Occurrence of the Stem End Rot Disease in Pomelo, *Citrus maxima* Caused by *Lasiodiplodia* Species in Ben Tre Province, Vietnam

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Abstract

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The study was conducted to isolate and select the fungal isolates causing stem end rot of pomelo fruit and characterized them by strong growth. Pomelos showing signs of stem end rot or stem wilt were collected. Sixteen fungal isolates were isolated from fruit samples collected from two districts Binh Dai and Mo Cay Bac of the Ben Tre province, Vietnam. The experiment with 16 fungal isolates was completely randomized, with 3 replicates, each replicate being a Petri dish. Nine fast growing fungal isolates were selected, with the diameter of fungal colonies ranging from 8.60 to 9.00 cm, 48 h after inoculation. These nine isolates when inoculated to pomelo fruits caused fruit rot symptoms and their pathogenicity was confirmed following Koch's postulates. The range of infection diameter on pomelo fruits was 0.85-3.00 cm. The three fungal isolates causing the earliest and the most severe pomelo fruit rot were identified as *Lasiodiplodia pseudotheobromae* PL-M01-A4-B and PL-B01-A7-B, and *L. theobromae* PL-M01-A6-B according to the ITS region, with 100% similarity.

Keywords: Fruit rot, *Lasiodiplodia pseudotheobromae, Lasiodiplodia theobromae*, pathogenic factor, pomelo, stem end rot.

Introduction

The genus *Citrus* includes many important plants such as tangerines, lemons, limes, oranges, and grapefruits (Kale & Adsule, 1995). *Citrus* is widely grown in tropical and subtropical regions, with economic production in more than 100 countries around the world (Saunt, 1990). Stem end rot disease (SERD) caused by *Lasiodiplodia theobromae* (Ma *et al.,* 2021), previously known as *Botryodiplodia theobromae* and *Diplodia natalensis* (Zhang *et al.,* 2014), is one of the most serious postharvest rot diseases of citrus trees (Zhang, 2014). Postharvest diseases significantly affect fruit quality, value and economic return (Lin *et al.,* 2020).

In China, stem rot disease has been reported on 4 citrus varieties, Satsuma, Ponkan, Nanfeng, and Tangerine, *Citrus reticulata* Blanco (Chen *et al.,* 2021). *L. theobromae* is found to cause fruit rot on oranges and mandarins grown in Bangladesh (Hasan *et al.,* 2020), limes in Argentina (Cerioni *et al.,* 2017), and all of citrus trees in 22 regions of Indonesia (Dwiastuti *et al.,* 2018). Since the 1940s, pomelo stem rot has been reported to be caused by the *L. theobromae* fungus (Miller *et al.,* 1940). One of the limitations in citrus cultivation is the infection with the *L. theobromae* fungi causing injury to the pomelo tree stem and the appearance of a yellow liquid that will harden over time to form yellow-brown crystals that tend to darken with time (Mahardika *et al.,* 2021). In China, *L. theobromae* was also recorded for the first-time causing fruit rot on grapefruit (Luo *et al.,* 2011). Transmission by wind and water from objects containing residues of *L. theobromae* causes a latent infection in the pre-harvest stage, making it difficult to prevent or treat the disease with chemicals or physical approaches (Punithalingam, 1976). Therefore, pathogenic fungi can infect and cause serious damage if not controlled in time (Zheng *et al.,* 2021).

Controlling temperature during storage is a fairly effective control method (Zhang, 2007). Fungicides are used to prevent the growth of fungal mycelium or spores. Fungicides have a protective role in agricultural products during storage, such as SERD caused by *L. theobromae* (Zhang, 2007). In addition, chemical fungicides are also used on a number of plant diseases caused by *L. theobromae* such as anthracnose on mango (Yang *et al.,* 2021), dieback on Cocoa caused by *L. pseudotheobromae* (Musdalifa *et al.,* 2021). In order to protect the yield and quality of crops, the excessive use of many chemicals can create resistance to pathogens and pollute the environment (Pal & Gardener, 2006). Apart from chemicals, biological products (Sharma *et al.,* 2009) and the use of antagonistic microorganisms against fungi (Yang *et al.,* 2021) are being considered, because they are environment friendly and safe for humans (Butt *et al.,* 2001). Thus, the current study aimed to identify the causing agents of stem end rot on pomelos in Ben Tre province, Vietnam and discuss possible control options for this disease.

