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Isolation and Identification of Fungi Associated with Contamination of Locally Processed Rice

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Abstract: Rice is the seed of the grass family *Oryza sativa* (Asian type) or *Oryza glaberrima* (African rice). As a cereal grain, it is the most widely consumed staple food worldwide, especially in Asia. It is the agricultural commodity with the third-highest worldwide production after sugarcane and maize. Current work isolated and identified the fungi associated with contamination of locally processed rice at the Rimi market in Kano Municipal Local Government, Kano State, Nigeria. Seven samples of rice grains were collected from the Rimi market and transported to the Microbiology laboratory of Kano University of Science and Technology, Wudil, in a sterile polyethylene bag for analysis. Five grains of each sample were placed on Petri dishes containing Sabouraud Dextrose Agar (SDA) media. The plates were subsequently incubated at room temperature for 5 days. The isolates obtained from the plates were *Aspergillus flavus* (22), *Aspergillus niger* (16), *Candida albicans* (30), *Cladosporium spp*. (9), *Fusarium oxysporium* (14), *Mucor spp.* (10), *Penicillium notatum* (12), and *Rhizophus stolonifera* (8). Based on the findings, *Aspergillus*, *Fusarium*, and *Cladosporium* spp. are among the most important genera of mycotoxigenic fungi.

Keywords: Aspergillus; Fusarium; mycotoxin; identification; *Oryza sativa*; locally processed; rice.

1. Introduction

Rice is an essential staple food crop cultivated and consumed by people from all walks of life [1-3] and ranks as the third-highest globally in production, after sugarcane and maize [4,5]. Its global consumption is expected to be 503.9 million tonnes (milled basis) [6], of which 80.5 percent is used for food, resulting in a per capita food intake of 53.9 kilograms [7,8]. Rice is farmed almost everywhere, including south of the Sahara and around the world [9]. Cereals encompass half of the world's arable land, with rice accounting for one-fifth of the same [10]. Rice is the most common staple in the tropics and one of the most cost-effective sources of calories and protein [11]. In 2009, rice met more than 20% of the daily calorie needs of more than 3.5 billion people in Asia and Sub-Saharan Africa (SSA) [12]. More than 50% of the energy needs of more than 520 million people in Asia and SSA [13,14], the majority of whom are in the poor-to-very poor strata [15]. Nigerians eat polished rice imported from Thailand and other rice-producing countries worldwide [16]. This is because it has been thoroughly cleaned, whereas the local variety requires homemakers and other rice consumers to pick out the stones and blow off the chaff before cooking [17], resulting in excessive labor for them [18]. Due to low yields in rice cultivation, demand for rice has been high in Nigeria and many other parts of the world for many years [19]. Since 1980, Nigeria has developed to become the third-largest rice producer in West Africa and Africa [20], behind Egypt and Madagascar [21]. Despite these advancements, poor processing techniques and gear have resulted in significant quality losses in rice processing [22].

Fungi are frequent plant pathogens that cause significant food and feed spoilage [23]. Rice, like other cereals, is vulnerable to mycotoxins in the fields and during storage [24]. An appropriate culture medium serves as a good substrate for fungal growth and toxinogenesis, allowing testing of isolated strains' toxigenic potential [25,26]. Mycotoxins such as aflatoxins, ochratoxin, deoxynivalenol [27-31], and fumonisins have been found in these basic cereals in several studies [32]. Ali *et al*. [33] observed that *Bipolaris oryzae* was the most predominant fungus, which was associated with 82.08 percent of seed samples [33], followed by *Alternaria padwickii* (63.36%), *Curvularia lunata* (46.08%), *Pyricularia oryzae* (44.64%), *Alternaria alternata* (34.56%), *Fusarium moniliforme* (27.36%), and *Curvularia pallescens* (21.6%), while *Aspergillus flavus* and *Curvularia oryzae* had an incidence of 15.84% [34,35]. Infection of plants by various fungi reduces crop yield and quality and contaminates grains with dangerous fungal secondary metabolites known as mycotoxins, resulting in major economic losses [36].

2. Materials and Methods

2.1. Study area.

Rimi Market is a sprawling open-air market known for discounted household items, including foodstuffs, fruits, vegetables, peppers, etc. This market is an excellent place to purchase locally woven materials, foodstuffs, local textiles, dyed materials, sculptures (mostly of animals like giraffes, elephants, ducks, etc.), carved stones, silver jewelry, beaded jewelry, jewelry boxes, leather shoes, bags, wallets, stuffed animals, and lots of goodies. The Local Rimi market is located in Kano Municipal LGA, along Murtala Muhammed Specialist Hospital, Kano, Nigeria.

