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Identification of Fungi Associated with Food Spoilage in Powdered Pepper (*Capsicum frutescens* L.) from Nigeria: A Case Study of Yaba Market

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ABSTRACT

Fungal contamination of pepper results in economic hazard and losses and changes in the taste and quality of foods when applied. This study is aimed at isolation and identification of fungi in powdered pepper sold in the market. Dry pepper samples were collected from fifteen different points of sale at Yaba market in Lagos, Nigeria and were treated in a stomacher for homogenization and serial dilution of 1:9. Isolation of fungi from the powered pepper was done by inoculation of 1mL from serial dilution of 10^{-6} on MEA (Malt Extract Agar) and CYA (Czapek Yeast Extract agar) plates and incubated for five days at 25^0C. Identification of the fungi was carried out by examining colony colour, hyphae, shape and arrangement of spores. Molecular identification of fungi to species level was carried out by DNA extraction of fungi, followed by Polymerase Chain Reaction (PCR) to prepare samples for sequencing in GenBank National Center for Biotechnology Information (NCBI). Agarose gel was used to assess DNA quality under UV light (PCR gel bands). The PCR products sequenced were analyzed first by electropherogram then compared with sequences in the GenBank (NCBI) and MycoBank blasted for highest probability, highest query cover and percentage identity matching EF (Exact Matches Found) and ITS (Internal Transcribed Spacer). Results show that *Aspergillus niger* was prevalent in all the samples. *Aspergillus niger* as a contaminant can cause economic hazard and losses by causing change of colour to the powdered pepper, making it difficult to sell and can also cause changes in the taste of foods when added as condiment.

Keywords: Pepper, market, fungi, DNA extraction, molecular identification, *Aspergillus niger*, GenBank, MycoBank

Introduction

Peppers are members of the Solanaceae family and are grown as annuals in commercial cultivation and is used not only as culinary but also as medicinal herbs (as it contains phytochemicals) to treat cough, toothache, sore throat, parasitic infections, rheumatism and wound healing (Ragunathan *et al*., 2021). Peppers contain lycopene and are made up of 80% water, 9% carbohydrate, 2% protein and 0.4% fat with a rich source of Vitamin C and Vitamin B_6.

Origin of cultivation of Peppers dates back to 6,000 years ago in East to Central Mexico (Kraft *et al*., 2014) although research by Kathrine and Christine (2014) in New York Botanical Gardens Press reported that chili peppers were first cultivated across different locations in Americas, Bolivia, Central Mexico and in the Amazon, but now they are commonly used across the world (Kraft *et al*., 2014).

Mode of preservation of pepper by the small scale farmers is by sun drying in open spaces or on the bare soil, but industrially preservation can be carried out by gamma γ-irradiation which has been applied to treatment of dried and milled pepper against toxin decontamination (Rahman, 2021) and microbial growth (Manivannan and Muthukumar, 2017). Gamma γ-irradiation has been used also to reduce colour and quality of the pepper during storage and to control emergence of mold growth and mycotoxins (Calado *et al*., 2014) and to extend shelf-life of peppers (Owoade and Ademola, 2014).

A lot of Fungi are associated with crop spoilage which includes *Aspergillus, Rhizopus, Penicillium, Alternaria, Fusarium, Mucor* and *Trichoderma*. Pepper is made up of 80% water in composition, which makes it susceptible to fungal disease during harvest, transportation of the fruits and mode of sales (packaging).

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Most of the products do not undergo proper phytosanitary conditions during drying on bare soil. Milling of the pepper in commercial industrial blenders can be a source of contamination and also during packaging to the end user (consumer).

Food products can become contaminated in the food chain process from production to consumption (Thakur and Kniel, 2018). Contamination can have diverse effects on small- and large-scale farmers economically and make the crop susceptible to potential mycotoxinogenic species (Aziz *et al*., 1998). In Nigeria, milled/powdered peppers are exposed in different sizes of plastic bags and bowls used for selling this product and are sold by measuring the desired quantity of the buyer, exposing the pepper to dust and other contaminants.