Material and Methods

Samples were collected from pomelo farms in Mo Cay Bac and Binh Dai districts of Ben Tre province. The fruit samples with SERD symptoms or wilt rot were collected, placed in plastic bags, marked with appropriate information, and transported to the laboratory (Figure 1).

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Incubation of diseased samples before isolation of fungal pathogens

After being transported to the laboratory, the diseased samples were stored in a cool, dry place, away from sunlight. Isolation steps were carried out when mycelia appeared on the diseased samples.

Isolation of fungal pathogens

The fruits were washed with distilled water (DW) for 1 min, dried and then washed again with 70 % alcohol. The isolation was carried out as previously described (Burgess *et al.,* 2021; Shivas & Beasley, 2005). Disease symptoms on the fruits were observed with the naked eye. Fruits affected by SERD showed brown or black spots and wrinkled skin. The fungal mycelium characteristics were observed under the microscope.

Fruit peel was disinfected with 70% alcohol, cut from the border between healthy and diseased tissue into small pieces 3 mm in diameter, and transplanted the cut part into a sterile Petri dish (90 mm diameter) containing potato dextrose agar (PDA) (Tafinta *et al.,* 2013). The pathogenic fungi were kept at room temperature for 2-7 days. The fungi were repetitively isolated until they had a pure culture. Isolates were cultivated in Petri dishes for 48 h to select the 3 strongest growing isolates. Koch's postulates were used to check the pathogenicity of the fungal isolates. The morphology of the isolates on PDA was observed and compared to the previous description (Burgess *et al.,* 2006).

Inoculation of fungal pathogens to pomelo fruits

Pomelo processing: The fruit was washed with DW for 2 min. It was washed again with alcohol 70% within 30 s and continuously rinsed with DW to remove some non-pathogenic exogenous fungi from the fruit. It was then dried and put in the safety cabinet to inoculate the fungi to the fruit. To determine pathogenicity of the fungal isolates*,* the fungi are cultured on PDA medium for 1 – 2 months. After that, its spore was collected by adding sterile distilled water to the Petri dishes containing the fungal cultures. Then, the spores are counted and adjusted to a concentration of 10^4 spores/mLNeedles were used to wound, and 1 mL of the prepared 1×10^6 suspension was pipetted to the wound. Nine wounds were made apart for each fungal isolate. The negative control pomelo fruits were injected with DW.

The pomelo fruits were placed in a cool, dry place, away from sunlight. The time of appearance and lesion diameter were recorded. Three fungal isolates causing stem rot and SERD on pomelo fruits with early appearance and large diameter of lesion were selected.

Identification of the fungal pathogens by molecular markers

DNAs of the fungal isolates causing SERD were extracted from filamentous fungal colonies. Fungal colony spores 5-8 days after culture on PDA medium were transferred into 2.2 mL Eppendorf tubes, shaken and kept at room temperature for 10 min. Tubes were centrifuged at 13,000 rpm for 5 min. The extract was transferred to a new Eppendorf tube. The cell pellet was washed with 500 µl 70% ethanol, centrifuged at 13,000 rpm for 5 min, and then dried under vacuum. The DNA preparation was stored in 100 µl of TE 0.1X buffer. Subsequently, PCR reaction was carried out with ITS1 and ITS4 primer pairs (White *et al.,* 1990) with the following sequences:

ITS1: 5'-TCCGTAGGTGAACCTGCGG-3'

ITS4: 5'-TCCTCCGCTTATTGATATGC-3'

PCR reaction was performed with a total volume of 50 µL and was carried out through the following thermocycles: denaturation at 95ºC for 5 min followed by 30 cycles at 95ºC for 90 s, annealing (52ºC for 60 s) × 30 cycles, elongation (72ºC for 90 s and 72ºC for 5 s) × 30 cycles and termination at room temperature. PCR product of the ITS amplified region was sequenced by an automated sequencing system. These sequences were compared with GenBank database on NCBI using BLAST tool.

Statistical analysis

Mean values of the fungal isolates were compared by Duncan's multiple range test through ANOVA analysis using SPSS 13.0 software.

Results

Isolation of fungi causing stem end rot disease on pomelo

Sixteen fungal isolates were isolated from 10 samples of pomelo with SERD symptoms collected from the two districts Binh Dai and Mo Cay Bac of the Ben Tre province. Observation of fungi on PDA medium showed that most of the fungal isolates had quite similar growth morphology. Fungal colonies were radial, and spread evenly on the surface of the medium. On the first day after the transfer of the isolates into the medium, the hyphae were white, filamentous, and crossed into each other. Depending on the fungal isolate, the time required for the isolate to completely cover the surface of the Petri plate was different, but the color of the mycelium was still white. After 5-7 days, the mycelium was spongy and soft, the color gradually changed from white to black, except for 2 isolates PL-M01-A1-A and PL-M01-A1- B (Table 1). Different isolates had different bright/dark black colors (Figure 1).

