FUNGI AND FUNGI-LIKE ORGANISMS
ГРИБИ І ГРИБОПОДІБНІ ОРГАНІЗМИ

https://doi.org/10.15407/ukrbotj82.05.453
RESEARCH ARTICLE

Molecular detection and symptomatology of *Lasiodiplodia theobromae* (*Ascomycota*) infecting rose apples (*Syzygium samarangense*) in Malaysia

Muhammad Naveed KHAN [1], **Mohammed Yahya ALLAWI** [2],
Ban Badran ADNAN [3], **Nurul Awatif AHMAD** [1], **Som Cit SINANG** [1],
Nor Nafizah MOHD NOOR [1], **Mazatul Azrin RAHMAN** [1], **Nurhaida KAMARUDDIN** [1],
Norasfaliza RAHMAD [4], **Raja Farhana R. KHAIRUDDIN** [1], **Jameel R. AL-OBAIDI** [1,5]*

[1] Department of Biology, Faculty of Science and Mathematics,
Universiti Pendidikan Sultan Idris, Tanjong Malim, Perak, 35900, Malaysia
[2] Environmental Science Department, College of Environmental Science and Technologies,
University of Mosul, 41002, Mosul, Iraq
[3] Department of Microbiology, College of Veterinary Medicine, 34001, Tikrit, Iraq
[4] Agro-Biotechnology Institute Malaysia (ABI), National Institutes of Biotechnology Malaysia (NIBM),
Jalan Bioteknologi, 43400, Selangor, Malaysia
[5] Applied Science Research Center, Applied Science Private University, Amman, Jordan

* Author for correspondence: *jr_alobaidi@yahoo.com*, *jameel@fsmt.upsi.edu.my*

Abstract. Rose apple (*Syzygium samarangense*, *Myrthaceae*) is considered one of the main tropical fruits native to Southeast Asia. In 2024, rose apples with fruit rot symptoms, characterized by dark, sunken circular lesions on ripening fruits, were observed in two locations in Perak, Malaysia. The identity of the pathogen that was responsible for the diseases was determined using morphological characteristics, DNA sequences, and phylogenetic analyses of internal transcribed spacer (ITS) and elongation translation factor 1-alpha (tef1-α). Morphological patterns of fungal isolates were observed on cultured potato dextrose agar (PDA) plates. The ITS and tef1-α of a fungal isolate were PCR amplified and sequenced, confirming the identity of *Lasiodiplodia theobromae* (GenBank accession nos. PQ584813 and PV155110, respectively). To fulfil Koch's postulates, the fungi were re-isolated and tested for pathogenicity on detached fruits. Symptoms similar to those observed in the market appeared on all inoculated fruits, while control fruits remained symptomless. To our knowledge, this is the first molecular-level report of *L. theobromae* infecting rose apples in Malaysia.

Keywords: fruit anthracnose, *Lasiodiplodia theobromae*, rose apple, *Syzygium samarangense*

Introduction

Rose apple (*Syzygium samarangense* (Blume) Merr. & L.M. Perry, family *Myrtaceae*) is one of the most important tropical fruits that is indigenous to Malaysia and other Southeast Asian countries,

ARTICLE HISTORY. Submitted 30 April 2025. Revised 27 August 2025. Published 30 October 2025

CITATION. Khan M.N., Allawi M.Y., Adnan B.B., Ahmad N.A., Sinang S.C., Mohd Noor N.N., Rahman M.A., Kamaruddin N., Rahmad N., Khairuddin R.F.R., Al-Obaidi J.R. 2025. Molecular detection and symptomatology of *Lasiodiplodia theobromae* (*Ascomycota*) infecting rose apples (*Syzygium samarangense*) in Malaysia. *Ukrainian Botanical Journal*, 82(5): 453–459. https://doi.org/10.15407/ukrbotj82.05.453

including Thailand and Indonesia, and cultivated in many other regions, such as Taiwan (Banadka et al., 2022). Rose apples are rich sources of nutrition, with carbohydrates, proteins, minerals, and vitamins (Venkatachalam et al., 2018). The rose apple is also high in vitamin C, polysaccharides, and antioxidant compounds, all of which have important health benefits (Cheng et al., 2022). Additionally, rose apples are used in many value-added products such as candies, jams, juices, and syrups, contributing significantly to their economic value (Banadka et al., 2022). In Malaysia, it is widely cultivated in an area of approximately 3 300 hectares, with an income of more than 9 million US dollars per year (Al-Obaidi et al., 2023). One of the main challenges facing Malaysia's rose apple industry is fungal-related postharvest losses. Due to their high moisture content, thin skin, and high chilling sensitivity, rose apples are susceptible to bacterial and fungal infections (Hasan et al., 2022). Anthracnose disease has significantly affected rose apple fruits, resulting in the loss of around one-third of annual production, leading to significant economic difficulties (Lii et al., 2017).

