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Temperature and pH Factor on Cocoa Bean-Based Ochratoxin-A (OTA) and Detoxification Potential of *Rhizopus stolonifer*

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Authors' contributions

This work was carried out in collaboration among all authors. Author AOA conceived and design the study, wrote the first draft of the manuscript and interpreted the data. Author ABF managed the literature searches, laboratory activities and processes. Author DOA design the study, performed the statistical analysis and wrote the protocol. Authors AOA and DOA managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To study the effect of temperature and pH on detoxification potential of *Rhizopus stolonifer* on Ochratoxin-A (OTA) from cocoa beans in Nigeria.

Place and Duration of the Study: This study was carried out in the Plant Pathology laboratory of Cocoa Research Institute of Nigeria (CRIN) for the period 2013-2017.

Methodology: Stored cocoa samples were collected from Cross-River, Ondo and Osun States in Nigeria, assayed on Potato Dextrose Agar. The pure culture of the isolate of *Rhizopus stolonifer* used as a detoxifying agent was obtained from CRIN Mycobank. *Aspergillus ochraceus, A. niger* and *Penicilium verrucossum* cultured from cocoa bean samples were used in the study. These ochratoxigenic fungi were cultured on Yeast Extract Sucrose (YES) agar in the dark for 14 days for detection and detoxification of toxin. Ochratoxin-A (OTA) productions were detected using retention factor (RF) on simple Thin Layer Chromatography (TLC) plate of solvent system Toluene: Ethyl

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acetate: Formic acid (5:4;1 v/v/v) while confirmation of OTA was done using 365nm wavelength of ultraviolet (uv) light.
Results: Five fungi genera: *Aspergillus, Penicilliun, Fusarium, Yeast* and *Rhizopus* were cultured from cocoa beans sample across the study locations*:* The tested temperature and pH indicated that *Aspergillus ochraceus* and *Penicillium verrucosum* had retention factor (Rf) value of 0.88 to 1.29 which showed green-blue colouration under Ultra Violet (UV) light confirming the presence of Ochratoxin-A (OTA) while *A.niger* isolate had RF of 0.88-0.55 with yellow to olive yellow colour confirming the absence of OTA. *Rhizopus stolonifer* isolate detoxify OTA when cultured with *A. ochraceus* and *P. verucossum* at pH 7.0 and 9.0 and 25°C-30°C with yellow to brownish-yellow colouration confirmed as a less toxic metabolites (Citreoviridin, Palutin and Palitantin) while no toxin was detected in *A. niger* plus *R. stolonifer* after detoxification.
Conclusion: There is variability in retention factor as temperature and pH changes. Production of OTA occurred between 25-30°C and pH of 4.0 to 9.0 but peaked at pH 4.0 with Rf 1.3 at neutral pH and 25°C enhanced detoxification potential of *R. stolonifer.* Thus *R.* s*tolonifer* can be developed to detoxify OTA from Cocoa beans at favourable eco-physiological factor.

Keywords: Ochratoxin-A; Cocoa beans; Temperature; pH; Detoxification; Rhizopus stolonifer.

1. INTRODUCTION

Mycotoxins are fungal metabolites commonly occurring in food, which pose a health risk to the consumer [1]. Ochratoxin-A (OTA) is a secondary fungal metabolite present in a wide variety of fruits [2]. An estimated 500 million of the poorest people in sub-Saharan Africa, Latin America and Asia are exposed to mycotoxin in staple food in the level above international standard, which resulted in increases in morbidity and mortality [3]. Over 25% of the world's agricultural commodities are estimated to be contaminated with mycotoxins to a certain degree [4]. Ochratoxin-A metabolic product of filamentous fungi; Aspergillus and Penicillium is known to be a potent toxin having hepatotoxic, carcinogenic, nephrotoxic and immuno-reducing properties [2,5]. Cocoa is an important crop, the raw material from which chocolate is manufactured, grown mainly in West Africa with significant quantity from Asia and Central and South America [6]. In the last decade, concern has increased about human exposure to OTA, a possible carcinogen to humans [7] found in cocoa beans, and consequently the interest in the study. Cocoa beans can become contaminated by these filamentous fungi during pre-processing at the farm, especially during drying and storage [8].

Fungal growth and metabolic secretion is strongly affected by a number of conditions, including pH [9], and temperature. The harmful effect of mycotoxin-producing fungi in food can be avoided by preventing contamination and/ removing contaminant from food [10]. Detoxification of mycotoxin is achieved by the removal of the contaminant or by inactivation of mycotoxin in these commodities [10,11]. There has been increasing interest in the use of microorganism for detoxification or decontamination of commodities due to their benefits.

