



Identification of Fungi Associated with Rotten Sweet Orange Fruits (*Citrus sinensis* L.) in University of Ilorin Botanical Garden

Olahan, G.S., Ajadi, I. and Issa, B.T

Department of Plant Biology, University of Ilorin, Nigeria.

ABSTRACT

The presence of fungi in oranges poses a significant risk to public health, impacting global economies and the well-being of both humans and animals. To effectively address this problem, it is important to identify the specific types of fungi to address this concern effectively. This study was aimed at isolating and characterizing fungal species associated with rotten sweet oranges (*Citrus sinensis* L.) in the University of Ilorin using phenotypic characterization and molecular tools. A total of five orange fruits were collected from different trees at the Unilorin botanical garden and immediately transported to the Biology laboratory, University of Ilorin. The rotten part of the fruits was cut out, surface-sterilized, and then inoculated onto potato dextrose agar. *Aspergillus flavus*, *Aspergillus niger* and *Rhizopus arrhizus* were the fungal isolated and identified. The identification of these pathogens is crucial for implementing effective post-harvest management measures. This will aid in eliminating the pathogens from sweet orange fruits during post-harvest handling, reduces spoilage and extending their shelf life.

Keywords: *Citrus sinensis*, Fungal analysis, Molecular analysis, Potato dextrose agar, University of Ilorin.

INTRODUCTION

Citrus sinensis L., commonly called sweet orange, is one of the most important agricultural products in the world. It is widely recognized for its nutritional benefits as well as its economic benefits (Seminara *et al.*, 2023). It belongs to the Rutaceae family, and primarily utilized for extraction and consumption of its fresh juice. *Citrus sinensis* is an important source of vitamin C as well as vitamin B (thiamin, riboflavin, pantothenic acid, pyridoxine, niacin, and folate) thus offers a lot of potential to serve as an antioxidant (Richa *et al.*, 2023). They are rich in metabolites such as flavonoids, carotenoids, alkaloid compounds, phenols as well and tannins (Richa *et al.*, 2023), so they can be used in the treatment of cardiovascular diseases and human malignancies. Minerals found in fruits, such as potassium and calcium lower blood pressure and reduce the risk of stroke (Madsen *et al.*, 2023). However, a number of diseases have been accountable for a significant decline in harvested fruits (Pétriacy *et al.*, 2018).

Microorganisms responsible for spoilage in crops might enter into the seed, during growing process in field, harvest, or even during transport and storage processes (Karanth *et al.*, 2023). The microorganism(s) involved proliferate rapidly and cause degradation owing to a certain environmental state, as it is the situation with the majority of the citrus fruits (Alegbeleye *et al.*, 2022). According to Mekonnen *et al.* (2023), *Phytophthora* sp., *Colletotrichum gloeosporioides* and other fungal diseases are the primary causes of pre-harvest

infections. *Aspergillus niger*, *A. flavus*, *Fusarium oxysporum*, *Mucor mucedo*, *Penicillium digitatum*, and *Rhizopus stolonifer* were found to be associated with sweet oranges (Samuel *et al.*, 2015). Isolation and identification of pathogens is essential for strategic planning of the control measures aimed at reducing disease related harms (Lorenzo *et al.*, 2018). Diseases are sometimes caused by new, previously unknown pathogens that need to be isolated and studied. If specific nutrient medium that allows only the growth of that pathogen is available, then the isolation of the particular pathogen is accomplished by growing a tiny portion of the diseased tissue on such medium (Venbrux *et al.*, 2023). Fungi such as yeasts and moulds are primarily associated with diseased and degraded citrus fruits (Mailafia *et al.*, 2016).

The existence of fungi, along with the contamination of orange fruits by mycotoxin, causes the spread of diseases in the growing area when the infected seeds are sown. The objective of this study was to isolate and identify fungi associated with rotten sweet orange fruits in the Botanical Garden, University of Ilorin, Ilorin, Nigeria through purely qualitative, not quantitative.

MATERIALS AND METHODS

Collection of Samples

Five rotten sweet orange fruits (*Citrus sinensis* L.) were plucked from five different trees at the University of Ilorin's Botanical Garden, Ilorin lying on the Latitude: 8° 31' 30.4295" N and Longitude: 5° 38' 31.6513" E. The oranges were collected using

*Corresponding author. E-mail: olahan.gs@unilorin.edu.ng; Tel: +2348065919065

Author(s) agree that this article remain permanently open access under the terms of the Creative Commons Attribution License 4.0 International license

sterile gloves and transported in polyethylene bags to the Unilorin Biology Laboratory for analysis.

