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RESEARCH ARTICLE

Mycobiota Associated with *Dacryodes edulis* H. J. Lam fruits sold In Rumokoro Market, Port Harcourt, Nigeria

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Dacryodes edulis H. J. Lam is a common fruit in Nigeria used to eat roasted or boiled corn. It can also be eaten alone by softening with hot water, hot ash, or by roasting on low heat. It is called "ube" in the eastern part of Nigeria. *D. edulis* fruits are prone to infection by fungal pathogens in the field, in transit and during storage. Proper identification of these pathogens is important in disease prevention and control. This study was carried out to isolate and identify the fungal species associated with *D. edulis* fruits. Fruits were obtained from the Rumokoro market, Rivers State, Nigeria in January 2020. Isolation of fungi was done by Potato Dextrose (PDA) method and identification was carried out by molecular method. Fungal DNA was extracted using the Zymo Fungal/Bacterial DNA Miniprep Kit. Polymerase chain reaction amplification of the internal transcribed spacer (ITS) region was carried out using the primer pair: ITS4 and ITS5. BLAST search was carried out on the National Centre for Biotechnology Information (NCBI) database and the fungi were identified as: *Rhizopus delemar*, *Aspergillus oryzae* and *A. welwitschiae*. The Phylogenetic tree was constructed to show the evolutionary relationship among the isolates and other fungal species retrieved from GenBank. The sequences of the isolates have been deposited in GenBank under the accession numbers: ON965489, ON965490 and ON965491 for *Rhizopus delemar*, *Aspergillus oryzae* and *A. welwitschiae* respectively. The molecular method used in this study enabled the isolates to be identified at the species levels.
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Keywords : *Dacryodes edulis*, DNA extraction, fungi, identification, sequencing

Introduction

Dacryodes edulis (G. Don) H.J. Lam, belonging to the Burseraceae family, is a tree plant with an oil-bearing fruit that is highly domesticated in the Central African Rainforest.[1] The tree is native to Nigeria, Central African Republic, Uganda, Angola, Ghana, Benin Republic, Cote d'Ivoire, Liberia, Democratic Republic of Congo, Togo, Cameroon, Equatorial Guinea, Gabon, Sierra Leone; and exotic to Malaysia.[2] Common English names for the plant include African plum, African pear, African palm, bush butter tree, and native pear. In the French language, it is commonly called prunier, atanga or safoutier.[2] African pear is a shade-loving plant and grows well at an altitude of 1000 m, with mean annual rainfall of 1400 to 4000 mm and mean annual temperature of 23 to 55° C. The tree adapts well to varying environmental conditions such as different soils, rainfall, altitude, temperature and day length. African pear tree is mostly known for its fleshy fruit which are produced annually or biannually and vary in size ranging from 4 to 12 cm in length and 3 to 6 cm in diameter.

African pear fruits are ellipsoidal drupes with thin exocarps that are pink in colour at the early stage of development but become dark blue or violet at maturity. The pulp is firm and thin. *D. edulis* fruit has a high nutritional value. The tree can produce up to 7 to 8 tonnes of oil per hectare of land.[2] The fruit pulp contains about 40 to 70% of lipids,[3] with a considerable proportion of linoleic and palmitic acid, 17.9% of fibre and 25.9% of protein.[4] Mineral contents include calcium, iron, magnesium, manganese, phosphorus and potassium.[5] Its oil is highly used in cosmetics, food and pharmaceutical industries.[6] The fruit's pulp or mesocarp can be

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Table 1. Frequency of occurrence of fungal isolates obtained from *Dacryodes edulis* fruits

Isolate ID	Frequency of occurrence
1	1.75 ± 0.21
2	2 ± 0.19
3	2.15 ± 0.15
4	2.5 ± 0.31

eaten raw, softened in hot water or roasted.[5] The pulp can be turned into powder and used as a butter substitute in biscuit making.[7] The bark of the tree produces a whitish resin that is locally used for treating parasitic skin diseases. In Congo, the bark is boiled and used as a mouthwash. It is also used to manage anaemia, leprosy and dysentery. It is mixed with palm oil and applied topically to relieve body pains and stiffness and to treat dermatological conditions. Leaf sap is introduced into the eardrum to treat ear problems. Leaf steaming is used as a vapour bath to manage fever and headache.

