

Isolation and Molecular Identification of Fungi in Stored Maize (*Zea mays* L) and Groundnuts (*Arachis hypogaea* L) in Ngaoundere, Cameroon

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Abstract A knowledge of the specific fungi responsible for food spoilage in an ecological zone is paramount for proper preservation. The main objective of this study was to isolate and identify at the molecular level the different fungal species from contaminated stored maize and groundnuts. Contaminated grains from local markets in Ngaoundere, Cameroon were used for the study. Their average percentage water content was 16.75 ± 0.27 and 11.24 ± 0.31 for maize and groundnuts respectively. Nine fungal strains were isolated and identified through 18S rDNA sequencing. It was found that *Rhizopus oryzae*, *Aspergillus flavus*, *Aspergillus oryzae* and *Cunninghamella polymorpha* were common in both maize and groundnuts. *Aspergillus tamari*, *Talaromyces purpureogenus* and *Penicillium citrinum* were present only in maize, while *Aspergillus parasiticus* and *Rhizopus stolonifer* were identified only from groundnuts. The novel information presented in this study will help to formulate measures to control the fungal contamination of stored maize and groundnut.

Keywords: stored grains, Fungal DNA, *Aspergillus*, *Rhizopus*, *Penicillium* and *Cunninghamella*

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1. Introduction

Filamentous molds are sometimes harmful to man: not only by causing human diseases but also through contamination and eventual spoilage of human food. From the time when primitive man began to cultivate crops and store food, spoilage fungi have demanded their tithe [1]. Food serves as a favorable medium for their growth and development. Several molds, notably the genera *Aspergillus*, *Penicillium* and *Fusarium* are known to be contaminants of agricultural produce whether stored or still in the farm and/or for their capacity to produce toxic secondary metabolites or mycotoxins [2]. Worthy of note is the fact that more than 25% of world harvests are contaminated by mycotoxins [3].

After wheat, and rice, maize is the third most important cereal grain worldwide [4] and is used as human food directly and animal food source indirectly. In Cameroon, maize and groundnuts are consumed throughout the year and as such the need for storage. In many practical situations, water activity is the dominant environmental factor governing their stability or spoilage. All micro-

organisms, including molds, require moisture to survive and multiply and growth is controlled in dried grains. Background knowledge of fungal water relations will enable prediction both of the shelf life of foods and of potential spoilage fungi. Water content plays a significant role; when grain has more moisture, it heats up and can have fungal attack [5]. The water content below which micro-organisms cannot grow is referred to as the safe water content [6]. If grain water content is too high, even the best aeration equipment and monitoring management will not help the grain from spoiling - it only delays the inevitable. In addition, climatic conditions are paramount for proper storage as fungal growth in stored grains is facilitated by hot and humid conditions [7]. Fungal infestation results in color change, decreases in nutritional values, and reduction of overall quality and quantity of the grain.

Research into fungal food spoilage and its prevention is clearly an urgent necessity: lacking in spectacular appeal, it is, however, often neglected. Worthy of note is the fact that research on the fungi which cause food spoilage can only be carried out effectively if based on accurate identification of the microorganisms responsible [1].

The objective of this study therefore, is to screen, isolate and identify at the molecular level, the major fungi strains that infest stored maize and groundnuts in Ngaoundere, Adamawa Region, Cameroon. The water content of the contaminated stored grains will be evaluated as a storage index.

2. Material and Methods

2.1. Sample (Contaminated Grain) Collection

Visibly contaminated stored maize and groundnuts were collected from four different sales point in the Ngaoundere town market in September 2014. They were then carefully put into separate sterile bags and carried to the laboratory. Bearing in mind that the objective was to isolate and identify fungi species involved in maize and groundnut spoilage, only contaminated grains were collected.

2.2. Determination of Water Content of Samples

The water content was determined according to the AOAC 925.10 (2000) method [8]. An empty weighing dish was cleaned, dried and weighed (M_0). Approximately 5g of milled sample was added into the dish and a new mass taken (M_1). The vessel containing the sample was then placed in an oven at 105°C for 24 hours. After drying, the dish was removed from the oven, cooled inside a desiccator before being weighed (M_2) again. The following formula was used to determine the water content:

$$\text{Water Content (WC)} = \frac{M_1 - M_2}{M_1 - M_0}$$

2.3. Isolation and Identification of Fungal Species

2.3.1. Enumeration

The standard protocol [9] recommended by the International Commission on Food Mycology (ICFM), was used for isolation and purification of fungal species. This technique involves several steps:

Surface Disinfection: Surface disinfection removes the inevitable surface contamination arising from dust and other sources and permits recovery of the fungi actually growing in the grains. This process provides an effective measure of inherent mycological quality.

