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Research Article
Investigation of Incidence of Aflatoxin on *Xylopia aethiopica* (Dunal) A. Rich in Seven States of Nigeria

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Abstract

Background and Objective: The plant *X. aethiopica*, which belongs to the family Annonaceae and commonly called Negro pepper/African pepper is widely used as spice, stimulant as well as for medicinal purposes in Nigeria is reported to be highly contaminated by moulds of *Aspergillus*: *A. flavus* and *A. niger*. These fungi are known for their remarkable ability to form aflatoxins which are poisonous carcinogens which after entering the body may be metabolized by the liver, leading to liver cancer. **Materials and Methods:** An investigation of incidence of fungi on *Xylopia aethiopica* (Dunal) A. Rich from 7 states of the Nigerian Deltaic zones and the subsequent detection of aflatoxin at 20 ppb on the dry seeds was carried out with Target™ Field Test Column (TFTC). The study was portended due to the considerable high incidence of *Aspergillus flavus* on the dried seeds of *X. aethiopica* from different locations in the Nigerian Deltaic zones. The seeds were surface sterilized with 1% chlorox and plated on blotter papers in Petri dishes and agar medium, respectively. Detection of seed fungal pathogens was carried out using the standard blotter method as described by the International Seed testing Association. Results implicated that 8 fungal species, namely: *Aspergillus flavus, A. niger, Penicilluim species, fusariurn moniliforuie, Chioridium viride, Setodochiuni caesariae*, *Rhizoctonia solani* and *Mucor mucedo* with varying degrees of incidence were associated with the seeds of *X. aethiopica*, irrespective of source of sample. *Aspergillus flavus* had a considerable high incidence of occurrence across the Deltaic zones and was found pathogenic and deep-seated in the seeds even after a prolonged 1 h treatment with Chlorox. **Results:** The results of the aflatoxin test were negative with Target™ column and as such no further test with TLC was carried out. It indicated that the presence of aflatoxin was possibly less than 20 ppb. **Conclusion:** In conclusion, results from this research showed that *X. aethiopica* is contaminated commonly by storage fungi which were prevalent across other parts of the Deltaic zones of Nigeria. Seeds of *X. aethiopica* should be surface sterilized with chlorox and thoroughly rinsed before use, if seeds are brought from stored stock and may also be boiled for at least $1^{1/2}$ h before use.

Key words: *Xylopia aethiopica*, aflatoxin, target™ field test column, *Aspergillus flavus*, chlorox

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The plant *X. aethiopica* belongs to the family Annonaceae. It is a spice, stimulant and as well aromatic and medicinal. It is commonly called Negro pepper/African pepper or Guinea pepper. In Nigeria and Africa, it is used as a spice in some area and as herb in other areas which are all under exploited. It is known as 'uda' by the Ibos. It can grow up to 20 m high and is native or indigenous to the rainforest and moist fringe forests of the savanna zones of Africa[1]. Being indigenous to the lowland forests, it is used indiscriminately in the Deltaic zones of Nigeria. Its follicle contains 3-5 brown to black coloured seeds which are peppery and hot when violated or beaten to loosen consistency[2,3]. It is highly preferred for its mild stimulant properties and efficacy in evacuating unwanted substances and debris from the womb of a mother after birth. It is also very essential ingredient in preparation of local soups to aid new mothers in breastfeeding[4]. It is useful in the treatment of several ailments, amongst which are bronchitis, dysenteric conditions, asthma, stomach ache, cough, rheumatism, mouth wash etc[5]. According to Ekundayo[6], *X. aethiopica* contains 4.3% essential oil.

Edlin[7,8] reported that *X. athiopica* improves flavor and adds zest to meals that are otherwise monotonous in taste. The follicle bark and seeds are directly used and consumed. It grows in the wild and among forest plants[9].

In Senegal, the fruit is used to flavor Café Touba, a coffee drink that is the country's spiritual beverage and traditional drink of the Mouride brotherhood[10]. The plant is loaded with macro mineral nutrients, such as phosphorus and some trace elements[1,11].