Previous studies have identified multiple *Lasiodiplodia* Ellis & Everh. (*Botryosphaeriaceae*, *Botryosphaeriales*, *Ascomycota*) species involved in similar infections in other *Myrtaceae* hosts such as guava (*Psidium guajava* L.) (Zee et al., 2021), but limited data exist on their specific impact on *Syzygium samarangense*. Beyond *Myrtaceae*, *Lasiodiplodia* species have been documented infecting a wide range of host plants in Malaysia, including cacao (*Theobroma cacao* L., *Malvaceae*) (Huda-Shakirah et al., 2022) and mango (*Mangifera indica* L., *Anacardiaceae*) (Munirah et al., 2017), highlighting their widespread occurrence and adaptability.

In June 2024, dark, sunken circular lesions on ripening fruits were observed in two locations in the state of Perak, Malaysia (Fig. 1). To date, no detailed etiological investigation has been conducted to characterize the causal pathogen responsible for these symptoms in Malaysian rose apple. The objective of this study was the identification of the causal agent of the postharvest disease in rose apples; this information will be helpful in developing suitable response mechanisms against postharvest fungal infections. This research aims to fill research gaps in the current understanding of fungal infections affecting this economically significant and promising fruit and to provide a foundation for integrated disease management strategies in the region.

Methodology

To identify the causal agent, infected rose apple fruit pieces (5×5×5 mm cubes) were surface sterilized with 1.5% sodium hypochlorite, followed by 70% ethanol, rinsed with sterile distilled water, and plated on potato dextrose agar (PDA). PDA plates were incubated at 25 °C, 50% relative humidity for 12 days. Fungal structures were observed under a microscope using safranin dye to enhance visualization. The stained samples were examined using a Trinocular Microscope Set Model CX33 (Olympus, Japan) to assess morphological characteristics. For molecular identification, internal transcribed spacer (ITS) regions 1 and 2 and elongation translation factor 1-alpha (tef1-α) were amplified by PCR and sequenced (Hami et al., 2021). The total reaction volume of 25 µl contained genomic DNA purified using an in-house optimized protocol, 0.5 pmol of each primer, deoxynucleotides triphosphates (dNTPs, 200 µM each), 0.5 U thermostable DNA polymerase, supplied PCR buffer, and water. The PCR conditions were as follows: one cycle of initial denaturation at 98 °C for 2 minutes; 25 cycles of denaturation at 98 °C for 15 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 30 seconds; and a final extension at 72 °C for 10 minutes (Al-Obaidi, 2017).

PCR products were analyzed using 1.5% agarose gel electrophoresis in 1X TAE buffer. A 100 bp DNA ladder was used as a molecular weight marker (Lane M). A total of two PCR amplicons (Lanes A and B) were loaded, and electrophoresis was performed at 100 V for 40 minutes. The gel was stained with ethidium bromide and visualized under a UV transilluminator using a Bio-Rad Gel Documentation System.

Sequence homology was analyzed using BLAST (http://blast.ncbi.nlm.nih.gov/) to identify the closest reference sequences from the NCBI GenBank database. Phylogenetic analysis of ITS and tef1-α sequences was performed using maximum likelihood in MEGA 11 (Çiftçi et al., 2023). The PCR products were purified by the standard PCR clean-up method. Pathogenicity tests of isolated *L. theobromae* were performed on detached fruits. Healthy fruits were washed thoroughly, surface sterilized with 6% sodium hypochlorite for 10 minutes, and rinsed with sterile distilled water (Karunamoorthy et al., 2023). Rose apple fruits were inoculated with 10 µl conidial suspension (1×10^6 spores/ml) of the pathogens prepared from 2-week-old cultures on PDA, while sterile water was used to inoculate