2.2. Materials and reagents used.

Sabouraud dextrose agar (SDA), Distilled water, Autoclave, Weighing balance, Petri dishes of 100x15mm dimension, Colony counter, Bunsen burner/ spirit lamp, Cotton wool, Incubator, Masking tape, Marker, Beaker, Glass slide, Measuring cylinder, Wire loop, Spatula, Conical flask, Droppers, Glass slide, Coverslip, Microscope, and Hand gloves.

2.3. Sample collection and transportation.

Seven samples of local rice varieties (Oryza sativa) were collected in sterile universal containers from various locations at Rimi market and promptly transferred to the microbiology laboratory of Kano University of Science and Technology, Wudil, for analysis.

2.4. Methods.

Samples were collected from the Rimi market for the isolation and identification of fungi associated with rice contamination. A compendium of methods for the microbiological analysis of foods, as described by the American Public Health Association (APHA), was used.

2.5. Preparation of Sabouraud Dextrose Agar Medium (SDA).

This was prepared by suspending 65g of the powder in 1 L of distilled water. The mixture was allowed to soak for 10 minutes, then swirled to mix thoroughly. It was then sterilized at 115°C for 15 minutes, then cooled at 47°C. It was then supplemented with an antibiotic (Chloramphenicol solution) at a 1:25 ratio to suppress bacterial growth.

2.6. Culturing of fungi.

Fungal isolation from collected rice samples was carried out using the direct plating method. Five rice grains were randomly inoculated into each Petri plate containing SDA medium. An antibacterial agent, Chloramphenicol (50 ppm), was used to inhibit the growth of bacteria. Petri plates were incubated at 27±2°C for 6-7 days and examined daily for fungal growth. Fungal colonies grown on inoculated samples were counted and subcultured on SDA for identification. Morphological and cultural characteristics of the growing cultures were evaluated for preliminary identification. Then, fungal colonies were subjected to microscopic identification [37].

2.7. Sub-culturing.

A pure culture of each colony type was obtained and subsequently maintained. The maintenance was performed by subculturing each colony onto SDA plates and incubating at room temperature for 5 days. In sub-culturing, the baiting method was used, which involves using a sterile wire loop to pick up the culture growth and then transferring it to the subculture media; after which, the Petri dishes were labeled accordingly.

2.8. Identification of fungi.

The mycelial structure techniques were studied [38] for the identification of unknown isolates using direct microscopy. The identification was achieved by placing a drop of normal saline on a clean, grease-free slide with a wire loop, then removing a small portion of mycelium from the fungal culture and placing it on the drop of normal saline. The mycelium was spread very well on the slide with the aid of a wire loop. A cover slip was gently applied with light pressure to eliminate air bubbles and prevent overflow. The slide was mounted and observed with 10X and 40X objective lenses, respectively. The species encountered were identified following the method adopted by [39].

The main characteristics employed in their identification are: 1. Hyphae: Septate or Non-septate 2. Mycelium: Colored or Non-colored 3. Spores: Types of asexual, nature of spores 3. Presence of special structures such as stolon, rhizoids, and foot cells.

3. Results and Discussion

Based on the results obtained in the present study (Table 1), a total of 121 fungi strains were isolated, comprising *Aspergillus flavus* (18.18%), *Aspergillus niger* (13.22%), *Candida* *albicans* (24.80%), *Cladosporium spp* (7.44%), *Fusarium oxysporum* (11.57%), *Mucor spp* (8.26%), *Penicillium notatum* (9.92%), and *Rhizopus spp* (6.61%). All identified fungi were reported to be present in all rice samples collected from Rimi market in Kano Municipal Local Government Area, though with varying frequencies (Table 2 and Figure 1).

All seven isolated samples were collected from different locations within the Rimi market and contained different fungi, indicating that fungi can survive and multiply in any environment. Also, according to the results of this research, the isolated organisms were important human-borne pathogens and caused disease, which is in agreement with the report by [39]. The macroscopic and microscopic characteristics of isolated fungi have been mentioned in Table 3.

Table 1. Fungal isolates were obtained from the rice sample.