It had been reported that *X. aethiopica* is contaminated by a considerable high incidence of the moulds of *Aspergillus: A. flavus* and *A. niger*. *Aspergilliis flavus* is known for its remarkable ability to form aflatoxins.

Aflatoxin of foods is a worldwide food safety concerns. Aflatoxin refers to a group of four mycotoxins, B1, B2, G1 and G2, produced primarily by two closely related fungi moulds, *A. flavus* and *A. parasitica*[12,13].

Aflatoxins are poisonous carcinogens which after entering the body may be metabolized by the liver[13-16].

Bulking of food items predisposes them to fungal infestation. visavis aflatoxin contamination[17]. Aftatoxin production is generally affected by genetical and environmental factors including moisture. That is to say that its production does not thrive in a dried state of food stuff[18-21].

Some study have been done on few indigenous species and stimulants from different parts of Nigeria, where a number of common or storage fungi have been implicated to be associated with and consumed with it.

This research was carried out to investigate the considerable high incidence of *Aspergillus flavus* on the dried seeds of *X. aethiopica* across various locations of the Nigerian Deltaic zones and to proffer solutions on how to handle the seeds to avoid the contamination, although bulking of food stuffs together with other goods could predispose it to fungi infection, especially when dry[22].

MATERIALS AND METHODS

This research was carried out in the International Institute of Tropical Agriculture (IITA) at Onne in Rivers State and Ibadan in Oyo state from April, 2016 to February, 2017. Apparently healthy dried seeds of *X. aethiopica* were procured from Port Harcourt (Rivers), Yenagoa (Bayelsa), Warri (Delta), Ikot-Ekpene (Akwa-Ibom), Owerri (Imo), Aba (Abia) and Calabar (Cross River) states. The seeds were surface sterilized with 1% chlorox and plated on blotter papers in Petri dishes and agar medium, respectively.

Detection of seed fungal pathogens was carried out using the standard blotter method as described by the International Seed testing Association[23,24]. Three layers of blotter paper were soaked in sterile distilled water and placed in sterile glass Petri dishes, (9 cm in diameter and agar medium (PDA). About 25 seeds were plated per dish into 16 dishes, totalling 400 seeds in each of the experiment. A set of untreated (control) seeds were also plated and labelled accordingly. The set ups were incubated at 29± °C for 7 days. At the end of the incubation period, the seeds were examined under a stereo-binocular microscope (6-50x) for fungal growth. Those grown on agar medium were constantly subcultured to maintain a pure culture. Identification and recording of fungal colonial growth were made based on their growth characteristics. Percentage Incidence of occurrence of fungi was calculated as:

$$\frac{\text{Infection}}{\text{Incidence}}\ (\%) = \frac{\text{No. of inflected seeds}}{\text{Total No. of seeds examined}} \times 100$$

Pathogenicity test: Spore suspensions of each fungus, *Aspergillus flavus*, *A. niger*, *Fusariumi moniliforme, Chloriduim viride, Setodochium caesariae, Rhizoctomia solani*, *Mucor mucedo* and *Penicillium* spp. were obtained by scrapping the fungal mycelia of their 10 days cultures.

Re-Isolation and identification: As typical of the associated fungi of the test *X. aethiopica* manifested, there was re-isolation of each of the fungus and reinoculation of seeds repeatably for 3 times as in above. These were done as confirmatory test of the *A. flavus* pathogenicity on *X. aethiopica*.

Detection of aflatoxin: As a result of the considerable high incidence of *A. flavus* contamination on the seeds of *X. aethiopica*, the need to test for the presence of aflatoxin became necessary.

Target™ Field Test Column (TFTC) device was used for the detection of the presence of aflatoxin at International Institute for Tropical Africa (TITA), Ibadan.

Target™ column kit was developed by Phillip[25], as a quick device for detection of presence of aflatoxin at 20 ppb in a short time. It is a quick fit device that detects the presence of aflatoxin at 20 ppb in 45 min, thereafter a more sensitive device such as Thin Layer Chromatography (TLC) could be used to determine the extent of aflatoxin contamination.