Although many species of *Fusarium*, *Aspergillus*, *Penicillium*, and *Alternaria* are known as plant pathogens, they are also sources of mycotoxins affecting animals and human health (Placinta *et al*, 1999). Miller (1995) categorized pathogenic fungi into two classes: fungi that affect the crops before harvest "field fungi or plant pathogens" and fungi that affect crops after harvest "storage or saprophytic fungi". Further Identification of the fungal infection using modern molecular techniques -- Polymerase Chain Reaction (PCR) amplification and Sequencing of PCR products are employed, as it is more reliable than the microscopy identification (Hariharan and Prasannath, 2021).

Peppers, deeply rooted in Nigerian culinary practices, face the persistent threat of pathogenic fungal contamination, leading to food spoilage. This study focuses on *Capsicum frutescens* L., known locally as "Ata wewe," aiming to unravel the intricate dynamics between this staple food condiment and prevalent fungi in powdered pepper sold in Yaba Market. The unique challenges posed by the Nigerian market, coupled with the cultural significance of pepper, necessitate a nuanced exploration of the identified fungal species and their implications.

Materials and Methods

Sample Preparation

Powdered pepper samples from fifteen different points of sales in Yaba Market were aseptically collected and stored in sterile bags, which were taken to the laboratory in University of Nebraska, Lincoln, USA for analysis.

From each of the fifteen bags, pepper powder samples of 25g were weighed out and dissolved in 225ml of 0.1% peptone water in sterile bags and homogenized in a stomacher for 2minutes. From each sample of homogenized 10^{-1} dilution, an aliquot (1.0 ml) was measured into 9ml of diluent (0.1% peptone in water) to make a serial dilution of 10^{-2} to10^{-7}. An aliquot 0.1 ml of the 10^{-6} dilution was pipetted and plated onto freshly prepared sterile CYA (Czapek Yeast Extract agar) and MEA (Malt Extract Agar) agar in Petri dishes. The spread plate technique was employed for the enumeration by spreading the inoculum evenly with a Jockey stick. Cultured plates were incubated at 25^{o}C for 3 days. Subculturing of fungal colonies which developed was carried out using sterile inoculation pins for the three-point inoculation and incubated at 25^{o}C for 3 days. Diluent used for homogenization and serial dilution was 0.1% Peptone water.

Fungal Identification

Pure cultures obtained were identified using Pitt and Hocking Keys, comparing growth patterns in the different media (Best growth) taking note of medium of growth, color of colony, diameter of colony, days of growth, morphology including stipe, conidia color, shape of colonies (branched or unbranched) were considered which linked to the inferred fungus and species.Further identification process was the molecular methods of identification using DNA extraction of the fungal molds as this provided a deeper understanding of the genetic makeup of the isolated fungi (White *et al*., 1990).

Extraction of Fungal DNA

DNA of the fungal isolates from milled pepper was extracted using the protocol of DNA^{TM} Fungal Kit (Zymo Research group, California, USA). DNA quality was further quantified using the Agarose gel electrophoresis performed according to the modified method of Saghai- Maroof *et al*. (1984). DNA was amplified and sequenced at the Molecular Biology Center of the University of Nebraska, Lincoln, U.S.A. The DNA sequence files were aligned using the Basic Local Alignment Search Tool for Nucleotides (BLASTN) of the National Center for Biotechnology Information (NCBI) Data Base. Each sequence was analyzed. First, the electropherogram was analyzed to determine the quality and size of the sequence. The purity of the extracted DNA was assayed with a Spectrophotometer (Model: ND-2000 Thermo Scientific, Wilmington, DE, USA).

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The sequence was copied in the GenBank and Mycobank webpages and blast used. The one with the highest probability, highest query cover, and percentage of identity was accepted. Each isolate gene provided copy was taken with the best sequence and sequences should be long as short ones do not give good results.

