

# Identification of Post Harvest Fungi Associated *Telfairia occidentalis* Hook F. Leaves Using Basic Molecular Techniques

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## ABSTRACT

**Background and Objective:** The leaf of *Telfairia occidentalis*, a highly nutritious and economically important vegetable in Nigeria, is threatened by fungal diseases that affect its quality and yield. This study was conducted to isolate and identify the fungal species associated with the leaves of *Telfairia occidentalis* using both conventional microbiological techniques and molecular methods. **Materials and Methods:** Fungal species were isolated from *Telfairia occidentalis* obtained from the Botanic Garden of the University of Port Harcourt, Choba Port Harcourt, Rivers State, Nigeria. Potato dextrose agar (PDA) was used as a growth medium for the fungi. Deoxyribonucleic Acid (DNA) was extracted from pure cultures of fungal isolates using Quick-DNA Fungal/Bacteria Mini Prep Kit. Polymerase Chain Reaction (PCR) amplification of the internal transcribed spacer (ITS) region of the fungal isolates was carried out using fungal universal primer pairs ITS4 and ITS5. Sanger sequencing was performed and the sequences obtained were aligned and compared with sequences on the National Centre of Biotechnology Information (NCBI) database. **Results:** The result of the nucleotide sequence analysis revealed that isolate 1 is *Trichoderma reesei* with 590 base pairs and 99.66% similarity while isolate 2 is *Aspergillus aculeatus* with 610 base pairs and 99.62% similarity. A phylogenetic tree was constructed using the best BLAST hits. The evolutionary tree was inferred using the neighbor-joining method. **Conclusion:** The study identifies *Trichoderma reesei* and the newly discovered *Aspergillus aculeatus* on post-harvest *Telfairia occidentalis* leaves, raising concerns about potential health risks from consuming contaminated leaves due to harmful metabolites.

## KEYWORDS

Fungi, DNA extraction, *Telfairia occidentalis*, *Trichoderma reesei*, *Aspergillus aculeatus*, vegetables

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## INTRODUCTION

Green leafy vegetables provide crucial minerals and trace elements that contribute to the proper effectiveness of the body system by sustaining regular metabolic processes in restoring damaged cells, necessary maintenance and prevention of diseases in humans<sup>1</sup>. The leafy vegetable, *Telfairia occidentalis* Hook F., commonly called fluted pumpkin, is native to Nigeria and is also found in the moist coastal areas



of West Africa but rarely occurs naturally in East Africa<sup>2</sup>. It is a warm-weather crop that grows well in lowlands and tolerates elevation of a few meters above the ground. It thrives best in soils rich in organic matter<sup>3</sup>. Fluted pumpkin belongs to the family Cucurbitaceae and is a strong climber and short-term perennial<sup>4</sup>. The leaves are harvested by pruning with a knife and harvesting by hand picking damages the plants and hinders the development of side shoots<sup>5</sup>.

This vegetable crop *T. occidentalis* has gained significant importance in its dietary practices and is now a prevalent component in regional households<sup>6</sup>. It is cultivated for its leaves and seeds which are good sources of organic acid, vitamins, proteins, oils and carbohydrates<sup>7</sup>. *Telfairia occidentalis* seeds and leaves are consumed because they are healthy sources of lipids, vitamins, fiber and minerals such as iron, potassium, phosphorus and mineral salt<sup>8</sup>. The fluted pumpkin leaf also contains 1.35% alkaloid, 1.50% flavonoid, 3.60% saponin, 12.20% phenol and 0.437% tannin<sup>9</sup>. Fluted pumpkin leaf contains iron which is usually extracted and given as blood tonic to patients lacking blood while the seeds are reportedly used for the treatment of arthritis<sup>10,11</sup>. The vegetable contains photosynthetic pigments called chlorophylls and carotenoids<sup>12</sup>. The study of Iweala and Obidoa<sup>13</sup> reveals that the long-term feeding of *T. occidentalis* supplemented diet caused a significant increase in the weight of animals which may be due to its content of rich nutrients.

