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Morphological Characterization and Molecular Identification of Three Fungal Pathogens Isolated from *Solanum melongena* L. in Sri Lanka

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ABSTRACT

Purpose: *Solanum melongena L. (brinjal/ eggplant) is a popular vegetable in Sri Lanka. Like many solanaceous crops, brinjal fruits and plants are susceptible to various diseases. The objective of this study was to isolate, confirm pathogenicity and characterize fungal pathogens causing leaf blight, yellowing and necrosis in S. melongena plants in selected localities in Gampaha district, Sri Lanka.*

Research Method: *Fungi associated with symptomatic S. melongena plants collected from three localities in Gampaha district, Sri Lanka, were isolated onto potato dextrose agar and their pathogenicity was tested on healthy S. melongena plants. Fungal isolates that were confirmed to be pathogenic were subjected to morphological and molecular characterization.*

Findings: *Fifteen fungal isolates were obtained from infected leaves of S. melongena plants, and four fungal isolates were confirmed to be pathogenic on S. melongena. Isolates H32a and H32b causing similar disease symptoms were identified as Lasiodiplodia theobromae with 99.81% sequence similarity by the analysis of the internal transcribed spacer region (ITS1-5.8S-ITS2). The remaining two pathogenic isolates were identified as Pseudopestalotiopsis theae and Diaporthe eugeniae, with 100% and 99.82% sequence similarities, respectively.*

Originality/ Value: *To our knowledge, this is the first report of leaf necrosis by L. theobromae, leaf yellowing by P. theae and leaf blight by D. eugeniae associated with S. melongena plants in Sri Lanka.*

Keywords: *Diaporthe, eggplant, internal transcribed spacer region (ITS), Lasiodiplodia, pathogenicity testing, Pseudopestalotiopsis*

INTRODUCTION

Solanum melongena L. (brinjal/ eggplant) is a vegetable belonging to family *Solanaceae* and is one of the most consumed vegetables around the world (Palia *et al.*, 2021). Eggplant was first domesticated in the South Asian/ South East Asian region (Solanke and Tawar, 2019), and its fruits are important due to their high nutritional value (Chioti *et al.*, 2022). *S. melongena* is also important for its medicinal properties against a range of ailments, including diabetes, heart diseases, asthma, and neuralgia due to the presence of an array of bioactive compounds including phenolics, alkaloids, carotenoids and reducing sugars (Solanke and Tawar, 2019).

S. melongena is commonly grown in the tropical and subtropical regions of the world (Kaniyassery *et al.,* 2022), and can be grown in all agro-climatic regions in Sri Lanka throughout the year (Thayaparan *et al.,* 2019). China was the

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leading *S. melongena* producer in the world in 2020, with an estimated yield of 36 million tons accounting for over 60% of world production. In comparison, Sri Lanka produced 141, 881.9 tons of *S. melongena* in the same year (FAOSTAT, 2020).

Different varieties of *S. melongena* are present with a wide range of fruit colors, sizes, and shapes (Arasakesary, 2019). Among them, elongated, ovoid or slender type with dark purple skin fruit type is the most common in Sri Lanka (Somawathi *et al.*, 2014). While several landraces of *S. melongena* are localized to different geographical regions in the country, recommended varieties include SM 164, Thinnaweli purple, Amanda F1, Anjalee-F1 and HORDI Lenairi 1 (Department of Agriculture Sri Lanka, 2022).

Each year, a high percentage of *S. melongena* crop production is lost due to diseases caused by a large number of pathogens and the majority of them are reported as fungal pathogens (Abdelaziz *et al*., 2022, Kaniyassery *et al.,* 2022). *S. melongena* leaf is much susceptible to diseases (Anand *et al*., 2016), interrupting photosynthesis and reducing plant growth, crop yield and crop quality (Jain *et al*., 2019). *Phomopsis vexans* causing phomopsis blight*, Cercospora melongenae* causing cercospora leaf spot*, Colletotrichum truncatum* causing anthracnose*, Fusarium oxysporum f.sp. radicis-lycopersici* causing vascular wilt and *Corynespora cassiicola* have been reported as fungal pathogens associated with *S. melongena* plants in Sri Lanka (Adikaram and Yakandawala, 2020). Thus, continued investigations to identify the emergence of new diseases and pathogens are required to prevent disease outbreaks or epidemics. Early recognition of fungal diseases and causative pathogens is important to implement suitable disease management strategies and reduce yield losses (Jain *et al*., 2019).

