Isolation and Identification of Fungi Associated with Post-Harvest Spoilage of Apple (*Pyrus Malus*) Traded in Kano Metropolis, Nigeria

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ABSTRACT

Storage of fruits at suboptimal condition provides fungi to grow and some produce mycotoxins, which can be dangerous/harmful for human consumption. This study was conducted to isolate and identify fungi associated with spoilage of apples sourced from Kano markets, Nigeria. A total of nine (9) fresh apple samples were collected randomly from different supermarkets on September of 2023 in Kano, Nigeria. Each apple was placed in a sterile plastic bag at room temperature (25–30°C) for fifteen days or until fungal growth was evident all over the samples. Growth of fungal colonies on Potato Dextrose Agar (PDA) was observed and molecular confirmation by Polymerase Chain Reaction (PCR) was conducted along with sequencing (sanger sequencing). Four fruit spoilage fungi were isolated, namely: *Aspergillus fumigatus, Aspergillus niger, Curvularia spp,* and *Yeast spp*. Out of the 9 samples that were analyzed, *A. fumigatus* and *Curvularia spp* were the most frequent isolates, *A. fumigatus* was seen in a total of 3 isolates (33.33%), *Curvularia spp* in 3 isolates (33.33%), *A.niger* in 2 isolates, and the least was Yeast spp seen in 1 isolate (11.11%). Sequence analysis of the Internal Transcribed Spacer regions of the nuclear encoded rDNA of the *A.fumigatus* isolate showed significant alignments for *Penicillium griseofulvum.* This is a potential refinement of the initial morphological characterization. The unexpected identification underscores the significance of incorporating molecular methods in fungal identification processes. The variation between initial morphological identification and molecular results emphasizes the need for precise and accurate identification techniques, particularly when dealing with closely related species. Most of these fungal isolates are pathogenic and can still produce severe illness in immune-compromised individuals, and sometimes otherwise healthy people may also become infected. The study highlights the limitations of traditional fungal identification techniques, as molecular analysis revealed *Penicillium griseofulvum* instead of the initially identified *Aspergillus fumigatus* in fungi from damaged apples. This underscores the importance of integrating molecular methods with conventional approaches for accurate fungal identification, vital for managing postharvest losses and ensuring food safety.

Key words: Apple, Fungi, Kano, Pathogenetic, *Penicillium griseofulvum,* Post-harvest spoilage

INTRODUCTION

Apples (*Pyrus malus*), like many fruits, are a rich source of essential nutrients. They provide a spectrum of macronutrients, including carbohydrates in the form of natural sugars and dietary fiber. The latter contributes to digestive health and metabolic regulation. Additionally, apples contain micronutrients such as vitamins (e.g., vitamin C) and minerals (e.g., potassium), each playing a distinct role in supporting physiological functions (Boyer and Liu, 2004).

In the broader scope of maintaining optimal health, incorporating apples into a balanced and varied diet is advantageous. The diverse array of nutrients in apples, combined with their low-calorie content, makes them a nutritious snack of choice. Consuming apples as part of a well-rounded diet contributes to overall nutritional balance, supporting growth, energy production, and disease prevention (Hyson, 2011).

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Apples, esteemed for their crisp texture and vibrant flavor, constitute a vital component of the global fruit market. However, post-harvest spoilage poses a significant challenge to the quality and shelf life of this esteemed fruit. Fungal pathogens are among the primary culprits responsible for the deterioration of apples during storage and distribution (Pitt and Hocking, 2009). The economic implications of post-harvest losses due to fungal spoilage necessitate a comprehensive investigation into the identification and characterization of these fungal agents.

The intricate interplay of environmental conditions, apple cultivars, and fungal communities within storage environments underscores the multifaceted nature of apple spoilage (Pesis *et al.,* 2017). Fungi from various genera, including *Penicillium, Botrytis, and Alternaria*, have been implicated in apple spoilage, each exhibiting distinct morphological and biochemical characteristics (Droby *et al.,* 2008). Fungal spoilage profoundly impacts the fruit's appearance, texture, flavor, and nutritional quality, undermining consumer satisfaction and market value. Furthermore, the proliferation of fungal pathogens can lead to mycotoxin production, posing potential health risks to consumers [International Agency for Research on Cancer (IARC), 1993]. Given the global trade of apples and the critical role they play in nutrition and commerce, the need to mitigate postharvest spoilage and its associated fungal agents is of paramount importance.