Results

The *Aspergillus niger* colonies from some isolates using the three point inoculum method are shown in Plate 1.

Plate 1: *Aspergillus niger* Colonies from some Isolates using the Three-Point Inoculum Method

From the morphological identification, the typical colour of *Aspergillus sp.* was observed, this is attributed to production of pigments, growth was observed on CYA (Czapek Yeast Extract agar) and MEA (Malt Extract Agar) but no growth on the G25N (Glycerol Nitrate Agar) media. Since no growth on G25N, *Aspergillus wentii* was ruled out (*A. wentii* was suspected due to the closeness in colour with greyish yellow to olive).

Hyphae were conspicuous with septae under the microscopic view with mature spores as seen under the microscope. Colonies were well spread in the Petri dishes on both CYA and MEA growth media. Conidiophores were observed with distinct conidia and appeared dark in colour and unbranched as seen in Plate 1. This is characteristic of *Aspergillus* sp.

Further clarification to species level using molecular identification, for DNA sequencing which was copied in the GenBank and Mycobank webpages clarified the presence of *Aspergillus niger* in all isolates. The result of the Alignment of fungal isolates sequence obtained in this study with NCBI database sequence is presented in Table 1. It shows the Basic Local Alignment Search Tool for Nucleotides (BLASTN). Least Isolate sequence was 99.69% and some were 100% identical to *Aspergillus niger*. From the analysis on sequence alignment, there is a marked relationship between isolates from this study and other comparable fungal isolates from GenBank.

Table 1: Alignment of Fungal Isolates Sequence with NCBI Database Sequence

Isolate code	Gen	Identification	Access code	Strain	Authors	Title	Journal	Country	Source of isolate	Query cover%	Evalue	% ID	Sequence
CI-A1	EF	*Aspergillus niger*	XM_02560	CBS 10188	Vesth,T.C et al	The genomes of	Not published	Sumatra, Indonesia	*Coffea robu*	100%	0	99.79%	>XM_025601974.1 Aspergillus
CI-A2	ITS	*Aspergillus niger*	OQ726570.1		Abu-Hussien,S. and Ahm		Submited	Cairo, Egypt	Oil paint Agricultura	100%	0	100%	>OQ726570.1
CI-B1	EF	*Aspergillus niger*	XM_02560	CBS 10188	Vesth,T.C et al	The genomes of	Not published	Sumatra, Indonesia	*Coffea robu*	100%	0	99.79%	>XM_025601974.1 Aspergillus
CI-B2	ITS	*Aspergillus niger*	XM_02560	CBS 10188	Vesth,T.C et al	The genomes of	Not published	Sumatra, Indonesia	*Coffea robu*	100	0	99.69	>XM_025601974.1 Aspergillus
CI-C1	ITS	*Aspergillus niger*	OQ726570.1		Abu-Hussien,S. and Ahm		Submited	Cairo, Egypt	Oil paint Agricultural Museum in Cairo, Egypt	100%	0	100%	>OQ726570.1 Aspergillus niger TTCCGTAAGGGIGAC
CI-C2	CMD	*Aspergillus niger*	MK193878.1		Fungaro,M.H.P. and Silva,J.J.			Brazil		99%	0	99.78%	8.1 Aspergillus
CI-D1	EF	*Aspergillus niger*	XM_00139	CBS 513.88						99%	0	99.90%	98905.2 Aspergillus
CI-D2	EF	*Aspergillus niger*	XM_00139	CBS 513.88						99%	0	99.90%	>XM_001398905.2 Aspergillus
CI-E1	CMD	*Aspergillus niger*	MK193878.1		Fungaro,M.H.P. and Silva,J.J.			Brazil		99%	0	99.78%	8.1 Aspergillus
CI-E2	EF	*Aspergillus niger*	XM_02560	CBS 10188	Vesth,T.C et al	The genomes of	Not published	Sumatra, Indonesia	*Coffea robu*	100%	0	99.79%	>XM_025601974.1 Aspergillus

