CTU Journal of Innovation and Sustainable Development
ISSN 2588-1418 | e-ISSN 2815-6412

DOI:10.22144/ctujoisd.2026.040

Biology and pathogenicity of *Pestalotiopsis microspora* and *Colletotrichum siamense* associated with circular leaf spot of rubber (*Hevea brasiliensis*)

Nguyen Don Hieu*, Doan Nhan Luan, and Nguyen Thi Kim Uyen

Department of Plant Protection, Rubber Research Institute of Viet Nam, Viet Nam

**Corresponding author (donhieurriv@gmail.com)*

Article info.

Received 17 Jan 2026
Revised 4 Jun 2026
Accepted 27 May 2026

Keywords

Circular leaf spot, Colletotrichum siamense, Hevea brasiliensis, pathogenicity, Pestalotiopsis microspora, rDNA-ITS

ABSTRACT

Circular leaf spot disease (CLSD) has recently emerged as an important foliar disease of rubber (Hevea brasiliensis) in Viet Nam, yet information on its associated pathogens remains limited. This study investigated the biological characteristics and pathogenicity of Pestalotiopsis microspora and Colletotrichum siamense associated with CLSD. Sixteen isolates of each species were obtained from diseased leaves collected in major rubber-growing regions of Viet Nam. Morphological characteristics were evaluated on different culture media and temperature regimes, while pathogenicity was assessed under laboratory and greenhouse conditions and confirmed through Koch's postulates. Pestalotiopsis microspora showed optimal growth on malt extract agar at 25°C, whereas C. siamense grew most rapidly on potato-based media at 25–30°C. ITS sequence analysis showed ≥99% similarity between all isolates and reference sequences of P. microspora and C. siamense in GenBank. Disease severity index values on detached leaves ranged from 34.4–52.1% for P. microspora, 54.5–99.8% for C. siamense, and 92.0–100% for mixed inoculations (ANOVA, $p < 0.01$). Greenhouse inoculations showed a similar pattern. Among six rubber clones, RRIV 124 was the most susceptible under laboratory conditions. These results indicate that CLSD in Viet Nam is associated with a pathogen complex in which C. siamense is more pathogenic than P. microspora, while co-inoculation further increases disease severity.

1. INTRODUCTION

Rubber trees (*Hevea brasiliensis*) are frequently affected by various diseases that reduce growth and latex productivity, among which foliar diseases play a particularly critical role in plantation performance (Kuruvilla, 2023). In addition to well-known foliar pathogens such as powdery mildew, Corynespora leaf fall, and black stripe, a newly emerging disease known as Circular Leaf Spot Disease (CLSD), or Pestalotiopsis leaf fall, has rapidly spread across major rubber-producing countries in Asia. The disease was first reported in Indonesia in 2016, followed by Malaysia and India in 2017, Thailand and Sri Lanka in 2019, China in 2020, Viet Nam in 2021, and the Philippines in 2022 (Kuruvilla, 2023).

Since its emergence, CLSD has spread rapidly across major rubber-producing regions in Asia and is increasingly recognized as an emerging threat to sustainable rubber production. Reports presented within the International Rubber Research and Development Board (IRRDB) network indicate that the disease has affected extensive plantation areas and may cause severe defoliation, substantial reductions in latex yield, and significant economic

losses under favorable epidemic conditions (Tajuddin & Ibadallah, 2019; Kuruvilla, 2023). However, most of these estimates are derived from industry reports and conference communications rather than peer-reviewed scientific publications and should therefore be interpreted with caution. Nevertheless, consistent observations reported across several rubber-producing countries highlight the potential importance of CLSD as a major foliar disease that warrants further scientific investigation.

In Viet Nam, CLSD was first detected in October 2021 and officially designated as Circular Leaf Spot Disease. By late 2022, the disease had spread to eight rubber companies in the Southeast region, affecting approximately 9,643 ha and showing variable susceptibility among more than 20 commonly cultivated rubber clones (Hieu et al., 2022).