Isolates	Sample													
	A1	A2	B1	B2	C1	C2	D1	D2	E1	E2	F1	F2	G1	G2
Aspergillus flavus	+	+	+	+	+	-	-	+	+	+	+	+	-	+
Aspergillus niger	+	-	+	+	+	+	-	+	+	+	+	-	+	+
Candida albicans	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cladosporium spp	+	-	+	-	+	+	+	-	+	+	+	-	+	+
Fusarium oxysporum	+	+	-	+	+	-	-	+	+	+	+	+	+	+
Mucor spp	+	-	-	-	+	-	-	+	+	-	+	+	-	+
Penicillium notatum	+	+	-	-	-	-	-	-	-	-	-	-	-	+
Rhizopur	+	+	+	-	+	-	+	+	+	+	+	+	-	-

(+) sign: indicate positive isolates; (-) sign: indicate negative isolates; (A1 and A2): Rice sample A plate 1 and 2; (B1 and B2): Rice sample B plate 1 and 2; (C1 and C2): Rice sample C plate 1 and 2; (D1 and D2): Rice sample D plate 1 and 2; (E1 and E2): Rice sample E plate 1 and 2; (F1 and F2): Rice sample F plate 1 and 2; (G1 and G2): Rice sample G plate 1 and 24.2

Table 2. Frequency of occurrence of fungal isolates.

Isolates	No. of isolates	Percentage of occurrence (%)
Aspergillus flavus	22	18.18
Aspergillus niger	16	13.22
Candida albicans	30	24.80
Cladosporium spp	9	7.44
Fusarium oxysporum	14	11.57
Mucor spp	10	8.26
Penicillium notatum	12	9.92
Rhizopur spp	8	6.61
Total	**121**	**100**

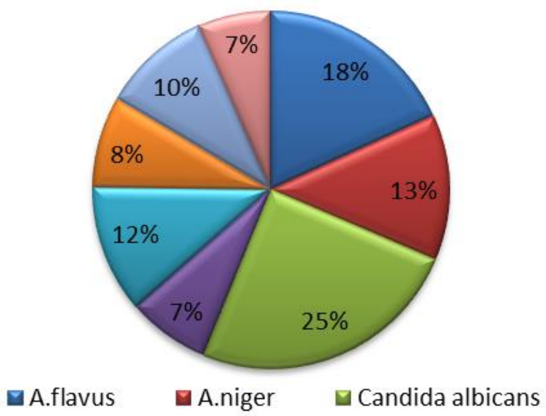

Figure 1. Frequency of occurrence of the fungal isolates.

Table 3. Macroscopic and microscopic characteristics of the isolated fungi.

S/N	Macroscopic examination	Microscopic examination	Inference
1	Velvet-like, yellow to green mold with a pale yellow on the reverse	The conidial heads radiate	*A. flavus*
2	The initial growth is white but later turns black as hyphae form. In the reverse, turning pale yellow.	Consists of smooth and colorless conidiophores and conidia. A closer look shows that the conidial head is dark brown.	*A. niger*
3	Colonies are white to creamy colored, smooth, and yeast-like.	Spherical to subspherical blastoconidia	*C. albicans*
4	Velvet-like; Colonies are slow growing and appear olive green to olive black, often becoming powdery due to the production of conidia.	Production of septate brown hyphae, erect and pigmented conidiophores, and conidia.	*Cladosporium*
5	Fast-growing colonies produce aerial mycelium that becomes white and forms discrete orange sporodochia.	Conidiospores are observed	*F. oxysporium*
6	Fast-growing colonies, cottony to fluffy, becoming dark grey.	Non-septate and broad (6-15 micrometers) hyphae are observed	*Mucor spp*
7	Fast-growing colonies in the shade of green, mostly consisting of dense conidiospores.	Chains of single-celled conidia	*Penicillium notatum*
8	Fast-growing and white cottony colonies spotted with black color	Sphorongiospores are smooth and non-septate	*Rhizopus stolonifer*

Based on the results obtained in the present study, a total of 121 fungi were isolated, comprising *A. flavus* (18.18%), *A. niger* (13.22%), *Candida albicans* (24.80%), *Cladosporium spp*(7.44%), *Fusarium oxysporum* (11.57%), *Mucor spp* (8.26%), *Penicillium notatum* (9.92%), *and Rhizopus spp* (6.61%). All identified fungi occurred at varying frequencies in all rice samples collected from the Rimi market in the Kano Municipal Local Government Area [37]. This is in line with the study of Alhendi *et al*. [40], who reported the presence of *A. niger*, *Mucor* spp, *Penicillium notatum*, *A. ochraceus*, *P. citrinum*, *A. parasiticus*, *Fusarium oxysporum*, and *Rhizopus* [39,40]. A similar study was conducted using a total of 127 durum wheat samples collected during the 2010-2012 season in Tunisia, indicating the presence of 6035 post-harvest fungal strains [41]. The most predominant post-harvest fungi genera isolates were *Alternaria* (28%), *Fusarium* (19%), *Penicillium* (19%), *Aspergillus* (14%), *Rhizopus* (7%), *Mucor* (6%), and other fungi (6%) [42].