The most important losses in agricultural production involving the greatest farm economy costs occur by postharvest diseases. For example, in developing countries like Nigeria, over 85% of food consumed is obtained from the farm and the inability of food to be available at all seasons is affected by postharvest losses, this unavailability of food at all seasons causes food insecurity. Despite the nutritive and market value of *T. occidentalis*, sustainable production is greatly constrained by various diseases each year, of which leaf spot causal agent *Phoma sorghina*<sup>14</sup>. Molecular-based technologies are the most reliable tools for characterizing microorganisms as they deal with the genetic composition of organisms. Molecular tools have made it possible to obtain in-depth information on analyses of systems subject to climate change<sup>15</sup>, foodstuffs, agriculture, industrial settings and across the environmental sciences<sup>16</sup>. This study was therefore aimed at isolating and identifying fungal species associated with fluted pumpkin leaves using conventional microbiological techniques and molecular methods.

## MATERIALS AND METHODS

**Study area and sample collection:** This study was carried out at the Regional Centre for Biotechnology and Bioresources Research Laboratory, University of Port Harcourt from January to February 2023, where the isolation of the organisms, extraction of DNA and amplification of Genomic DNA were carried out. The leaf samples were collected in April from the Botanic Garden of the University of Port Harcourt, Choba Port Harcourt, Rivers State, Nigeria. The 10 samples were collected using a sterilized trowel and were transferred to clean, dry and labeled plastic containers. The samples were authenticated at the Regional Centre for Biotechnology and Bioresources Research and used for further analysis.

### Isolation of fungal organisms

**Serial dilution:** All glassware used was autoclaved at 121°C for 15-20 min. The work area was sterilized with 70% ethanol. Several dilution blanks were prepared, transferred to test tubes and labeled  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$  and  $10^{-5}$ . They were separately filled with 9 mL of water except the first test tube labeled  $10^{-1}$  which served as the stock solution and was filled with 10 mL of water. One gram of the leaf sample was transferred into the first test tube ( $10^{-1}$ ) and mixed properly; this was to aid even distribution of the sample in the tube. A micropipette was used to transfer 1 mL of the sample into the next test tube ( $10^{-2}$ ) and mix. One milliliter of the dilution was transferred from a test tube ( $10^{-2}$ ) into ( $10^{-3}$ ) and 1 mL was aseptically transferred from test tube  $10^{-3}$ - $10^{-4}$ . Finally, 1 mL of the dilution from the test tube containing  $10^{-4}$  dilution was transferred to the test tube labeled ( $10^{-5}$ ). The test tube labeled with  $10^{-3}$  was used for further investigation.

**Inoculation and incubation of fungal organisms:** After making a dilution of leaf sample, 0.5 mL of the desired dilution ( $10^{-3}$ ) was pipetted onto the surface of freshly prepared PDA in a Petri dish. A sterilized bent glass rod was used to spread the sample evenly. Each Petri dish was well labeled with dates and sealed with masking tape.

The plates were incubated at room temperature ( $27\pm 2^{\circ}\text{C}$ ) for 7 days under a laminar flow (ESCO Technologies Inc., Horsham, Pennsylvania, USA.) to obtain the fungal organisms. Fungal organisms when obtained were sub cultured to obtain pure cultures of fungi. Pure cultures were stored at  $4^{\circ}\text{C}$  in a refrigerator till when needed.

**Extraction of fungal DNA:** The DNA was extracted using the protocol of Zymo Quick-DNA Fungal/Bacterial Mini-Prep Kit (Zymo Research Group, California and USA), the protocol of the kit mentioned above was used with modifications.