Fungal spores usually appeared 12-18 days after culturing, and were oval and hyaline (Figure 2). Through morphological observation, although there were many similarities with *L. theobromae*, it was still difficult to determine the fungal species. Therefore, based on the growth characteristics of mycelium on PDA medium, mycelia characteristics, color and spores, there were 14 possible isolates of *L. theobromae* roughly identified. To determine the exact species name, PCR technique and gene sequencing of the ITS region were used.

Table 1. Characteristics of color, mycelium and spores of fungal isolates obtained from pomelo infected fruits collected from Mo Cay Bac and Binh Dai districts, Ben Tre province, Vietnam.

Fungal isolates	Location	Characteristics		
		Color	Mycelium	Spore
PL-M01-A1-A	Mo Cay Bac	White	Radiating and interweaving	Oval and transparent
PL-M01-A1-B	Mo Cay Bac	White	Radiating and interweaving	Oval and transparent
PL-M01-A4-A	Mo Cay Bac	White, then black	Radiating and interweaving	Oval and transparent
PL-M01-A4-B	Mo Cay Bac	White, then black	Radiating and interweaving	Oval and transparent
PL-M01-A6-A	Mo Cay Bac	White, then black	Radiating and interweaving	Oval and transparent
PL-M01-A6-B	Mo Cay Bac	White, then black	Radiating and interweaving	Oval and transparent
PL-B01-A1-A	Binh Dai	White, then black	Radiating and interweaving	Oval and transparent
PL-B01-A1-B	Binh Dai	White, then black	Radiating and interweaving	Oval and transparent
PL-B01-A7-A	Binh Dai	White, then black	Radiating and interweaving	Oval and transparent
PL-B01-A7-B	Binh Dai	White, then black	Radiating and interweaving	Oval and transparent
PL-B01-A8-A	Binh Dai	White, then black	Radiating and interweaving	Oval and transparent
PL-B01-A8-B	Binh Dai	White, then black	Radiating and interweaving	Oval and transparent
PL-B01-A9-A	Binh Dai	White, then black	Radiating and interweaving	Oval and transparent
PL-B01-A9-B	Binh Dai	White, then black	Radiating and interweaving	Oval and transparent
PL-M02-A2-A	Mo Cay Bac	White, then black	Radiating and interweaving	Oval and transparent
PL-M02-A2-B	Mo Cay Bac	White, then black	Radiating and interweaving	Oval and transparent

The acronyms of the fungal isolates were derived from the fungal names, farm codes, fruit codes, and codes for isolates obtained from the same fruits, respectively.

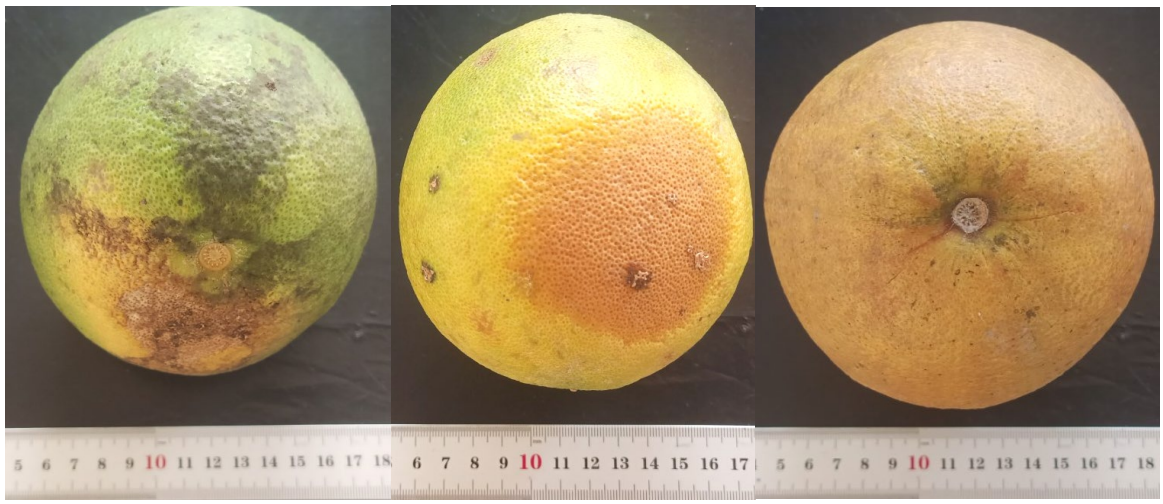

Figure 1. Pomelo fruit samples with symptoms of stem end rot disease.