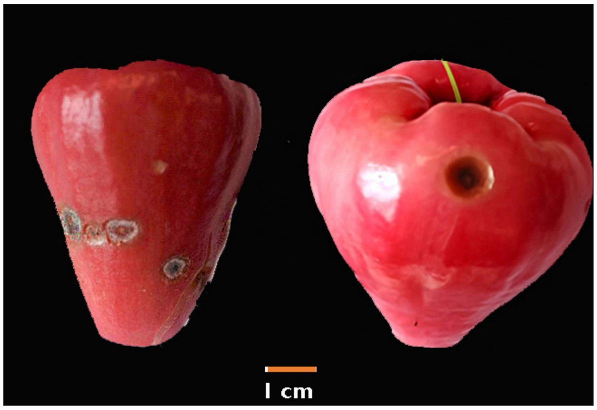

Fig. 1. Early symptoms of rose apple fruit rot caused by *Lasiodiplodia theobromae*

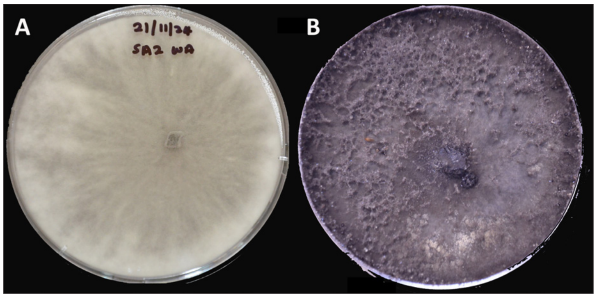

Fig. 2. Morphological characteristics of *Lasiodiplodia theobromae* on potato dextrose agar medium, showing a white to light-cream colony at 6 days (A) and a dark grey to black colony with fluffy aerial mycelium at 14 days (B)

control fruits using the pin-prick method (Chaudhary et al., 2020). Inoculated fruits were placed in plastic containers and incubated at 27 °C for 7 days.

Results

In the cultured plate, a morphological pattern of fungi is shown growing from the pieces of rose apple, initially appearing white and later developing a darker, textured surface (Fig. 2). Fungal conidia were predominantly straight, with lengths ranging from 21–24 μm and widths between 2.2–4.3 μm.

The purified PCR products were subjected to bidirectional sequencing with universal primers M13F (-20) and M13R-pUC (-26) using a BigDye® Terminator v3.1 Cycle Sequencing Kit. The sequence from the plate identified as *L. theobromae* (546 bp) (GenBank accession nos. PQ584813 and PV155110) showed 100% similarity with *L. theobromae* (Fig. 3).

Agarose gel electrophoresis confirmed the successful amplification of the target gene regions, as shown in Fig. 4. The DNA ladder (Lane M) provided size reference bands, with the most relevant markers at 100 bp, 500 bp, and 1000 bp. PCR products in Lanes A and B exhibited strong bands, indicating successful amplification of the target regions. The absence of non-specific bands or primer dimers suggests the high specificity of the primers used (Fig. 4). This result confirms that the ITS and TEF-1α regions were successfully amplified, allowing for further molecular characterization of the *Fusarium* strains through sequencing and phylogenetic analysis.

Phylogenetic clustering revealed a close relationship between our fungal isolates and those from other countries (Fig. 5A, B). The phylogenetic relationships among the *Lasiodiplodia* isolates were analyzed using Maximum Likelihood (ML) based on two genetic regions: the translation elongation factor 1-alpha (tef1-α) gene (Fig. 5A) and the internal transcribed spacer (ITS) regions 1 and 2 (Fig. 5B). In both trees, the isolates consistently clustered within the *L. theobromae* clade, supported by high bootstrap values (ranging from 92% to 100%), strongly confirming their species identity. In the tef1-α tree, representative isolates such as *L. theobromae_PLSB370* and *L. theobromae_CMMP4054* grouped tightly with known *L. theobromae* reference strains, indicating close evolutionary relatedness. Similarly, in the ITS-based tree (Fig. 5B), the isolates *L. theobromae_PLSB370* and *L. theobromae_PDS88* also clustered with authenticated *L. theobromae* sequences, showing congruent topologies with the tef1-α-based tree. The inclusion of other *Lasiodiplodia* species and outgroup taxa, such as *Diplodia seriata* De Not. and *Lasiodiplodia brasiliensis* M.S.B. Netto, M.W. Marques & A.J.L. Phillips, allowed for clear delineation of clade boundaries and rooting of the phylogenetic trees. The consistent placement of isolates in both gene trees provides robust molecular evidence that the studied strains belong to *L. theobromae*. The presence of minor sub-clustering within the *L. theobromae* group may reflect underlying genetic variation, potentially linked to geographical origin or host specificity.