Despite numerous studies being conducted within the IRRDB network, the causal agents of CLSD have not yet been fully agreed upon. *Pestalotiopsis* spp. has been identified as the primary pathogen in Indonesia and Malaysia, whereas studies in Thailand and India have emphasized the dominant role of *Colletotrichum* spp. Co-infections involving both fungi have been reported in Sri Lanka and Viet Nam, while *Neopestalotiopsis* spp. has been associated with the disease in China and the Philippines (Kuruvilla, 2023). Recent research in Viet Nam confirmed the involvement of a pathogen complex comprising *Pestalotiopsis microspora* and *Colletotrichum siamense* (Hieu et al., 2023).

To date, scientific information on the biology, diversity, epidemiology, and pathogenic mechanisms of CLSD-associated fungi remains limited, particularly in Viet Nam where the disease was only recently detected. Although preliminary reports have identified *P. microspora* and *C. siamense* as major fungal species associated with CLSD, their relative contributions to disease development, interactions during co-infection, and pathogenic variability among rubber clones have not been comprehensively evaluated under Vietnamese conditions. Furthermore, much of the currently available information comes from regional surveys and conference reports, underscoring the need for experimentally validated studies supported by morphological, molecular, and evidence of pathogenicity.

The present study was conducted as part of the project entitled “Development of an Integrated Management Protocol for Circular Leaf Spot Disease Newly Detected in Viet Nam”. Therefore, this study aimed to characterize the biological characteristics, molecular identity, and pathogenicity of *P. microspora* and *C. siamense* associated with CLSD in Viet Nam, thereby providing scientifically validated information to support disease diagnosis, resistance screening, and the development of sustainable management strategies for the rubber industry.

2. MATERIALS AND METHODS

2.1. Fungal isolates and GenBank sequence deposition

A total of 32 isolates of *Pestalotiopsis microspora* and *Colletotrichum siamense* were collected in 2023 from four major rubber-growing provinces in the Southeast and Central Highlands of Viet Nam (Dong Nai, Lam Dong, Tay Ninh, and Gia Lai). The fungal isolates were collected from symptomatic rubber leaves representing four commercial clones. Isolates No. 1, 7–11, and 13–16 originated from RRIV 124; isolates No. 5, 6, and 12 from RRIV 209; isolates No. 2 and 3 from RRIV 1; and isolate No. 4 from RRIV 106. Isolate codes indicate their collection sites, namely: BL (Binh Long), BT (Binh Thuan), DN (Dong Nai), DP (Dong Phu), LN (Loc Ninh), PR (Phu Rieng), TB (Tan Bien), TN (Tay Ninh), and TNg (Tay Nguyen, Gia Lai Province). The rDNA-ITS sequences of all isolates were successfully generated and deposited in GenBank. The *P. microspora* isolates were registered under the accession numbers PV562640 (PesBL03), PV562643 (PesBL06), PV562634 (PesBT03), PV562635 (PesBT04), PV562662 (PesDN52), PV562668 (PesDN59), PV562645 (PesDP01), PV562646 (PesDP02), PV562630 (PesLN05), PV562632 (PesLN07), PV562652 (PesPR20), PV562654 (PesPR22), PV562672 (PesTB02), PV562636 (PesTN01), PV562637 (PesTN02), and PV562669 (PesTNg01). The corresponding *C. siamense* isolates were deposited under the accession numbers PV562544 (CoBL03), PV562547 (CoBL06), PV562538 (CoBT03), PV562539 (CoBT04), PV562566 (CoDN52), PV562572 (CoDN59), PV562549 (CoDP01), PV562550 (CoDP02), PV562534 (CoLN05), PV562536 (CoLN07), PV562556 (CoPR20), PV562558 (CoPR22), PV562576 (CoTB02), PV562540 (CoTN01), PV562541 (CoTN02), and PV562573 (CoTNg01). These publicly available sequences provide a validated molecular resource supporting species identification as well as subsequent phylogenetic and pathogenicity analyses.