The fungal mycelium was scrapped off from the surface of the plates using a sterilized surgical blade and transferred into a sterilized mortar and pestle. Bashing bead buffer (750  $\mu\text{L}$ ) was added to the sample before homogenizing with liquid nitrogen ( $-196^{\circ}\text{C}$ ). Homogenized samples were transferred into Eppendorf tubes of 1.5 mL and promptly put on ice. The Eppendorf tubes containing the homogenized samples were centrifuged in a refrigerated centrifuge at 10,000 g for 1 min. About 40  $\mu\text{L}$  of the supernatant was transferred to a Zymo-Spin III-F Filter in a collection tube and centrifuged at 7,000 g for 1 min. The Zymo-Spin III-F Filter was discarded after use. About 1,200  $\mu\text{L}$  of Genomic Lysis Buffer was added to the filtrate in the collection tube and properly mixed, 800  $\mu\text{L}$  of the mixture was then transferred to a Zymo-Spin IIC Column in a collection tube and centrifuged at 10,000 g for 1 min. The flow-through was discarded from the collection tube and step 6 was repeated. About 200  $\mu\text{L}$  of DNA Pre-Wash Buffer was added to the Zymo-Spin IIC Column and centrifuged at 10,000 g for 1 min. About 500  $\mu\text{L/g}$  DNA of Wash Buffer was added to the Zymo-Spin IIC Column and centrifuged at 10,000 g for 1 min. The Zymo-Spin IIC Column was transferred to a clean 1.5 mL microcentrifuge tube and 50  $\mu\text{L}$  of DNA Elution Buffer was added directly to the column matrix and centrifuged at 10,000 g for 30 sec to elute the DNA.

**Determination of DNA concentration and purity using NanoDrop:** DNA Concentration and purity were measured using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, Delaware, USA). Purity is measured as a ratio of absorbance at 260 nm to that of 280 nm. The Nanodrop was connected to a computer system and the sensor was sterilized using 70% ethanol, 1  $\mu\text{L}$  of Elution buffer was dropped directly on the NanoDrop sensor to create a blank. The DNA samples were loaded separately and wiped after obtaining the result to avoid contamination.

**Determination of quality of the DNA using gel electrophoresis:** The gel electrophoresis allows the movement of samples from the cathode (-) to the anode (+) charge through agarose gel connected to a power source. To check the quality of the DNA, gel electrophoresis was performed. Half a gram (0.5 g) of agarose powder was mixed with 50 mL of Tris Boric EDTA (TBE) IX in a measuring flask and microwaved for 2 min to get a clear solution. A 3  $\mu\text{L}$  of EZ-vision gel (blue light) was added to the content in the conical flask and then poured into the casting tray. The comb was inserted for the creation of wells. The content was allowed to sit between 20-30 min at room temperature for the gel to solidify. The gel electrophoresis machine (C.B.S. Scientific EPS-300X (C.B.S. Scientific Company, California, USA) was set up and the plate holding the gel was put into the tank. The TBE IX was poured into the gel tank until the gel was completely submerged. Each DNA sample which was mixed with 3  $\mu\text{L}$  of EZ-vision gel blue light 2X was loaded separately into the wells made by the comb. The setup was allowed to run for 40 min. At the end of the run, the DNA fragments were viewed with a UV transilluminator (Lab net International, Edison, New Jersey, USA). Where bands appeared, the DNA was said to be of good quality.

**Fungal DNA amplification and sequencing:** The primers used for the fungal PCR were: ITS4- forward (5'TCCTCCGCTTATTGATATGS-3') and ITS5: Reverse (5'GGAAGTAAAAGTCGTAACAAGG-3'). The PCR was done in a total volume of 25  $\mu$ L containing 2.5 mL of 10X PCR buffer, 1  $\mu$ L of 25 mM MgCl<sub>2</sub>, 1  $\mu$ L each of 5  $\mu$ M forward and reverse primers, 1  $\mu$ L of DMSO, 1  $\mu$ L of 2.5 mM dNTPs, 0.1  $\mu$ L of Taq DNA polymerase, 3  $\mu$ L of 10 ng/ $\mu$ L genomic DNA and 13.4  $\mu$ L nuclease free water. Amplification was done in a thermocycler with initial denaturation at 94°C for 5 min, followed by 36 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec and elongation at 72°C for 45 sec. Followed by a final elongation step at 72°C for 7 min and hold temperature at 10°C. Amplified fragments were visualized on safe view-stained 1.5% agarose gel.