Preparation of Culture Media and Isolation of Fungi from the Rotten Sweet Orange Fruits

Potato Dextrose Agar (PDA) culture medium was prepared following the Manufacturer's instructions. Nineteen grams of the powder was weighed into 500 ml of sterile distilled water inside a conical flask. It was allowed to dissolve on a hot plate and then autoclaved for 15 minutes at 121 °C and 15 lb/inch² of pressure. After cooling, Streptomycin was added to it at 1 ml to prevent the growth of bacteria. The molten culture medium (15 ml) was poured into 3 sterile Petri dishes. The rotten portion of the sweet orange was surface sterilized using Bleach, and then rinsed in 3 changes of sterile distilled water. It was then blotted dry with sterile cotton wool. The diseased tissue was cut with a sterile scapel and inoculated on the PDA plates. The inoculated Plates were incubated at ambient temperature for 5-7 days. They were observed for fungal growth during the incubation period.

Morphological and Microscopic Identification and Characterization

The morphological identification of isolates was conducted by visually observing the mycelium and comparing their colony colors and texture, and color of the reverse sides of the plates. The isolates were later subjected to microscopic analysis and their micrographs taken with an electron binocular microscope at X40 for their morphological identification with reference to Kidd *et al.* (2023).

Molecular Characterization

The Genomic DNA of the isolates from the rotten sweet orange fruits were extracted following the protocol of Quick-DNA™ Fungal/Bacterial MiniPrepKit (Zymo Research Group, California, USA), with little modifications. The quantity and concentration of the extracted DNA were measured using Nano-Drop 2000c spectrophotometer (Thermo Fisher Scientific Inc. Wilmington, Delaware, USA) and the extracted DNA sent to Inqaba biotec, Ibadan, Nigeria, for amplification and sequencing in both directions. The primers used to amplify the nuclear ribosomal DNA (rDNA) fragments of the isolates were the Internal Transcribed Spacer 1 (ITS1) with the sequence TCCGTAGGTGAACCTGCGG and ITS4 with the sequence TCCTCCGCTTATTGATATGC. Sequence quality was verified by reviewing the electropherograms. The "forward" and "reverse" sequences obtained after sequencing were aligned using the BioEdit v. 7.2.5 software to obtain the consensus sequences. The sequences of the isolates were compared with those deposited in the National Center for Biotechnology Information (NCBI), using the BLAST tool.

RESULTS AND DISCUSSION

Morphological Identification of the Fungal Isolates

Fungal infection of fruits, such as sweet oranges (*C. sinensis*), generally occurs during harvest, on the field, as well as during handling, transportation, storage, marketing, or after purchase by the consumer (Yusuf *et al.* 2022). In this study, three fungal species tagged Isolates A, B and C. which were morphologically suspected to be *Aspergillus flavus* (Fig. 1a and b), *A. niger* (Figs. 2a and b) and *Rhizopus stolonifer* (Figs. 3a and b) based on their microscopic and macroscopic features, were isolated from sweet oranges collected from the University of Ilorin Botanical Garden. Their identity was based on the criteria proposed by Kidd *et al.* (2023).

Each of them aligned with 100 sequences deposited in the National Center Biotechnology Information (NCBI) composite biological database. The isolate A's consensus sequence was 99.64% identical to *Aspergillus flavus* (ON365673.1), isolate B's consensus sequence was 100% similar to *Aspergillus niger* (JX501365.1), while isolate C's consensus was 100% identical to *Rhizopus arrhizus* (MT603963.1). These confirmed the true identities of the fungal isolates associated with sweet orange fruits in this study. Molecular techniques have demonstrated greater reliability compared to conventional approaches, enabling examination of DNA sequence data for comparisons between known and unknown fungal species. Relying on visual observation of spores and mycelium for fungal identification, a common practice in traditional morphology, has frequently resulted in misidentifying fungal isolates (Bhunjun *et al.*, 2021).

These results are in alignment with reports of Tafinta *et al.* (2014) and Oviasogie *et al.* (2015) in which *Aspergillus fumigatus*, *A. niger*, *A. flavus*, *Rhizopus stolonifer* and *Neurospora crassa* were isolated from rotten sweet orange fruits. They reported further that *Aspergillus* sp. is the principal organism associated with the post-harvest rot of sweet oranges, and they are responsible for the production of several toxic metabolites such as aflatoxins. When consumed, these aflatoxins are harmful to animals and humans. Ojo *et al.* (2013) also isolated *Aspergillus niger*, *Fusarium oxysporum*, *Penicillium digitatum* and *Rhizopus stolonifer* from the spoilt orange fruits displayed for sale in five different market places in five major cities in Oyo State in South Western Nigeria. Akintobi *et al.* (2011) isolated *Aspergillus niger*, *Penicillium digitatum* and *Rhizopus stolonifer* from deteriorating citrus fruits in Ibadan, Southwestern Nigeria. Similarly, Samuel *et al.* (2015) reported the incidence of *Aspergillus niger*, *A. flavus*, *Fusarium oxysporum*, *Mucor mucedo*, *Penicillium digitatum*, and *Rhizopus stolonifer* on