Whole grains were completely immersed in 0.4% excess sodium hypochloride (NaOCl) solution for about two minutes, with occasional stirring. The grains were then drained and rinsed with sterile distilled water. This procedure was repeated thrice, each time with fresh NaOCl solution.

Plating: Potato Dextrose Agar (PDA) enriched with Chloramphenicol of mark HIMEDIA was used. Chloramphenicol, being an antibiotic, will inhibit the growth of bacteria in the medium.

10 disinfected surface sterilized grains were placed sparsely on Petri dishes containing solidified medium.

Incubation: Petri plates were incubated upright at 28±2°C for 5-10 days. To reduce evaporation, the dishes were sealed with parafilm. Distinct colonies representing

different species were sampled for sub-culturing and eventual purification.

Purification of species: With the help of a sterile platinum wire, a few spores (or clump of mycelium in some cases), were harvested from distinct colonies and inoculated on a freshly prepared PDA/Chloramphenicol medium. This was done successively until pure cultures were obtained. The pure cultures were then incubated on PDA/Chloramphenicol agar slant, coded and stored at 4°C prior to DNA extraction.

Identification of isolated fungi species was by DNA Barcoding using universal ITS primers.

2.3.2. Extraction of DNA

Fungal DNA was isolated using a NucleoSpin Plant II Kit (Macherey-Nagel) following the manufacturer's protocol. The quality of the DNA isolated was checked using agarose gel electrophoresis. 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front had migrated to the bottom of the gel.

2.3.3. PCR Amplification

PCR amplification reactions were carried out in a 20 µl reaction volume containing 1X Phire PCR buffer (contained 1.5 mM MgCl₂), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 1 µl DNA, 0.2 µl Phire Hotstart II DNA polymerase enzyme, 0.1 mg/ml BSA and 3% DMSO, 0.5M Betaine, 5pM of forward and reverse primers. The forward primer used for amplification was ITS-1F (5'-TCCGTAGGTGAACCTTGCGG-3') and the reverse primer ITS-4R (5'-TCCTCCGCTTATTGATATGC-3'). PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) with the following profile

| | | |
|------|--------|--------------|
| 98°C | 30 sec | |
| 98°C | 5 sec | } 40 cycles. |
| 62°C | 10 sec | |
| 72°C | 15 sec | |
| 72°C | 60 sec | |
| 4°C | ∞ | |

2.3.4. Agarose Gel ELECTROPHORESIS

PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. 1 µl of 6X loading dye was mixed with 5 µl of PCR products, was loaded and electrophoresis performed with 0.5X TBE buffer for about 1-2 hours, until the bromophenol blue front migrated to almost the bottom of the gel. The molecular standard used was 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

2.3.5. DNA Sequencing and Phylogenetic Analysis

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using

the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol. The PCR mix consisted of the following components: ExoSAP treated PCR Product (10-20 ng), either Forward or Reverse Primer (3.2 pM), Sequencing Mix (0.28 μ l), 5x Reaction buffer (1.86 μ l) and Sterile distilled water (make up to 10 μ l). The sequencing PCR temperature profile consisted of a first cycle at 96°C for 2 minutes followed by 30 cycles at 96°C for 30 sec, 50°C for 40 sec and 60°C for 4 minutes for all the primers. The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 [10]. The obtained DNA sequence was then blasted in the NCBI gene library. The sequence obtained was submitted to NCBI and accession numbers were obtained. Neighbor joining phylogenetic tree was constructed using MEGA software with the sequence obtained and related sequences retrieved from the GenBank.

3. Results and Discussion

3.1. Water Content

The average water content was 16.75 \pm 0.27 % and 11.24 \pm 0.31 % respectively for contaminated maize and

groundnuts. The water content of the groundnut sample was in line with the 11.5% postulated by Highley *et al.* [11]. Despite the relatively low water content, groundnut grains were still visibly infected. The minimum water content to prevent fungal contamination is 6.5% [12]; values of 16.75 \pm 0.27 % and 11.24 \pm 0.31 % favor fungal growth.

