Research Article

Molecular Identification and Characterization of Lasiodiplodia theobromae Associated with Post-Harvest Rot Disease in Soursop Fruits

Okon G. Okon[1], Abdelhak Rhouma[2]*, Lovina I. Udoh[1], Yakubu I. Uwaidem[1], Ukponobong E. Antia[3], Bright F. Archibong[1], Johnson E. Ita[1], Iniubong S. Akpan[1]

[1]Department of Botany, Faculty of Biological Sciences, Akwa Ibom State University, Nigeria; [2]Regional Centre of Agricultural Research of Sidi Bouzid, CRRA, Gafsa Road Km 6, B.P. 357, 9100, Sidi Bouzid, Tunisia; [3]Department of Microbiology, Faculty of Biological Sciences, Akwa Ibom State University, Nigeria.

Abstract | The characterization of Lasiodiplodia theobromae, a fungal species causing fruit rot in Annona muricata (soursop), is a vital aspect of understanding postharvest fruit losses and enhancing fungal identification. This study aimed to identify the fungal pathogen and assess its pathogenicity using biotechnological tools. L. theobromae was isolated from decaying soursop fruit pulp obtained from street vendors in Ikot Akpaden Junction, and its characteristics were analysed using molecular techniques. Genomic DNA was extracted from the fungal isolate and stored in a 1.5 ml micro centrifuge tube. The DNA concentration, measured with a Gene Quant Pro spectrophotometer, was 380 ng. Quality assessment using 1.5% agarose gel electrophoresis revealed a sharp DNA band with no smearing, indicating high-quality DNA. PCR amplification was conducted using forward and reverse primers targeting the rbel gene. The amplicons were visualized on a 1.5% agarose gel using a 100 bp molecular ladder. A band observed at approximately 600 bp confirmed successful amplification. However, the PCR results were partially inconclusive due to the time gap between fungal isolation and amplification, with a success rate of 1.5% for rbel gene amplification. Despite this limitation, L. theobromae was successfully identified and characterized. The study confirms that this fungal species is associated with fruit rot in A. muricata, providing a foundation for developing targeted postharvest disease management strategies.

Keywords | Annona muricata, Fruit rot, Fungal characterization, Lasiodiplodia theobromae, Molecular identification, Postharvest disease

Editor | Muhammad Nauman Zahid, Quality Operations Laboratory, University of Veterinary and Animal Sciences, Lahore, Pakistan.
Received | May 27, 2025; **Accepted** | November 10, 2025; **Published** | June 25, 2026
***Correspondence** | Abdelhak Rhouma, Regional Centre of Agricultural Research of Sidi Bouzid, CRRA, Gafsa Road Km 6, B.P. 357, 9100, Sidi Bouzid, Tunisia;
Email: abdelhak.rhouma@gmail.com
Citation | Okon OG, Rhouma A, Udoh LI, Uwaidem YI, Antia UE, Archibong BF, Ita JE, Akpan IS (2026). Molecular identification and characterization of Lasiodiplodia theobromae associated with post-harvest rot disease in soursop fruits. S. Asian J. Life Sci. 14: 24-28.
DOI | https://dx.doi.org/10.17582/journal.sajls/2026/14.24.28
ISSN (Online) | 2307-8316; **ISSN (Print)** | 2309-3331

INTRODUCTION

Soursop (*Annona muricata* L.) is a tropical fruit-bearing tree widely cultivated in the Caribbean, South America, and parts of Africa for its edible fruit, which is valued for its unique flavour, nutritional properties, and medicinal uses (George *et al*., 2019). The fruit has gained increasing economic importance due to its rising demand in local and international markets. However, the postharvest shelf life and market value of soursop are severely constrained by fungal diseases, particularly fruit rot, which leads to significant yield and quality losses (Okereke and Wokocha, 2020).

Among the major pathogens implicated in fruit rot, *Lasiodiplodia theobromae* (Pat.) Griffon and Maubl. (Dothideomycetes, Botryosphaeriaceae). It is a well-documented fungal species affecting many tropical and

subtropical fruit crops. This pathogen is known for its aggressive nature, broad host range, and capacity to cause pre- and postharvest infections (Ismail *et al*., 2020). In A. muricata, L. theobromae has been associated with soft rot, fruit necrosis, and premature fruit drop, particularly under warm, humid conditions that favour disease development (Akinbode *et al*., 2018).

