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Effect of Neem Leaf Extract as Treatment on the Occurrence of *Aspergillus* Species on Maize Seeds

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ABSTRACT

Assessment of the occurrence of *Aspergillus flavus* on maize seeds collected from different markets and the effect of treatment with Neem leaf extract was undertaken in Makurdi, Benue State. Detection of seed borne fungi was generally by standard blotter method while effect of seed treatment on occurrence of *Aspergillus flavus* was assessed with Neem Leaf extract at the rate of 0, 10, 20, 30, 40 and 50% w/v. Two species of *Aspergillus* species namely *Aspergillus flavus* and *Aspergillus niger* were identified with *Aspergillus flavus* having a higher percentage of occurrences than *Aspergillus niger*. Percentage occurrence of *Aspergillus flavus* was significantly higher ($P<0.005$) at Wurukum market compared with modern market which recorded the lowest incidence. Percentage occurrence at Kanshio, Railway and Wadata markets were not different from that of Wurukum but were also significantly higher compared with modern market. Inhibition of radial growth of *Aspergillus flavus* by neem leaf extract was significantly higher at 10% w/v concentration compared with the other concentrations. There was no significant difference in percentage germination. The relatively high occurrence of Aspergillus flavus points to the possibility of aflatoxin contamination and possibly aflatoxicoses in the near future if appropriate preventive measures are not implemented by farmers and marketers of maize products. Farmers who intend to prevent fungal attack of their maize seeds should adopt Neem leaf extract at 10% w/v as this effectively prevents the growth of *Aspergillus* species.

Keywords: Maize seeds, *Aspergillus*, Neem, Extract

INTRODUCTION

Maize *(Zea mays L.)* is one of the most important staple food and feed crops in the world. It is one of the world's widely cultivated cereal crops and ranks third in world production after wheat and rice [1]. Maize is a great contributor to the enhancement of household food security for both urban and rural areas in many low and middle-income countries. Its production has been rising but still, maize production globally must increase even more to meet future demands [2].

Wangari [1] reported that fungal infections particularly those of the *Aspergillus* species are responsible for about 50-80% of damage on farmer's maize during storage, especially if conditions are favorable for proliferation resulting to subsequent aflatoxin production in food and feeds. Aflatoxin, a metabolite of *Aspergillus flavus* and *Aspergillus niger* are the most popular, widespread and of great economic importance in the agricultural sector.

Fungicides are the primary means of controlling postharvest diseases. However, repeated use of certain fungicides in maize storage structures has led to the appearance of fungicide-resistant populations of storage pathogens [3]. In recent years there has been considerable pressure by consumers to reduce or eliminate chemical fungicides in foods. Furthermore, the use of synthetic chemicals to control postharvest diseases has been restricted due to their carcinogenicity, teratogenicity, high and acute residual toxicity, long degradation period, environmental pollution and their adverse effects on human health. Natural plant extracts have the potential of providing an alternative way to protect maize from fungal contamination. They provide a source of inspiration of novel drug compounds, as plant derived medicines have made large contributions to human health and well-being [4].

MATERIALS AND METHODS

Study area

The study was carried out in the botany laboratory of the

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Benue State University Makurdi. Makurdi, the capital of Benue State, lies between latitude 07°15' – 07°45'N Longitude 08°15' – 08°, 40E. The town lies in the guinea savanna vegetative belt and on the bank of the 2nd largest river in Nigeria, River Benue. The river divides the town into North and South banks and the town covers an area of 16 km^2.

Collection of maize samples

Maize seed samples were collected in polythene envelopes from five markets namely; Wurukum, Wadata, Kanshio, Modern and high level in Makurdi and taken to the botany laboratory of the Benue State University for further studies.

Preparation of Potato Dextrose Agar (PDA)

Potato dextrose agar was prepared by suspending 39 grams of Potato Dextrose Agar in 1000 ml of distilled water. This was then heated using a heating mantle to dissolve the medium completely. The agar was afterwards sterilized by autoclaving at 15 lbs at 121°C for 15 min and allowed to cool before dispensing into sterile Petri dishes.