2.2. Morphological characterization of the causal fungi

This study aimed (i) to characterize the morphological features of the causal fungi and compare them with published taxonomic descriptions for species identification, (ii) to classify isolates based on morphological similarity, and (iii) to determine suitable culture media and temperature conditions for fungal growth.

2.2.1. Growth of Pestalotiopsis microspora and Colletotrichum siamense on different culture media

Sixteen (16) isolates of *Pestalotiopsis microspora* and sixteen (16) isolates of *Colletotrichum siamense* were initially cultured on potato dextrose agar (PDA) for 7 days, then transferred to experimental media. Two factorial experiments were conducted using a completely randomized design, each with three replications and two Petri dishes per replicate.

- Experiment 1: 48 treatments consisting of 16 *P. microspora* isolates × 3 culture media (PDA, PSA and MEA).
- Experiment 2: 48 treatments consisting of 16 *C. siamense* isolates × 3 culture media (PDA, PSA, and MEA).

All media were sterilized and poured into sterile Petri dishes (20 mL per dish). Mycelial plugs (8 mm in diameter) taken from the actively growing margins of 7-day-old colonies were placed at the center of each dish. Cultures were incubated at 25°C under controlled environmental conditions.

Data collection

- Colony color and morphological characteristics were recorded at 6 days after inoculation (DAI).
- Mycelial growth was assessed by measuring colony diameter at 6 DAI, when at least one treatment reached the edge of the Petri dish. Colony diameter was measured along two perpendicular axes (d_1 and d_2), and the mean colony diameter (D) was calculated as:

$$D = (d_1+d_2)/2$$

(where D denotes the average colony diameter, and d_1 and d_2 are two perpendicular diameters measured for each colony)

Spore morphology and size were examined for all isolates grown on the best-performing medium. Sterile distilled water was added to the colony surface, and conidia were gently dislodged using a sterile swab. Spore suspensions were observed under a light microscope (40X objective) connected to a computer. Images were captured, and the length and width of 10 randomly selected spores per isolate were measured using Euromex ImageFocus Alpha software.

Colony diameter and spore size data were analyzed using the General Linear Model (GLM), and mean comparisons were performed using SAS software version 8.1.

2.2.2. Effect of temperature on mycelial growth of P. microspora and C. siamense

The optimal culture medium identified in the previous experiment was used to evaluate the effect of temperature on fungal growth. Two factorial experiments were established using a completely randomized design with three replications and two Petri dishes per replicate.

- Experiment 1: 64 treatments consisting of 16 *P. microspora* isolates × 4 temperature regimes (20, 25, 30, and 35°C).
- Experiment 2: 64 treatments consisting of 16 *C. siamense* isolates × 4 temperature regimes (20, 25, 30, and 35°C).

Mycelial plugs (8 mm in diameter) were placed at the center of Petri dishes and incubated in programmable incubators set to the respective temperatures.

Data collection

- Colony color and morphology were recorded at 6 DAI.
- Colony diameter was measured at 6 DAI using the same procedure described above.

Data were analyzed using GLM, and treatment means were ranked using SAS software version 8.1.

2.3. Observation of spore germination and infection structure formation

One representative isolate of *P. microspora* (PesDP01) and one representative isolate of *C. siamense* (CoDP01) were selected for microscopic observation of spore germination and the development of infection structures. These isolates were chosen because they exhibited typical colony and conidial morphology for their respective species, showed stable sporulation under laboratory conditions, and were among the highly pathogenic

isolates identified during preliminary pathogenicity screening. The objective of this experiment was to describe the general infection process of the two fungal species rather than to compare variation among isolates.

- In vitro: Spore suspensions prepared in sterile distilled water were placed onto glass slides, which were maintained in moist chambers (Petri dishes lined with moist filter paper). Observations were conducted at 4, 8, 12, 16, 20, and 24 hours after inoculation.
- In planta: Rubber leaves of clone RRIV 124 at the 7-day-old developmental stage were artificially inoculated with spore suspensions following slight wounding. Inoculated leaves were placed in plastic chambers (26×17×8 cm) under high-humidity conditions at 25°C. Spore germination and appressorium formation at the inoculation sites were observed at 2, 3, 4, 5, and 6 days after inoculation using a light microscope (40X objective) connected to a computer.