Growth of fungal pathogens on PDA

The results obtained (Table 2) showed that the average diameter growth of fungal isolates was 3.96 cm at 24 h and 7.16 cm at 48 h. Specifically, the fungal isolates with fast growth were PL-M01-A1-A, PL-M01-A1-B, PL-M01-A4-A, PL-M01-A4-B, PL-M01- A6-A, PL-M01-A6-B, PL-B01-A1-A, PL-B01-A7-A, PL-B01-A7-B and PL-M02-A2-A, with diameters of 9.00 cm, 9.00 cm, 8.75 cm, 9.00 cm, 9.00 cm, 8.90 cm, 8.60 cm, 8.63 cm and 8.63 cm, respectively, 48 h after inoculation. The pathogenicity of these 9 fungal isolates causing SERD on pomelos was proven following Koch's postulates (Figure 2).

Evaluation of the pathogenicity of fungal isolates on pomelo fruit

After infection, it was found that all nine fungal isolates could cause stem end and fruit rot disease on pomelos. Among them, three fungal isolates PL-M01-A6-B, PL-B01-A7-B and PL-M01-A4-B caused the fastest rot, in only 4 days after inoculation, and the diameter of the rotted area was 2.1 cm, 1.8 cm and 1.8 cm, respectively. The same three fungal isolates with the fastest time to cause whole fruit rot, were selected for identification (Table 3). The control fruit still had the disease symptoms on the stem end. This might be due to the fact that fungal spores had entered the fruit through the wound in the stem end during harvesting or postharvest storage. Alcohol could have only partially

disinfected the diseased fruit but could not destroy all fungal spores remaining in the wound. Inoculation with distilled water created moist conditions that helped the fungi that survived to develop. Although mycelium developed at the stem end, it did not cause fruit rot in the control. The results of the fruit rot diameter were taken 2 days after the fruit rot appeared.

Identification of three selected fungal isolates which caused SERD

Three selected fungal isolates with fast growth on media and with proven pathogenicity on pomelos were identified by sequencing the ITS region. The results of the identification of the three fungal isolates were as follows: PL-M01-A4-B and PL-B01-A7-B were *Lasiodiplodia pseudotheobromae* and PL-M01-A6-B is *L. theobromae* with 100% similarity as shown in Figure 3.

Table 2. Mycelial growth diameter of different fungal isolates on PDA medium.

No.	Fungal isolates	Mycelium diameter (cm)	
		24 h	48 h
1	PL-M01-A1-A	4.57 c	9.00 a
2	PL-M01-A1-B	5.35 b	9.00 a
3	PL-M01-A4-A	3.90 d	8.43 bc
4	PL-M01-A4-B	5.57 b	8.75 ab
5	PL-M01-A6-A	6.80 a	9.00 a
6	PL-M01-A6-B	6.85 a	9.00 a
7	PL-B01-A1-A	5.50 b	8.90 ab
8	PL-B01-A1-B	0.97 g	4.43 e
9	PL-B01-A7-A	5.23 b	8.60 ab
10	PL-B01-A7-B	5.30 b	8.63 ab
11	PL-B01-A8-A	1.70 f	4.17 e
12	PL-B01-A8-B	2.30 e	5.37 d
13	PL-B01-A9-A	0.50 g	1.83 g
14	PL-B01-A9-B	0.77 g	2.77 f
15	PL-M02-A2-A	4.53 c	8.63 ab
16	PL-M02-A2-B	3.63 d	8.03 c
Level of significance		*	*
CV (%)		2.58	1.05

Values followed by the same letters in the same column are not significantly different according to Duncan post-hoc test at P=0.05. The acronyms of the fungal isolates were derived from the fungal names, farm codes, fruit codes, and codes for isolates obtained from the same fruits, respectively.

