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Molecular characterization of filamentous fungi associated with spoilage of sweet oranges sold in Choba Market, Port Harcourt, Nigeria

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Abstract. *Ikechi-Nwogu CG, Odogwu BA, Edumasam MA. 2023. Molecular characterization of filamentous fungi associated with spoilage of sweet oranges sold in Choba Market, Port Harcourt, Nigeria. Asian J Agric 7: 52-56.* Post-harvest spoilage of sweet oranges is one of the major causes of post-harvest losses. This study was conducted to identify the fungi associated with post-harvest spoilage of sweet oranges using molecular techniques in Choba Markets, Rivers State, Nigeria. The DNA of the fungal isolate SO-1 and SO-2 were characterized using Internal Transcribed Spacer 4 and 5 molecular markers and aligned by the Basic Local Alignment Search Tool for Nucleotide (BLASTN) 2.8.0 of National Centre for Biotechnology Information (NCBI) database. Based on the sequence similarities, it was observed that isolate SO-1 was 98.96% identical to *Neurospora crassa* Shear & B.O.Dodge while SO-2 was 99.48% identical to *Aspergillus flavus* Link. These findings showed that *N. crassa* and *A. flavus* are some of the causal fungal pathogens of spoilt sweet oranges. It is anticipated that this result will provide information that will be helpful in the deployment of the appropriate post-harvest management of these fungi during post-harvest handling to minimize the spoilage of sweet oranges and thus reduce post-harvest losses.

Keywords: *Aspergillus flavus,* Choba Market, *Neurospora crassa,* RBCL marker, spoilage, sweet orange

INTRODUCTION

Fruits are the edible part of flowering plants' developed ovaries, which are generally eaten raw (Uji 2007; Ikhiwili 2012). Fruits are an important class of food needed for normal growth and development; humans and animals depend on them. Aside from humans, organisms such as bacteria and fungi also rely on fruits as a food source (Haase 2008). High sugars and low pH values make fruits desirable for fungal spoilage (Kuyu and Tola 2018).

In botany, an orange is referred to as any small, evergreen tree or shrub bearing round fruits (Daubenton 2013). However, it is called sweet orange to differentiate it from the *Citrus aurantium* Lour., referred to as bitter orange. Sweet orange is botanically known as *Citrus sinensis* (L.) Osbeck is one of Nigeria's main money making fruits (Gorinstein et al. 2001).

Sweet orange belongs to the family Rutaceae because they are often characterized as trees and shrubs and have strong scents. They appear to be one of the major commercial fruit crops that are widely eaten fresh, processed into juices or peels used as a fragrance. It is a principal source of vitamin C; nevertheless, once cut or squeezed, it speedily begins to be dispensed. After only eight hours at room temperature or 24 hours in a refrigerator, there is about a 20 percent loss of vitamin C (Pathy 2018). Moreover, oranges are a good source of folate, a source of vitamins A and B1, and dietary fiber (Kubala and Arnarson 2021). It comprises calcium, potassium, carotenoids, magnesium, flavonoids, antioxidants, acids, essential oils, folate, iron, manganese, zinc, sodium, and iodine (Dhalaria et al. 2020). Some other products made from oranges include: orange oil, orange tea, orange blossom honey, marmalade, and slug repellent. According to Barros et al. (2012), Taylor et al. (2020), and Marcene (2021), sweet oranges have been reported to have anti-inflammatory, and anti-tumor properties, prevent cancer, asthma, obesity, arthritis, kidney stones, coronary heart diseases, high blood pressure, and stroke, and help maintain healthy mucus membranes, skin, and vision. It also boosts our immune system and helps our body to fight diseases. In addition, various fruits have naturally occurring polyphenolic compounds (Nile and Park 2014) that function as free radical inhibitors and play vital roles in preventing aging (Arif et al. 2022) and their associated diseases.

Orange has a thick, bitter rind that can be processed into animal feed. It is also used as a flavoring or for garnishing. The white portion of the rind (pericarp or albedo) is a source of pectin and has almost the same amount of vitamin C as the flesh. The crop is mainly cultivated in subtropical and tropical regions (Encyclopedia of Food and Culture 2022), globally in over 137 countries on six continents. In Nigeria, its production spreads throughout the country.