The ripe fruit is naturally highly perishable, lasting only between 2 to 3 days at ambient temperature.[8] This makes it difficult for the fresh fruits to be exported to other countries where they are not produced. Fungal diseases have been reported on *D. edulis* trees and harvested fruits. Disease symptoms include fruit and leaf drop, necrotic spots and galls on fruits and leaves, dieback of branches etc.[2] Fruits are usually attacked by post-harvest rots pathogens among which *Rhizopus stolonifer* Vuillemin, 1902; family Mucoraceae and *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., 1909; family Botryosphaeriaceae are of greatest concern, contributing to about 80% rot of the affected fruits. *Erwinia* spp. and *Aspergillus niger* van Tieghem, 1867; family Aspergillaceae are the other rot pathogens of African pear. Many fruits are eaten by birds on the tree causing fruit spoilage. Young leaves are eaten by some insects and this leads to continuous growth of the shoot because the leaflets fall off the tree before they mature. The larvae of a nitidulid beetle, *Carpophilus* sp. Stephens, 1830; family Nitidulidae, eats the seed of African pear fruit in Cameroon, and when the adult finds its way out of the fruit, secondary infections usually occur and this leads to fruit decay. A caterpillar of the pyralid moth, *Patania balteata* Fabricius, 1798; family Crambidae is the most destructive pest of *D. edulis* tree in Congo, leading to a burnt appearance of the leaves.[2]

Considering that people may consume fruits with high levels of pathogen infestation since they are sold cheaper than healthy ones, these fruits represent a potential reservoir for these pathogens which can be transmitted to humans when consumed. Monitoring the occurrence of pathogens in fruits is important for a profound assessment of human exposure to food-borne risks, as well as providing data for improved prevention/ control strategies.[9] In this study, we assessed the post-harvest fungal organisms causing deterioration of *D. edulis* fruits in Rumokoro market, Rivers State, Nigeria.

Materials and Methods

Healthy and diseased *Dacryodes edulis* fruits were obtained in May 2022 from Rumuokoro market, Port Harcourt, Rivers State, Nigeria. The study was carried out at the Regional Centre for Biotechnology and Bio-resources Research, University of Port Harcourt, Rivers State, Nigeria. Amplified products were sequenced at the International Institute of Tropical Agriculture (IITA), Ibadan.

Fungi were isolated from the healthy and diseased *D. edulis* fruits using Microbiology conventional cultural method. The fruits were sliced into pieces and surface sterilized by rinsing with tap water and soaking in 70% ethanol for 3 minutes. The sliced fruits were then rinsed with sterile distilled water for three consecutive times.

Several dilution blanks were prepared and sterilized test tubes were labeled 10^1, 10^2, 10^3, 10^4 and 10^5. The test tubes were filled with 9ml of sterilized distilled water except the first test tube labeled 10-1 which served as the stock solution. This was filled with 10ml of water. One gram of both the diseased and healthy fruits was separately weighed, meshed and transferred into the first test tube (10^1). The tube was vigorously shaken to properly mix the constituents in the tube. A micropipette was used to transfer 1000µl of the sample into the next test tube (10^2), and the content was properly mixed by inversion. One thousand microlitre of the dilution was transferred from the second test tube (10^2) into the third test tube (10^3). One thousand microlitre of the dilution was aseptically transferred from the third test tube (10^3) to the fourth test tube labeled 10^4. Finally, 1000µl of the dilution from 10^4 test tube was transferred to the test tube labeled 10^5. The test tube labeled with 10^3 was used for further analysis. This procedure was carried out separately for the diseased and healthy fruits. Using a micropipette, 100µl of the content from the 10^3 dilution for the diseased and healthy fruits were separately transferred into sterilized Petri dishes containing already prepared Potato dextrose agar (PDA) medium. These were incubated for 7 days at room temperature. Each fungal colony observed on Petri dishes was sub-cultured on PDA to obtain pure cultures of fungi. Pure cultures when obtained were used for DNA extraction. *D. edulis* fruits are shown in Fig 1.

Figure 1. *Dacryodes edulis* fruits

Molecular Techniques for Identification of Microorganisms

Quick-DNA Fungal/Bacterial MiniPrep Kit (Zymo Research Group, California, USA) was used for the extraction of fungal DNA. Nanodrop 2000c Spectrophotometer by Thermo Fisher Scientific Inc. Wilmington, Delaware, USA was used to determine the concentration and purity of extracted DNA. DNA quality check was by agarose (1.5%) gel electrophoresis.