Hence, this investigation was conducted to isolate fungal pathogens, confirm pathogenicity of each isolate, characterize and identify each pathogen that caused leaf blight, yellowing and necrosis in *S. melongena* plants at three localities in Gampaha district of Sri Lanka.

MATERIALS AND METHODS

Study Site and Sampling

Small scale fields with more than ten *Solanum melongena* plants at varied maturity levels, from flowering to fruit setting stages, in Yonganmulla (7° 4' 11.856'' N, 80° 3' 42.9012'' E), Hiswella (7° 1' 14.52'' N, 80° 9' 48.132'' E) and Amithirigala (7° 1' 54.228'' N, 80° 11' 7.872'' E) in Gampaha District were visited in August, 2019. A total of seven symptomatic leaf samples (with leaf blight, yellowing and necrosis) were collected from each field in each locality, placed in polythene bags with appropriate labels, and brought to the laboratory of the Department of Plant and Molecular Biology, University of Kelaniya.

Isolation of Fungal Pathogens

Symptomatic plant parts were cut into small sections (1 cm^2) and aseptically transferred to 70% ethanol solution for 15 seconds for surface sterilization. Afterwards, sterilized plant sections were washed three times with sterilized distilled water for 30 seconds to remove excess ethanol. They were transferred onto a sterilized filter paper and after the edges were trimmed small sections were aseptically placed on Potato Dextrose Agar (PDA) medium. The inoculated plates were incubated at room temperature (approximately 30 ± 2 ^oC) for two to three days. The obtained fungal cultures were sub-cultured on new PDA plates to obtain pure cultures.

Pathogenicity Confirmation Test

Ninety-six, healthy one-month-old *S. melongena* plants under greenhouse conditions were used for pathogenicity confirmation. Wounded and non-wounded inoculations of the fifteen isolates were carried out using mycelial plugs (Lim *et al*., 2019) or by placing a drop or spraying a spore suspension at 1 x 10^7 conidia/ ml concentration (Stahr and Quesada-Ocampo, 2020) with three

replicates per fungal isolate. Non-colonized PDA plugs or sterilized distilled water was used for wounded and non-wounded control treatments.

All the plants were covered with a polythene bag containing cotton wool soaked with sterilized distilled water to provide moisture. Control and treated plants were arranged in a randomized complete block design (RCBD) in a greenhouse at the Department of Plant and Molecular Biology, University of Kelaniya. All inoculated leaves were observed after the 2nd day of treatment up to the 14th day, and symptom development was recorded. Fungal pathogens were re-isolated from infected leaves.

Morphological Identification of Fungal Pathogens

Microscopic slides were prepared for each fungal isolate using the sticky tape with and without lactophenol cotton blue stain. Pycnidia developed on two weeks old fungal isolates were gently teased apart and examined under high power (100x) of a phase-contrast microscope (Olympus CX41, Olympus, Japan).

Molecular Identification of Fungal Pathogens

Genomic DNA was extracted from the four pathogenic fungal isolates by a modified CTAB method (Daranagama *et al*., 2015). Polymerase chain reaction (PCR) was carried out for fungal DNA using universal primer pairs ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), for identification of fungal species (White *et al*., 1990). The PCR mixture (25 µl) contained 5 µl of 1x GoTaq® Flexi buffer, 1.5 µl of 25 mM of $MgCl_2$, 0.5 µl of 0.2 mM dNTPs, 1 µl of 0.4 µM forward and reverse primers 0.25 µl of 5 U/µl of GoTaq® DNA polymerase (Promega Inc., USA) and 2 µl of template DNA. Amplification was carried out in a thermal cycler (Veriti® 96-well Thermal Cycler, ABI Biosystems, USA) with an initial denaturation of 5 min at 95 °C, followed by 37 cycles of 30 seconds at 95 °C, 30 seconds at 54 °C, 90 seconds at 72 °C, and a final extension of 10 min at 72 °C. PCR products were visualized on 1% agarose gel and sequenced bidirectionally by the Sanger sequencing method at Macrogen Inc, South Korea. Sequences were manually checked and edited using BioEdit software (version 7.2.5) and aligned with the nucleotide database (blastn) in National Centre for Biotechnology Information (NCBI) to find the best match for the fungal isolates. The sequence data obtained in this study were deposited in GenBank, under accession numbers MT990527, MT990528, MT990526, and MT990529.