Apart from environmental factors such as high temperatures and relative humidity, fungi deploy pectic enzymes that degrade apple pectin, thereby exposing the nutrients within apple cells to the voracious fungi (Blevea *et al.,* 2006). Fruits like apples, abundant in sugars and nutrients, serve as fertile grounds for fungal proliferation (Prasad, 2007). Fungal infestation usually begins when the apple's skin is compromised, be it through punctures or other wounds.

Toxigenic fungi have been successfully isolated from spoiling fruits (Pose *et al.,* 2004). Suboptimal storage conditions further exacerbate fungal growth and mycotoxin production (Tournas and Memon, 2009). The primary culprits responsible for apple rot are the fungi *Penicillium expansum* and *Monilinia fructigena* (Fiori *et al.,* 2008). Additionally, other fungal genera, including *Colletotrichum, Xylaria, Botryosphaeria* (Camatti-Sartor *et al.,* 2005), and *Rhizopus oryzae* (Kwon *et al.,* 2011), have been identified in apples. *Aspergillus spp*. has also been isolated and known to cause infections and allergies (Mons, 2004). *Cladosporium spp*. was frequently found in stored apples in some studies, along with *Penicillium, Acremonium, Aspergillus, Aureobasidium, Cryptococcus, Sporobolomyces, and Alternaria spp* (Robiglio and Lopez, 1995; Watanabe, 2008).

Traditional methods for studying fungi involve conventional cultivation and microscopic identification, which require the expertise of skilled taxonomists (Frisvad *et al.,* 2006). Identification of fungal species relies on mycelial characteristics such as color, size, and shape, as well as morphological traits like conidia size and conidiophore morphology (Pitt and Hocking, 2009; Al-Hindi *et al.,* 2011). Nevertheless, slight variations in the composition of growth media can hinder accurate comparisons of mycelial features (Larone, 1995).

Molecular techniques have proven effective and convenient for fungal identification. DNA-based assays offer reliability in detecting various fungi. Different molecular approaches have been employed to detect *Aspergillus* in environmental and clinical samples (Einsele *et al.,* 1997; Leinberger *et al.*, 2005; Liu, 2011). These methods have targeted various genetic regions, including the 18S rRNA gene, mitochondrial DNA, intergenic spacer regions, and the internal transcribed spacer (ITS) regions. The ITS regions, situated between the 18S and 28S rRNA genes, present distinct advantages due to their high copy number, approximately 100 copies per genome. The sequence variation within the ITS regions has made them invaluable in phylogenetic studies of various organisms (Anaissie *et al.,* 2009). Some studies have utilized the nucleotide sequences of ITS 1 and 2 for clinically significant *Aspergillus* species, assessing their variability for species- level identification (Henry *et al.*, 2000; Hinrikson *et al.*, 2005).

There is an increase in cases associated with food borne diseases in Kano state, most of which are caused by fungal pathogens. Fungal spoiled apples are consumed daily by the inhabitants of the community, which considers it as an alternative to the good ones that are expensive leading to serious health complications. Identification and characterization of these fungal pathogens that are associated with post-harvest spoilage of apple may be an initial step in developing anti-fungal agents that can be used to eradicate or minimize post-harvest losses and maintain apple quality throughout the supply chain. Although attempts have been made to conduct research on microorganisms leading to the post-harvest spoilage of different fruits and vegetables in

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Kano metropolis, there is a paucity of research on fungal spoilage of apples in Kano metropolis in the existing literature. It is in view of this, that this research work was set up to identify the fungal species responsible for the post-harvest losses of apples traded in Kano metropolis macroscopically, microscopically and molecularly.

MATERIALS AND METHODS

Study Area and Sample Collection

The study was conducted at the Post graduate Laboratory, Department of Microbiology, Bayero University Kano, Nigeria. DNA extraction, quantification, Polymerase Chain Reaction (PCR), and gel electrophoresis were conducted at the Biotechnology Research Lab Unit, Nigerian Institute for Trypanosomiasis Research (NITR) in Kaduna, Nigeria. PCR product sequencing was carried out at the Inqaba Biotec West Africa in Ibadan, Nigeria. A nine (9) fresh apple samples were collected randomly from different supermarkets in September of 2023 in Kano, Nigeria. A total of three apples each of the Empire variety, the Honey crisp variety and the Granny Smith variety were collected. The collected apples had some obvious signs of fungal spoilage, including softening discoloration and visible mold growth. Each apple was placed in a sterile plastic bag at room temperature (25-30° C) for fifteen days or until fungal growth was evident all over the sample (after approximately fifteen days).