The polymerase chain reaction was carried out by amplification of the fungal internal transcribed spacer (ITS) regions using the universal primer pair, ITS4, forward: (5´-TCCTCCGCTTATTGATATGS -3´) and ITS5, reverse: (5´-GGAAGTAAAAGTCGTAACAAGG-3´). A PCR mixture of 25µL total volume was prepared and this contained 1.0µL of each primer (concentration of 5µM), 1.0 µL of DMSO, 1.0µL of 2.5mM DNTPs, 0.1µL of Taq polymerase, 1.0µL of 25mM $MgCl_2$ (Promega), 3µL of genomic DNA (10ng/µL), 2.5µL of 10X PCR buffer and 13.4µL of Nuclease-free water. Amplifications were performed in a thermal cycler (GeneAmp® 9700 PCR System, Applied Biosystems, California, USA) using thirty-six cycles with each cycle consisting of a denaturation step at 94°C (30s), an annealing step at 54°C (30s), and an elongation step at 72°C (45s). A final extension step of 7 mins was done at 72°C after the last cycle. The reaction products were held at 10°C in the machine cycler till when needed for gel electrophoresis. The reaction products were separated on agarose gel, purified using a gel band purification kit and sequenced on ABI 3500 Genetic Analyzer (Thermo Fisher Scientific, Massachusetts, U.S.A).

Raw sequences were cleaned and edited on Molecular Evolutionary Genetics Analysis (MEGA) software, version 10.0.1 (MEGA X).10 Identification of edited sequences was done on BLAST alogrithm on National Centre for Biotechnology Information (NCBI) database. Best blast hits obtained were aligned using Clustal W alogrithm and a neighbour -joining phylogenetic tree was constructed to determine the evolutionary relationship among the fungal species isolated and other species on GenBank.

Results

Fungal Isolates of Dacryodes edulis Fruits

Four fungal organisms were isolated and found to be associated with *D. edulis*. The frequency of occurrence of the fungal species grown on PDA was determined. Sample 4 had the highest frequency of occurrence (2.5) while sample 1 had the least (1.75) as presented in Table1. Fig 2 shows the pure cultures of the fungal isolates.

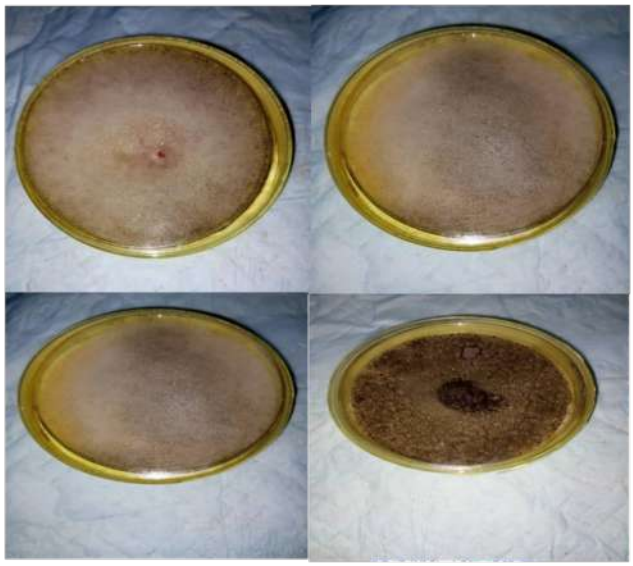

Figure 2. Fungi isolates obtained from *Dacryodes edulis* fruit and grown on potato dextrose agar

DNA Concentration and Purity

Nanodrop 2000C spectrophotometer showed that the concentration and purity of the fungal DNA obtained were of good quality for other downstream experiments. Genomic DNA purity was calculated as a measure of the ability of DNA to absorb UV light with an absorption peak at 260/280nm.