Even though sweet orange encompasses numerous components which are beneficial to man, fungi affect their economic, medicinal, traditional, and nutritional value, thereby shortening their shelf life. In developing countries, where protection and handling of fresh fruit are inadequate, losses during storage and transit can represent the harvested crop by over 50% (Eckert and Ogawa 1985).

When fruits become less palatable, and there is a change in the usual taste, it indicates that pathogens have invaded the fruits and changed their natural conditions. Aside from the alterations of taste, there are texture, smell, and physical appearance changes owing to the actions of pathogen invasion. These fungi are known tof destroy fruits, reducing consumption and income. Therefore, microorganisms need to be identified to reduce the risk of contamination and infection from handling and consuming these fruits. The objective of this research was to isolate and classify the filamentous fungi associated with the spoilage of sweet orange fruits sold in Choba Market, Port Harcourt, to reduce losses.

Orthodox approaches have been used to identify pathogens; however, according to Lutzoni et al. (2004), these methods are bulky and inefficient. Furthermore, morphological characters can be contentious or problematic even for trained mycologists, as they may not always provide accurate groupings within an evolutionary framework, mainly at the species level. Effective identification of disease-causing fungal pathogens of sweet orange is proper because, according to St-Germain and Summerbell (2003), diseases caused by fungi have become a noteworthy medical problem and are increasing at a disturbing rate. In addition, the increase in the number of patients that are not immuno-competent has emphasized the significance of the precise identification of fungi. Therefore, to accurately identify these pathogens, a molecular characterization technique has been employed to verify the identification of the fungi pathogens (Gontia-Mishra et al. 2013; Bechem and Afanga, 2017).

MATERIALS AND METHODS

Study area and sample collection

The study was conducted at the Regional Centre for Biotechnology and Bioresources Research, University of Port Harcourt, Choba, Rivers State, Nigeria. Polymerase Chain Reaction (PCR) product sequencing was done at the International Institute for Tropical Agriculture (IITA) in Ibadan, Nigeria. Twenty (20) fresh samples with infected parts were collected randomly from the Choba Market in Port Harcourt, Rivers State, Nigeria, in February 2020. The samples were collected into sample bags, labeled, and immediately taken to the laboratory.

Isolation of fungi from sweet orange using the blotter method

The modified standard blotter method of Ikechi-Nwogu et al. (2019) was used to isolate fungi associated with sweet oranges. The filter paper, distilled water (placed in a conical flask), and petri dishes (wrapped in foil paper) used for the work was first autoclaved at 121°C for 15 minutes. Next, the petri dishes were lined with three layers of sterilized 9 cm Whatman's filter paper, the filter paper was soaked with little water, and then the petri dishes were covered immediately. Next, four (4) small bud clusters were surface sterilized in a beaker (Plate 2.1) using 70% ethanol for 2-3 minutes. Next, the ethanol was discarded, rinsed with sterile distilled water twice, and plated per petri dish. Then the plates were wrapped with masking tape and then labeled. After this, they were 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+ 2°C).

Morphological and microscopic identification and characterization

The morphological identification of isolates KN-01 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.

Molecular characterization using the Internal Transcribed Spacer (ITS) marker and identification

The Genomic DNA of the two isolates SO-1 and SO-2 found on the oranges were extracted following the protocol of Quick-DNATM Fungal/Bacterial MiniPrepKit (Zymo Research Group, California, USA). The manufacturer described the protocol by modifications at the Regional Center for Biotechnology and Bioresources (RCBB), the University of Port Harcourt, Choba, Rivers State, Nigeria. The isolate DNA quantity and concentration were measured using Nano-Drop 2000c spectrophotometer (Thermo Fisher Scientific Inc. Wilmington, Delaware, USA). The DNA purity was measured as a ratio of absorbance at 280 nm to that of 260 nm. The DNA samples were sent to the International Institute of Tropical Agriculture (IITA) Bioscience Center, Ibadan, Nigeria, for sequencing and amplification. Furthermore, the primers used to amplify the nuclear ribosomal DNA (rDNA) fragments of the SO-1 and SO-2 isolates were the Internal Transcribed Spacer 4 (ITS4) with the sequence TCCTCCGCTTATTGATATGS and ITS5 with the sequence GGAAGTAAAAGTCGTAACAAGG. The ABI 3500 capillary electrophoresis sequencer sequenced the amplicons. Then, the DNA sequence file was saved in the Bioedit file with an extension. ab1. The Molecular Evolutionary Genetics Analysis (MEGA) version 7.0.26 software analyzed the sequence. Next, the sequence was aligned by the Basic Local Alignment Search Tool for nucleotide (BLASTN) 2.8.0 version to the database of the National Center for Biotechnology Information (NCBI).