The PCR products were sent to the International Institute of Tropical Agriculture (IITA), Ibadan for purification and sequencing. The PCR products were sequenced on ABI 3500 Genetic Analyzer (Thermo Fisher Scientific, Massachusetts, USA).

**Phylogenetic analysis:** Sequences were cleaned and identified on the BLAST algorithm on the National Centre for Biotechnology Information (NCBI) database. Best blast hits were used for the construction of a neighbor-joining phylogenetic tree which was used to determine the evolutionary relationship among the fungal species isolated and other species on GenBank. Evolutionary analysis was conducted on Molecular Evolutionary Genetics Analysis (MEGA) software, version 10.0.1 (MEGA X).

## RESULTS

**Fungal isolates of *Telfairia occidentalis* leaves:** Two fungal organisms were isolated from *Telfairia occidentalis* leaves (Fig. 1a-b). The mean value of fungi colony on plated *T. occidentalis* leaves was calculated and the frequency of occurrence of each organism on PDA was determined. Isolate 1 had a higher frequency of occurrence (2.25) than isolate 2 which had 2.0.

**Polymerase Chain Reaction (PCR):** The PCR profiles of isolates 1 and 2 showed bands on gel when viewed under UV light. The PCR product of each isolate is presented in Fig. 2.

**ITS sequences obtained:** Amplified PCR products after sequencing and blasting revealed the species identity of the fungal isolates to be *Trichoderma reesei* (isolate 1) and *Aspergillus aculeatinus* (isolate 2) in Fig. 2.

Accession numbers of isolates were obtained from GenBank and each fungal isolate was assigned a strain number. The accession numbers are in parentheses below:

- **Isolate 1:** *Trichoderma reesei* (ON965496) strain number RCBBR\_AEAN9
- **Isolate 2:** *Aspergillus aculeatinus* (ON965497) strain number RCBBR\_AEAN10

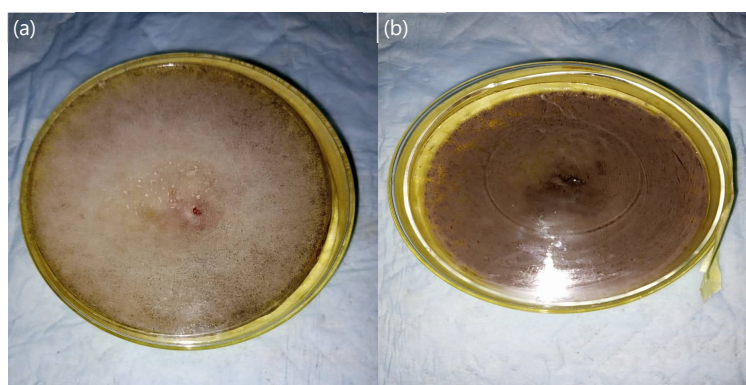


Fig. 1(a-b): Pure culture plates of the fungal isolates obtained from *Telfairia occidentalis* grown on potato dextrose agar, (a) Isolate 1 and (b) Isolate 2