2.4. rDNA-ITS region sequencing and molecular identification

Genomic DNA was extracted from 16 isolates morphologically identified as belonging to the genus Colletotrichum and 16 isolates of *P. microspora*. The rDNA internal transcribed spacer (ITS) region was amplified using the universal primers ITS1 and ITS4 following White et al. (1990). PCR reactions were carried out in 25 µL volumes containing standard buffer, $MgCl_2$, dNTPs, primers, GoTaq® Green DNA polymerase, and template DNA. Amplification was performed under conventional cycling conditions, and PCR products were verified by electrophoresis on 1.0% agarose gels. Successful amplicons were purified and commercially sequenced. Raw sequences were edited and assembled using BioEdit, aligned with ClustalW, and compared with reference sequences in the NCBI GenBank database using BLASTn.

Species-level identification of Colletotrichum isolates was inferred based on ITS sequence similarity together with morphological characteristics and comparison with previously reported CLSD-associated Colletotrichum species in Viet Nam. Because ITS sequences alone may not provide sufficient resolution to distinguish species within the *C. gloeosporioides* species complex, the identification of these isolates as *C. siamense* should be regarded as provisional pending confirmation through multi-locus phylogenetic analyses.

2.5. Pathogenicity evaluation of *Pestalotiopsis microspora* and *Colletotrichum siamense* isolates on rubber clone RRIV 124 under laboratory conditions

The pathogenicity of *P. microspora* and *C. siamense* isolates was evaluated on detached leaves of rubber clone RRIV 124 under controlled laboratory conditions. The objectives of this experiment were (i) to compare the virulence of individual fungal isolates and (ii) to select representative isolate pairs exhibiting the highest pathogenicity on RRIV 124 for subsequent pathogenicity and host–pathogen interaction studies.

2.5.1. Plant material and fungal isolates

Symptom-free, detached leaves of rubber clone RRIV 124 at the 20-day-old developmental stage were used as plant material. Five isolates of *P. microspora* (PesDP02, PesBT03, PesPR22, PesBL06, and PesDN59) and five isolates of *C. siamense* (CoDP02, CoBT03, CoPR22, CoBL06, and CoDN59) were selected based on their geographical origin and relatively high virulence levels identified in preliminary screening assays conducted prior to Koch's postulates.

2.5.2. Experimental design and inoculation procedure

The experiment was arranged in a completely randomized design with three replications. Each replicate consisted of two detached leaflets placed in plastic boxes lined with moist filter paper to maintain high relative humidity. Three independent pathogenicity assays were conducted:

- Inoculation with five *P. microspora* isolates;
- Inoculation with five *C. siamense* isolates; and
- Inoculation with five mixed inocula of *P. microspora* and *C. siamense* (1:1, v/v).

Sterile distilled water was used as the negative control in all assays.

Prior to inoculation, detached leaves were surface-sterilised by washing once with an antifungal solution, rinsing three times with sterile distilled water, and air-drying under aseptic conditions. Leaves were placed with the abaxial surface upward on wire-mesh supports inside plastic boxes.

Conidial suspensions were adjusted to 1×10^4 conidia mL^{-1}. This inoculum density was selected based on preliminary trials and was consistent with the inoculation protocol previously developed for

CLSD of rubber under detached-leaf conditions (Hieu et al., 2026). Preliminary observations indicated that higher inoculum concentrations (10^5–10^6 conidia mL^{-1}) led to rapid tissue necrosis and lesion coalescence, thereby hindering accurate assessment of disease progression and isolate-to-isolate variation. Therefore, 1×10^4 conidia mL^{-1} was considered the most appropriate concentration for comparative pathogenicity evaluation in this study. Inoculation was performed by applying 10 µL droplets of the suspension onto six artificially wounded sites on the abaxial surface of each leaflet, located within the interveinal lamina. Following inoculation, the plastic boxes were sealed and incubated in a controlled-environment chamber at 25°C under ambient light conditions.