About 50 g ground sample of the dried seeds of *X. aethiopica*, 5 g iodized pack salt ($NaCl_2$) and 10 mL extraction solution (from the kit package) were placed into a blender jar and blended at high speed for 1 min. The jar was uncovered to allow the mixture settle and layers separated, showing clear top solvent layer from the clear top solvent layer, 2 mL from the clear zone solvent was transferred into an uncapped mixing vial using red disposable pipette, 6 mL of water with blue disposable pipette and 6 mL toluene with yellow pipette. At this point the cap of the vial was screwed unto it and inverted gently for 10 times and allowed to stand until layers are separated. Then 1 m of the top solvent was transferred from the vial to the Target™ column with silver pipette. A rubber tube syringe was used to apply air to the top of the Target™ column forcing down the solution in it. Pressure from the syringe was removed when the solution reached the column packaging.

The residual solvent dripped through the bottom into a waste collecting vial. The sample was forced down and 2 mL wash solution was added to the content in the column with green pipette. The syringe forced down completely the solution through the column until air bubbles were visible at the bottom of the column and excess water collected into the waste vial.

The test result was read by holding the narrow portion of the column at 5 cm (2 inches) from UV lamp, 365 nm (long wave) with protective lenses and hand gloves. Observation of fluorescence blue band on the column indicates positive result in a sample contaminated with aflatoxin at or greater than 20 ppb, total aflatoxin: If positive, a further work could he done with TLC plate to determine the extent of aflatoxin contamination.

Characteristics of fungal isolates: The identification of seed borne fungi colonial growth was made using the description by Dashak and Llewellyn[26], through a stereo binocular microscope (6-50x).

Statistical analysis: All data were analyzed using one way analysis of variance (ANOVA) followed by *post hoc* analysis using Waller Duncan test for comparison between treated and control groups. All results were presented as mean±SEM (standard error of mean). The statistical significance was accepted at $p<0.05$.

RESULTS

Features of fungal isolates: The features of the fungi showed that the conidiophores of the *Aspergillus* species colonies were upright and simple, with globose swelling. The position of the phialides occurred at the apex of the entire surface with various colours *en mass*.

Identification of fungal isolates: The test results on *X. aethiopica* showed that the seeds were infected by 8 fungi species irrespective of source of sample and the fact that they looked apparently healthy.

Results from Table 1 indicated that *Aspergillus* species (*A. flavus* and *A. niger*) had the highest incidence of fungi on the seeds of *X. aethiopica*. The presence of *A. flavus* was highest in the untreated seeds from Delta state, while there was no significant difference in the percentage occurrence of *A. niger* in seeds of *X. aethiopica* collected from Rivers, Bayelsa, Delta, Imo and Abia states, respectively.

Other fungi isolated were *Fusarium moniliformis*, *Mucor mucede, Penicillin* spp. *Rhizoctonia solani* and *Setoddium caesariae* with no significant difference in the occurrence of the fungi in all the states where the seeds were collected.

The results in Table 2 showed percentage incidence of occurrence of isolates of *A. flavus* in the seeds of *X. aethiopica* in the seeds collected from the different states. In the untreated seeds, the occurrence of *A. flavus* was highest in seeds from Delta state (66.50±0.70) followed by those from Imo (62.30±0.14), Rivers (52.15±0.07), Cross River (49.15±0.07), Abia (48.25±0.07), Bayelsa (42.85±0.07) and Akwa Ibom (37.85±0.07) states, respectively.