Pathogenicity tests of the fungi

Healthy orange fruits were inoculated with each fungus to assess their ability to induce spoilage on the healthy oranges. That was achieved using the methods described by Chukwuka et al. (2010). Twenty healthy oranges were washed and rinsed with distilled water and surface-sterilized using 70% ethanol. A sterilized cork borer was used to bore holes in each of the fruits to enable inoculation. The holes were sealed with petroleum jelly to avoid infection. Afterward, the fruits were each placed in sterilized polythene bags, incubated at 25 + 2°C in the laboratory, and observed for five days. When symptoms developed and were followed by the appearance of visible fungi, the fungi were sub-cultured from the inoculated fruits and compared with the original isolates.

Morphological and microscopic characterization and identification

The morphological identification of the 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 using an electron binocular microscope at X40.

RESULTS AND DISCUSSION

Morphological identification

The post-harvest shelf-life of fresh fruits is one limiting factor affecting their economic value. However, during post-harvest handling in developed and developing countries, about 20-25% of harvested fruits are decomposed by pathogens (Zhu 2006; Strano et al. 2017). Fungal infection of fruits, such as sweet oranges (*C. sinensis*), generally occurs during harvest and growing seasons, handling, transportation, storage, marketing, or after purchase by the consumer (Yusuf et al. 2022). Several organisms have been associated with the post-harvest decay of sweet oranges. Among the organisms are *Aspergillus fumigatus*, *Aspergillus niger*, *Rhizopus stolonifer*, *Neurospora crassa,* and *Aspergillus flavus* (Tafinta et al. 2013).

In this study, two fungal organisms coded SO-1 and SO-2 were isolated and found to be associated with sweet oranges sold in the Choba Market. From the morphological visual observation, the isolates SO-2 (Figure 1 *left*) developed powdery yellowish-green spores on the surface and a reddish-gold colored mycelium at the reverse side of the Petri dish, which indicated that it was similar to *Aspergillus* spp. At the same time, SO-1 (Figure 1 *right*) produced orange-colored spores that are both visible to the naked eye, similar to *Neurospora* spp. (Figure 1).

Molecular characterization using the Internal Transcribed Spacer (ITS) marker and identification

The molecular study was conducted to determine the specific species of the fungal genera *Neurospora* spp. and *Aspergillus* spp., and the genomic DNA of the isolates SO-1 and SO-2 were successfully extracted. The Nano Drop result showed that the isolates' DNA concentrations were 130.3ng/µL and 128.7ng/µL. While the absorption peak of the 260nm/280nm readings was 1.387 and the 260nm/230nm readings 1.357, respectively (Table 1).

Polymerase chain reaction (PCR)

The result of the amplified DNA band of the isolates SO-1 and SO-2 are presented in Figure 2. From the result, the SO-1 and SO-2 isolates sequence had over 587 and 600 base pairs, respectively.

The SO-1 and SO-2 isolate sequences aligned with 100 sequences deposited in the National Center Biotechnology Information (NCBI) composite biological database. The SO-1 isolate sequence was 98.96% identical to *N. crassa* (MH790549.1), while SO-2 was 99.48% identical to *A. flavus* (MH591447.1). The phylogenetic tree result showed the relationship between the isolates SO-1 from this study and other fungal isolates on the NCBI database is shown in Figures 3A and 3B. In addition, the phylogenetic analysis showed that SO-1 was related to other *Neurospora* species, such as *N. tetrasperma, N. intermeda,* and *N. dictyophora.*

Table 1. The concentration of DNA extracted from fungal isolates of sweet oranges using a Nano-drop (2000c) spectrophotometer

Fungi sample ID	DNA concentration (ng/µL)	Absorbance at 260nm/280nm (purity)
SO-1	130.3	1.387
SO-2	128.7	1.357