Preparation of Collected Samples

The apple fruits were divided into species. The samples, which were apparently diseased, were cut from the advancing edges of the lesion with a sterilized knife. These cut samples were washed with sterile distilled water and surface sterilized by using 1 % sodium hypochlorite for 3-5 minutes and then washed with five successive changes of distilled and sterile water. After the surface sterilization, the moisture on each of the cut samples was removed before inoculation onto the surface of agar plates.

Media Preparation

Potato Dextrose Agar (PDA) was prepared containing Chloramphenicol (30 mg/l) to prevent the growth of bacteria for 9 plates according to the manufacturer's instruction and was allowed to gel for fungal isolation.

Fungal Isolation and Amplification

The sterile petri dishes containing the already prepared PDA were opened and the sterilized apple pieces were placed onto the surface of the agar plates at different positions. The plates were labeled and incubated at 25± 2°C in the laboratory for seven days. Finally, all identified fungi were sub-cultured on Potato Dextrose Agar (PDA) medium under darkness at room temperature (25± 2ºC).

Morphological and Microscopic Identification and Characterization

The morphological identification of isolates was conducted by visually observing the mycelium and comparing their colonies for their diameters, colors of conidia, reverse colors, and texture. The isolates were later subjected to microscopic analysis for identification by an electron binocular microscope at X40. Endophytic or epiphytic nature of fungal isolates was determined through morphological and molecular analyses.

Molecular Characterization using the Internal Transcribed Spacer (ITS) Marker and Identification

Genomic DNA Extraction and Quantification

The Genomic DNA of the most frequent fungi isolates G-3, E-2 and H-3 found on the apples was extracted following the protocol of BIONEER Accu Prep® Genomic DNA Extraction Kit (BIONEER Corp, Daejeon34301, Republic of Korea).

The isolate DNA quantity and concentration were measured using Nano-Drop 2000c Spectrophotometer. The DNA purity was measured as a ratio of absorbance at 280 nm to that of 260 nm.

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume IX Issue VIII August 2024

Polymerase Chain Reaction (PCR)

The nuclear ribosomal DNA (rDNA) gene fragments of G-3, E-2 and H-3 isolates were then amplified by PCR using the universal primer pairs Internal Transcribed Spacer 1&2 (ITS1/ITS2) (Glass, N.L and Donaldson, G.C 1995; Borneman, J and Hartin, R . J 2000). PCR products were purified using the QIA quick PCR purification kit (QIAGEN, GmbH, Germany), and sequenced in both directions using the respective PCR primers. Furthermore, the sequences of primers used to amplify the nuclear ribosomal DNA fragments of the above isolates are Internal Transcribed Spacer1 (ITS1) 5'-TCCGTAGGTGAACCTGCGG-3' and Internal Transcribed Spacer2 (ITS2) 5'-GCTGCGTTCTTCATCGATGC-3' (White *et al.*, 1990). A 20 µl (final volume) of the PCR mixture was prepared using the BIONEER Accu Power ® Hot Start PCR Pre Mix, as follows: 1µl each of diluted forward and reverse primer, 5µl of DNA sample (Template DNA), and the volume was made by addition of 13µl of sterile nuclease-free water. The mixture was subjected to an initial denaturation temperature of 94ºC for 5 min, 35 cycles each of 0.5 min denaturation at 94ºC, 1 min annealing at 65°C, and 1 min elongation at 72°C, steps. Reactions were terminated with a final extension step at 72ºC for 5 min. The amplification process was carried out in a Thermal Cycler (MG96G, GERMANY).