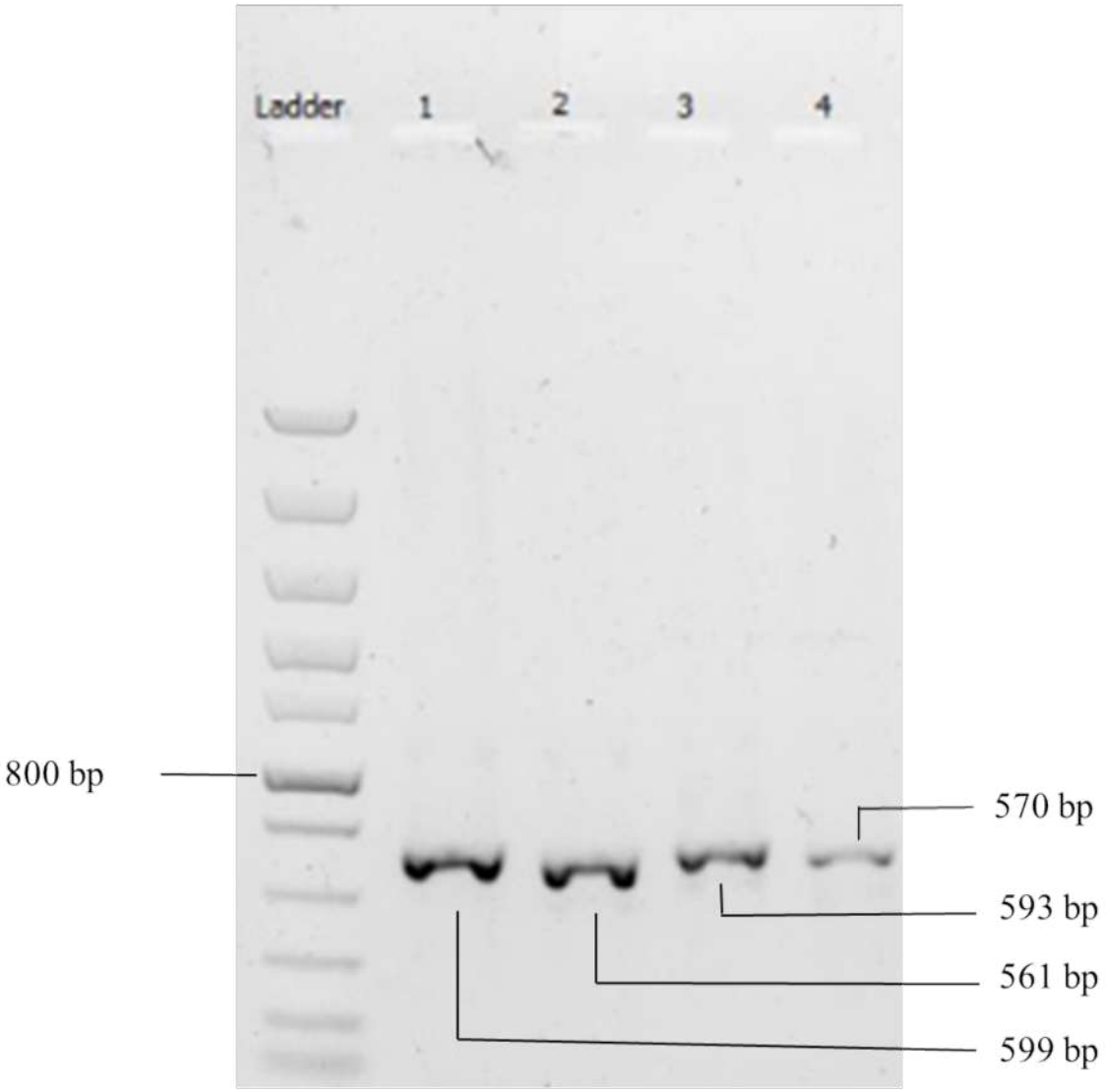

Figure 3. PCR products obtained from ITS1-2 region of fungal isolates

Polymerase Chain Reaction (PCR)

DNA amplification was carried out using fungal universal primer pair; ITS4- forward (5'TCCTCCGCTTATTGATATGS3') and ITS5- reverse (5'GGAAGTAAAAGTCGTAACAAGG3'). Amplified DNA showed bands on gel when viewed under UV light. The PCR product of each isolate was visible as presented in Fig 3.

Sequences of ITS region of Fungal Isolates

Isolates that were amplified after PCR were sequenced. Sequences were blasted on National Centre for Biotechnology Information (NCBI) database and the species identity of isolates was revealed.