Collection of neem (*Azadirachta indica*) **leaves**

Fresh leaves of *Azadirachta indica* (Neem) were collected from different locations in Makurdi metropolis. A cutlass was used to cut branches while the leaves were harvested by handpicking. The leaves were put in clean polythene bags and taken to the laboratory. In the laboratory, the leaves were first prewashed carefully under a gentle stream of tap water for one to two minutes to remove surface dirt. This was followed by washing in sterile distilled water containing 1% sodium hypo chloride for thirty seconds. The leaves were then removed and rinsed in three successions of sterile distilled water as reported [5].

Extract concentrations

Concentrations of the plant species was prepared to give 10 g/ml, 20 g/ml, 30 g/ml, 40 g/ml and 50 g/ml respectively. Extract concentration of 40 g/ml was obtained by dissolving 40 g of the plant leaf powder of each plant species respectively in 100 ml of sterile distilled water in a beaker. Extract concentration of 60 g/ml was obtained by dissolving 60 g of the plant leaf powder of each plant species respectively in 100. ml of sterile distilled water in a beaker. The same principle was applied to all other extract concentrations.

EXPERIMENTS

Experiment 1. Assessment of *Aspergillus* species on maize seeds collected from market in Makurdi

The experiment was set up in a completely randomized design (CRD) with markets being the treatments while number of samples per market formed the replications. Detection of seed-borne fungi was done by standard blotter method highlighted [6]. In this method, three pieces of blotter papers were soaked in distilled water and placed in 9 cm sterile plastic Petri dishes after draining out excess water. For each treatment, 120 seeds were plated (40 seeds per replication). Working samples of these seeds were plated out on the moist blotter papers after surface sterilization with 0.5% sodium hypochlorite solution for 1 min and incubated at room temperature. After seven days of incubation, the seeds were examined for fungal growth under a microscope. Slides of different fungal spores encountered were prepared and examined with Olympus microscope at ×40 magnification to establish identity of individual fungus. Identification aids are included [7-9]. Percentage occurrence of *Aspergillus* species were determined by counting the number of times each individual fungus occurred divided by the total number of fungi and expressed as a percentage:

$$\text{Percentage occurrence of fungi} = \frac{Number\ of\ times\ each\ fungi\ occured}{Total\ number\ of\ fungi\ per\ plate} \times 100 \quad (1)$$

Additionally, seed germination was determined by using the formula below,

$$\text{Percentage Germination} = \frac{Number\ of\ seeds\ germinated}{Total\ number\ of\ seeds\ per\ plate} \times 100 \quad (2)$$

Experiment 2. Assessment of effect of neem leaf extract on radial growth of *A. flavus*

The method of Liamngee et al. [10] was used. Two (2) ml of the extract concentration was dispersed in Petri dishes and 15-20ml of molten PDA was added. The petri dishes were swirled gently on the work bench to ensure even dispersion of the extracts. The agar-extract mixture was allowed to solidify and used for the inhibition of mycelia growth of the test fungi and 4 mm diameter of mycelia disc obtained from the colony edge of 5 days old pure culture of *A. flavus* was inoculated centrally onto the medium using a 5 cm cork borer. Four replications were used for each extract concentration. Control where Petri dishes containing PDA with no botanical extracts were also inoculated with *A. flavus.* The plates were arranged on laboratory desk following completely randomized design and incubated at room temperature. Measurement of growth was done using a meter rule on days 3, 5, 7. Inhibition of fungi growth was calculated using the formula:

$$\text{Growth inhibition of fungi} = \frac{R_1 - R_2}{R_1} \times 100 \quad (3)$$

Where; R_1 = Radial growth of the pathogen in the control plates and R_2 = Growth of the pathogen with treatment.

Experiment 3. Effect of seed treatment with neem leaf extract on occurrence of *Aspergillus* species on maize seeds

The effect of seed treatment with Neem leaf extract was assessed using Neem leaf extract at rates of 10, 20, 30, 40 and 50 g/ml. Untreated maize seeds were used as control. Seeds were treated by soaking in neem solution as described [11] and plated using standard blotter method as described in Experiment 1. For each extract concentration, 10 maize seeds were plated and replicated 5 times in completely randomized design. The seeds were incubated for 7 days as earlier

described, after which percentage occurrence of *Aspergillus species* and percentage seed germination were calculated and recorded.

Data analysis

Data obtained were analyzed using analysis of variance (ANOVA) and the Fisher's least significant difference was used to separate the means at 5% level of significance.