Table 1: Fungal isolates (incidence %) on untreated samples of *X. aethiopica* in the different states investigated

Fungal isolates (Mean±SD)	Rivers state	Bayelsa	Delta	Imo	Abia	Akwa Ibom	Cross rivers
A. flavus	52.05±0.01	42.85±0.01	66.5±0.01	62.25±0.01	48.45±0.35	37.85±0.01^{e}	45.55±5.02^{d}
A. niger	70.60±0.14^{g}	45.15±0.01^{g}	58.50±0.07^{g}	41.65±0.07^{g}	41.65±0.07^{g}	43.35±0.07	42.15±0.07^{d}
Fusarium moniliforme	11.25±0.07^{b}	9.85±0.07^{a}	19.25±0.07^{g}	10.45±0.07^{a}	14.25±0.07^{b}	31.25±0.07^{d}	16.25±0.07^{b}
Mucor mucedo	15.35±0.07	18.15±0.07	16.05±0.07^{b}	16.05±0.07	17.35±0.07	15.35±0.07^{b}	25.45±0.07
Penicillin spp.	21.25±0.07^{d}	18.55±0.07^{d}	20.25±0.07^{d}	31.50±0.07^{d}	34.150±0.07^{g}	45.50±0.07	22.85±0.07
Rhizoctonia solani	7.85±0.07^{a}	11.65±0.07^{b}	13.55±0.07^{a}	15.35±0.07^{b}	11.15±0.07^{a}	12.35±0.07^{a}	8.25±0.07^{a}
Setoddium caesariae	28.45±0.07	22.25±0.07	28.30±0.00	21.65±0.07	26.75±0.07	28.35±0.07	25.25±0.07

Means with the same alphabet across the column shows no significant difference at p<0.05

Table 2: Incidence (%) of occurrence of isolates of *A. flavus* in the different states

Samples (Mean±SD)	Rivers state	Bayelsa	Delta	Imo	Abia	Akwa Ibom	Cross rivers
Re-isolated	41.15±0.07^{a}	39.60±0.00^{a}	46.50±0.70^{a}	56.10±0.00^{a}	42.15±0.07^{a}	40.25±0.07^{a}	37.75±0.07^{a}
Treated	45.55±0.07^{b}	50.15±0.07	48.50±0.70^{a}	58.15±0.07	42.85±0.07^{b}	41.25±0.07	47.25±0.07^{b}
Untreated	52.15±0.07^{a}	42.85±0.07^{b}	66.50±0.07^{b}	62.30±0.14	48.25±0.07	37.85±0.07^{a}	49.15±0.07

Means with the same alphabet across the column shows no significant difference at p<0.05

Table 3: Effects of treatment on the occurrence of *A. flavus*

Treatment time (%) (Mean±SD)	Rivers state	Bayelsa	Delta	Imo	Abia	Akwa Ibom	Cross rivers
1% chlorox (5 min)	45.45±0.07^{a}	50.15±0.07^{b}	49.05±0.70	34.75±0.07+	47.25±0.07	58.15±0.07	42.850±0.07^{g}
1% chlorox (1 h)	41.50±0.70^{a}	39.65±0.07^{b}	46.50±0.70	40.25±0.07^{d}	37.70±0.07	56.15±00.07	42.150±0.07

Means with the same alphabet across the column shows no significant difference at p<0.05

In the treated seeds, percentage (%) occurrence of *A. flavus* was highest in seeds collected from Imo states, followed by seeds from Bayelsa and Delta states. There was no significant difference in the occurrence of *A. flavus* in the seeds collected from Cross river, rivers and Abia states, respectively. It was lowest in the seeds from Akwa Ibom state. In the re-isolated seeds, there was no significant difference in the percentage occurrence of *A. flavus* in seeds collected from Imo, Delta, Abia , rivers, Akwa Ibom, Bayelsa and Cross River states, respectively.

Results from the effect of treatment of the seeds with 1% chlorox on the occurrence of *A. flavus* (Table 3) showed that at 5 min treatment, there was significant difference of the effect of treatment on *A. flavus* in the different states with the treatment of the seeds from Akwa Ibom state having the highest effect on *A. flavus*, followed by seeds from Bayelsa, Delta, Abia, Rivers, Cross River and Imo states, respectively.

Treatment of the seeds with 1% chlorox at 1 h had significant difference in all the states with the highest effect on *A. flavus* in seeds from Akwa Ibom state followed by Delta, Cross River, Rivers, Imo, Bayelsa and Abia states, respectively. Treatment of samples with chlorox showed no efficiency and did not show any significant effect on elimination of the fungal isolates at $p \leq 0.05$ degrees of judgment (DMRT).