2.5.3. Disease assessment and data analysis

Lesion development was evaluated at 6 days post-inoculation. Lesion areas were quantified using ImageJ software, and disease severity was scored on a 5-point scale ranging from 0 (no visible symptoms) to 4 (large, expanding lesions with visible mycelial growth). The disease severity index (DSI) was calculated as:

$$DSI\ (\%) = \sum(a \times b) \times 100 / (n \times t)$$

where *a* is the number of inoculation sites at each disease grade, *b* is the corresponding disease grade, *n* is the total number of observation sites, and *t* is the maximum disease grade.

DSI values were arcsine-square-root-transformed prior to statistical analysis. Pathogenicity levels were classified as non-pathogenic (DSI = 0), mild (≤25%), moderate (>25–50%), severe (>50–75%), or very severe (>75%). Data were subjected to analysis of variance (ANOVA) using SAS software version 8.1, and treatment means were compared using Fisher's Least Significant Difference (LSD) test at the 5% significance level ($P \leq 0.05$).

2.6. Evaluation of resistance and susceptibility of rubber clones

Based on the results of the pathogenicity assay conducted on rubber clone RRIV 124, the three most aggressive isolate pairs of *P. microspora* and *C. siamense* were selected to evaluate the resistance and susceptibility of six rubber clones, namely RRIV 209, RRIV 114, RRIV 1, RRIV 106, RRIV 103, and RRIV 124. Evaluations were conducted under both laboratory (detached-leaf assay) and screenhouse conditions.

2.6.1. Laboratory conditions (detached-leaf assay)

Plant and fungal materials

Three isolates of *P. microspora* (PesDP02, PesBL06, and PesPR22) and three isolates of *C. siamense* (CoDP02, CoBL06, and CoPR22) were used. Detached, symptom-free leaves at approximately 20 days of age were collected from six rubber clones: RRIV 209, RRIV 114, RRIV 1, RRIV 106, RRIV 103, and RRIV 124.

Experimental design

The experiment was arranged in a completely randomized design with two factors, three replications, and two detached leaflets per replication. Leaflets were maintained in plastic boxes lined with moist filter paper to ensure high relative humidity. Three independent experiments were conducted:

- Experiment 1: 18 treatments comprising factor A (three *P. microspora* isolates) and factor B (six rubber clones);
- Experiment 2: 18 treatments comprising factor A (three *C. siamense* isolates) and factor B (six rubber clones);
- Experiment 3: 18 treatments comprising factor A (three mixed inocula of *P. microspora* + *C. siamense* at a 1:1 ratio, v/v) and factor B (six rubber clones).

Control treatments consisted of inoculation with sterile distilled water on all six rubber clones.

Inoculation and disease assessment

Inoculation procedures, lesion measurement, disease severity scoring, DSI calculation, and pathogenicity classification were performed. DSI values (%) were arcsine square-root transformed prior to statistical analysis and analyzed using the generalized linear model (GLM) procedure in SAS software version 8.1.

2.6.2. Greenhouse conditions

Plant and fungal materials

The same fungal isolates used in the laboratory assay, three *P. microspora* isolates (PesDP02, PesBL06, PesPR22) and three *C. siamense* isolates (CoDP02, CoBL06, CoPR22), were employed. Leaves aged 20 ± 2 days from the six rubber clones (RRIV 209, RRIV 114, RRIV 1, RRIV 106, RRIV 103, and RRIV 124) were used for inoculation.

Experimental design

A randomized complete block design with two factors was applied. Each treatment was inoculated on three plants, corresponding to three replications (one plant per replication). For each plant, three fully expanded middle leaflets were selected for inoculation. Three experiments were conducted as follows:

- Experiment 1: 18 treatments comprising factor A (three *P. microspora* isolates) and factor B (six rubber clones);
- Experiment 2: 18 treatments comprising factor A (three *C. siamense* isolates) and factor B (six rubber clones);
- Experiment 3: 18 treatments comprising factor A (three mixed inocula of *P. microspora* + *C. siamense* at a 1:1 ratio, v/v) and factor B (six rubber clones).