Despite its economic impact, there is limited region-specific information on the identity, pathogenicity, and characteristics of *L. theobromae* strains infecting soursop, especially in developing countries where this fruit is widely cultivated. Characterizing the fungal pathogen at both morphological and molecular levels is crucial for accurate diagnosis, understanding epidemiology, and implementing effective disease management strategies (Slippers and Wingfield, 2007). Therefore, this study aimed to isolate and characterize *L. theobromae* associated with fruit rot in *A. muricata*, contributing to improved plant health management and postharvest handling practices.

MATERIALS AND METHODS

Sample collection

Plant material for this study was collected from the local community of Ikot Akpaden in Mkpat Enin L.G.A. Microbial analysis was carried out at the Microbiology Laboratory, Akwa Ibom State University, Ikot Akpaden. In contrast, the molecular analysis was conducted at the Genomics Training Centre and Laboratory Limited, Uyo, Akwa Ibom State.

Soursop fruits were purchased from roadside vendors stationed at Ikot Akpaden Junction and were kept for some days to avoid spoiling. After spoilage, the fruit was transferred to the Microbiology Laboratory, Akwa Ibom State University.

Microbial analysis

The spoilt soursop was mashed using a sterile mortar and pestle, after which, 1 g of the sample was weighed and put in a beaker containing 9 mL of sterile distilled water to make up the aliquot to be used for serial dilution of the sample.

For the serial dilution, 1 mL from the aliquot was used to carry out a dilution of 10^{-1} to 10^{-5} for the soursop sample. After serial dilution, 1 mL from the dilution factors 10^{-3} and 10^{-4} was pipetted into sterile petri dishes (pure plate method); this was done in duplicates. Sterilized PDA medium was poured into the inoculated plate and allowed to solidify. The plates were incubated at 28°C for 5 to 7 days, after which purification was performed. After obtaining a pure culture, the isolates were sub-cultured into sterile stock bottles, kept slant and stored in the refrigerator at 4°C after growth of the fungi.

Lasiodiplodia theobromae where identified microscopically with phenotypic criteria (growth, color, aspect of the colony) (mycelium, conidiophore, conidia, resistance structures, sexual form), after a series of sub-culturing until purification of the fungus using cotton blue as mounting medium and with reference to different identification keys (Domsch *et al*., 1980). The names of authors of fungal taxa are abbreviated according to Kirk and Ansell (1992). The systematic arrangement in follows Kirk *et al*. (2008). Name corrections, authorities, and taxonomic assignments were checked against Index Fungorum.

Molecular identification

The soursop fruits were harvested from the mother plant and were taken to the laboratory, where 500 mg was weighed out using a sensitive scale for DNA isolation. DNA extraction was carried out using a DNA extraction kit (Genomic DNA Mini kit by Geneaid). The fruit pulp was lysed using a laboratory mortar and pestle in 400 µL of extraction buffer. It was transferred into 1.5ml microcentrifuge tubes, 400 µL of the same buffer, and 50ul of RNase was added to it and was mixed by vortex. The mixture was incubated at 60°C for 10 min. 100 µL of GP2 buffer was added and then mixed by vortex, and was incubated again on ice for 3 min. The mixture was then transfer to a filter column in a 2 mL collection tube and the mixture was transfers to the filter column. This mixture was centrifuged for 1 min at 1000xg, then the filter column was discarded. The supernatant was carefully transferred from the 2 mL collection tube to a new 1.5 mL microcentrifuge tube. 1.5 volumes of GP3 buffer were then added and vortexed immediately for 5 s (Chen *et al*., 2021).

The GD column was placed in a 2 mL collection tube. 700 µL of the mixture was transferred to the GD column and centrifuged at 14-16,000 × g for 2 min. The flow-through was discarded, and the GD column was placed back in the 2 mL collection tube, and the remaining mixture in the GD column was transferred and then centrifuged at 14-16,000 xg for 2 min. The flow-through was discarded, and the GD column was placed back in the 2 mL collection tube. 400 mL of WI buffer was added to the GD column and centrifuged at 14-16,000 xg. The flow-through was discarded, and the GD column was placed back in the 2 mL collection tube. 600 µL of wash buffer was added to the GD column and centrifuged at 14-16,000×g. The flow-through was discarded, the GD column was placed in the 2 mL collection tube, and then it was centrifuged for 3 min at 14-16,000 × g to dry the column matrix (He *et al*., 2024).