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Discussion

In this study, dominance of *Aspergillus niger* in all isolates can be related to the works of Ng *et al*. (2011) who reported that *Capsicum frutescens* pepper seeds produce lectin which is able to inhibited spore germination and hyphal growth of *A. flavus* and *Fusarium moniliforme.* This may be a probable reason for the absence of other toxinogenic causing fungi of *Aspergillus* infections which contaminates stored grains when prevailing abiotic factors (such as temperature and water activity) are favourable. Interactions between environmental stress factors, such as water activity and temperature, may have an influence on growth, expression of biosynthetic regulatory genes and mycotoxin production by mycotoxigenic fungal species. *A. niger* dominance aligns with previous research findings, underscoring its concerning association with peppers and other spices in Indonesia and the Phillipines on the high side (Pitt and Hocking, 2022).

The repercussions of pepper contamination extend beyond economic implications. The contamination of food products in the production-to-consumption food chain process has diverse effects on both small and large-scale farmers. It renders crops susceptible to potential mycotoxinogenic species, adding a layer of complexity to food safety concerns (Yogendrarajah *et al*., 2014). *A. niger* impacts on pepper quality, as contamination not only jeopardizes health but also negatively affects the quality and market value of pepper. Its presence can cause undesirable color changes (blackening) and alter taste, impacting their ability to sell the product and resulting in potential economic losses and making the product less appealing to consumers.

Though *Aspergillus niger* is generally recognized as safe (GRAS) and is commonly used in industrial and in food processing, but consumption of large amounts can lead to gastrointestinal discomfort and can lead to respiratory issues as well as affect people with compromised immune systems (Cinar and Onbasi, 2019). Although *A. niger* holds a GRAS status, however, two of 19 *Aspergillus niger* isolates were reported by Abraca *et al*. (1994) to produce ochratoxin A. Although a low percentage of *A. niger* has been reported to produce ochratoxin A however, 20% of 150 *A. niger* strains studied by Leong *et al*. (2006b) and Frisvad *et al*. (2011), produced ochratoxin A.

The observed prevalence of *Aspergillus niger* emphasizes the need for vigilant monitoring and control measures throughout the pepper production and distribution chain as a closer examination of the interplay between environmental stress factors and fungal contamination. The Nigerian climatic condition is characterized by high temperatures and humidity levels, which enhances an environment conducive to fungal proliferation as was also observed by Makhlouf and Boudjenah (2012) in Algerian spices.

According to FAO (2015) Proper drying techniques by rapid drying under appropriate temperature and humidity conditions can prevent fungal growth. Hygienic storage practices by storing pepper in clean, dry, and pest-free containers can further minimize contamination risks and also regular market surveillance and monitoring of spices is crucial for ensuring food safety and consumer protection.

In conclusion, this study revealed that *Aspergillus niger* is a wide spread fungal disease associated with post-harvest diseases of milled /powdered *Capsicum frutescens* L.in our markets, which affirms that *Aspergillus niger* is a pervasive fungal disease associated with post-harvest contamination of milled/powdered *Capsicum frutescens* L. in Yaba market.

Although *Aspergillus niger* is commonly used in the industry and food production, phytosanitary storage conditions during drying and storage of the pepper products have to be carried out to minimize the risk of mycotoxin contamination.

This can be carried out by good postharvest practices avoiding high temperatures by avoiding storage in polythene bags and also rapid drying of crops can avoid a decrease in the pepper quality and reduce the health risk due to mold growth and regular monitoring and adherence to quality control measures to guarantee safety of the final product to the consumers and enhanced food security.

Also, knowledge of the environmental stressors is important for carrying out targeted interventions to control fungal contamination, using the local climate and market conditions, which can better enhance the resilience of the pepper supply chain against fungal threats in the relationship between environmental factors and fungal dynamics.

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