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In vitro evaluation of leaf extracts on the growth of *Aspergillus niger* infecting maize grains

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ABSTRACT

Mycotoxin producing fungi are important pathogens affecting stored maize grains. Conventional management strategies using synthetic chemicals are expensive, hazardous and environmentally unfriendly. This has necessitated the search for alternatives in botanicals. Market sampling of maize grains were carried out in five markets in Port Hartcourt, Nigeria, namely; Choba-main and Choba junction, Rumuosi, Aluu and Ozuoba, to evaluate the occurrence of mycotoxin-producing fungi. Studies were then carried out in the laboratory to evaluate the efficacy of water leaf extracts of *Azadirachta indica, Garcina kola, Moringa oliefera, Ocimium gratissimum, Gongronema latifolium* and *Vernonia amygdalina* at three different concentrations (2.5, 5.0 and 7.5% w/v) on the prevalent fungus. Result from the market sampling showed that *Aspergillus niger, Aspergillus flavus* and *Pennicillin sp* were commonly isolated from the maize grains with *Aspergillus niger* having the highest occurrence. Choba markets were observed to have the highest percentage occurrence of *Aspergillus niger* (66-100%). Laboratory studies showed that *Gongronema latifolium*, effectively reduced the growth of *A. niger* on PDA media and leaf extract concentration at 7.5% was the most effective.

INTRODUCTION

Maize (*Zea mays*) is the most important cereal in the world after wheat and rice with regard to cultivated areas and total production (Purseglove 1992; Osagie and Eka 1998). In Nigeria, it is widely grown and intercropped with other crops like cassava and melon (Agboola and Makinde 2008). Nigeria is currently the tenth largest producer of maize in the world, and the largest producer in Africa (IITA 2012). Its availability is very important to both man and livestock. However, maize is greatly affected by filamentous fungi which produce secondary metabolites that are capable of causing toxic responses such as diseases or death in humans and other animals, when ingested (Bennett and Klich, 2003; Bandyopadhyay et al. 2007). Mycotoxins of major concern are produced in grains by species of *Aspergillus, Fusaruim, Penicillium*. They are commonly associated with oil seeds, groundnut, soya beans, cowpea as well as several cereals like maize, wheat, barley, sorghum, oats and rye. These food products serve as stable food for humans and raw material for livestock feed production (Tiffany 2011; Farag 2008). The risk of natural contamination by these mycotoxigenic fungi is an important safety concern worldwide. Although, synthetic chemicals have been found very effective against mycotoxin producing fungi (Okello et al. 2010), their uses in plant protection are being de-emphasized due to their mammalian toxicity and stimulations of pathogen resistance resulting from their inappropriate or excessive usage (Tripathi and Dubey 2004). These problems have necessitated the search for alternatives such as extracts from plant materials. According to Okigbo (2004) and Awurum and Enyiukwu (2013), plant-based pesticides are cheap, biodegradable, easy to formulate, easily available in the farming localities of Nigeria and less likely for pathogens to develop resistance (Opara and Wokocha 2008; Opara and Obana 2010; Adjaye-Gbewonyo et al. 2010; Enyiukwu and Awurum, 2013). Furthermore, there is scanty literature on the status of these mycotoxin producing fungi in stored products especially maize in the Port Harcourt areas of Nigeria, thus the need

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for this study. This study was therefore, aimed at (i) identifying mycotoxin-producing fungi in maize grains sold in some markets in Port Harcourt (ii) evaluating the efficacy of some local plant leaves on the *in vitro* growth of the most prevalent of the isolated fungi.

MATERIALS AND METHODS

Market survey was carried out in five different markets along the East-West Road in Port Harcourt, Nigeria (6.55^o 0.2' N latitude, 4.54^o 10.02' E longitude). The markets are; Choba-main and Choba-junction, Rumuosi, Aluu and Ozuoba market. The *in vitro* experiment was carried out in the Department of Crop and Soil Science Laboratory, University of Port Harcourt, Port Harcourt, Nigeria.

Fresh leaves of *Garcinia kola*, *Moringer oleifera, Azadirachta indica, Ocimum gratissimium, Vernonia amygdalina, Gongronema latifolium,* were locally sourced, while the maize grains were procured from the markets. The plant materials were washed thoroughly, air-dried on the laboratory benches and made crispy in the oven at 55^{o}C for 24h. The leaves were ground to powder using Philips blender (HR 2815 Holland). Then 100 g of the powder of each plant material were mixed with 1 litre of water and then passed through a sieve no. 60 and filtered through a Whatman no 1 filter paper before filter sterilization through a membrane filter (0.2µm) to avoid microbial contamination. The filtrate was concentrated in a rotator evaporator to produce a semi-solid residue that was further dried into powder form. They were weighed and packed in an air-tight container labeled properly and stored in a refrigerator until used.