Control plants were inoculated with sterile distilled water.

Inoculation and disease assessment

Conidial suspensions were prepared and adjusted to a concentration of 1×10^4 conidia mL^{-1}. Leaf surfaces were surface-sterilized with 70% ethanol and air-dried prior to inoculation. A 100 µL droplet of spore suspension was applied to each wounded inoculation site, and a sterile moist cotton pad (5 mm in diameter) was placed over each site to maintain localized humidity.

The lesion area was measured at 7 days post-inoculation using ImageJ image analysis software. Data were analyzed using the GLM procedure in SAS software version 8.1.

Following the final disease assessment, three diseased leaf samples were randomly collected for pathogen re-isolation to confirm the identity of the causal agents.

3. RESULTS AND DISCUSSION

3.1. Growth characteristics and morphology of *P. microspora* on different culture media

3.3.1. Colony morphology and mycelial growth

All 16 *Pestalotiopsis* isolates exhibited satisfactory growth on MEA, PDA, and PSA at 25°C. Colonies were generally circular, with white mycelia slightly elevated above the agar surface. Despite these common features, substantial inter-isolate variability was observed after 6 days of incubation in terms of colony diameter, mycelial texture, pigmentation, and sporulation time.

Based on colony architecture, isolates were classified into four major morphological types: (i) uniformly circular colonies with abundant aerial mycelia; (ii) colonies with multilayered, undulate margins and aerial mycelia; (iii) undulate colonies with sparse mycelia closely appressed to the agar surface; and (iv) isolates exhibiting inconsistent morphology across different media. These traits were largely isolate-specific and not strictly medium-dependent (Table 1 and Figure 1).

Several isolates produced yellowish-brown pigments that diffused into the surrounding agar, resulting in visible discoloration near the colony center. Sporulation time varied widely among isolates and culture media, ranging from 7 to 21 days. In general, sporulation occurred earlier and more abundantly on PDA and PSA than on MEA.

Table 1. Colony morphological characteristics of 16 *P. microspora* isolates

Morphological trait	Description	Isolate codes
Colony texture and elevation	White mycelium, raised, circular colony	PesDP01, PesDN52, PesDP02, PesPR20, PesTNg01
	White mycelium, raised, multilayered, undulate colony	PesBL06, PesLN05, PesTN02
	White mycelium, appressed, multilayered, undulate colony	PesBL03, PesBT03, PesPR22
	White mycelium, raised, irregular colony	PesBT04, PesDN59, PesLN07, PesTB02, PesTN01
Pigment production	Yellow–brown pigmentation	PesBL03, PesBT03, PesDN59, PesPR22
Time to sporulation	Approximately 7 days	PesBT03, PesDP01, PesPR20, PesPR22
	Approximately 14 days	PesBL03, PesBL06, PesBT04, PesDP02, PesLN05, PesLN07, PesTB02, PesTN01, PesTN02, PesTNg01
	Approximately 21 days	PesDN52, PesDN59

Morphological observations were conducted on potato dextrose agar (PDA) at 25 ± 1°C.

Table 2. Colony diameter (mm) of *P. microspora* isolates grown on different culture media at 25°C (6 days after inoculation)