Isolates were identified as *Rhizopus delemar* (Boidin) Wehmer & Hanzawa, 1912; family Mucoraceae (isolate 1), *Aspergillus oryzae* (Ahlburg) E. Cohn, 1884; family Aspergillaceae (isolate 2) and *Aspergillus welwitschiae* (Bres.) Henn., 1907; family Aspergillaceae (isolate 4). Isolate 3 could not be identified maybe due to sequencing errors as the BLAST for this particular isolate did not obtain any hit.

Strain numbers were assigned to each fungal isolate and the ITS sequences were submitted on GenBank. An accession number was assigned to each fungal organism. The accession numbers are in parentheses below.

Sample 1: *Rhizopus delemar* (ON965489) Strain number RCBBR_AEAN1
Sample 2: *Aspergillus oryzae* (ON965490) Strain number RCBBR_AEAN2
Sample 4: *Aspergillus welwitschiae* (ON965491) Strain number RCBBR_AEAN4

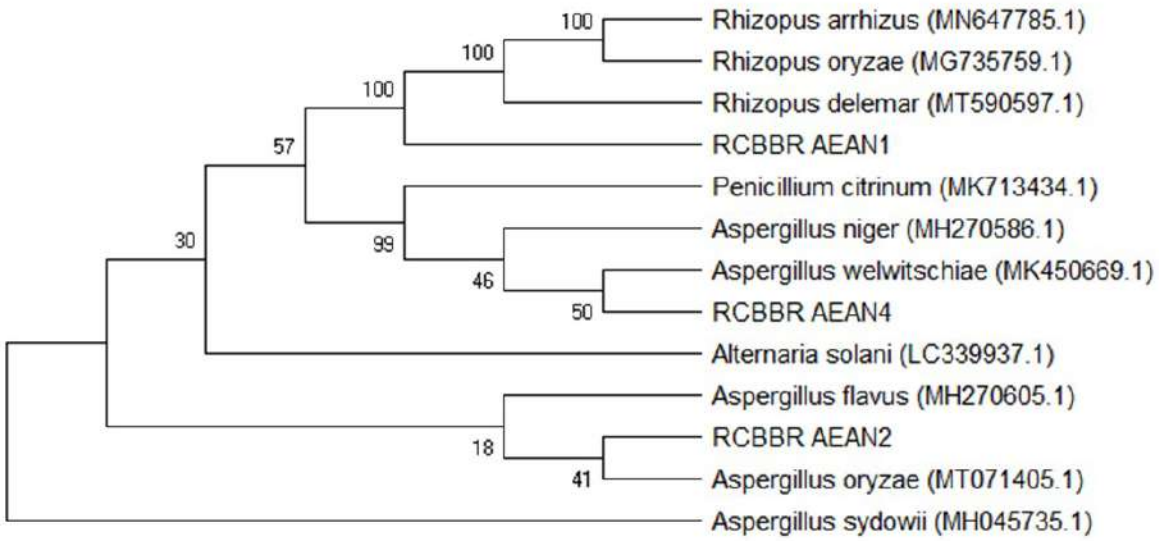

Figure 4. Neighbor-joining tree based on the sequenced ITS1-2 region of isolates

Phylogenetic Analysis of Fungal Isolates

Sequence data of other closely related fungal isolates available on GenBank were aligned with the ITS sequences of isolates obtained from this study using Clustal W alogrithm on MEGA X software. A phylogenetic tree was built to show the evolutionary relationship that exists among these organisms as presented in Fig 4. The length of the vertical lines determines the level of the relationship.

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Discussion

The prime objective of this study is to characterize the fungal organisms associated with *Dacryodes edulis* using polymerase chain reaction to amplify the Internal Spacer (ITS) regions of the fungal genome with the motive of sequencing and eventual species identification. This led to the isolation and identification of *Rhizopus delemar*, *Aspergillus oryzae* and *A. welwitschiae* with 100, 97.83 and 94.44 percent similarity.