Hence the aim is to study the effect of temperature and pH on the detoxification potential of *R. stolonifer* on OTA detected from stored cocoa beans

2. MATERIALS AND METHODS

2.1 Sourcing for Cocoa Beans Samples

Twenty-seven cocoa beans samples collected for the study were the dried and ready for sale in farmer's stores and warehouses in Cross-River, Ondo and Osun States, Nigeria. The beans were collected and kept in an airtight bag for laboratory assay.

2.2 Isolation of Ochratoxigenic Fungi from Stored Cocoa Beans

The cocoa beans samples were randomly selected and dissected into smaller pieces; surface sterilized with 10% sodium hypochlorite (NaOCl), rinsed in 3 changes of sterile distilled water and blotted dry by sandwich in between sterile filter paper before inoculation into the culture media. Potato Dextrose Agar (PDA) used as a medium for the isolation of the mycoflora of the samples was routinely prepared in the laboratory. The medium cooled to 45°C, acidified with 10% lactic acid after sterilization and poured into sterile Petridish to set.

The PDA plates were inoculated directly with the sterilized cocoa bean samples using the method of Pitt [12] and incubated at 28+2°C for 7days. The growth of colonies was observed and counted on the 7th day after which pure cultures were obtained. Fungi isolates were identified using identification manual by Singh et al. [13] and identification keys for fungi by Bannet and Hunter [14] and Raper and Fennell [15].

2.3 Source of *Rhizopus stolonifer* (Detoxifying Agent)

A pure culture of local isolate of *R. stolonifer* used as detoxifying agent was obtained from the Mycology laboratory of Cocoa Research Institute of Nigeria (CRIN) Ibadan.

2.4 Production of Ochratoxin-A from the Ochratoxigenic Fungi Isolates

Agar plate method was used to detect and detoxify OTA isolated from three ochratoxigenic fungi (*A. ochraceus, A. niger* and *P. verrucosum*) isolated for the study using the method of Masoud and Kaltoft [16]. Yeast Extract Sucrose (YES) agar was used as medium [13] and 20 g of Yeast extract, 150 g of sucrose, 0.5 g of Magnesium sulphate and 1 ml of trace element ($CuSO_4.H_2O$) were dissolved in 1 liter of sterile water. The media was homogenized and the pH varied to 4.0, 7.0 and 9.0 using 1N of either HCl or NaOH. Sterilization of media was done at 121°C for 15min in an autoclave. Cooled to 45°C and dispensed into sterile plates to set. A 7-day old culture of *A. ochraceus, A. niger* and *P. verrucosum* were inoculated into the YES agar and incubated in the dark at 25°C and 30°C for 14 days [17].

2.5 Detoxification of Ochratoxin-A Using *Rhizopus stolonifer*

The YES agar plates were inoculated with 5mm mycelia disc each of 7-day old cultures of *A. ochraceus, A. niger* and *P. verrucosum* along with the detoxify agent (*R. stolonifer*) and incubated in the dark at 25°C and 30°C for 14 days as carried out for OTA production.

2.6 Detection of Ochratoxin-A

Agar plugs were obtained using sterile cork borer to cut mycelia disc from the cultures after incubation [13]. The mycelia disc obtained was removed with inoculating needle and flooded with 2ml chloroform: methanol (2:1, v/v) and placed mycelia down on a Thin Layer Chromatography (TLC) plate [16]. The TLC plates with the spotted mycelia disc were allowed to dry in cold air and the plates were developed in solvent system of Toluene: Ethyl acetate: Formic acid (5:4:1, v/v/v) for 45 minutes in the dark [18]. The migration was then interrupted when the solvent reached 10 cm mark on the plate and air dried. The plates were observed in the dark and under 365 nm ultra violet light [19,17,13,20].

3. RESULTS AND DISCUSSION

3.1 Microbiological Analysis of Cocoa Beans Samples

Five fungi genera; *Aspergillus, Penicillium, Fusarium, Yeast* and *Rhizopus* were isolated from Cross-River, Ondo and Osun States in Nigeria. All the five genera were cultured from Cross-River and Ondo while three genera were isolated from Osun State. Yeast was the major fungi present in the cocoa samples across all sample locations, *R. stolonifer* was found in samples containing no ochratoxigenic fungi. *Aspergillus sp* including *A. niger, A. flavus, A. wentii, A. ochraceus* and *A. fumigatus* were among the fungi isolated from the samples, likewise *Penicillium sp* and *Fusarium sp.* (Table 1).