The inoculated rose apple fruits displayed typical white/grey (*L. theobromae*) lesion symptoms after four days, while the control fruits displayed no symptoms (Fig. 6). Koch's postulates were fulfilled when the pathogen was re-isolated from inoculated fruits and confirmed to be identical to the original isolates used in the pathogenicity tests (Al-Obaidi et al., 2023).

Discussion

The identification of *L. theobromae* as the causal agent of postharvest fruit rot in Malaysian rose apples represents a significant advancement in understanding the fungal pathogens affecting this economically important crop. This study provides the first molecular-level confirmation of *L. theobromae* infecting rose apples in Malaysia, complementing previous reports of fungal pathogens such as *Pestalotiopsis samarangensis* in Thailand (Maharachchikumbura et al., 2013) and *Colletotrichum siamense* associated with leaf spot disease in the same region (Khuna et al., 2023). The morphological and molecular characterization of *L. theobromae* isolates, supported by phylogenetic analyses of ITS and tef1-α regions, is consistent with current best practices in fungal taxonomy, as tef1-α has been shown to offer better resolution among species of *Lasiodiplodia*. In our study, BLASTn analysis revealed 100% sequence identity between our isolate (PLS2S20) and reference sequences of *L. theobromae* for both ITS and tef1-α. Furthermore, our phylogenetic trees placed the isolate firmly within a well-supported *L. theobromae* clade, with high bootstrap values confirming its close relationship to authenticated global isolates. Overall, the combined morphological and molecular evidence provides a solid basis for attributing the observed postharvest rot in *Syzygium samarangense* to *L. theobromae*. To the best of our knowledge, this represents the first confirmed report of *L. theobromae* infecting rose apple in Malaysia, filling a significant gap in the regional plant pathology literature and contributing to the broader understanding of this pathogen's host range and geographic distribution.

The pathogenicity tests conducted in this study fulfilled Koch's postulates, confirming *L. theobromae* as the causative agent of the observed symptoms (Úrbez-Torres et al., 2008). The development of dark, sunken lesions on inoculated fruits, coupled with the successful re-isolation of the pathogen, provides robust evidence of its role in postharvest decay. These findings are consistent with previous studies highlighting *L. theobromae* as a significant postharvest pathogen in tropical and subtropical regions, affecting a wide range of fruit crops, including papaya, mango, and citrus (Al-Obaidi et al., 2023; Karunamoorthy et al., 2025). The economic implications of *L. theobromae* infection in rose apples are profound, given the crop's growing contribution to Malaysia's

Fig. 3. Developmental stages of *Lasiodiplodia theobromae* observed under a microscope. A: mycelium; B: mature conidia. Magnification ×100, scale bar 30 μm

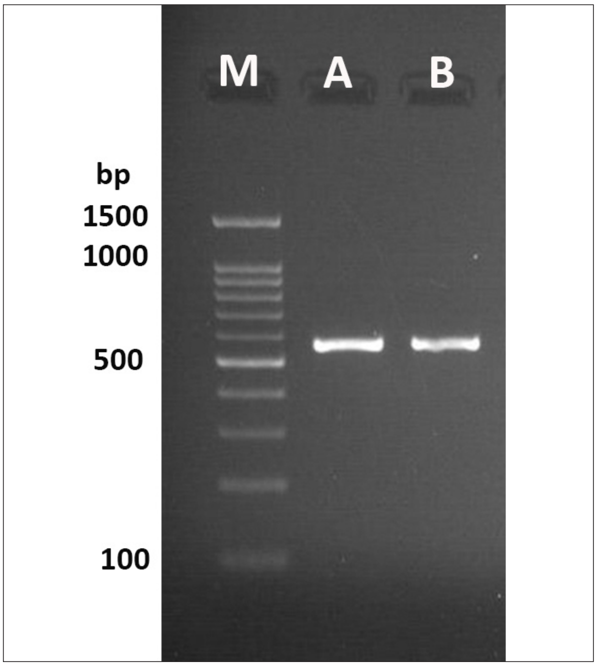

Fig. 4. Agarose gel electrophoresis of PCR-amplified DNA. A: ITS region (546 bp) using universal ITS1 and ITS2 primers; B: tef1-α gene (533 bp)