Total aflatoxin at 20 ppb: The results of the aflatoxin test were negative with Target™ column and as such no further test with TLC was carried out. The Target™ column was to quickly detect the presence of aflatoxin. Thin Layer Chromatography (TLC) plate was to be used to determine the extent of contamination if aflatoxin was found present.

DISCUSSION

An investigation of incidence of fungi on *X. aethiopica* from 7 states of the Nigerian Deltaic zones and the subsequent detection of aflatoxin at 20 ppb on the affected seeds was carried out. From the study, a total of 8 fungal isolates were found associated with the *X. aethiopica.* They are *Aspergillus flavus*, *A. niger*, *Rlzizoctouia solani*, *Fusarium moniliforme*, *Penicillium* spp. *Chloridium viride*, *Setodochium caesariae* and *Mucor mucedo*. *Aspergillus flavus* was of a considerable high incidence across the 7 states.

Percentage incidence of *F. moniliforme* was low, possibly because of low moisture content of the seeds hence they were in their dried state. This seems to agreed with the report of Dashak and Llewellyn[26], that moisture content predisposes crops or foods to fungal pathogens. The seeds harboured these fungi possibly because they were bulked with other food items or goods in store hence it was reported by Wheeler[17] that bulking of goods or different kinds of food items in store predisposes them to fungal infections.

Considering the high incidence of *A. flavus,* it may be claimed that the internal warmth environment of the seeds was conducive for *A. flavus* deep-seatedness.

Re-infection of the seeds by *A. flavus* after prolonged treatment with chlorox for 1 h showed that at p<0.05 degree of judgment, the treatment was ineffective (DMRT)[26].

The study showed a considerable high incidence of *A. flavus* a group of fungi that produces aflatoxin. It was

with this in mind that aflatoxin test even at 20 ppb test was carried out, since they have been reported to be carcinogenic, mutagenic and teratogenic and there was the speculation that they contained this toxin which has been implicated for the increase in liver cancer in our society today as reported by Singh *et al.*[15].

The negative test result of aflatoxin could be that the *A. flavus* isolated was a non-toxigenic strain. This may be likened to agree with the report of Dorner[13]. It reported that toxigenic strain of *A. flavus* typically produce aflatoxins B1 and B2. This means that if there is toxigenic strain, there is also likely to be non-toxigenic strain as well.

Another reason why the aflatoxin test was negative could as well be that the natural soil where the seeds of *X. aethiopica* were harvested contained the non-toxigenic strain of *A. flavus*. This supports the report of Ogundana and Ajulo[21] which reported on the success of strategies of reduction of aflatoxin by the use of non-toxigenic strain of *A. flavus* to compete in the soil to exclude the toxigenic strain of *A. flavus*.

The *X. aethiopica* was in dried state as such there may be some genetic factor in addition to moisture that did not enhance the production of aflatoxin. This also agrees with the report of Catty[18], Brown *et al.*[19] and Palumbo *et al.*[20], which emphasized on the effect of genetic factors and moisture state for production of aflatoxin.

It could also be that *X. aethiopica* is loaded with some mineral nutrients in it. This agrees with Corner[2] and Deepak *et al.*[12], who altogether at different times reported that abundance of phosphate, nitrogen and some trace elements which occur in such quantity as cannot be limited to give way to the synthesis of aflatoxin could result in non-production of aflatoxin. Generally, it could be that the presence of aflatoxin in *Xylopia aethiopica* is very minute and insignificant, less than 20 ppb to be detected by the quick device used.

CONCLUSION AND RECOMMENDATION

Based on the findings in this research, it may be concluded that *X. aethiopica* is contaminated commonly by storage fungi. Fungi isolated from one source were prevalent across other parts of the Deltaic zones of Nigeria. Seeds of *X. aethiopica* should be handled with care to reduce rate of infection. The seeds should be surface sterilized with chlorox and thoroughly rinsed before use. Researchers are encouraged to do more toxicological works using Thin Layer Chromatography (TLC) for detection and quantification of presence and extent of aflatoxin contamination.

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