GEL Electrophoresis

Electrophoresis of the amplified ribosomal DNA (rDNA) PCR products was performed on the Bio-Rad Contour-Clamped Homogenous Electric Field DRII electrophoresis cell (Hemel Hempstead, UK), using 1.0% (w/v) agarose gel (general purpose, Biogene, Kimbolton Cambs, UK) in 0.5× Tris-Acetate-EDTA (TAE) buffer at 100 V for 1hr. A 100-bp (Promega G210A, Madison, WI, USA) ladder was used as a molecular size marker. The result of the gel was viewed in the G; BOX Gel documenter (Syngene G: BOX Chemi XRQ, USA). The purified DNA was then sequenced. The specific nucleotide sequences obtained were subjected to Basic Local Alignment Search Tool (BLAST) search programme at GenBank (http://www.ncbi.nlm.nih.gov/blast/) to determine the closest known relatives of the most frequent isolates.

RESULTS AND DISCUSSION

Results

Morphological and Microscopic Characterization and Identification

In this study, four (4) fruit spoilage fungi were isolated and found to be associated with spoilage of apples sold in Kano markets. Based on the colony morphology and microscopic examination, the isolated fungal species were identified as follows: *Aspergillus fumigatus, A. niger*, *Curvularia spp*, and Yeast spp. Out of the 9 samples that were analyzed, *A. fumigatus* was seen in a total of 3 isolates (33.33%) (coded G-3, E-2 and H-3), *Curvularia spp* in 3 isolates (33.33%)(coded E-1, H- 1 and H-2), *A.niger* in 2 isolates (22.22%)(coded G-1 and E-3), *Yeast spp* in 1 isolate (11.11%)(coded G-2). Table 1 presents the frequency of occurrence of isolated fungi and the microscope photos of the fungal isolates are presented in Figure 1.

Molecular Characterization Using the Internal Transcribed Spacer (ITS) Marker and Identification

Genomic DNA Extraction and Quantification

The genomic DNA of the most frequent isolate *A.fumigatus* coded G-3, E-2, and H-3 was extracted. The Nano Drop result showed that the isolates DNA concentrations of G-3, E-2 and H- 3 were 50.3ng/µL, 26.0 ng/µL and 98.0ng/µL respectively. While the absorption peak of the 260nm/280nm readings of G-3, E-2 and H-3 were 1.83, 1.27 and 1.94 respectively (Table 2).

Polymerase Chain Reaction (PCR) and Gel Electrophoresis

The result of the amplified DNA band of the isolates G-3, E-2 and H-3 is presented in Figure 2. From the result, typical PCR amplimers of the ITS regions of the nuclear encoded rDNA genes in the fungal isolates are represented each amplimer was approximately 500-600bp in size.

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume IX Issue VIII August 2024

DNA Sequence and Alignment

The DNA sequence result of the H-3 isolate which was identified as *A.fumigatus* in microbiological analysis is presented in Figure3. The H-3 isolate sequence aligned with 100 sequences deposited in the National Center for Biotechnology Information (NCBI) composite biological database. Sequence analysis of the ITS regions of the nuclear encoded rDNA showed significant alignments of 100% in sample H-3 for *Penicillium griseofulvum* with 579/579(100%) identities (Accession NO: KT898744.1). The phylogenetic tree showing the evolutionary relationship between isolate BUK_BCH_IYU (H-3) and reference related fungal isolate base on the ITS gene sequence is presented in Figure 4.

Table 1. Frequency of occurrence of isolated fungi and percentage frequency from 9 samples on Potato Dextrose Agar (PDA) containing chloramphenicol (30 mg/l).

Fungal isolates	Occurrence	Percentage (%)
A.fumigatus (G-3, E-2 and H-3).	3	33.33
Yeast spp (G-2).	1	11.11
Curvularia spp (E-1, H-1 and H-2).	3	33 33
A.niger (G-1 and E-3).	2	22.22
Total	9	≈100