RESULTS AND DISCUSSION

Pathogenicity Confirmation of Isolated Fungi

Fifteen fungal isolates were obtained from infected *S. melongena* leaves in selected localities in Gampaha district. Four fungal isolates (H32a, H32b, U10, and U11) were identified as pathogenic fungi based on the pathogenicity confirmation test.

Necrotic spots were initiated at the site of wound as well as non-wound inoculation with isolates H32a and H32b three days after inoculation. In addition, premature leaf senescence was observed in some replicates while all leaves were detached within four to six days of inoculation. Isolate U10 did not respond to wound and non-wound fungal mycelial plug inoculation and spore suspension inoculation methods. Leaf chlorosis was observed with isolate U10 in wounded leaves inoculated by spore suspension spraying method four days after inoculation. In contrast, symptoms were not observed in non-wounded leaves, suggesting that wounding may be necessary for infection. This observation was confirmed by the report of Maharachchikumbura *et al*. (2011). Wound inoculated leaves were detached 5th to 6th day after inoculation. Leaf blight was observed six days after wound and non-wound inoculation of isolate U11 (Figure 01).

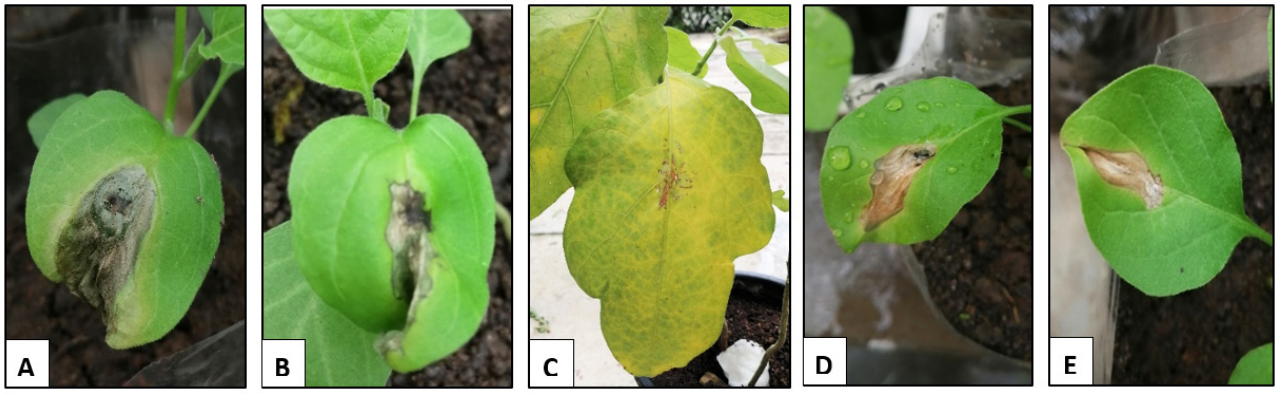

Figure 01: **Symptoms of pathogenicity confirmation test of fungal isolates H32a, H32b, U10 and U11, A: Leaf necrosis in wound inoculated leaf of isolate H32a four days after inoculation; B: Leaf necrosis in non-wounded leaf inoculated with isolate H32b four days after inoculation; C: Leaf chlorosis on spray inoculated wounded leaf with isolate U10 spore suspension four days after inoculation; D: Leaf blight on wound inoculated isolate U11 six days after inoculation; E: Leaf blight on non-wounded leaf inoculated with isolate U11 six days after inoculation.**

The symptoms in the pathogenicity test of each isolate were similar to infected leaves collected from the field, and all re-isolated fungal isolates were morphologically similar to their respective original fungal isolates.

Molecular Identification and Morphological Characterization of Isolated Fungal Pathogens

Four fungal pathogens isolated from *S. melongena* belonged to three species based on morphological and molecular characterization. The most commonly used DNA barcoding marker, ITS region was used for the species level identification of isolated fungal pathogens in this study (Raja *et al*., 2017). Sequence analysis of ITS region showed 99.81% sequence similarity in isolates H32a and H32b to previously reported *Lasiodiplodia theobromae* isolate IRNKB244. ITS sequence of isolates U10 and U11 showed 100% and 99.82% sequence similarity to *Pseudopestalotiopsis theae* isolate P3BS3_B and *Diaporthe eugeniae* isolate ASHM304 respectively (Table 01).

