ISOLATION AND IDENTIFICATION OF FUNGAL ASSOCIATION WITH STORED MAIZE GRAIN IN AFIKPO

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ABSTRACT

The study was aimed at isolating and identifying fungi associated with the storage of maize grain in Afikpo, Ebonyi State. Maize grains were purchased from different storage centres and market in Afikpo town. A total of five different fungi – Rhizopus spp, Mucor spp, Penicillium spp, Fusarium spp and Aspergillus spp were isolated from the samples collected. The frequency of the isolates reviewed that Aspergillus spp had the highest frequency of 33.3% followed by Fusarium spp, 21.4%, then Penicillium spp 19.0% while the least frequency of 11.9%, was Rhizopus spp. The thin-layer chromatography method was employed in detecting mycotoxins production, which, are secondary metabolites of molds with adverse effects on humans, animals, and crops that result in illnesses and economic losses. The result showed that all the fungi isolated produced more than one toxins; this include the identified aflatoxin B1, zearalenone and fumonisin B1. Factors influencing the presence of fungi and mycotoxins in foods or feeds include environmental conditions related to storage. Other extrinsic factors such as climate or intrinsic factors such as fungal strain specificity, strain variation and instability of toxigenic properties also play a part.

INTRODUCTION

Maize scientifically called *Zea mays* is one of the most important cereal food crops. Krishnamurthi (2004), opined that its grains are important for the production of oil, starch and glucose. It is an important component of both human and animal diet. During storage, grains undergo quantitative and qualitative losses. The losses occur mainly because of improper storage. A large number of pathogenic fungi, bacteria, viruses and insects infecting maize grain cause combined worldwide annual losses of 9.4% (Shurtleff, 2000). Fungi affect the quality of grain through increase in fatty acid reduction in germination, mustiness and finally spoilage of grain. The importance of fungi is also due to production of toxins that causes health hazard in human and animals (Hiscocks, 2001). Fungal development in grains is influenced by temperature humidity and period of storage.

The contamination of maize grains by mould and mycotoxin are very frequent. Jay (1998) observed that this contamination could lead to nutrient losses and detrimental effect on animals and production. Drought, humidity, temperature, insect, infestation and rough handling have been suggested as factors which contribute to the presence of fungi and subsequently toxins in agricultural products. Fungal spoilage of corn reduces the nutritional value and palatability of the feed, thereby

Mycotoxicoses is a disease of animal and humans following consumption of feeds and foods invaded by fungi that produce toxic substances called mycotoxins (Agrios, 1978; Moss, 1989). Some common Mycotoxicoses caused by common and wide

spread fungi such as *Aspergillus*, *Penicillium, Fusarium* and starchy botry's result in severe illness and death.

Fungal spoilage of maize grains reduces the nutritional value and palatability of the feed, thereby increasing its allergic potential and may result in mycotoxic contamination (Scudamore and Livesay, 1998).

Jay (1998) remarked that some fungi species particularly *Aspergillus fumigatus* present in maize have been linked to mystic infection of cattle. However, corn grows in ears, each of which is covered in rows of kernel that are protected by the silk like threads called corn silk. Maize comes in a host of different varieties of colors. Pitt and Hocking *et al.*, (2009) noted that there are red, pink, purple and blue corns (maize).

Infected cones show visible signs. These signs help in the identification of infected maize. Fuzzes, powders and slimes of white, black, green, orange, red and brown colors are signs of silently invading, acidifying, fermenting, discoloring and disintegrating microbes that render commodities unpalatable and unsafe (Pitt and Hocking, 2002).

Maize (*Zea mays*) is a grain grown by farmers in various parts of the world especially in Africa. Its spoilage could limit its availability to consumers thereby causing economic waste to the farmers. The aim of this research was to isolate and identify fungi associated with the spoilage of maize grains in Afikpo, Ebonyi State, where its cultivation goes back to prehistoric time.