The dried GD column was transferred to a clean 1.5 mL microcentrifuge tube. 100 µL of pre-heated elution buffer or TE buffer was added to the center of the GD column matrix. It was allowed to stand for 3-5 min to ensure the elution buffer is completely absorbed. Then it was finally centrifuged at 14-16,000 for 30 s to elute the purified DNA (Salvatore *et al*., 2020).

DNA concentration was determined using a Spectrophotometer (Gene Quant GO). The presence and quality of DNA were also evaluated by agarose gel electrophoresis. DNA was quantified on a 1.5% agarose gel. Electrophoresis was conducted in a 1X TAE (Tris-base glacial acetic acid, EDTA) gel buffer at 120V or for 20 min. The gel was stained with Sul of Safe View dye. After the gel electrophoresis ran for 20 min, the TAE buffer was drained off the gel. The gel was then visualized under a UV transilluminator (Delgado Gómez *et al*., 2023).

Primers from the rbcL gene used for PCR amplification include rbcL 1F. For the amplification of the desired loci, the PCR master mix contained PCR amplification buffer, MgCl, DMSO, DNTPs, and Taq polymerase. The PCR final reaction Volume was made up to 25 µL, using 0.5 µL of One Taq master mix, 0.5 forward primer, 0.5 reverse primer, 2ul of template DNA, and 9.5 µL of nuclease-free water. PCR reaction conditions conducted at BIO-RAD thermocycler were the following: Initial denaturation at 94˚C for 30 s, 30 cycles of denaturation at 94˚C for 30 seconds, annealing at S5˚C for 30 s, initial elongation at 68 ˚C for minutes, and final elongation at 68˚C for 5 min. Amplicons were separated on a 1.5% agarose gel electrophoresis for 20 min at 120V. A DNA ladder of 100 bp was used as a molecular weight standard (Suwannarach *et al*., 2022).

RESULTS AND DISCUSSIONS

The front view of *L. theobromae* colonies typically appears filamentous and cottony or woolly in texture. The fungal mycelium, which consists of intertwining hyphae, gives this appearance. The colour of the front view can vary depending on the medium and growth conditions employed in the laboratory. Initially, it may appear white or cream-coloured and then gradually turn brown or black with the production of darkly pigmented conidia and conidiomata. The reverse side of *L. theobromae* colonies typically appears dark, ranging from brown to black. This visible coloration is due to the production of pigments by the fungus, including melanin. Colony morphology, under a microscope, the colony of *L. theobromae* appears as a dense mass of mycelium, which is the vegetative part of the fungus. The mycelium consists of elongated, branched hyphae that intertwine to form a mat-like structure (Figure 1).

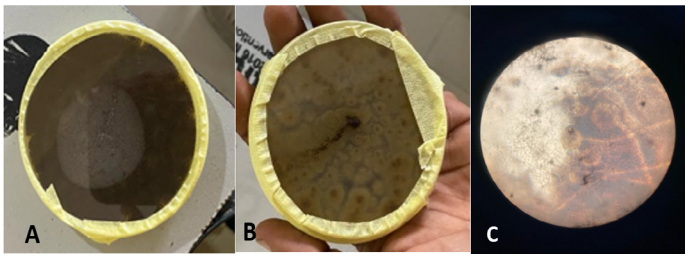

Figure 1: Morphological aspect (A and B) and microscopic characteristic (C) of L. theobromae.

The extracted DNA from A. muricata was stored in a 1.5 mL microcentrifuge tube, and DNA concentration was determined using a Spectrophotometer (Gene Quant Pro). The concentration of DNA was 380ng. The DNA was loaded on a 1.5% agarose gel to verify the DNA quality. The DNA appeared as a discrete band without a smear on the gel, indicating good-quality DNA. PCR amplification was performed using 0.5ul of Forward and 0.5ul of Reverse primers on the rbel gene. Amplicons were separated on a 1.5% agarose gel by electrophoresis. A DNA ladder of 100 bp was used as a molecular weight standard. After running the PC product on electrophoresis, the band indicated at about 600bp.