Aspergillus welwitschiae has been reported on *Agave sisalana* Perrine, 1838; family Asparagaceae (sisal) as the causative agent of bole rot disease which hampers the production of sisal fibres in Brazil. Brazil is the chief producer of sisal fibres globally. *A. welwitschiae* is an opportunistic pathogen with necrotrophic behaviour[11] and also a saprotroph with a wide host range. Host range of *A. welwitschiae* includes tomato, root-mustard, carrot, pepper, Chinese flowering cabbage, cucumber, lettuce, broccoli, oilseed rape, radish, kidney bean and cabbage.[12] Previous research established *A. welwitschiae* as the only causative agent of bole rot in the field, but little is known about the evolution of this species and its strains.[13] *Aspergillus welwitschiae* belongs to the phylum Ascomycota.[11] It was named after *Welwitschia mirabilis*, a plant species native to the Namib desert (South Africa), where the fungus was first encountered.[14,15] *A. welwitschiae* occurs in the soil and disperses its spores into the air. It may inhabit plants and cause diseases symptoms, followed by plant death.[16,17] The bole rot disease was previously reported to be caused by *Aspergillus niger* and related species.[18,19] This misclassification may be because both *A. niger* and *A. welwitschiae* belong to section *Nigri* of the *Aspergillus* genus. *Aspergillus* section *Nigri* (black aspergilli) is one of the most complex, confusing, and challenging groups to classify and identify.[20] The section is made up of six different clades[21] and twenty-six distinct species.[22] Species of *Aspergillus* section *Nigri* are closely related, indistinguishable by almost any morphological features, and form a monophyletic clade.[23] They can only be accurately identified by the calmodulin (CaM) gene and not by the universal inter-transcribed spacer region (ITS) gene.[24] *A. welwitschiae* has also been reported to be responsible for post-harvest rot on Chinese cabbage and vegetable cellars in Harbin, China in 2019 and 2020. Symptoms include; water-soaked spot at the base of the leaves midrib which later extends and turns into rot. The diseased part changes colour and becomes dark brown to black with some black spores on the leaf surface.[12]

Aspergillus oryzae is a filamentous fungus that belongs to the *Aspergillus flavus-oryzae* group. It has been used in the fermentation of different foods in many countries all over the world for many centuries. *A. oryzae* genome is made up of

genes involved in primary and secondary metabolism, biomass degradation, cell signalling and transcriptional regulation.[25] *A. oryzae* is rich in many bio-active secondary metabolites belonging to different chemical classes such as fatty acids, terpenoids, oxylipins and coumarins.[26] The aspirochlorine and heterotetracyclic gliotoxin produced by *A. oryzae* have been reported to show anti-fungal and anti-microbial activities.[27] Isocoumarin derivatives of *A. oryzae* in solid cultures showed moderate anti-cancer activities against many human cancer cell lines.[28] Furthermore, *A. oryzae* has a secretory system that allows it to secrete high concentrations of proteins into its culturing medium. Its use as biotechnological tool in industrial, food, veterinary and pharmaceutical fields is as a result of this characteristic.[29] *Aspergillus oryzae* has been isolated from cocoa leaves as an endophyte and found to produce koji acid (KA) in culture. It also synthesized kojic acid when inoculated into cocoa seedlings, and led to high levels of caffeine production. Since this fungus grows as an endophyte, without infecting the coffee plant, it may be seen as a useful fungus since it induces a caffeine defense response that may confer tolerance on plants against pathogens and insects.

Fungal diseases are one of the most destructive diseases of vegetables and fruits, with *Rhizopus* spp. having a major effect.[31,32] *Rhizopus* species cause both field and post-harvest diseases. They rapidly establish themselves on several plant species, especially vegetables and fleshy fruits. During infection, they produce a large quantity of fungal spores and this increases the probability of infecting open wounds on plants in subsequent disease cycles.[33] Acidic pH and high host nutrients are necessary for the optimal growth of *R. delemar*. The acidic environment and presence of glucose, induce spore swelling which leads to increase in spore diameter of up to three folds. *Rhizopus delemar* also causes the fatal disease, mucormycosis in immuno-compromised individuals. The annual incidence of mucormycosis is estimated to be 1.7 per million people.[34] The main route of entry of *R. delemar* is by inhalation of sporangial spores. Nasal-brain and pulmonary infections are the most common symptoms of the diseases.[35]

Fungal species are associated with *Dacryodes edulis*. Molecular characterization through Polymerase Chain Reaction amplification and sequencing of Internal Transcribed Spacer (ITS) regions was effective in the identification of fungal samples. This study will help to promote knowledge of the fungal species associated with the fruit of *Dacryodes edulis* which will help in disease control and lead to an increase in crop productivity.

For adequate classification and identification of subsequent pathogenic organisms affiliated with different crops, the use of molecular tools is therefore recommended. Sequencing errors should be highly prevented to successfully classify/identify all organisms isolated.

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