* species were isolated from cocoa samples collected in Ondo State as shown in Table 1. *Aspergillus niger, A. fumigatus, A. flavus, A versicolor, A. ochraceous,* and *A. carbonarius* were found in all the collection centers while *A. alutaceus* were found in Ikpenmen Owo only.

3.2 Ochratoxin A Producers

Aspergillus ochraceus, A. niger, A. niger aggregate, *A. carbonarius* , *A. aculeatus, A terreus, A vesicolor* and *A. flavus* of the forteen Aspergilli species isolated from the cocoa beans gave evidences of ochratoxin A production. The isolates all showed bluish green fluorescence light under TLC at 360 nm (Table 2). Of the toxigenic isolates, *Aspergillus ochraceus, A. niger,* and *A. carbonarius* were the only species present in all the collection venue.

3.3 Ochratoxin A Production by Aspergilli

Aspergilli ochratoxin A production were not according to the location where the fungi were found. *A. ochraceus* found in cocoa beans from Ikpenmen town produced the highest amount of OTA (372 µg/kg) while sample infested with *A. niger* aggregate from Akure alone produced OTA 109 µg/kg which were not produced by *A. ochraceus* found in Ondo, Idanre and Akure. *A. carbonarius* collected from all the sample collection areas possess the capacity to produce OTA as shown in Table 3. The limit of detection (LOD) of ochratoxin A is less than 0.01 µg/kg culture while the limit of quantification (LOQ) is less than 0.10 µg/kg culture.

Table 1. Isolated fungi at different collection location

S/N	Isolated fungi	Ondo	Idanre	Akure	Ile-Oluji	Ikpenmen	Oba-Akoko
1	*Aspergillus flavus*	+	+	+	+	+	+
2	*A. fumigatus*	+	+	+	+	+	+
3	*A. nidulans*	+	-	+	+	+	+
4	*A. niger*	+	+	+	+	+	+
5	*A. glaucus*	-	-	+	+	+	+
6	*A niger* aggregate	+	-	+	-	+	+
7	*A. versicolor*	+	+	+	+	+	+
8	*A. terreus*	-	-	+	+	+	-
9	*A. aculeatus*	+	-	+	+	-	-
10	*A. carbonarius*	+	+	+	+	+	+
11	*A. alutaceus*	-	-	-	-	+	-
12	*A. granulosus*	-	-	-	-	+	-
13	*A. ustus*	-	+	-	+	-	-
14	*A. ochraceous*	+	+	+	+	+	+

Key: + shows fungi isolated at the location, - shows it is absent

Table 2. Ochratoxigenic Isolates

S/N	Suspected OTA producer	Coloration
1	*Aspergillus ochraceus*	+
2	*Aspergillus niger*	+
3	*Aspergillus niger* aggregate	+
4	*Aspergillus carbonarius*	+
5	*Aspergillus terreus*	+

+Key: + means it's present and – means it's absent

Aspergillus carbonarius from all the sample collection venue were able to produce ochratoxin A while *A. niger* aggregate specie that produced the toxin were those collected from Akure. OTA produced in Oba-Akoko by specie *A. carbonarius* had the highest value of 539 µg/kg culture.

4. DISCUSSION

Results of ochratoxin A producers showed *A. niger, A. ochraceous*, and *A. carbonarius* were the main toxin producers in Ondo State while *A. niger* aggregate and *A. terreus* produces insignificant quantities of OTA. The results were in agreement with the results of Leong et al. [39] and Amezqueta et al. [15] that *A. carbonarius* produces OTA in grape and Mounjouenpou *et al*. [18] that *Aspergillus niger* produces OTA in cocoa beans. *A carbonarius* from the study showed potency to produce reasonable amount of OTA but this was against the report of Amezqueta et al. [15] that the fungi produces negligible content in cocoa.

The result of the study agrees with Mounjouenpou et al. [40] that *Aspergillus carbonarius* and *A. niger* produces ochratoxin A though the former produces more toxin than other OTA producers. The author added that *A. niger* aggregate isolated along the other Aspergilli in cocoa collected from Cameroun is another OTA producer. In addition, the report of the study also showed that *A. terreus, A. niger* and *A. carbonarius* produced OTA in varying quantity which was collaborated by the work of Mounjouenpou et al. [18]. The report of Rizzo et al. [41] that *A. versicolor* and *A. fumigatus* are OTA producers contrasted our result as these fungi species were not found to produce the toxin.

Production of ochratoxin A by fungi had been attributed to a lot of factors among which are temperature, water activity, substrate composition, pH and competing agents in the environment [42-45]. Cocoa beans from the report of Mounjouenpou et al. [18] may not

Table 3. Amount of OTA produced (µg/kg) by toxigenic fungi from each location in Ondo State

***Aspergillus* species**	**Ondo**	**Idanre**	**Akure**	**Ile-Oluji**	**Ikpenmen**	**Oba-Akoko**
A. ochraceus	<LOQ	<LOQ	<LOQ	87±6.3	372±8.6	217±3.8
A. niger	300±11.6	173±8.1	<LOQ	<LOD	73±4.1	216±5.7
A. niger aggregate	<LOQ	<LOD	109±4.2	<LOQ	<LOQ	<LOQ
A. carbonarius	274±7.9	253±6.6	203±7.7	91±4.9	217±5.3	539±9.4
A. terreus	32±4.8	72±2.8	<LOQ	<LOQ	<LOQ	<LOQ

Key: - <LOD=0.01 µg/kg culture; <LOQ=0.10 µg/kg culture

be a very good medium for ochratoxin A production *in-vitro.* The author compared rice and cocoa beans as media and after 20 days incubation with *Aspergillus carbonarius* inoculum, fungi grown on rice produced 2,772 ng/g OTA while from cocoa bean were produced, 84.5 ng/g OTA. This may be responsible for the low quantity of ochratoxin A produced by the fungi *in-vitro.*

5. CONCLUSION

The study showed that higher than tolerable values were recorded in the towns where samples were collected and analyzed for ochratoxin A presence and concentration. This point to the need for improved post-harvest management practices by cocoa farmers, and government intervention through continued advocacy and enlightenment programmes for the farmers in Ondo State.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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