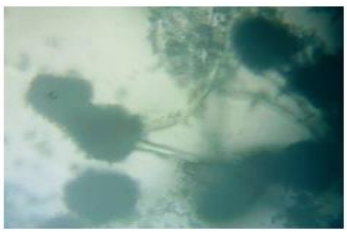

Plate a: *A.niger*

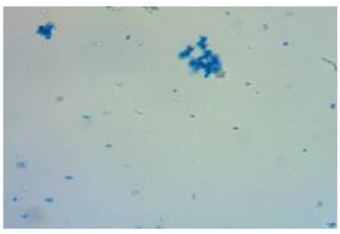

Plate b: Yeast spp

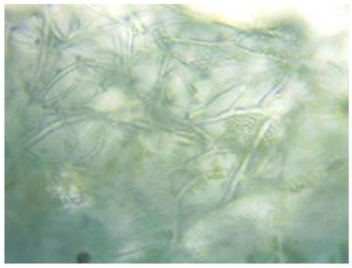

Plate c: *Curvularia*

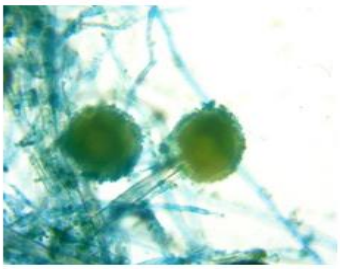

Plate d: *A.fumigatus*

Figure 1: Microscopic presentation of the isolates from damaged apples.

Table 2. The concentration of DNA extracted from fungal isolates of apple using a Nano-drop (2000c) spectrophotometer.

Fungi sample ID (purity)	**DNA concentration (ng/µL)**	**Absorbance at 260nm/280nm**
G-3	50.3	1.83
E-2	26.0	1.27
H-3	98.0	1.94

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume IX Issue VIII August 2024

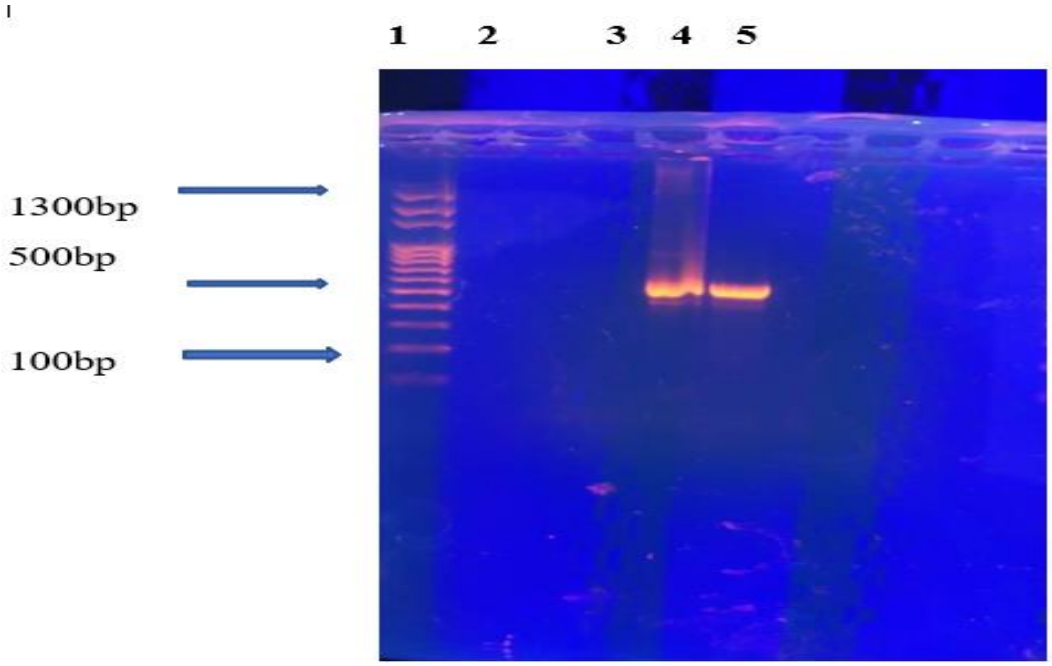

Figure 2. Typical PCR amplimers of the ITS regions of the nuclear encoded rDNA genes in fungal isolates obtained from apples in 1% agarose gel

Legend

1: 100bp DNA ladder, 2: DNA negative control, 3: Isolate G-3, 4: Isolate E-2, 5: Isolate H-3