RESULTS & DISCUSSION

Occurrence of *Aspergillus* species on maize seeds collected from markets in Makurdi

Table 1 shows the occurrence of *Aspergillus flavus* on maize seeds collected from markets in Makurdi. Two species of Aspergillus namely *Aspergillus flavus and Aspergillus niger* were isolated from maize collected from the various markets in Makurdi, Benue State. Percentage occurrence of *A. flavus* was significantly higher (P<0.05) at wurukum market compared with modern market which recorded the lowest incidence. Percentage occurrence at Kanshio, railway and Wadata markets were not different from that of Wurukum but were also significantly higher compared with Modern market. For *A. niger*, Wurukum market also recorded significantly higher (P<0.05) percentage occurrence compared with railway market, which had the lowest incidence. There was no significant difference in percentage germination.

Table1. Occurrence of *Aspergillus* species on maize seeds collected from markets in Makurdi.

	Percentage Occurrence		
Markets	***A. flavus***	***A. niger***	**Percentage seed germination**
Kanshio	25.00a	11.70a	59.20
Modern	16.70b	24.20a	40.80
Railway	24.20a	7.50b	40.80
Wadata	27.50a	19.20a	40.80
Wurukum	30.80a	18.30a	57.20
LSD (0.05)	**7.99**	**9.72**	**NS**

Means followed by the same letters in a column are not significantly different at p=0.05

Effect of neem leaf extract on the radial growth of A. flavus of maize in vitro in Makurdi

The effect of neem leaf extract on the radial growth of *A. flavus* of maize in Makurdi, Benue State is shown in **Table 2**. Varying concentrations of neem leaf extract results in the inhibition of growth of *A. flavus*. Three days after inoculation (DAI), all the concentrations of neem extract; 10, 20,30, 40 and 50% w/v significantly (P<0.05) reduced radial growth of *A. flavus* compared with the control. There was no significant difference in the radial growth of *A. flavus* at 5DAI.

At 7 DAI, all the concentrations significantly (P<0.05) reduced the radial growth of *A. flavus* compared with control. Amongst the various concentrations, radial growth was significantly higher at 10% compared with the other concentrations (20, 30, 40, and 50% w/v) tested.

Table 2. Effect of neem leaf extract on the radial growth of *A. flavus in vitro*.

	Radial growth (cm)		
Concentrations (w/v)	**Day 3**	**Day 5**	**Day 7**
0	1.79a	2.31	6.68a
10	0.36c	0.52	1.15b
20	0.45bc	0.52	0.65c
30	0.49b	0.52	0.68c
40	0.43bc	0.52	0.68c
50	0.40bc	1.97	0.65c
LSD (0.05)	**0.118**	**NS**	**0.405**

Means followed by the same letters in a column are not significantly different at p=0.05

Effect of seed treatment with neem leaf extract on occurrence of *Aspergillus* species on maize seeds in Makurdi

The effect of seed treatment with Neem leaf extract on the occurrence of *Aspergillus* species of maize seeds is shown in **Table 3.** Percentage occurrence of *A. flavus* was significantly higher (P<0.05) in the control compared with the occurrence in seeds treated with neem leaf extract. All the concentrations (10, 20, 30, 40 and 50% w/v) tested significantly reduced percentage occurrence of the fungus with no significant difference amongst them.

There was no significant difference in the percentage occurrence of *A. niger* in the control compared with the treated seeds though values for the control was higher than those treated. There was no significant difference in percentage germination.

This study reveals a percentage occurrence of *Aspergillus species* ranging from 11-30% in maize samples collected from five different markets in Makurdi, Benue State. Two species of *Aspergillus* namely *Aspergillus flavus* and *Aspergillus niger* were isolated in this study with higher percentage occurrence recorded for *Aspergillus flavus* than for *Aspergillus niger* in most of the markets sampled. A higher occurrence of *Aspergillus flavus* agrees other reports [12] which reported *A. flavus* as the most dominant species of

Table 3. Effect of seed treatment with neem leaf extract on occurrence of *Aspergillus* species on maize seeds in Makurdi.