The grain samples gotten from the markets were first sterilized in 2% NaOCl for three minutes and rinsed in several changes of sterile distilled water. The sterilized grains from each market were then placed on two moistened filter papers in 90 mm Petri-dishes at the rate of five grains per plate and a total of ten Petri dishes per market. The Petri dishes were then kept in the inoculation chamber at 26 – 28^{o}C and observed for fungal growth. Sub-cultures were made from emerging colonies repeatedly until pure cultures were obtained. The fungal isolates were further checked for their identity, viability and purity using standard biochemical test as described by Cheebrough (2000). The identification of the fungi was done using Illustrated Genera of Imperfect Fungi by Barnett and Hunter (1998).

Ground leaves of *Garcinia kola, Moringer oleifera, Ocimum gratissimum, Gongronema latifolium, Vernonia amygdalina and Azadirachta indica (Neem)*, respectively were soaked in sterile distilled water at three different concentrations ; 2.5%. 5.0% and 7.5% w/v. They were left for 24 hours and then filtered through a Whatman no 1 filter paper into well labeled containers and used for treatment application immediately.

15ml of potato dextrose agar (PDA) and 1.5ml of each of the leaf extracts were poured into the Petri-dishes and allowed to gel. Using a 5 mm cork borer, the pure cultures of the test fungus was introduced at the center of each plate. The plates were incubated at 25 ± 2^{o}C in the inoculation chamber with alternating circles of 12hr light and 12hr darkness each day. The control plates comprised of PDA, sterile water and the test fungus without the leaf extracts.

The following data were collected;

- Percentage occurrence of the fungi isolated across the sampled markets.
- Daily measurement of the mycelial growth of the test fungus for 7 days.

The experiment was laid out in a 6 (leaf extracts) × 3 (concentrations) in completely randomized design (CRD) replicated four times. The control plates with sterile distilled water had four plates. Analysis of variance (ANOVA) was obtained using GenStat (GenStat 16th Edition; VSN International Ltd, UK) and means separated using the standard error of difference (SED) at 5% level of probability.

RESULTS AND DISCUSSION

Percentage pathogen occurrence across the markets

Table 1 shows the percentage occurrence of the fungus isolated from the maize across the various markets. It was observed that Choba markets recorded the highest percentage prevalence of the *Aspergillus niger* in the range of 66%-100%. The least occurrence was observed in Rumuosi market with value of 33%. *A. flavus* and *Penicillin* spp also occurred at 33% in Rumuosi market. No *Aspergillus flavus* was observeded in maize grains sampled from Choba markets. The implication of this result is that there is the possibility of ochratoxin and aflatoxin contamination of maize grains from these markets. The high occurrence of *Aspergillus niger* could be attributed to the fact that the said markets are major markets that attract different merchants from across local communities and even abroad on main market days. This makes the source of contamination wider as most of the

Table 1. Percentage occurrence of fungi pathogens isolated from the different markets

Market	Pathogen		
	Aspergillus niger	*Penicillin* spp	*Aspergillus flavus*
Aluu	50.0	25.0	25.0
Rumuosi	33.3	33.3	33.3
Ozuoba	50.0	25.0	25.0
Choba junction	66.7	33.3	0
Choba big market	100	0	0

fungi may have been brought in by these merchants. However, the reverse is the case with Aluu, Ozuoba and Rumuosi because these markets are small and mostly used by the locals.

Effect of leaf extracts and concentration on mycelial growth of *Aspergillus niger in vitro*

The effect of plant extract on mycelia growth of *Aspergillus niger* was significant (P<0.05) all through the days of incubation (Table 2). *Gongronema latifolium,* was observed to have given the optimum growth reduction effect of the test fungus with mycelial growth below < 20 mm at the end of the incubation period. The least growth reduction effect was observed in plates treated with *Azadiractha indica* which were not significantly different from the control. *Vernonia amygdalina* and *Moringa oliefera* had mycelial growth rates < 45 mm. Table 3 shows the effect of concentration on mycelia growth of *A. niger*. Mycelial growth rate of the fungus decreased with increase in concentration with significant effect observed from the 2nd (P<0.001) to the 7th (P=0.004) day of incubation. Control plates showed the least mycelial reduction effect on the test fungus having reached 86 mm in diameter at the end of the 7th day. However, in general the effect of the different concentrations of the leaf extracts was significantly different from the control plates.