The findings confirmed that L. theobromae is a major causal agent of fruit rot in A. muricata, consistent with previous reports on its pathogenicity in various tropical fruit crops. The morphological and molecular characterization of the isolated fungus aligns with typical diagnostic features of *L. theobromae*, including dark pigmented, septate conidia and rapid colony growth on PDA medium, as described by Slippers *et al*. (2013) and Ismail *et al*. (2020). The high level of virulence observed in pathogenicity tests supports its role as a primary pathogen rather than a secondary invader, underscoring its economic significance in soursop production systems (Salvatore *et al*., 2020).

The widespread occurrence and adaptability of *L. theobromae* can be attributed to its opportunistic nature and ability to survive in plant debris and as an endophyte in healthy tissues (Slippers and Wingfield, 2007). Environmental conditions in tropical and subtropical regions, especially high humidity and warm temperatures, favour the infection cycle, sporulation, and disease spread. This makes postharvest infection particularly challenging, as fruits harvested under such conditions are more susceptible to rapid spoilage (Akinbode *et al*., 2018).

Moreover, the study highlighted the importance of accurate identification using both morphological and molecular tools. While classical methods based on conidial morphology remain useful, they can be confounded by the morphological plasticity of the fungus, especially under varying culture conditions (Netto *et al*., 2014).

DNA sequencing of the internal transcribed spacer region provided more precise identification, corroborating studies that advocate molecular techniques for accurate diagnosis of *Lasiodiplodia* spp. (Alves *et al*., 2008).

The implications of these findings are twofold. Agriculturally, they point to improving field sanitation, postharvest handling, and storage conditions to limit fungal spread. The pathogen's endophytic phase suggests that surface sterilization alone may not suffice, and integrated disease management strategies including pruning, fungicide application, and resistant cultivars should be explored (Mahunu *et al*., 2016). Secondly, from a research perspective, further studies are required to assess the genetic variability of *L. theobromae* isolates from different regions, as pathogenicity and response to control measures may differ between strains.

CONCLUSIONS

This study successfully isolated and characterized *L. theobromae* as the primary fungal pathogen responsible for fruit rot in soursop. Morphological and molecular tools enabled accurate identification, affirming the pathogen's role in postharvest losses. The insights gained from this study contribute to a deeper understanding of soursop pathology and provide a foundation for developing sustainable disease management practices to improve fruit quality, marketability, and farmer income. Given its aggressive pathogenicity and widespread distribution, managing this disease requires an integrated approach that combines good agricultural practices, proper postharvest handling, and early detection methods. Additionally, future research should focus on assessing the genetic diversity of *L. theobromae* populations, evaluating potential resistant soursop cultivars, and developing environmentally friendly control strategies such as biocontrol agents or natural antifungal compounds.

ACKNOWLEDGEMENTS

The authors are grateful to the review editor and the anonymous reviewers for their helpful comments and suggestions to improve the clarity of the research paper.

NOVELTY STATEMENT

This study presents the first comprehensive molecular and phenotypic characterization of *L. theobromae* as the causative agent of postharvest fruit rot in A. muricata (soursop) from the Ikot Akpaden region of Nigeria, filling a critical gap in the understanding of fungal pathogens affecting this economically important tropical fruit. While *L. theobromae* has been documented in other crops, this research is novel in its region-specific focus, providing empirical evidence of the pathogen's role in soursop spoilage within a previously understudied agroecological zone, thereby enhancing localized disease surveillance and management strategies. A key advancement of this work is the successful integration of molecular techniques, including DNA extraction and PCR amplification of the rbel gene, despite technical challenges such as low amplification efficiency (1.5% success rate), which underscores the need for optimized protocols in resource-limited settings. By confirming the pathogen's identity through both morphological and molecular approaches, this study not only validates *L. theobromae* as a major postharvest threat but also highlights the importance of accurate fungal diagnostics in mitigating fruit losses. Furthermore, the findings emphasize the broader implications for soursop production, particularly in tropical regions where postharvest handling practices are often inadequate, and propose the need for integrated disease management strategies, including improved storage conditions, field sanitation, and potential biocontrol solutions. This research lays a critical foundation for future investigations into the genetic diversity of *L. theobromae* isolates, host resistance mechanisms, and sustainable control measures tailored to soursop-growing regions in West Africa, ultimately contributing to enhanced food security and economic resilience for smallholder farmers.

AUTHOR'S CONTRIBUTION

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Generative AI and AI-assisted technology statement

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Conflict of interest

The authors have declared no conflict of interest.

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