Colony morphology, growth rate, fungal hyphal and conidial characters of the three fungal pathogens were observed for morphological characterization of the three species (Figure 02, Figure 03, Figure 04), Table 02

Table 01: **Identification of pathogenic fungal species isolated from symptomatic leaves of S. *melongena* in Gampaha District, Sri Lanka in 2019 by ITS1-5.8S-ITS2 rDNA sequence analysis.**

Isolate	Accession number (GENBANK)	Highest match with NCBI database	Percent identity	Query cover	E value
H32a	MT990527	*Lasiodiplodia theobromae* isolate IRNKB244 (MN634046)	99.81	100	0
H32b	MT990528	*Lasiodiplodia theobromae* isolate IRNKB244 (MN634046)	99.81	100	0
U10	MT990526	*Pseudopestalotiopsis theae* isolate P3BS3_B (MK120119)	100	100	0
U11	MT990529	*Diaporthe eugeniae* isolate ASHM304 (MK110370)	99.82	100	0

Table 02: Morphological characteristics of L. theobromae, P. theae and D. eugeniae isolated from symptomatic leaves of S. melongena collected at the three localities in Gampaha District, Sri Lanka in 2019.

Morphological character	Fungal pathogen		
	Lasiodiplodia theobromae	*Pseudopestalotiopsis theae*	*Diaporthe eugeniae*
Colony morphology	Blackish-grey mycelium	White colour mycelium and yellowish orange on lower surface	White colour mycelium
Colony margin	Entire	Slightly undulate	Entire
Growth	4.08±0.23 cm per day	0.87±0.08 cm per day	1.25±0.06 cm per day
Fungal hypha	Septate and branching	Septate and branching	Septate and branching
Special structures	Arthroconidia present	Not observed	Not observed
Pycnidia	Ash color, scattered arrangement	Black color, clustered arrangement, form black concentric circles	Black and brown color, scattered arrangement, form black concentric circles
Conidia	Dark brown color ellipsoid oval form; One septum in the middle	Fusiform shaped, three brown color median cells together two hyaline terminal cells as apical and basal cell, separated by four septa	Alpha conidia were aseptate, hyaline, multi-guttulate and fusiform in shape, Beta conidia were not observed, Gamma conidia were hyaline and cylindrical in shape, with acute ends
Conidial appendages	Not observed	One to three, unbranched and tubular apical and basal appendages.	Not observed
Conidial length	20 µm -23 µm	29 µm -15 µm	24 *µm* -32 *µm*
Conidial width	10 µm to 14 µm	4 µm -2 µm	2 µm -3 µm

L. theobromae species, *Diaporthe* and *Phomopsis* genera have been reported as pathogens as well as endophytes in different plants with wide host ranges and geographical distribution (Lim *et al.*, 2019; Salvatore *et al.,* 2020; Silva *et al.,* 2020). While the exact transition process of endophytic to pathogenic/parasitic form of fungi or *vice versa* is not fully explained, changes in the tropic states are believed to derive from distinct fungal gene expressions in response to plant species, physiology and plants' ability to perceive fungal presence (Pillai, 2017; Wheeler *et al.,* 2019).

Woodward *et al.* (2005) first demonstrated direct and wound penetration of *L. theobromae* causing *S. melongena* fruit rot in USA, while Vieira *et al.* (2018) reported the same in Brazil. However, in this study, the isolated pathogens H32a, and H32b identified as *L. theobromae* formed a brown color necrotic lesion on leaves of *S. melongena.* and to the best of our knowledge, this is the frst record of this disease symptoms caused by *L. theobromae* in *S. melongena* plants.

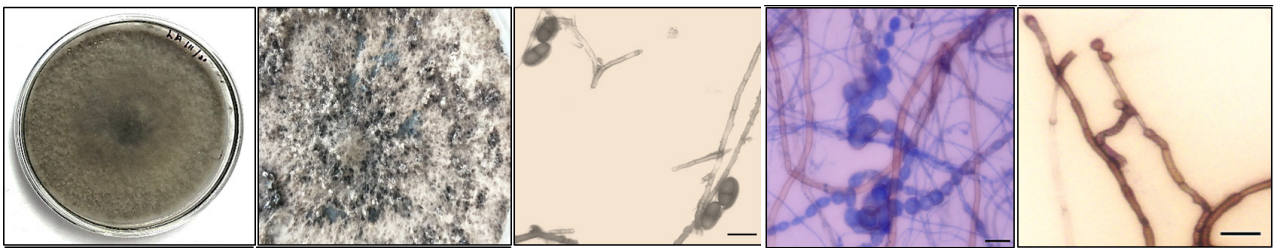

Figure 02: **Colony and hyphal morphology of L. *theobromae* (Isolate H32a) isolated from symptomatic leaves of S. *melongena*, A: Seven days old pure culture- upper surface; B: Pycnidia formed on colony surface; C : Conidia; D: Chains of arthroconidia; E: Swollen segments in pigmented fungal hypha; Scale bar: B= 1 cm, C, D, E= 20 µm, FH= Fungal hypha, SC= Septum in the conidia.**