1. TTCCTCCGCT TATTGATATG CTTAAGTTCA GCGGGTATCC

41.CTACCTGATC CGAGGTCAAC CTGAGATAAT TAAAGGTTGG

81. GGGTCGGCTG GCGCCGGCCG GGCCTACTAG AGCGGGTGAC

121. GAAGCCCCAT ACGCTCGAGG ACCGGACGCG GTGCCGCCGC

161. TGCCTTTCGG GCCCGTCCCC CCGGCGGGGG GGACGGGGCC

201. CAACACACAA GCCGGGCTTG AGGGCAGCAA TGACGCTCGG

241. ACAGGCATGC CCTCCGGAAT ACCAGAGGGC GCAATGTGCG

281. TTCAAAGACT CGATGATTCA CTGAATTCTG CAATTCACAT

321. TAGTTATCGC ATTTCGCTGC GTTCTTCATC GATGCCGGAA

361. CCAAGAGATC CGTTGTTGAA AGTTTTAACT AATTTCGTTA

401. TAGGTCTCAG ACTGCAACTT CAGACAGCGT TCAGGGGGGC

441. CGTCGGCGGG CGCGGGGCCC GCCGAGGCAA CATAGGTTCG

481. GGCAACACGG GTGGGAGGTT GGGCCCCGAG GGGCCCGCAC

521.TCGGTAATGA TCCTTCCGCA GGTTCACCTA CGGAAACCTT

561. GTTACGACTT TTACTTCCA

Figure 3: Sequence and alignment of H-3 isolate

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume IX Issue VIII August 2024

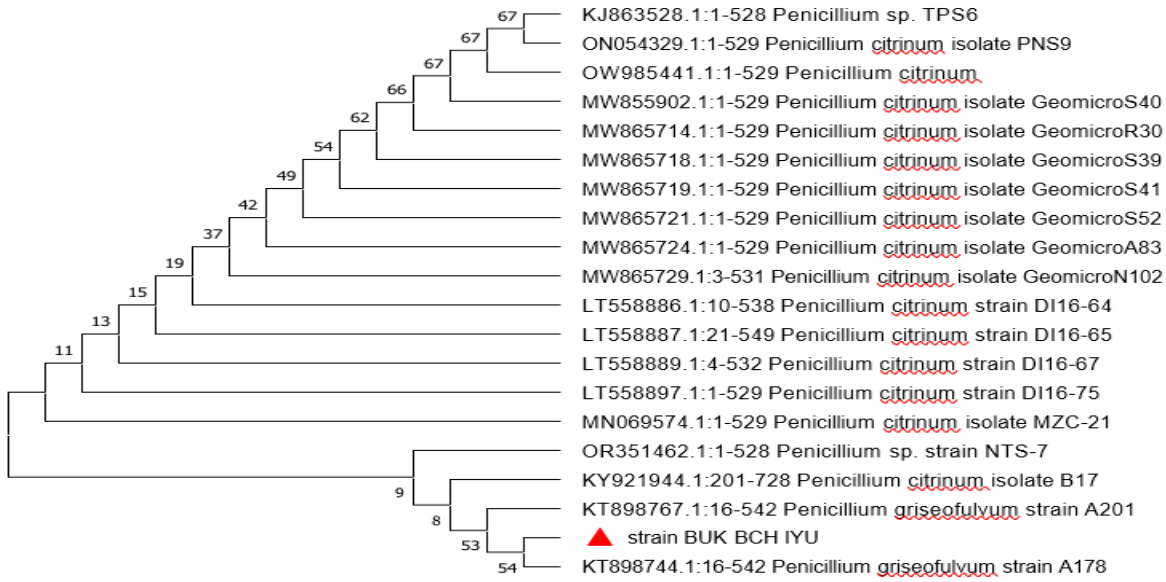

Figure 4: Neighbour-joining method indicating the evolutionary relationship between Isolate BUK_BCH_IYU (H-3) and reference related fungal isolate base on the ITS gene sequence.

Discussion

Morphological and Microscopic Characterization and Identification

Penicillium and *Aspergillus* species have been reported before as pathogens of fruit spoilage (Fiori *et al.*, 2008). However, in this study, a different species was found**.** The microbiological analysis of the experiment yielded the isolation of diverse fungal species from the tested samples. The identified species are: *Aspergillus fumigatus*, *Aspergillus niger*, a *yeast species*, and *Curvularia.* Each of these fungi has distinct characteristics and implications in various contexts.

Aspergillus fumigatus is a filamentous fungus commonly associated with environmental sources and is known for its rapid growth on culture media. Its presence in the analyzed samples could indicate environmental contamination. *A. fumigatus* is also recognized for its potential to cause respiratory infections in immunocompromised individuals (Latgé, 1999).