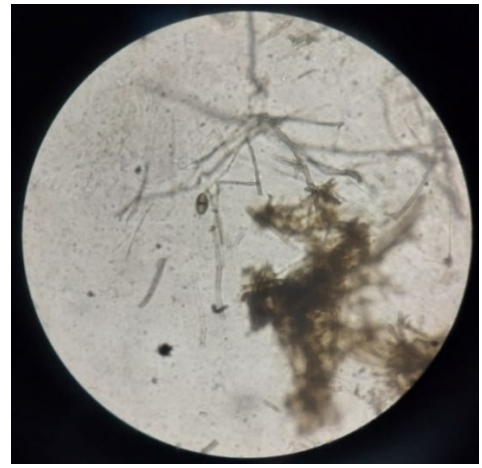

Figure 2. Spore of the fungal pathogens under 10X magnification.

Table 3. Time required for the appearance of fruit rot, whole fruit rot and diameter of fruit rot during a 20 days investigation.

Fungal isolates	Time required (days after infection)		Diameter of fruit rot (cm)
	for the appearance of fruit rot	for whole fruit rot	
PL-M01-A1-A	13	17	1.00
PL-M01-A1-B	14	>20	0.85
PL-M01-A4-B	4	7	1.80
PL-M01-A6-A	14	20	3.00
PL-M01-A6-B	4	7	2.10
PL-B01-A1-A	10	>20	1.90
PL-B01-A7-A	5	>20	1.25
PL-B01-A7-B	4	8	1.95
PL-M02-A2-A	6	14	1.10
Control	No fruit rot recorded	No fruit rot recorded	No fruit rot recorded

The acronyms of the fungal isolates were derived from the fungal names, farm codes, fruit codes, and codes for isolates obtained from the same fruits, respectively.

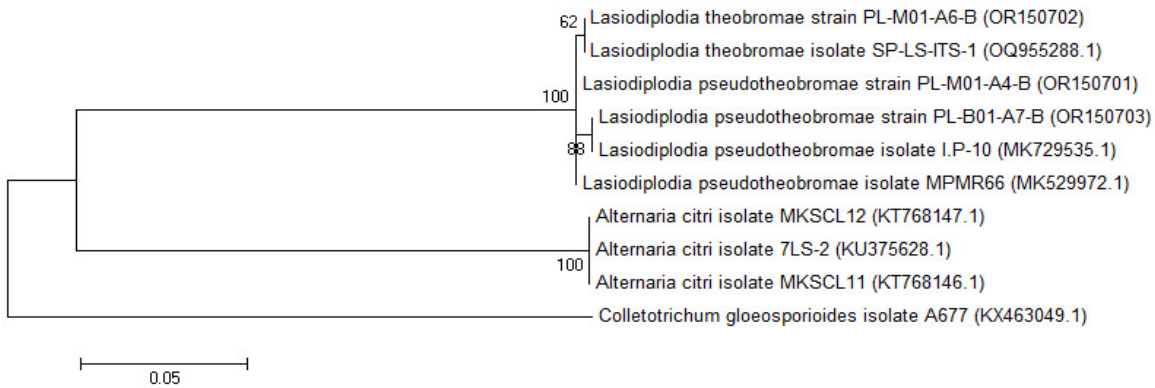

Figure 3. Phylogenic tree of the three identified fungal isolates.