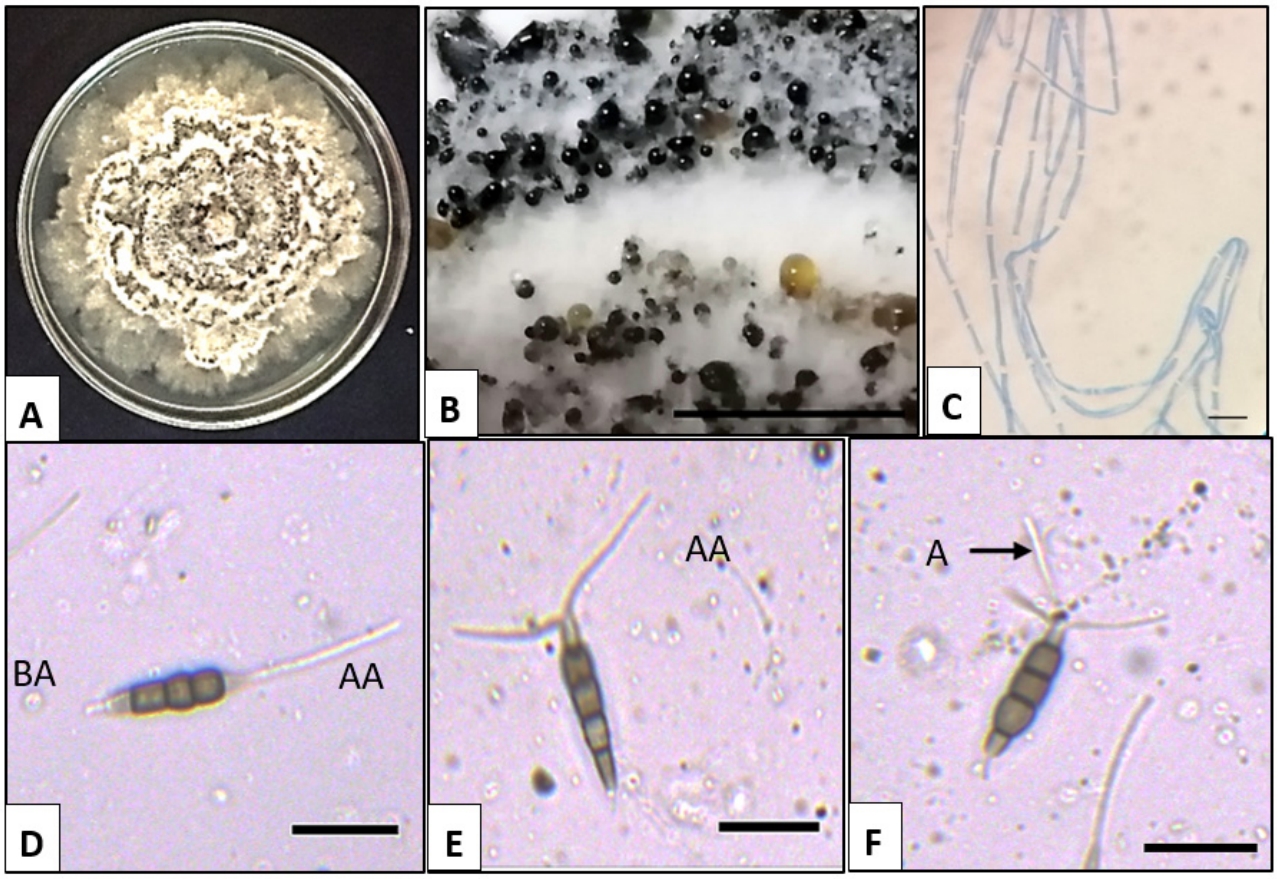

Figure 03: **Colony and hyphal morphology of P. theae (Isolate U10) isolated from symptomatic leaves of S. melongena, A: Two weeks old pure culture- Upper surface; B: Pycnidia; C: Septate branching fungal hypha; D: Conidia with one apical appendage; E: Conidia with two apical appendages; F: Conidia with three apical appendages; Scale bar: B= 1 cm, C, D, E, F = 20 µm, AA=Apical appendage/s, BA = Basal appendage.**