MATERIALS AND METHODS

MATERIALS

Colony counting chamber, Incubator, Autoclave, Refrigerator, Hot air Oven, Wire loop Bunsen Burner, Test tube rack, staining Rack, Cotton Wool, Spatula, Beakers, Electronic Weighing Balance, Washing bottles, Swab sticks, Slides, Cover slip, Glass rod, Conical flask, Aluminum Foil, Petri-dishes, Pipettes and Test tubes.

REAGENTS/MEDIA

Potato dextrose agar (PDA), methyl red indicator, phenol red, distilled water, peptone water, ethanol, lactorphenol cotton blue and urea.

SAMPLE COLLECTION

The samples of stored maize grain were collected from different sellers in Afikpo market and were brought to the microbiology laboratory of science laboratory technology.

SAMPLE PREPARATION

With the aid of sterile forceps, the spoilt corn samples were removed from the cob and about 10g of it was weighed and soaked in 90ml of phosphate buffer saline and was vigorously shaken to homogenize. Ten fold serial dilutions of the sample were prepared.

MEDIA PREPARATION

Potato dextrose agar for isolation and identification was aseptically prepared according to the manufacturer's instruction and autoclaved for 15 minutes.

INOCULATION

Serially diluted sample was used to inoculate the prepared media using pour plate method. The agar plates were allowed to solidify and placed in an inverted position for 7 days at 25^0C, thereafter, their colonies were observed.

IDENTIFICATION OF ISOLATES

Colony morphology

A drop of lactophenol was placed on a clean microscopic slide. A small portion of the isolate was placed in the drop of lactophenol (LP) and suspended. A clean cover glass was placed over the suspension and observed microscopically.

Spore Staining

A smear of the isolate was made on a clean, grease free microscopic slide, placed and heat fixed and placed over a steaming water bath and placing of the blotting papers over the area of the smear without sticking out past the edges of the slide. The blotting paper was then saturated with 5.6% solution of malachite green and steamed for 5 minutes. Following this, the slide was cooled to room temperature and then rinsed thoroughly with tap water. Safari was then applied for one minute and rinsed briefly but thoroughly before blotting dry. It was then examined microscopically.

Motility Test

The motility test was determined by transferring a small drop of live isolates to the centre of a slip of a depression slide using petroleum jelly or 2-3 drops of peptone water with growth of the

organism replaced on a clean slide with wire 100p. Then cover slip was placed over the slide, the slide was left for sometime and then examined microscopically with the high power objective. Motile organisms were seen swimming around.

BIOCHEMICAL IDENTIFICATION OF ORGANISM

(i) Carbohydrate Assimilation Test

Filtered and sterilized carbohydrates were added to the medium at concentration of 1% while the HCl. 2ml of the media were dispended into 10ml test tube. The tubes were also inoculated with isolated at 20°c for 14 days. A change in the colour of the medium of orange and yellow were taken as positive result. A change to pink or purple was considered negative result.

(ii) Amino-Acid Assimilation Test

Medium preparation and indication were as described for the carbohydrate assimilation test. 10mm test tubes containing 2ml of the media were inoculated with the isolate and control tubes for each fungus and amino-acid. Also tubes were incubated at 20°c for 14 days. A change to pink or purple was considered positive result while a change in colour of the medium to orange was taken as negative result.

(iii) Lipase Activity Test

At 121T^{0}C for 10 minutes, the medium of 0.5% peptin, 0.3% yeast extract and 1.0% agar were autoclaved. It was then filtered and dispensed into sterilized test tubes. Isolates were inoculated into the surface of the medium and incubated at 20°C for 7 days.

Positive result is an indication of formation of clearance zone in the medium.

(iv) Hydrolysis Test

The basal medium was similar to that of the amino acid assimilation test with addition of 0.05mg milk and 1.2 mg agar.