Aspergillus niger, identified in this study, is a versatile fungus with both industrial applications and potential implications in food spoilage. *A. niger* is known for its black pigmentation due to melanin production, which imparts a distinctive appearance to its colonies. In addition to its role in food processing, *A. niger* can produce various enzymes with applications in biotechnology (Machida *et al.*, 2005).

Yeasts, unicellular fungi identified, are diverse and can have both positive and negative impacts. While some yeasts are essential for fermentation processes in the food and beverage industry, others may contribute to spoilage. Further characterization and identification of the yeast species would provide insights into its specific role and impact.

Curvularia, another fungus identified in this study, is a dematiaceous fungus commonly found in soil and plant materials. Its presence might suggest environmental exposure or contamination. *Curvularia* species are known for causing plant diseases and occasionally human infections, particularly in immunocompromised individuals (de Hoog *et al.*, 2005).

Finally, the diverse array of fungi isolated in this study highlights the complexity of microbial communities in the analyzed samples. The presence of potentially pathogenic fungi like *A. fumigatus* and *A. niger* raises considerations for environmental monitoring and potential health risks. Further investigations, including molecular techniques and more detailed characterization, would provide a comprehensive understanding of the fungal composition and their roles in the tested samples.

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume IX Issue VIII August 2024

Molecular Characterization Using the Internal Transcribed Spacer (ITS) Marker and Identification

The use of molecular techniques was pertinent in elucidating the specific fungal species causing the rot of apples. Molecular techniques have proven more dependable than traditional methods as they allow comparing DNA sequence information between known and unknown fungal species DNA sequences from public repositories. The morphological (traditional) description using visual observation of the spores and mycelium commonly used for identifying fungi has led to the wrong identification of fungal isolates (Ikechi-Nwogu *et al.,* 2019).

The molecular analysis revealed a 100% identity match between the initially identified *Aspergillus fumigatus* isolate and *Penicillium griseofulvum*. Many authors have reported similar results. It was shown that ITS regions analyses detected a greater number of species than selective plating (Mansfield and Kuldau, 2007). Peterson also isolated 2 new *Penicillium* species from peanut field soils and used ITS to identify the novel species (Peterson and Horn, 2009). This is a potential refinement of the initial morphological characterization. The unexpected identification underscores the significance of incorporating molecular methods in fungal identification processes. Misidentifications can occur due to similarities in colony appearance or microscopic features, leading to erroneous species assignments. Molecular techniques, such as DNA sequencing, provide more accurate identification by examining specific genetic markers, overcoming the limitations of traditional methods (Balajee *et al*., 2007). The identification of *Penicillium griseofulvum*, a different fungal species from the initially presumed *A. fumigatus*, indicates the significance of molecular analysis in confirming and correcting initial identifications. It is important to note that both *A. fumigatus* and *P. griseofulvum* belong to the class Eurotiomycetes and can share certain morphological traits, potentially leading to misidentification.

Relating to the earlier discussion, the presence of *Penicillium griseofulvum* instead of *A. fumigatus* alters the interpretation of the fungal community in the samples. *P. griseofulvum* is commonly found in various environments and has implications in food spoilage, mycotoxin production, and biotechnological applications (Houbraken *et al.,* 2010).

Further investigations could explore the reasons for the misidentification, including potential morphological similarities between *A. fumigatus* and *P. griseofulvum*, as well as the implications of the presence of *P. griseofulvum* in the analyzed samples. While molecular characterization was performed for the most dominant fungal isolate, future studies would benefit from extending this analysis to all isolated species to provide a more comprehensive understanding of their genetic properties and confirm their identities.

CONCLUSION

In conclusion, the initial morphological characterization of the fungi isolated from damaged apples sourced from Kano markets identified *Aspergillus fumigatus*, *Aspergillus niger*, a *yeast species*, and *Curvularia*. However, the subsequent molecular analysis revealed a 100% identity match with *Penicillium griseofulvum* instead of *A. fumigatus.*

The presence of *Penicillium griseofulvum* in the analyzed samples suggests potential implications for postharvest losses, as this fungus is known for its involvement in food spoilage and mycotoxin production. The refinement of *Aspergillus fumigatus* initially observed in the macroscopic and microscopic analyses indicates the challenges in accurate fungal identification based solely on traditional methods. There is the need for a comprehensive approach that integrates traditional and molecular techniques for more reliable and conclusive results in similar studies.

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