sweet oranges obtained Awka major markets, Anambra, Nigeria. Also, Muhammed *et al.* (2021) in their study on fungi associated with sweet orange fruits in Pakistan reported *Aspergillus niger*, *A. flavus*, *A. fumigatus* and *Penicillium digitatum*.

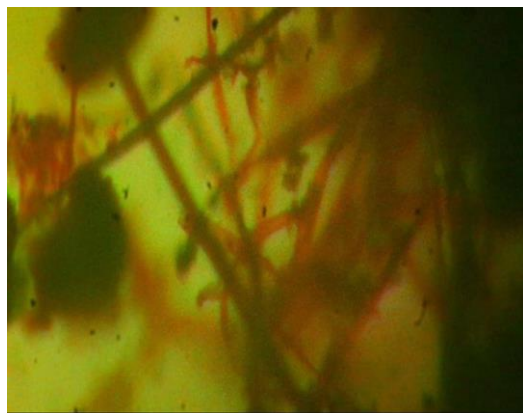


Fig. 1a: Micrograph of Isolate A



Fig. b: Culture of Isolate A on PDA

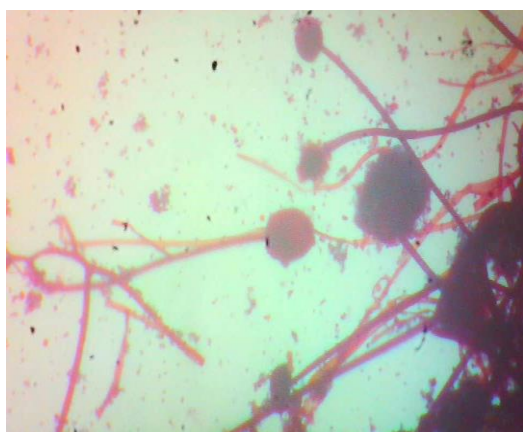


Fig. 2a: Micrograph of Isolate B



Fig. 2b: Culture of Isolate B on PDA

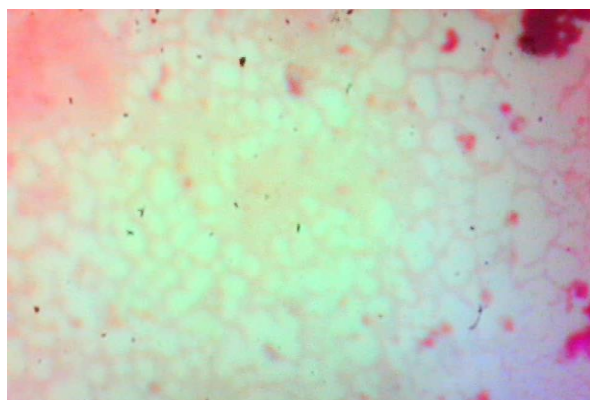


Fig. 3a: Micrograph of Isolate C

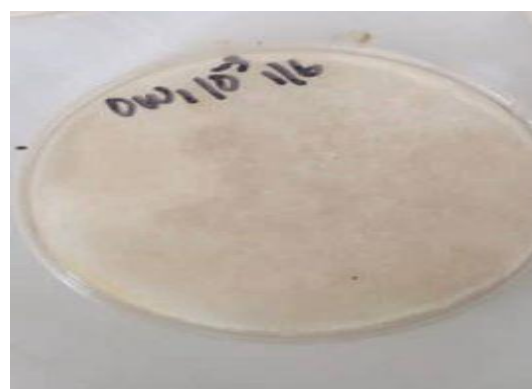


Fig. b: Culture of Isolate C on PDA

Molecular Characterization

The consensus DNA sequences for isolates A, B and C are represented in Figs. 4, 5 and 6 below:

TACGGCACCTGAGTGGTTCTAGCGAGCCACCTCCCACCCGTGTTTACTGTACCTTAGTTGCTTCGG
CGGGCCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGGCCGCGCCCGCCGGAGACACCAC
GAACTCTGTCTGATCTAGTGAAGTCTGAGTTGATTGTATCGCAATCAGTTAAACTTTCAACAATGG
ATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAATTC
CGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAG
CGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTTGGGTCGTCGTCCTCTCCGGGGGGGACGGG
CCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTA
GGCCCGGGCCGGCGCTTGCCGAACGCAATCAATCTTTCCAGGTTGACCTCGGATCAGGTAGGGAT
ACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA

Figure 4: Consensus DNA sequence of *Aspergillus flavus*

ATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCCGGCT
TGTGTGTTGGGTCGCCGTCCCCCTCTCCGGGGGGACGGGCCCCGAAAGGCAGCGGCGGCACCGCGT
CCGATTCCGTAGGTGAACCTGCGGAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAG
CGAAATGCGATGGGATAACCGCTGAACAAGGATCATTACCGAGGGGGGGTCTTTGGGCCCCAACC
TCCCATCCGTGTCTATTGTACCCTGTTGCTTCGGCGGGCCCCGCGCTTGTGCGCCGCCGGGGGGG
GCCTCTGCCCCCGGGCCCCGTGCCCGCCGGAGACCCCAACACGAACACTGTCTGAAAGCGTGCAG
TCTGAGTTGATTGAATGCAATCAGTTAAACTTTCTTAAGCATATCAATAAGCGGAGGAACTAAT
GTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACTCCTCGAGCGTATGGGGCTTTGT
CACATGCTCTGTAGGATTGGCCGGCGCCTGC

Figure 5: Consensus DNA sequence of *Aspergillus niger*

GTCGTAACAAGGTTTCCGTAGGTTAGAGGACGGAAGGATCATTAATTATGTAGTAAAAGAACCTGTA
AAGCGCCTTACCTTAGGGTTTCTCTGGGGTAAGTGATTGCTTCTACACTGTGAAAATTTGGCTGAG
AGACTCAGACTGGTCATGGGTAGACCTATCTGGGGTTTGATCGATGCCACTCCTGGTTTCAGGAGCA
CCCTTCATAATAAACCTAGAAATTCAGTATTATAAAGTTTAATAAAAAACAACCTTTTAACAATGGATC
TCTTGGTTCTCGCATCGATGAAGAACGTAGCAAAGTGCGATAACTAGTGTGAATTGCATATTCAGTG
AATCATCGAGTCTTTGAACGCAGCTTGCACTCTATTAAGCATATCAATACAAACCTATGATCTGAAGTC
TGGTTTTTCTATAGAGTACGCCTGCTTCAGTATCATCACAAACCCACACATAACATTTGTTTATGTGGT
AATGGGTCGCATCGCTGTTTTATTACAGTGAGCACCTAAAATGTGTGTGATTTTCTGTCTGGCTTGCT
AGGCAGGAATATTACGCTGGTCTCAGGATCTTTTTCTTTGGTTCGCCCAGGAAGTAAAGTACAAGAG
TATAATCCAGCAACTTTTGAAC

Figure 6: Consensus DNA sequence of *Rhizopus arrhizus*

Conclusion

Aspergillus flavus, *A. niger* and *Rhizopus arrhizus* were isolated from rotten sweet oranges. The identification of these pathogens is crucial for implementing effective post-harvest control measures. This will aid in eliminating the pathogens from sweet orange fruits during post-harvest handling, reducing spoilage and extending their shelf life.

Authors' contributions

Olahan, G.S., conceived the idea and researched literature, Olahan, G.S., Ajadi, I. and Issa, B.T., involved in the protocol development, result analysis, and draft of the manuscript. All authors reviewed, edited and approved the final version of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

REFERENCES

- Akintobi, A.O., Okonko, I.O., Agunbiade, S.O., Akano, O.R. and Onianwa, O. (2011). Isolation and identification of fungi associated with the spoilage of some selected fruits in Ibadan, South Western, Nigeria. *Academia Arena*, 3(11): 1-10
- Alegbeleye, O., Odeyemi, O. A., Strateva, M. and Stratev, D. (2022). Microbial spoilage of vegetables, fruits and cereals. *Applied Food Research*, 2(1):100122. <https://doi.org/10.1016/j.afres.2022.100122>