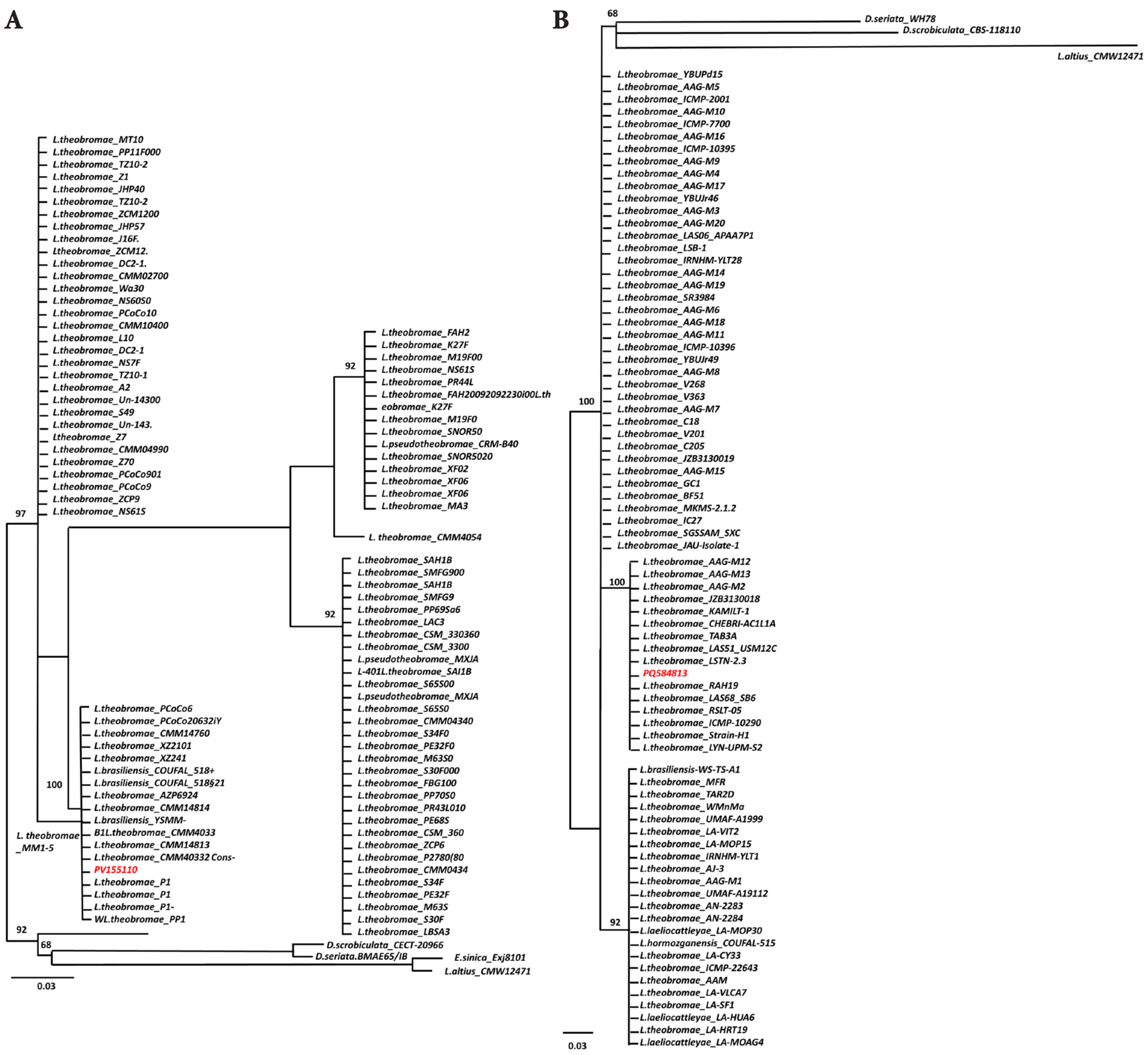

Fig. 5. Phylogenetic tree for *L. theobromae* generated using translation elongation factor 1-alpha (tef1-α) region (A), and internal transcribed spacer (ITS) regions 1 and 2 (B)

agricultural economy. The high moisture content, thin skin, and chilling sensitivity of rose apples make them particularly susceptible to fungal infections, exacerbating postharvest losses during storage and transportation (Zee et al., 2021). Our findings highlight the urgent need for effective management strategies to mitigate the impact of *L. theobromae* on rose apple production. Potential approaches include the development of resistant cultivars through breeding programs, the application of biocontrol agents, and the use of antifungal treatments during postharvest handling. Additionally, enhancing the activity of defence-related enzymes in rose apples could improve their resistance to fungal infections, thereby extending shelf life and reducing losses (McLaughlin et al., 2023). This study also underscores the importance of molecular tools in accurately identifying and characterizing fungal pathogens. The use of ITS and tef1-α sequences for phylogenetic analysis not only confirmed the identity of *L. theobromae* but also revealed its genetic relationship with isolates from other regions (Pornsuriya et al., 2023).