After autoclaving at 110°c for 30 minutes, the medium was poured into the Petri-dish. Isolates were inoculated at the centre of the plate and incubated at 20°c for 14 days. The appearance of a clear zone around the fungal colony was taken as a positive result.

FUNGAL IDENTIFICATION

With reference to De Hoog *et al.*, (2000); Jay (1992), the isolates were identified using their cultural and morphology characteristics.

Percentage Frequency (%) of Visible Colonies

Calculation of percentage of isolates is gotten from the formula

$$\text{Percentage frequency (\%)} = \frac{X}{Y} \times \frac{100}{1}$$

Where: X = number of particular isolate

Y = total number of isolates

RESULT

The fungi isolated from the maize grain samples together with their frequency of occurrences are presented in tables. The isolated organisms are *Mucor* spp, *Aspergillus spp, Rhizopus spp* and *Penicillium spp* and *Fusarium spp.*

TABLE 1: Cultural, Morphological Characteristics and Identification

The isolation show five genera of fungi namely *Rhizopus spp, Mucor spp, Penicillium spp, Fusarium spp* and *Aspergillus spp*

(Table 1).

Isolates	Cultural characteristics	Morphological characteristics
Rhizopus spp	Large fluffy white milky colonies which later turns black as culture ages.	Non-septate hyphal with up right *sporagioshere* connected by stolon and rhizoids, dark pear-shaped *sporaregium* on hemispherical columella.
Mucor spp	Cream white/large fluffy white colonies almost covering the covering the whole surface.	*Sporangium* comes out directly from the hyphal without stolon or rhizoids collumella.
Penicillium spp	Large fluffy white colonies almost covering the whole surface.	Non–septate branched hyphal enlarge at the apex to form *cornidophorex* they produce brownish black *ceridia* in chains.
Fusarium spp	Rapidly growing wooly to cottonly lemon and yellow	Multicellular distinctive sickle shaped macro *coniclia.*
Aspergillus spp	Very common colours of colony (black and white)	*Conidia* borne in 360 arrangements covering the upper 2/3 of the *conidiophores.*

Isolates	Carbohydrate assimilation	Spore formation	Amino acid assimilation	Motility	Hydrolysis
Lipases activity					
Mucor spp	+	+	-	-	-
Rhizopus spp	+	+	+	-	-

Penicillium spp	+	-	+	-	-
Fusarium	+	-	+	-	-

Key: + = positive; - Negative

The isolated organisms showed that Fusarium, *Penicillium specie* and *Aspergillus species* are positive for carbohydrate assimilation, lipase and amino acid tests. Rizopus specie showed positive results for Carbohydrate assimilation, Spore formation and Amino acid assimilation tests. While *Mucor specie was* positive for Carbohydrate assimilation and Spore formation texts (table 2).

TABLE 3: Frequency of Visible Colonies

Isolates	(X) Frequency/Number of Occurrence	Frequency (%)
Rhizopus spp	5	11.9
Mucor spp	6	14.3
Penicillium spp	9	21.4
Aspergillus spp	14	33.3
Total (Y)	42	

Frequency of Visible Colonies indicates that *Aspergillus species* (33.3%) was the highest number of isolate among the five fungi genera observed, which goes to indicate involvement of this specie in spoilage of stored grains (table 3).

DISCUSSION

Five genera of fungi viz.: *Rhizopus spp, Mucor spp, Penicillium spp, Fusarium spp* and *Aspergillus spp* were identified to be responsible for the spoilage of maize grains in Afikpo and they were identified by the cultural and morphological characteristics (Table 1).

The isolates showed varying biochemical characteristics in the course of their biochemical identification. *Fusarium, Penicillium specie* and *Aspergillus species* are positive for carbohydrate assimilation, lipase and amino acid tests. *Rizopus* specie showed positive results for Carbohydrate assimilation, Spore formation and Amino acid assimilation tests. While *Mucor specie* was positive for Carbohydrate assimilation and Spore formation texts (table 2).