3.2. Enumeration and Purification of Fungal Species

Nine distinct pure isolates were obtained based on their morphology, pigmentation, growth rate and spore formation. Seven different strains were isolated from maize, and six strains from groundnuts. Four fungal strains were common in both maize and groundnuts. The high contamination rate was probably due to the relative high water content of the grains

3.3. DNA Isolation, Amplification and Sequencing

Figure 1 shows a photo of the gel obtained after gel electrophoresis. Clear and unique bands per sample implies excellent DNA quality from a pure fungal culture. Amplification was done based on the protocol by White *et al.* [13]. Figure 2 gives the outcome of DNA amplified products against a control DNA of known number of base pairs.

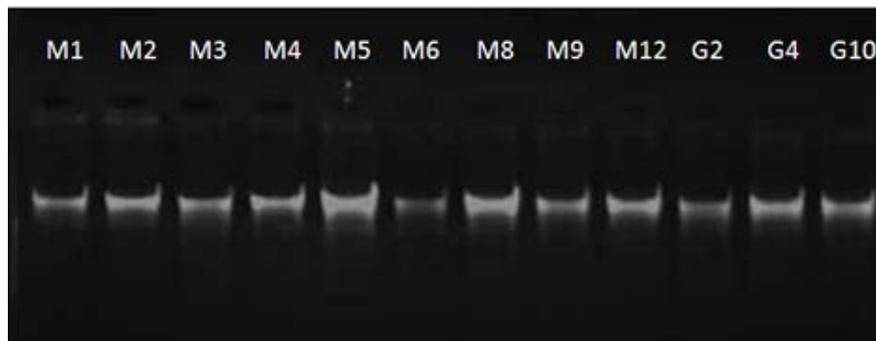


Figure 1. Isolated DNA on agarose gel after electrophoresis

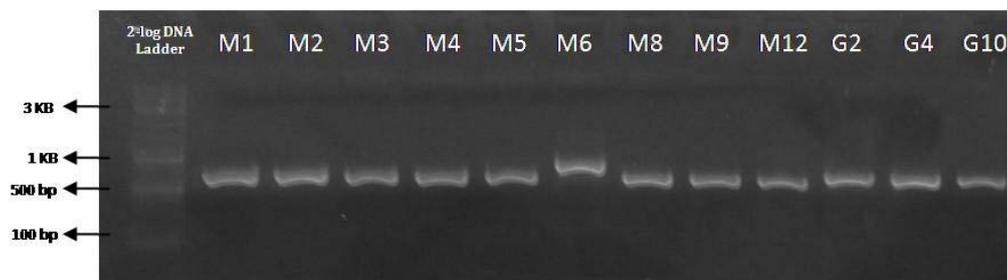


Figure 2. Amplified DNA against control DNA of known concentration

The concentration of DNA after amplification was above 500 base pairs, a value suitable enough for proper sequencing.

DNA sequencing was carried out in order to obtain the respective DNA sequence of the different isolated and amplified fungal DNA. Once obtained, the sequences were blasted and submitted in the NCBI gene library. Table 1 gives the different fungal strains identified from maize with attributed gene bank numbers. An elaborated phylogenetic tree of the identified fungal strains is shown in Figure 3.

Table 1. Identified fungal strains in maize with NCBI gene bank numbers

| No | Organism | Gene Bank Number |
|----|---|------------------|
| 1 | <i>Rhizopus oryzae</i> strain NIIST-ET-01 | KT152017 |
| 2 | <i>Aspergillus flavus</i> strain NIIST-ET-01 | KT159914 |
| 3 | <i>Aspergillus tamarii</i> strain NIIST-ET-01 | KT159915 |
| 4 | <i>Aspergillus oryzae</i> strain NIIST-ET-01 | KT159916 |
| 5 | <i>Cunninghamella polymorpha</i> strain NIIST-ET-01 | KT159917 |
| 6 | <i>Talaromyces purpureogenus</i> strain NIIST-ET-01 | KT159918 |
| 7 | <i>Penicillium citrinum</i> strain NIIST-ET-01 | KT159919 |