- Bhunjun, C. S., Phillips, A. J. L., Jayawardena, R. S., Promputtha, I. and Hyde, K. D. (2021). Importance of Molecular Data to Identify Fungal Plant Pathogens and Guidelines for Pathogenicity Testing Based on Koch's Postulates. *Pathogens*, 10(9): 1096. <https://doi.org/10.3390/pathogens10091096>
- Karanth, S., Feng, S., Patra, D. and Pradhan, A. K. (2023). Linking microbial contamination to food spoilage and food waste: The role of smart packaging, spoilage risk assessments, and date labeling. *Frontiers in Microbiology*, 14: 1198124. <https://doi.org/10.3389/fmicb.2023.1198124>
- Lorenzo, J. M., Munekata, P. E., Dominguez, R., Pateiro, M., Saraiva, J. A. and Franco, D. (2018). Main Groups of Microorganisms of Relevance for Food Safety and Stability. In *Innovative Technologies for Food Preservation* (53–107). Elsevier. <https://doi.org/10.1016/B978-0-12-811031-7.00003-0>
- Madsen, H., Sen, A. and Aune, D. (2023). Fruit and vegetable consumption and the risk of hypertension: A systematic review and meta-analysis of prospective studies. *European Journal of Nutrition*, 62(5): 1941–1955. <https://doi.org/10.1007/s00394-023-03145-5>
- Mailafia, S., Okoh, G. R., Olabode, H. O. K. and Osanupin, R. (2016). Isolation and identification of fungi associated with spoilt fruits vended in Gwagwalada market, Abuja, Nigeria. *Veterinary World*, 10(4): 393–397. <https://doi.org/10.14202/vetworld.2017.393-397>
- Mekonnen, D. A., Adeyemi, O., Gilbert, R., Akerele, D., Achterbosch, T. and Herforth, A. (2023). Affordability of healthy diets is associated with increased food systems performance in Nigeria: State-level analysis. *Agricultural and Food Economics*, 11(1): 21. <https://doi.org/10.1186/s40100-023-00263-w>
- Muhammad, A.S., Mohammed, I.U., Ameh, M., Bello, I., Haliru, B.S., Bagudo, H.A. and Sanda, A. (2018). Isolation and identification of fungi associated with the spoilage of sweet orange (*Citrus sinensis* L) and banana (*Musa sapientum* L) in Sokoto Metropolis. *Journal of Applied Biotechnology and Bioengineering*, 5(3):172–182
- Muhammad, I., Asif, R. and Gulshan, I. (2021). Fungi associated with postharvest deterioration of sweet orange fruits in Rawalpindi and their management by biocontrol agents. *Jammu Kashmir Journal of Agriculture*, 1 (1): 07-15.
- Ojo, O.A., Olaniran, O.A. and Odelade, K.A. (2013). Survey of Postharvest Diseases and Aflatoxin contamination of marketed orange fruit (*Citrus* sp) in major cities in Oyo State, Nigeria. *International Journal of Applied Plant Pathology*, 10(6): 110-117.
- Oviasogie, F. E., Ogofure, A. G., Beshiru, A., Ode, J. N. and Omeje, F. I. (2015). Assessment of fungal pathogens associated with orange spoilage. *African Journal of Microbiology Research*, 9(29): 1758–1763. <https://doi.org/10.5897/AJMR2014.7246>

- Pétriaccq, P., López, A. and Luna, E. (2018). Fruit Decay to Diseases: Can Induced Resistance and Priming Help? *Plants*, 7(4): 77. <https://doi.org/10.3390/plants7040077>
- Richa, R., Kohli, D., Vishwakarma, D., Mishra, A., Kabdal, B., Kothakota, A., Richa, S., Sirohi, R., Kumar, R. and Naik, B. (2023). Citrus fruit: Classification, value addition, nutritional and medicinal values, and relation with pandemic and hidden hunger. *Journal of Agriculture and Food Research*, 14:100718. <https://doi.org/10.1016/j.jafr.2023.100718>
- Samuel, O., Ifeanyi, O. and Ugochukwu, O. (2015). Filamentous fungi associated with the spoilage of sweet oranges sold in major Awka Markets, Nigeria. *Bioengineering and Bioscience*, 3(3): 44-49.
- Seminara, S., Bennici, S., Di Guardo, M., Caruso, M., Gentile, A., La Malfa, S. and Distefano, G. (2023). Sweet Orange: Evolution, Characterization, Varieties, and Breeding Perspectives. *Agriculture*, 13(2): 264. <https://doi.org/10.3390/agriculture13020264>
- Tafinta, I., Shehu, K., Abdulganiyyu, H., Rabe, A. and Usman, A. (2014). Isolation and Identification of Fungi Associated with the Spoilage of Sweet Orange (*Citrus Sinensis*) Fruits in Sokoto State. *Nigerian Journal of Basic and Applied Sciences*, 21(3): 193. <https://doi.org/10.4314/njbas.v21i3.4>
- Venbrux, M., Crauwels, S. and Rediers, H. (2023). Current and emerging trends in techniques for plant pathogen detection. *Frontiers in Plant Science*, 14: 1120968. <https://doi.org/10.3389/fpls.2023.1120968>