The frequency of visible occurrence of the individual isolates indicates *Fusarium* to be 21.4%, *Penicillium specie* 19.0%, *Aspergillus* 33.3%, Rizopus *specie* 11.9% and Mucor *specie* 14.3% (Table 3).

The isolation of *Rhizopus spp, Mucor spp, Penicillium spp, Fusarium spp* and *Aspergillus spp* in this study agrees with the work of Onyeze *et al.,* (2013) in their study of "isolation and characterization of fungi associated with the spoilage of corn (*Zea mays*) in Enugu." Also the presence of these fungi (table 1) is in line with the works of Amadi and Adeniyi (2009) who isolated *Rhizopus spp, Penicillium spp, Fusarium spp* and *Aspergillus spp.* in their study.

However, the frequency of visible occurrence as recorded in this present study is in line with the works of many authorities. The percentage of *Aspergillus spp* 33.3% as recorded in this work (table 3) is greater than that observed by Onyeze *et al.,* in 2013, who had the percentage of occurrence of *Aspergillus spp at* 9.09%. The percentage of *Fusarium spp* 21.4% as recorded in

this work is less than that recorded by Onyeze *et al.,* (2013) who recorded a percentage of 36.4%.

The presence of these isolates will not be unconnected with the fact that this important food stuff is poorly stored leading to its contamination either from the environment or handlers. This position is in line with the works of Onyeze *et al.,* (2013) when they opined that the conditions to which corn is exposed in the field and store, as well as the storage method used to preserve it have effects on the type, rate, and extent of infection of the corn by fungi. However, the rate and degree of spoilage has been shown to be higher under moist or high humidity conditions (Onyeze *et al.,* 2013).

There is need for caution in the storage and use of this all important food stuff as its contamination with these organisms implies health risk on the consumers of this maize. This position concurs with the views of Onyeze *et al.,* (2013) when they noted that all the fungal organisms identified, characterized, and isolated in their study are capable of causing death to man and animals resulting from mycotoxins which they are capable of producing. Harrigan *et al.*, (1988) buttressed that fact when they observed that insufficient drying and precarious condition of storage could promote macor growth as mucor genera need water for growth.

Moreover, all the fungi organisms identified, characterized and isolated in this study are capable of causing toxins which they are capable of producing. Also, the result of this study shows

that the conditions to which corn is exposed in the field and store, as well as the storage method used to preserve it, have effects on the type, rate, and extent of infection of the corn by fungi.

Most of the organisms isolated in this work are cable of producing mycotoxins as identified in *Fusarium spp. and Aspergillus spp.* This finding is in consonance with the work of Larone (1998) who noted that *Fusarium spp.* includes diverse producing toxigenic lineage in maize and derived products. Agrios, (1978) opined that aflatoxin is about the most popular and widespread mycotoxins produced by species of *Aspergillus*. Mycotoxins are secondary metabolites produced by filamentous fungi which may contaminate food, feeds or raw materials used in producing them. By this implication, the consumption of any of these contaminated maize grains will pose health risk to the unsuspecting consumer. This position is in line with the position of Ciegler and Bennett (1980), who observed that mycotoxins have been implicated as causative agents of different human and animal health disorders. Amadi and Adeniyi (2009) concluded that both the toxigenic fungi and the mycotoxins they produce are potential problem for both health and economic perspectives.

CONCLUSION

Conclusively, maize may be infected by myriad of fungi which produce dangerous mycotoxins that posse health risk to its consumer.

RECOMMENDATION

(i) Maize should be properly dried before storage so as to minimize fungi infection which is harmful to both man and animal.

(ii) There is need for pre-harvest treatment of maize so as to reduce the rate of infection and spoilage by fungi.

(iii) There is need to avoid physical damage on the maize as this will not only reduce the economic value but also act as vehicle for the transmission of mycotoxins.

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