3.2 Detected Toxins

Aspergillus ochraceus, A. niger and *P. verrucosum* were the three ochratoxigenic fungi selected for OTA production and detoxification in the study. These fungi were tested for their ability to produce OTA at pH 4.0, 7.0 and 9.0 under 25°C and 30°C using YES agar at 14 days incubation under 12 hourly dark and light environment. *Aspergillus ochraceus* and *P. verrucossum* isolates produced OTA in all the tested temperature and pH at varied retention factors (Rf). *Aspergillus ochraceus* isolates had between 1.10 and 1.29 as Rf while *P. verucossum* had between 0.88 and 1.20 as the Rf. All of which have a blue green color under 365nm ultraviolent light which confirm the presence of OTA (Table 2).

Aspergillus niger isolates had Rf between 0.5 and 0.88 with a yellow to olive yellow color under 365 nm ultraviolent light which is a pointer to absence of OTA but presence of other mycotoxins (Table 2).

The detoxification potential of local isolate of *R. stolonifer* was tested at different pH and

temperature on fungi isolates. The results showed that the Rf of *A. ochraceus* plus *R. stolonifer* was at highest at pH 4 and 30°C with 1.03. *P. verrucossum* plus *R. stolonifer* had peak Rf of 0.97 at pH 4 at 25°C with no toxin detected at pH 7 at 25°C. *Aspergillus niger* plus *R. stolonifer* had Rf of 0.57 at pH 7 alone. Colour appearance of detoxified toxin under the 365nm UV light for *A. ochraceus* plus *R. stolonifer* at pH 4.0, 25°C and pH 7.0, 30°C (Table 3) with yellow to yellowish brown and also for *P. verucossum* at pH4 and pH7 at 30°C. No toxin was detected for *A. niger* plus *R. stolonifer* in all the tested pH and temperature.

In Table 4, the mycotoxins detected from *A. ochraceus, A. niger* and *P. verrucossum* include OTA found in *A. ochraceus*, and *P. verucossum* while Citreoviridin and Kojic acid was found in *A. niger* confirmed by the Rf and blue green fluorescence under the Ultra Violet light.

Table 1. Fungi isolated from cocoa bean samples

Sample location	***Yeast***	***Aspergillus niger***	***A. flavus***	***A. wentii***	***A. fumigatus***	***A. nidulans***	***A. ochraceus***	***P. verrucosum***	**Fusarium sp**	***Rhizopus sp***
Cross river	+	+	+	-	-	+	+	+	+	+
Ondo	+	-	+	-	-	-	+	+	+	+
Osun	+	-	+	+	+	-	-	+	-	-
% Occurrence	100	16.7	91.7	58.3	41.7	50.0	75.0	66.7	41.6	66.7

Present Absent (+/-)

Table 2. Retention factor of OTA detection and detoxified

Fungi isolate	**25°C (RF)**			**30°C (RF)**		
	pH 4.0	**pH 7.0**	**pH 9.0**	**pH 4.0**	**pH 7.0**	**pH 9.0**
Aspergillus ochraceus	1.20	1.15	1.10	1.29	1.13	1.15
A. niger	0.5	ND	ND	0.5	0.88	0.87
P. verrucossum	1.10	0.88	0.97	1.20	0.97	0.96
A. ochraceus plus *R. stolonifer*	0.97	0.57	0.87	1.03	0.93	0.94
A. niger plus *R. stolonifer*	ND	ND	ND	ND	0.57	ND
P. verrucossum plus *R. stolonifer*	0.97	ND	0.95	1.03	0.87	0.95

Not Detected (ND:)

Table 3. Colour appearance of toxin extracts under 365nm UV light

Fungi extracts	**Temperature**					
	25°C			**30°C**		
	pH 4.0	**pH 7.0**	**pH 9.0**	**pH 4.0**	**pH7.0**	**pH 9.0**
Aspergillus ochraceus	Blue Green	Blue Green	Blue Green	Blue Green	Blue Green	Blue Green
A. niger	Olive Yellow	ND	ND	Olive Yellow	Olive Yellow	Yellow
P. verrucossum	Blue Green	Blue Green	Blue Green	Blue Green	Blue Green	Blue Green
A. ochraceus plus *R. stolonifer*	Blue Green	Yellow	Yellow Brown	Blue Green	Yellow	Blue Green
A. niger plus *R. stolonifer*	ND	ND	ND	ND	ND	ND
P. verrucossum plus *R. stolonifer*	Blue Green	ND	ND	Light Yellow	Yellow Brown	Blue Green

Not Detected (ND)