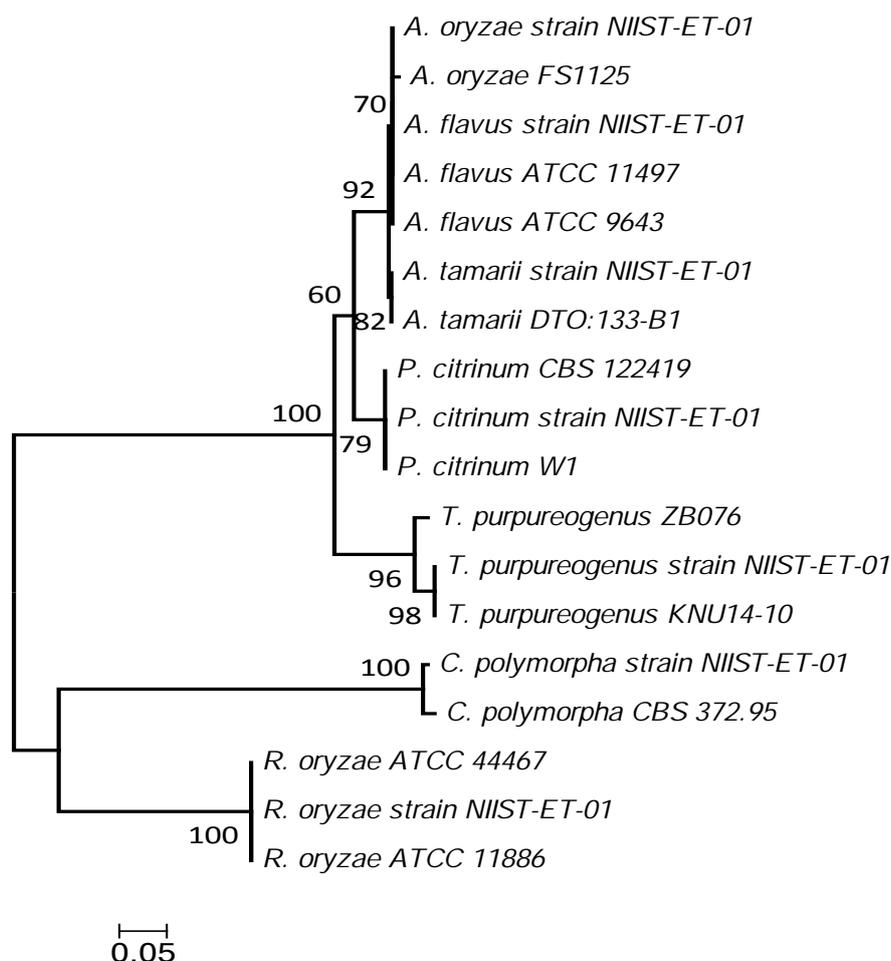


Figure 3. Neighbor-joining phylogenetic tree of isolated fungi strains and related strains

Aspergillus parasiticus and *Rhizopus stonilifer* were identified only from the groundnut samples. The presence of these different fungi strains signifies a high level of contamination, partly due to the relatively high water content in the stored grains above the minimal 6.5%. On the other hand, climatic and environmental conditions also favored the proliferation of fungi. Ngaoundere has a mean annual temperature of 22 °C and an annual rainfall of over 1500mm, coupled with its tropical wet and dry savanna climate with two seasons. These climatic conditions are ideal for fungi growth especially within the collection period (September) when the atmospheric humidity of Ngaoundere is very high due to heavy rains.

Though the presence of mycotoxins were not tested in this study, the presence of *Aspergillus* species, especially *A. flavus*, is an indication of a probable presence of aflatoxins.

Identification of *Aspergillus*, *Rhizopus* and *Penicillium* were in line with outcomes of most authors [1], [2] and [14]. There was no incidence of contamination by *Fusarium* species in our samples. These might be due to the relative low water content of our samples, as contamination by *Fusarium* species increase if the level of seeds moisture reaches at least 18 to 20% [15].

4. Conclusion

The different fungal species identified in stored maize and groundnuts in Ngaoundere, Cameroon, is a major step

in the fight against fungal contamination. The identified species is a proof of contamination during storage. *Aspergillus* species, especially *A. flavus* is a source of deadly mycotoxins, notably aflatoxins. There is the need for proper preservation technique in addition to adequate environmental and storage conditions in order to prevent contamination. If preservation is properly implemented, especially with natural antifungal agents with little or no side effect, fungal contamination and hence fungal food poisoning will be greatly subdued.

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