	Percentage Occurrence		
Concentration (w/v)	***A. flavus***	***A. niger***	**Percentage seed germination**
0	20.00a	8.00	72.00
10	0.80b	4.00	60.00
20	1.00b	6.00	50.00
30	1.20b	4.00	66.00
40	0.60b	0.00	34.00
50	1.00b	2.00	52.00
LSD (0.05)	**6.74**	**NS**	**NS**

Means followed by the same letters in a column are not significantly different at p=0.05

Aspergillus in Makueni County in Kenya. It is also in agreement with research [13] which reported same in Nandi County in Kenya and another study [14] who reported same in their study involving selected counties of Kenya. The occurrence of *Aspergillus flavus* in maize in most of the markets sampled implies that *Aspergillus flavus* is widely distributed in Makurdi, Benue State and that most of the commonly grown maize varieties are susceptible to fungal spoilage and possibly aflatoxin contamination. Thaddeus [4] reported a high occurrence of 58.50% for *Aspergillus* species in Eastern Kenya while another research [15] showed that occurrence of *Aspergillus* species was high in post-harvest maize of West Africa.

The varying percentage of *Aspergillus* species observed in this study can be attributed to several factors; the most striking ones being poor storage structures and pre-harvest infections which greatly influenced the mycoflora in storage since some of the structures used for maize storage are mostly woven bags heaped on the floor, the verandah and sometimes in outdoor storages like granaries. The nature of these structures most times does not guarantee a moisture-free storage, mould infection and hence low protection of grains against *Aspergillus* contamination [16].

Occurrence of *Aspergillus flavus* and *A. niger* in this study differed markedly from market to market with Wurukum having the highest percentage occurrence of 30.80% while modern market had the least percentage occurrence of 16.70%. Despite the low occurrence of *Aspergillus flavus* in Modern market, highest prevalence of *Aspergillus niger* was observed in samples from this market. A possible explanation for this may be differences in maize varieties and genetic differences which support the ability of one species to thrive more than another in a given area. Also, there was significant difference in percentage occurrence between Wurukum and Modern market when compared to other markets. This contrasts with another report [14] who reported that frequency of occurrence of *Aspergillus* isolates across all the five districts of Kenya do not significantly differ from each other when analyzed through analysis of variance at 95% level of significance. It is however in agreement with other research [17] who reported that incidence of *Aspergillus* species among regions sampled differed significantly.

Percentage seed germination revealed that none of the samples from the various markets had germination higher than 60%. This might be due to improper handling and processing of seeds after harvesting, differences in storage conditions and kind of seed-borne fungi associated with them. This is similar to other reports [18-20] whose findings showed that no maize seed have germination higher than 80%. Highest germination percentage despite variability within markets was however highest for maize from Wurukum while those from Modern market, Wadata and Railway markets had least percentage seed germination.

As part of the objectives of this study, neem leaf extract was tested for its effectiveness in reducing the growth of species of *Aspergillus* in maize collected from markets in Makurdi, Benue State. Results obtained showed that different concentrations of Neem leaf extract reduced the radial growth of *A. flavus* in maize seed examined. The ability of Neem leaf extract to inhibit the growth of *A. flavus* as show that neem leaf extract possesses strong antifungal properties which are also dependent on the concentration of the plant extract.

In terms of effect of concentration of neem leaf extract on percentage occurrence of *Aspergillus* species, it was observed from this study that varying concentrations of neem leaf extract affected significantly, the percentage occurrence of the two species of *Aspergillus* identified. It was observed that increasing concentrations of neem leaf extract resulted in reduction in percentage occurrence of *Aspergillus* species. This is an implication of the effect concentration has on occurrence of *Aspergillus* species in maize collected from Makurdi since Neem leaf extract reduces the abundance of *Aspergillus* on maize. This is so since the control maize seeds

without Neem leaf extract treatments recorded the highest abundance of *Aspergillus* species. The effect of concentration of Neem leaf extract on percentage occurrence of *Aspergillus* species also differed significantly. Ability of neem leaf extract to affect the relative abundance of *Aspergillus* species by lowering its abundance is similar to other reports [4] which reported that abundance of *Aspergillus* is affected by several plant extracts due to their antifungal nature.

CONCLUSION

Two major species of *Aspergillus* are associated with maize seeds in Makurdi, Benue State. This species are *Aspergillus flavus* and *Aspergillus niger*; the most abundant of which is *Aspergillus flavus*. The relatively high prevalence of *Aspergillus flavus* in this study points to the possibility of aflatoxin contamination and possibly aflatoxicoses in the near future if appropriate methods of prevention and control of this fungus are not implemented by farmers and marketers of maize products.

Application of neem leaf extract inhibits greatly the growth of fungi on maize and the degree of inhibition increases with increasing concentration.

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