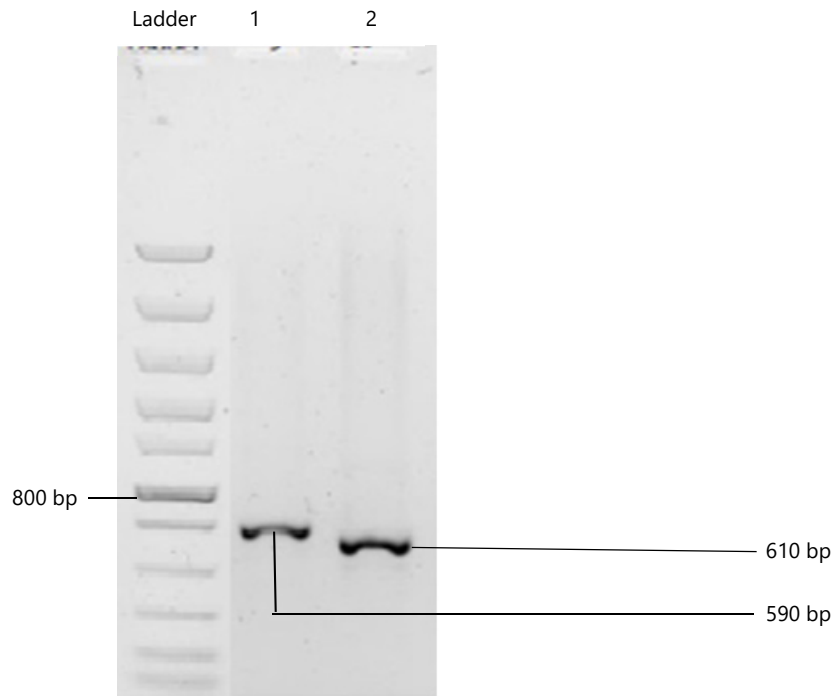


Fig. 2: PCR products generated from fungal DNA samples

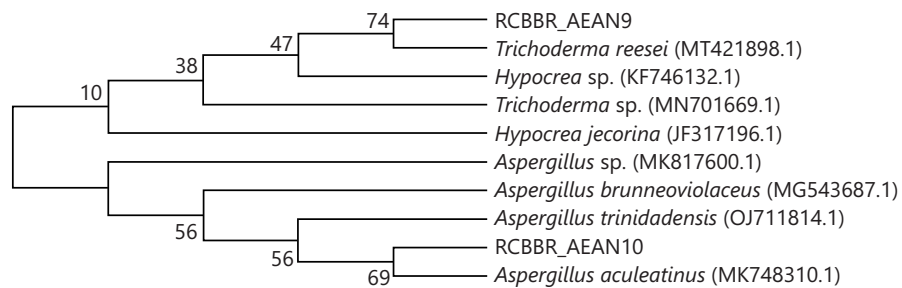


Fig. 3: Phylogenetic tree of fungal isolates based on the sequenced ITS region using the neighbor-joining method

**Phylogenetic analysis:** The ITS sequences data of other fungal isolates evolutionarily related to the isolates obtained from *T. occidentalis* were obtained from GenBank and used to construct a phylogenetic tree. The closest relatives of the fungal isolates obtained from *T. occidentalis* are *Hypocrea jecorina*, *Hypocrea* sp., *Trichoderma* sp., *Aspergillus brunneoviolaceus*, *A. trinidadensis* and *Aspergillus* sp., as shown in Fig. 3.

## DISCUSSION

The morphology of the isolated fungi was yellow to brown dark spores and a frequency of  $2 \pm 0.71$  and green sporulation and a frequency of  $2.25 \pm 0.25$  for *Trichoderma reesei* and *Aspergillus aculeatinus*, respectively. Molecular characterization tools offer insights into the identification and analysis of unknown species, allowing for the comparison of DNA sequences between known and unknown organisms. This molecular data enhances understanding of phylogeny and provides valuable information for studying the taxonomy and evolution of species. Molecular methods for identifying organisms are highly reliable, accurate, dependable and quick. Specifically, molecular characterization using Polymerase Chain Reaction (PCR) amplification and sequencing of internal transcribed spacer (ITS) regions was utilized to effectively identify fungal organisms isolated from *Telferia occidentalis*. This method identified two fungi *Trichoderma reesei* and *Aspergillus aculeatinus*.

*Trichoderma reesei* is a mesophilic and filamentous fungus that belongs to the phylum Ascomycota, class Sordariomycetes, order Hypocreales and family Hypocreales. *Trichoderma reesei* was originally isolated from Solomon Island during World War II because it degraded canvas and garments of the US army and all strains currently used in biotechnology and basic research were derived from this isolate<sup>17</sup>. *Trichoderma reesei* is an efficient producer of extracellular proteins hence it is widely used in cellulase and biofuel production. According to previous study of Bischof *et al.*<sup>18</sup> *Trichoderma reesei* is a widely utilized organism in the biotechnology industry, serving as a key producer of cellulase enzymes. Its dominance as a workhorse in enzyme production has made it essential for various industrial applications. The fungus is also known to have antagonistic properties. Cellulases produced by *T. reesei* strongly inhibit Pepper Mild Mottle Virus infections in plants<sup>19</sup>. *Trichoderma reesei* was found to be an effective antagonistic microorganism against the plant pathogen, *Rhizoctonia solani*. Grosch *et al.*<sup>20</sup> and Harish *et al.*<sup>21</sup> Studied *Bipolaris oryzae*, which causes brown spot disease in rice. *Trichoderma reesei* is capable of mycoparasitism against *Pythium ultimum*, an aggressive soil-borne plant pathogen<sup>22</sup>. According to Sánchez-Montesinos *et al.*<sup>23</sup>, *Trichoderma* fungi can negatively impact certain areas of agriculture and human health. In agriculture, their adverse effects are primarily associated with their mycoparasitic capabilities, leading to a condition referred to as green mold, which poses a significant threat to the cultivation of mushrooms such as shiitake, oyster and champignons.