Discussion

In the study by Chen *et al.* (2021), *Lasiodiplodia pseudotheobromae* was identified by ITS, translation elongation factor 1-α gene *(TEF*), and beta-tubulin (*TUB*) regions. However, fortunately, in the current study, the causing agents of the SERD in pomelo were successfully identified as isolates of *L. pseudotheobromae* and *L. theobromae* according to the ITS regions only. Moreover, both *L. pseudotheobromae* and *L. theobromae* were also detected on to cause both canker and gummosis in nectarine (Endes *et al.,* 2016) and leaf necrosis on tea plants (Li *et al.,* 2019). Nevertheless, according to Phillips *et al.* (2013), combining ITS with other regions can distinguish the species in a cluster. Thus, the selected three pathogenic isolates should further have their *TEF* or *TUB* regions amplified. *L. theobromae* is more common and is a main pathogen of post-harvest diseases on fruits (Mascarenhas *et al.,* 1996). On pomelo, the *L. theobromae* has been found earlier to cause fruit rot (Luo *et al.,* 2011) and stem rot (Puspita *et al.,* 2023). However, although it is potentially harmful in Vietnam in general and on pomelos in particular, there are not currently many studies to clarify the spread, occurrence and extent of damage the *L. theobromae* fungus can cause. On the other hand, *L. pseudotheobromae* also affects a variety of plants and trees (Bragard *et al.,* 2023). The *L. pseudotheobromae* has been found to cause fruit rot on mandarin and orange (Chen *et al.,* 2021), stem canker in hackberry (Liang *et al.,* 2020), cankered stems, blighted branches and decayed kernels on walnut (Li *et al.,* 2015), coffee dieback (Freitas-Lopes *et al.,* 2020), trunk canker on acacia (Castro-Medina *et al.,* 2014), mango dieback (Kwon *et al.,* 2017), and cacao pod rot (Serrato-Diaz *et al.,* 2019). The *L. pseudotheobromae* has been morphologically observed with white, soft and spongy mycelia then black with oval and hyaline spores (Li *et al.,* 2019), which is consistent with the current study. Noticeably, this is the first report demonstrating *L. pseudotheobromae* as the pathogen of SERD on pomelo. Thus, suitable approaches should be made to reduce the infection of both *L. pseudotheobromae* and *L. theobromae* on pomelo in Vietnam. Many approaches have been made in the world to reduce damage caused by *Lasiodiplodia* spp. For instance, an immersion in warm water of 49°C for 3 min inhibits or reduces the growth of pathogens (Schirra & D'hallewin, 1997), which limits the penetration ability of pathogenic fungi through the wound from the stem end without affecting the quality of pomelo fruit. Moreover, using chemical fungicides is an advantageous method to quickly destroy pathogens but it disadvantageously affects the environment and human health (Rani *et al.,* 2021). Fungicides that are still safe such as carvacrol and thymol, have the ability to control SERD on pomelos and maintain their fruit quality during storage (Yan *et al.,* 2020). Moreover, using antagonistic microorganisms is an environmentally friendly method and has a certain importance in pathogenic fungal control (Eljounaidi *et al.,* 2016). Therefore, using microorganisms as a biocontrol agent is a potentially sustainable way to control the SERD on pomelo caused by *L. pseudotheobromae* and *L. theobromae,* and needs to be investigated for the control of SERD on pomelo citrus.

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الملخص

ثوان، ف.م، ب.ت.ب كوين، ه.ن.د خاو، د.ت. اكسان، ل.ت. كوانج، ل.ن.ت اكسان ون.ك. خونج. 2025. حدوث مرض تعفن نهاية الساق في الليمون الهندي/البوملي (*Citrus maxima*) المتسبب عن أنواع الفطر *Lasiodiplodia* في مقاطعة بن تره، فيتنام. مجلة وقاية النبات العربية، 43(3): 310–316. https://doi.org/10.22268/AJPP-001333

أجريت هذه الدراسة بهدف عزل وتحديد العزلات الفطرية المسببة لتعفن نهاية الساق على ثمار الليمون الهندي/البوملي والتي تتميز بقوة نموها. تمّ جمع ثمار الليمون الهندي التي يظهر عليها علامات تعفن نهاية الساق أو ذبول الساق. تمّ عزل ست عشرة عزلة فطرية من عينات الثمار التي تم جمعها من منطقتين هما بينه داي ومو كاي باك في مقاطعة بن تري، فيتنام. أجريت التجربة على 16 عزلة فطرية عشوائية تماماً، بواقع 3 مكررات كلٌّ منها عبارة عن طبق بتري. تمّ اختيار تسع عزلات فطرية سريعة النمو، حيث تراوح قطر المستعمرات الفطرية من 8.60 إلى 9.00 سم، بعد 48 ساعة من التلقيح. تسببت هذه العزلات التسعة عند تلقيحها لثمار الليمون الهندي بظهور أعراض تعفن الثمار وتمّ تأكيد قدرتها على التسبب بالمرض وفقاً لفرضيات كوخ. كان نطاق قطر العدوى على ثمار الليمون الهندي 0.85–3.00 سم. تمّ تشخيص العزلات الفطرية الثلاثة التي تسبب تعفن ثمار الليمون الهندي وتعطي أعراضاً أبكر وأكثر شدّة على أنها *Lasiodiplodia pseudotheobromae* PL-M01-A4-B و*L. theobromae* PL-M01-A6-B؛ PL-B01-A7-B من خلال تحديد تسلسل منطقة ITS، مع تشابه بنسبة 100%.

كلمات مفتاحية: تعفن الفاكهة، *Lasiodiplodia pseudotheobromae*، *Lasiodiplodia theobromae*، العامل الممرض، الليمون الهندي، تعفن طرف الساق.

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