Table 4. Mycotoxin detected and the detoxified toxin

Fungi extracts	Detected toxin	Rf/colour	Detoxified toxin	Rf/colour
Aspergillus ochraceus	Ochratoxin-A	1.10-1.29, B/G	Palitantin	0.57/ Yellow brown
			Citreoviridin	0.87/ Yellow
A. niger	Citreoviridin	0.87/ Yellow	ND	ND
	Kojic acid	0.37/ Yellow	ND	ND
P. verrucosum	Ochratoxin-A	0.88-1.2/ B/G	Palutin	0.98/Yellow
			Citreoviridin	0.87/Yellow

**Blue/Green (B/G)*

In the detoxification study OTA produced by *A. ochraceus* was detoxified to Palitantin (yellow brown colour at Rf 0.57) and Citreoviridin (yellow colour at Rf 0.87). OTA produced by *P. verrucossum* was detoxified to Palutin (yellow fluorescence at Rf 0.95-0.97) and Citreoviridin (yellow at Rf 0.55). While the Citreoviridin and Kojic acid produced by *A. niger* was totally detoxified as no migration or color appeared on the spotted area under the UV light after detoxification.

Influence of culture condition such as pH, temperature, culture media (micronutrients) and other physiological factors affect the production of OTA by *Aspergillus* and *Penicillium* [2] as was observed in this study.

In the microbiological analysis of stored cocoa sample, Yeast was majorly isolated from all samples tested while *R. stolonifer* was found in samples containing no ochratoxigenic fungi. Amezqueta et al. [2] reported the occurrence of *R. stolonifer* isolates after yeast in stored cocoa sample, which collaborate with the result from this study.

Isolates of *A. ochraceus* and *P. verrucossum* isolated from stored cocoa beans produce OTA on YES agar in this study which corroborates with findings of Koteswara et al. [21] on his study of Culture Media and Factors Influencing Ochratoxin-A production by Species of *Penicillium* isolated from poultry feeds [22] with YES agar for the detection of OTA.

Ochratoxin-A was produced from *A. ochraceus* and *P. verrucossum* in this study at pH 4 with a blue green fluorescence under 365nm UV light at both 25°C and 30°C while *A. niger* did not produce OTA at all tested pH and temperature. Khalesi and Khatib [23] studied the effect of different eco-physiological factors on OTA production and reported changes in pH have a notable effect on OTA production as observed in this study, [24] reported highest OTA production between 20°C and 30° and Amezqueta et al. [2] reported *P. verrucossum* and *A. ochraceus* were able to synthesize OTA at lower temperature than *A. niger. A. niger* produce mycotoxin other than OTA from cocoa in the study this was substantiate by different research on evaluation of Mycotoxins from coffee beans where as low as 1-9% were reported [25,26,27, 28].

Effect of varied temperature and pH on OTA production was observed as OTA were produced at temperature between 25°C and 30°C and pH between (4-9) in both *A. ochraceus* and *P. verrucossum* which collaborate with the study of Koteswara et al. [21] on culture media and factors influencing Ochratoxin A production by two species of *Penicillium* isolated from poultry feeds. The optimum conditions for *Penicillium* growth and OTA production was recorded at pH 5.5–7.5 by two species of *Penicillium* on Yeast extract as reported by Koteswara et al. [21] which was closely similar to pH4 and 7 reported in this study, though *P. verrucossum* was able to grow and produce toxin at pH 9. The detoxification potential of *R. stolonifer* as reported in the study collaborate the work done by Varga et al. [29] on biodegradation of OTA in wheat using different *Rhizopus* species, of which *R. stolonifer* was able to degrade OTA in moist spiked wheat. This study recorded detoxification of OTA produced by *A. ochraceus* to Citreoviridin and Palitantin and OTA produced by *P. verrucossum* detoxified to Palutin and Citreoviridin which are less toxic metabolites, while the citreoviridin and Kojic acid produced by *A. niger* were detoxified. The effect of varied temperature and pH had an impact on the detoxification potential of *R. stolonifer. Rhizopus stolonifer* was able to detoxified OTA from *A. ochraceus and P. verrucossun* at pH 7 and 9 at 25°C and pH 7 at 30°C and only at pH 4 at 30°C on OTA from *P. verrucossum*. This is a pointer that optimum temperature of 25°C - 30°C favours efficacy and detoxification potential of *R. stolonifer.*

4. CONCLUSION

This study revealed that *R. stolonifer* has the potential to detoxify OTA when there are favourable eco-physiological factors. The effect of varied temperature and pH had an impact on the detoxification potential of *R. stolonifer.* The microbiological analysis showed that cocoa beans are not only prone to OTA but other toxins. With the right solvent system, simple TLC proved to be an effective tool for preliminary evaluation of cocoa beans for OTA. The study is more of a qualitative work, further research need is required to confirm and quantify OTA detoxified at different temperatures and pH by *R. stolonifer*

COMPETING INTERESTS

This study declares no existence of any competing interests

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