The genus *Aspergillus* is widely distributed across various ecosystems and substrates, including soil, textiles and food<sup>24</sup>. Molds like *Aspergillus* can negatively impact human health due to their potential to cause toxicity, trigger allergic reactions and lead to infections<sup>25</sup>. *Aspergillus aculeatinus*, a species within this genus, belongs to the group of black aspergilli and is closely related to *Aspergillus aculeatus*<sup>26</sup>. Through genome sequencing combined with phylogenetic and phenotypic analyses, *A. aculeatinus* was identified as a new species<sup>27</sup>. This species has been isolated from Thai coffee beans and shows potential for industrial use, producing bioactive compounds such as neoxaline, the antifungal compound aculeacin and the antitumor compound paclitaxel, initially known as Taxol by Bristol-Myers Squibb<sup>28</sup>. To date, only one genome of *A. aculeatinus* has been sequenced<sup>30</sup>. Species in *Aspergillus* section Nigri, which includes the black aspergilli, are known for causing food spoilage, plant diseases and for producing industrially significant compounds like lipases, amylase, citric acid and gluconic acid<sup>29</sup>. Due to the fermentation capabilities and high level of protein secretion in the solid-state culture of *Aspergillus* spp., it is often used by the industry for the production of pectin-degrading enzymes. Hence, *A. aculeatinus* has an extensive and highly conserved set of genes encoding cell wall degrading enzymes, although their precise biochemical characterization and specificities have to be determined<sup>28,30</sup>.

Morphological and microscopic characteristics form the foundation of traditional microbial identification techniques. However, this method can result in misidentification and is inadequate for pinpointing microorganisms at the species level. Molecular techniques, based on the genetic composition of organisms, provide precise identification since the genetic makeup of all living beings varies to some degree. Many scientists in developing countries primarily rely on traditional methods for identifying microorganisms, which can lead to incorrect interpretations of the fungal community. In this study, molecular techniques were successfully employed to identify fungal isolates from *Telfairia occidentalis* leaves. The study also highlights the ecology and economic significance of these fungal isolates, providing essential information for researchers to develop strategies for improving plant health.

## CONCLUSION

Fungal species are associated with the *T. occidentalis*. Molecular characterization using Polymerase Chain Reaction (PCR) amplification and sequencing of internal transcribed spacer (ITS) regions proved effective in identifying fungal samples. This study will enhance understanding of the fungal species associated with *Telfairia occidentalis* and also aid plant pathologists make informed decisions on disease control resulting in a decrease in post-harvest loss and promoting crop protection.

## SIGNIFICANCE STATEMENT

This study discovered the fungi species associated with postharvest *Telfairia occidentalis* leaves. Despite their significant nutritional benefits, *Telfairia occidentalis* leaves are compromised by pathogens that reduce their shelf life. Molecular identification of fungal organisms is crucial as it is faster, more reliable and more accurate, giving plant pathologists the knowledge needed to make informed decisions. *Trichoderma reesei* and *Aspergillus. aculeatinus* have been documented to cause spoilage and can be harmful to animals and humans when consumed. Proper storage and handling practices should be adopted to minimize fungal contamination and public awareness campaigns should be conducted to educate consumers on the potential health risks associated with consuming contaminated leaves.

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