Also, from the results obtained in this research, *C. albicans* has a high percentage of occurrence (24.80%), followed by *Aspergillus flavus* (18.18%). The results agree with those of [43], who isolated 96 Candida (50%), Aspergillus (25%), and Fusarium (25%) from 36 stored rice samples [44]. In another study, Alhendi *et al*. (2013) in Tabriz, Iran, reported that 31.5% of the samples analyzed were contaminated with fungal genera belonging to *Candida* (50%), *Aspergillus* (7%), *Penicillium* (3.5%), *Acremonium* (14.5%), *Cladosporium* (3.5%), and *Alternaria* (3.5%) [45].

According to this investigation, the organisms recovered were major human foodborne pathogens that cause disease. According to estimated figures, foodborne illness affects 5.8 million Ghanaians annually [46]. In many underdeveloped nations, the high prevalence of diarrheal diseases indicates serious food safety issues. Food handling errors are a major contributor to the spread of foodborne infections. Proper food handling, for example, is linked to 97 percent of all foodborne illnesses related to catering establishments, with Africa accounting for 90 percent of all cholera cases worldwide [47]. In Ghana, 27,000 cases were reported, with Kumasi in the Ashanti Region being the most impacted [48,49].

Furthermore, all seven samples collected from various locations within the Rimi market were discovered to contain numerous fungi, demonstrating that fungi can live and grow in any environment. Fungi are eukaryotic microorganisms that can be found in various situations when organic material is present [50]. Molds are significant in food because they can grow in environments that many bacteria can not, for example, low pH, low water activity (Aw), high osmotic pressure, and low temperature [51]. Molds, in general, may develop at pH levels between 1.5 and 9.0 and require a water activity (Aw) of 0.80 or less, allowing them to grow on partially dried surfaces (including food) [52]. Compared with bacteria and other microorganisms, molds are also less thermophilic. Molds are crucial spoilage organisms, but many also generate mycotoxins, which have been linked to food poisoning [53]. Some of these mycotoxins are carcinogenic or mutagenic, causing pathology in specific organs such as the liver and kidneys [54].

Finally, the study found that all the samples examined contained various types of fungi, most of which are key food spoilage organisms that secrete poisons. The findings agree with the research by Kellerman *et al*. [55], who suggested that mycotoxins present in low-quality feed are responsible for the bulk of animal-related diseases (e.g., chronic aflatoxicoses) on farms. Aflatoxin B1, for example, is extremely toxic and can cause cancer in both people and animals. Fumonisin B1 (FB1) is produced by *Fusarium moniliforme* and has been linked to equine leucoencephalomalacia and porcine pulmonary edema [55]. After consuming tainted corn, several illnesses were discovered in animals. Mycotoxins have caused very few human illnesses in Australia, although they have caused animal diseases. It is vital to remember that fungus can produce extremely high mycotoxin concentrations in small pockets of grain [56], contaminating large amounts of grain at levels that exceed permitted limits for domestic and export markets.

4. Conclusions

Toxigenic or pathogenic fungi can cause food deterioration by producing mycotoxins. The fungi isolated in the present investigation are known to produce secondary metabolites. Humans and animals may be exposed to these secondary metabolites. Aflatoxin, for example, has been linked to human liver cancer (hepatoma), aflatoxicosis, and acute hepatitis, particularly in developing countries. On the other hand, pathogenic fungi can cause infections and allergies. Aspergillus produces aflatoxins, which are linked to a variety of ailments in cattle and humans worldwide. Some Aspergillus species are the primary producers of the well-known carcinogenic aflatoxins, and their presence in food is a major concern for food safety, as they are dangerous even at low levels. The presence of Aspergillus in rice samples could pose a health risk if not cooked properly. *Fusarium* and *Cladosporium spp.* are two of the most common mycotoxigenic fungal genera. Thus, the rice should be well-cooked before being tossed. Because rice and other agricultural goods play a vital role in human nutrition, appropriate handling of the output from harvest to consumption is required. Farmers who harvest rice into bags for transportation, marketers, and consumers should all take care to avoid contamination and endeavor to establish an environment that discourages the growth or multiplication of microorganisms.

Author Contributions

Conceptualization, A.K. and A.K.S.; methodology, A.K. and A.K.S.; data curation, A.I.A. and V.S.; formal analysis, A.K. and A.K.S.; investigation, A.I.A. and V.S.; resources, Y.A.B.; writing—original draft preparation, I.A.K. and U.U.Z.; writing—review and editing, A.K., A.K.S., and all authors; visualization, I.A.K., U.U.Z., and Y.A.B.; supervision, A.K. and A.K.S.; project administration, A.K.; All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

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Conflict of Interest

The authors declare no conflict of interest.

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