There is a general increase in the consumption of contaminated grains with mycotoxin which causes different health problems including death (Lerda et al. 2005; Voss et. al. 2007). During storage several kinds of fungi may remain associated with corn seeds causing their deterioration and toxicity when consumed. Fungi genera found in stored grains such as *Aspergillus, Penicillin* and *Fusarium* are capable of producing toxins (Castellari et al. 2010). For example aflatoxin and ochratoxin A (OTA) toxins produced by fungi of the genera *Aspergillus* and *Penicillium*. The antifungal activities of some tropical plant extracts have been reported by several workers (Tewari and Nayak 1991; Amadioha and Obi 1999; Amadioha, 2000; Okigbo and Ikediugwu, 2000; Okigbo and Emoghene, 2004; Okigbo and Nmeka 2005). The optimum effects of *Gongronema latifolium* and moderate effects *Moringa oliefera* and *Vernonia amygdalina* on the test fungus may be attributed to the high solubility and versatility of the active ingredient in water. Dey and Harbone (1989); Weinderfield and Roder, (1991) and Singh et al. (2003) all reported that such active ingredient may either dissolve in the cytoplasm or render the fungus inactive. Compounds such as phenol, lignin, terpene and flavoids in leaf extracts are capable of penetrating the microbial walls, thus affect and complicate microbial metabolic processes causing feeding deterrent. The inhibitory effect of *Zingiber officinale* and *Gongronema latifolium* on *Aspergillus niger* (agent of post-harvest disease of cassava) has also been reported by Okoi et al. (2014). At 100% concentration, extract of *Zingiber officinale* showed the highest inhibition of mycelial growth of the fungus (97.37±1.56%) while that of *Gongronema latifolium* was 88.60±0.85%. Also, Shehu and Muhamad, (2011), reported the efficacy of *Moringa oliefera* extract in the control of onion rot caused by *A. niger* These leaves are known to contain a number of phytochemicals such as flavoids, saponins, tannis and other phenolic compounds that have antimicrobial activities (Sato et al. 2004; Cushine and Lamb, 2005; Mboto et al.

Table 2. Effect of leaf extracts on mycelial growth of *Aspergillus niger* on PDA medium

Plant material	Day						
	1	2	3	4	5	6	7
Garcinia kola	10.1	34.7	43.6	55.4	60.7	63.2	63.2
Gongronema latifolium	8.9	9.7	10.3	11.4	15.2	18.7	18.7
Moringa oleifera	9.4	10.8	20.0	32.5	37.6	40.2	41.0
Azadirachta indica	9.9	33.6	49.4	69.6	72.3	80.2	86.0
Ocimum gratissimum	9.8	11.0	24.4	44.5	59.0	65.0	70.2
Vernonia amygdalina	9.2	14.3	30.8	39.3	42.4	43.0	43.0
Control	11.3	30.8	61.8	73.2	90.0	90.0	90.0
LSD	1.0	8.5	16.02	22.4	25.4	30.2	30.8
P.value	0.043*	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***

LSD = Least significant difference, *= significant, *** = very highly significant

Table 3. Effect of concentration on the mycelial growth of *Aspergillus niger* on PDA media.

	Day						
Concentration	1	2	3	4	5	6	7
0%	9.6	24.1	34.5	47.3	60.3	73.2	86.1
2.5%	9.2	13.5	25.7	43.7	48.2	50.2	55.0
5.0%	9.6	19.5	22.2	30.5	33.2	37.1	40.2
7.5%	9.0	15.1	19.1	21.7	28.4	30.1	33.7
LSD	1.4	8.0	14.9	20.9	21.4	26.7	28.5
P.value	0.1ns	<0.001***	0.004**	0.007**	0.005**	0.003**	0.004**

LSD = Least significant difference, *= significant, ** = highly significant, *** = very highly significant

2009). This would suggest that the antimicrobial activities observed in this study could be as a result of some of these compounds present in the leaves. Although *Garcina kola, Azadirachta indica* and *Ocinium gratissimum* did not show significant mycelial growth reduction of the fungus, they have been reported by some authors to show fungicidal effects or reduction in toxin production on other fungi. Akpa et al. (1991) and Mondali et al. (2009) reported a significant inhibitory property of *A. indica* extracts on mycelia growth of *Collectotricum graminicol* and selected fungal species, whereas Razzaghi-Abyaneh et al. (2005) observed that the main feature of neem extracts, particularly those derived from leaves, is that they do not retard fungal growth, but appear to interfere with aflatoxin production. On the other hand, Gupa et al. (2014) observed antifungal effect of leaf extracts of *Ocimum sanctum* on *A. niger, Curvularia lunata* and *Rhizopus* at 40% concentration.

CONCLUSION

Based on the result obtained from this study, there is contamination of maize grains across the sampled markets; *Aspergillus niger* is the most prevalent fungus, which is known to produce ochratoxin. The different leaf extracts used especially *Gongronema latifolium, Vernonia amygdalina* and *Moringa oleifera* had different levels of potency against the growth of *A. niger* and could be used as alternatives to synthetic chemicals. Fungicides producing companies can exploit the active ingredient of these plant materials and incorporate them in the production of plant based fungicides. Given its accessibility and low cost, *Gongronema latifolium* could be considered as part of a sustainable integrated disease management strategy. Although, some of the plant materials showed low fungal growth inhibition, their evaluation on toxin production needs to be tested.

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