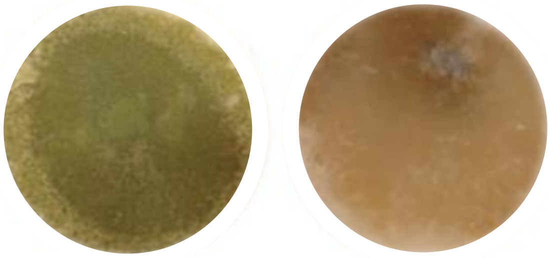


Figure 1. Pure culture of fungus isolated from sweet orange, S0-2 (*left*) and SO-1 (*right*)

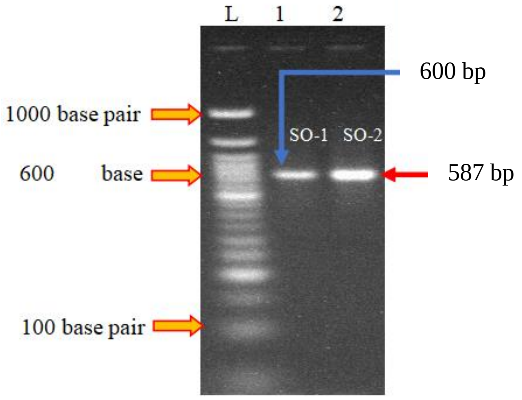
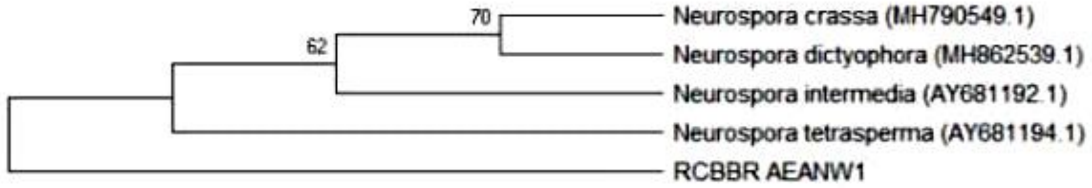


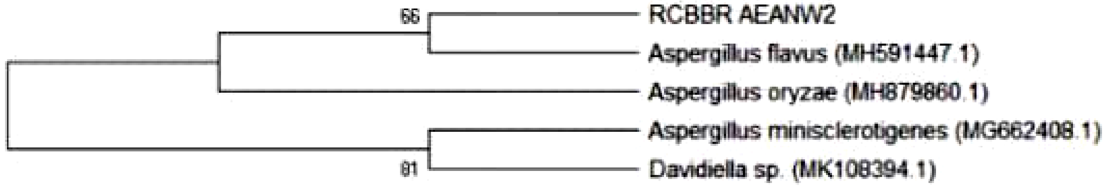
Figure 2. Amplified PCR product generated from SO-1 and SO-2 isolates using ITS marker. L: DNA Ladder, L=1 KB Ladder Sequence Alignment

Sample 1
Strain number – RCBBR_AEANW1
Neurospora crassa



A

Sample 2
Strain number – RCBBR_AEANW2
Aspergillus flavus



B

Figure 3. Phylogenetic tree generated by maximum composite likelihood analysis based on the ITS 4-5 gene sequences. A. *Neurospora crassa,* B. *Aspergillus flavus*

The identification of *Aspergillus* spp. as a causal pathogen for post-harvest rot in sweet oranges is similar to the work by Tafinta et al. (2013) and Oviasogie et al. (2015). They reported that *Aspergillus* spp. is the principal organism associated with the post-harvest rot of sweet oranges and that they produce several toxic metabolites like aflatoxins that are hazardous to human health. The use of molecular techniques was pertinent in elucidating the specific fungal species causing the rot of sweet oranges. 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 techniques employed in this study led to the successful characterization of two (2) fungi isolated causing rot of sweet oranges, namely *A. flavus* and *N. crassa,* which belong to the division Magnoliophyta, class Magnoliopsida, order Sapindales, and family Rutaceae. *Aspergillus* species are producers of mycotoxins. When consumed, these mycotoxins are secondary metabolites harmful to animals and humans.

In conclusion, findings from the study identified *A. flavus* and *N. crassa* as the causal pathogens associated with post-harvest rot of sweet oranges using molecular techniques. Therefore, this technique's use in identifying the fungal pathogen is pertinent. Also, the identification of these pathogens will be helpful in the deployment of the appropriate post-harvest management and control of these fungi during post-harvest handling to minimize the spoilage of sweet oranges and thus reduce post-harvest losses.

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