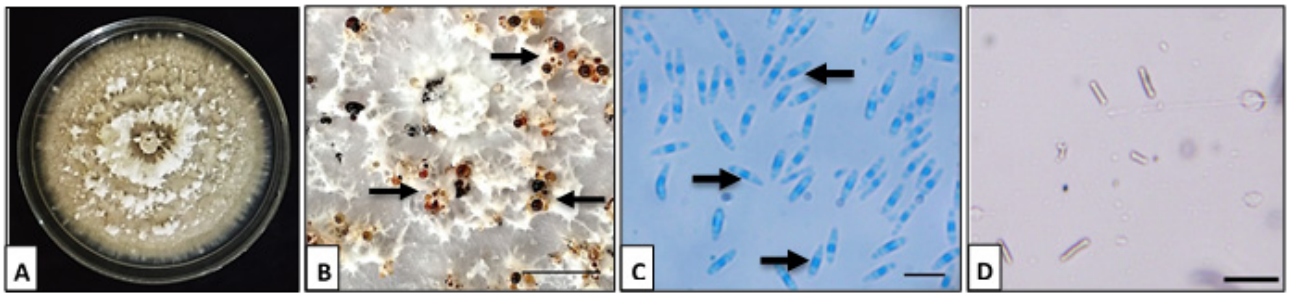

Figure 04: **Colony and hyphal morphology of D. eugeniae (Isolate U11) isolated from symptomatic leaves of S. melongena, A: Seven days old pure culture- Upper surface; B: Pycnidia on colony surface; C: Alpha conidia (guttules indicated by arrows); D: Gamma conidia; Scale bar: B= 1 cm, C, D= 20 µm.**

Genus *Diaporthe* has been described to consist of many plant pathogens, saprophytes as well as endophytes (Mena *et al.,* 2022), though less records are available on the pathogenicity of the species *D. eugeniae*. Mahendranathan *et al*. (2010) reported that *Phomopsis vexans* (anamorph of Genus *Diaporthe*) caused phomopsis blight in *S. melongena* leaves in Sri Lanka, while the same caused leaf blight and fruit rot in *S. melongena* in India (Udayashankar *et al.,* 2019). Similarly, the isolated fungal pathogen identified as *D. eugeniae,* caused leaf blight of *S. melongena*.

According to Maharachchikumbura (2014), *Pestalotiopsis* species cause a range of diseases in a wide range of host plants, including cankers, shoot dieback, leaf spots, blights, severe chlorosis and fruit rot. Fungal pathogen *P. theae* isolated from chlorotic *S. melongena* leaves in this study caused chlorosis in healthy plants after re-inoculation, confirming its pathogenicity.

Pseudopestalotiopsis is a new genus which was recently grouped out from genus *Pestalotiopsis* (Lateef *et al*., 2015). According to Maharachchikumbura *et al.* (2014), *Pestalotiopsis* is categorized into 3 main clades as *Neopestalotiopsis, Pestalotiopsis,* and *Pseudopestalotiopsis.* The color of the median cells in conidia was the major delimiting character in this classification and there are no differences in their culture morphology other than this conidial difference. The species with lightly pigmented concolorous median cells belonged to the genus *Pestalotiopsis* while species with dark concolourous median cells were classified under genus *Pseudopestalotiopsis.*

Early identification of plant diseases and their causative agents is crucial in the development and timely application of disease management strategies to reduce yield and quality loss of commodities. *L. theobormae, P. theae* and *D. eugeniae* have not been previously reported to be associated with *S. melongena* plants in Sri Lanka. Therefore, this study would be beneficial in identifying minor diseases of the plants and associated fungal pathogens, which might contribute to yield loss in *S. melongena* and other related Solanaceous crops.

CONCLUSIONS

Out of 15 fungi isolated from symptomatic leaves of *S. melongena* sampled at Hiswella, Yonganmulla and Amithirigala fields in Gampaha district, Sri Lanka in 2019, *Lasiodiplodia theobromae, Pseudopestalotiopsis theae* and *Diaporthe eugeniae* cause leaf necrosis, leaf chlorosis and leaf blight respectively, on *Solanum melongena* plants.

The observed morphological characters along with obtained ITS nucleotide sequence evidence confirmed the presence of pathogenic fungi *L. theobromae*, *P. theae* and *D. eugeniae* in symptomatic leaves of *Solanum melongena* plants.

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