Such insights are crucial for understanding the global distribution and evolution of fungal pathogens, informing the development of targeted control measures. Future research should focus on exploring the environmental and physiological factors influencing *L. theobromae* infection in rose apples. Investigating the role of temperature, humidity, and fruit maturity in disease development could provide valuable insights for optimizing storage conditions and reducing postharvest losses. Furthermore, extending this research to other species could broaden our understanding of *L. theobromae*'s host range and pathogenicity mechanisms. In conclusion, this study provides the first molecular evidence of *L. theobromae* as the causal agent of postharvest fruit rot in Malaysian rose apples. Our findings contribute to the growing body of knowledge on fungal pathogens affecting tropical fruits and highlight the need for integrated management strategies to safeguard rose apple production and enhance its economic value.

Fig. 6. Pathogenicity test of *L. theobromae* on rose apple fruits: control fruit (left); fruit with signs and symptoms (right)

Acknowledgements

The authors sincerely acknowledge the Research Management and Innovation Center (RMIC) at Universiti Pendidikan Sultan Idris (UPSI) for their valuable support. Special appreciation is extended to the Department of Biology, Faculty of Science and Mathematics, UPSI, for providing essential facilities and research assistance. This work is part of the Master's degree research requirements of M.N.K., and the support of all contributing individuals and institutions is deeply appreciated.

ETHICS DECLARATION

Authors have no competing interests to declare.

ORCID

M.Y. Allawi https://orcid.org/0000-0002-0897-4577
B.B. Adnan https://orcid.org/0009-0006-2944-4088
S.C. Sinang https://orcid.org/0000-0003-3502-5470
N.N. Mohd Noor https://orcid.org/0000-0002-4424-1231
M.A. Rahman https://orcid.org/0000-0001-5726-1744
N. Kamaruddin https://orcid.org/0000-0001-7557-2060
R.F.R. Khairuddin https://orcid.org/0000-0002-6253-2913
J.R. Al-Obaidi https://orcid.org/0000-0002-5705-0223

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Молекулярне визначення і симптоми ураження *Syzygium samarangense* грибом *Lasiodiplodia theobromae* (*Ascomycota*) у Малайзії

М.Н. ХАН [1], М.Й. АЛЛАВІ [2], Б.Б. АДНАН [3], Н.А. АХМАД [1], С.С. СІНАНГ [1],
Н.Н. МОХД НООР [1], М.А. РАХМАН [1], Н. КАМАРУДДІН [1], Н. РАХМАД [4],
Р.Ф.Р. ХАЙРУДДІН [1], Дж. Р. АЛЬ-ОБАЇДІ [1,5]
[1] Освітній університет Султана Ідріса, Танджонг-Малім, Перак, Малайзія
[2] Мосульський університет, Мосул, Ірак
[3] Тікритський університет, Тікрит, Ірак
[4] Національний Інститут біотехнології в Малайзії, Селанґор, Малайзія
[5] Університет прикладних наук в Аммані, Йорданія

Реферат. Плоди *Syzygium samarangense* вважаються одними з основних тропічних фруктів, що походять із Південно-Східної Азії. У 2024 р. у двох локаціях у штаті Перак, Малайзія на зрілих плодах виявили симптоми плодової гнилі з характерними округлими бурими плямами. Ідентифікацію збудника гнилі проводили з урахуванням морфологічних особливостей і філогенетичного аналізу послідовностей ITS-регіону ДНК із залученням генів tef1-α. Морфолого-культуральні ознаки вивчали при культивуванні гриба у чашках Петрі на картопляно-декстрозному агарі (PDA). Ампліфіковані за допомогою ПЛР та секвеновані послідовності підтвердили належність досліджених культур гриба до *Lasiodiplodia theobromae* (реєстраційні номери у базі даних GenBank PQ584813 і PV155110). Відповідно до постулатів Коха, гриби повторно виділили в культуру та перевірили їхню патогенність на окремих плодах. Подібні до початкових симптоми з'явилися на всіх інокульованих плодах, тоді як контрольні плоди залишалися безсимптомними. Наскільки нам відомо, це перше підтверджене молекулярними методами повідомлення про ураження плодів *S. samarangense* грибом *L. theobromae* в Малайзії.

Ключові слова: *Lasiodiplodia theobromae*, *Syzygium samarangense*, антракноз плодів, “яванське яблуко”