Isolate	Culture medium			Mean (Isolate)
	MEA	PDA	PSA	
PesBL03	59.2^{nop}	53.1^{p}	55.5^{op}	55.9^{H}
PesBL06	76.8^{d-k}	82.4^{a-h}	79.8^{a-j}	79.7^{CDE}
PesBT03	79.8^{a-j}	81.6^{a-i}	75.8^{e-l}	79.1^{CDE}
PesBT04	83.6^{a-g}	72.5^{g-m}	79.1^{a-j}	78.4^{CDE}
PesDN52	89.0^{abc}	88.9^{abc}	85.0^{a-f}	87.6^{A}
PesDN59	85.3^{a-f}	26.1^{q}	65.3^{l-o}	58.9^{H}
PesDP01	90.0^{a}	87.7^{a-d}	70.8^{i-m}	82.8^{ABC}
PesDP02	71.3^{h-m}	74.7^{f-m}	66.5^{k-n}	70.8^{FG}
PesLN05	78.1^{b-j}	72.4^{g-m}	76.7^{d-k}	75.7^{DEF}
PesLN07	82.8^{a-g}	78.3^{b-j}	83.1^{a-g}	81.4^{BCD}
PesPR20	66.1^{k-n}	63.9^{mno}	70.5^{i-m}	66.8^{G}
PesPR22	76.2^{e-l}	80.2^{a-j}	85.1^{a-f}	80.5^{CDE}
PesTB02	87.8^{a-d}	70.2^{j-m}	86.3^{a-e}	81.4^{BCD}
PesTN01	83.0^{a-g}	72.5^{g-m}	69.8^{j-m}	75.1^{EF}
PesTN02	74.8^{f-m}	77.7^{c-j}	78.3^{b-j}	76.9^{CDE}
PesTNg01	89.4^{ab}	80.9^{a-j}	88.8^{abc}	86.4^{AB}
Mean (Medium)	79.6^{A}	72.7^{C}	76.0^{B}	

*CV = 5.7%; $F_{isolate}$ = 38.3**; F_{medium} = 30.2**; $F_{isolate*medium}$ = 11.9**. Means followed by the same letter are not significantly different at p < 0.01 for isolate, culture medium, and the isolate × medium interaction.*

Analysis of variance revealed highly significant effects of isolate, culture medium, and their interaction on colony diameter ($p < 0.01$). MEA supported the greatest mean colony diameter, whereas PDA and PSA supported slower vegetative growth but favored earlier or more prolific sporulation in certain isolates (Table 2).

3.3.2. Conidial morphology

Conidia produced by all 16 isolates on MEA exhibited morphological characteristics typical of the genus *Pestalotiopsis*. Conidia were fusiform, straight to slightly curved, and composed of five cells separated by four septa, including one apical cell, three median cells, and one basal cell, with appendages present at one or both ends (Figure 2).

The three median cells were pigmented, ranging from gray to dark brown, whereas apical and basal cells were hyaline. Considerable variation in pigmentation intensity was observed both among and within isolates. Conidial length ranged from 19.24 to 27.34 µm, and width from 6.46 to 9.55 µm, with statistically significant differences among isolates ($p < 0.01$). Additional variability was observed in apical cell shape and in the number and length of appendages (Table 3).

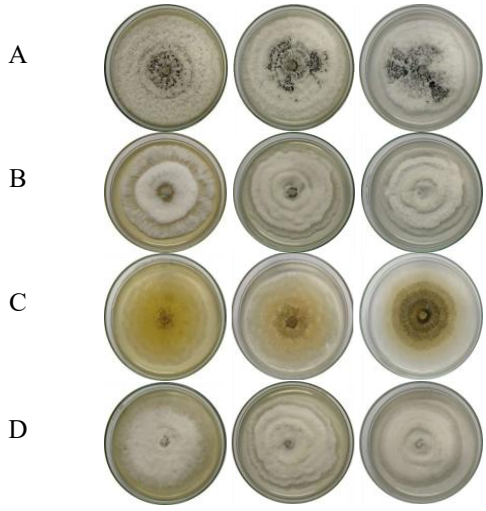

Figure 1. Representative colony morphologies of *Pestalotiopsis microspora* isolates grown on MEA, PDA, and PSA (left to right). Colonies were incubated at 25 ± 1°C and photographed after 7–14 days.

(A) Raised, cottony, circular colony (PesDP01); (B) raised colony with multilayered undulate margins (PesTN02); (C) sparse, appressed mycelium with undulate margins and yellow–brown pigmentation (PesPR22); (D) irregular and heterogeneous colony structure (PesTN01).

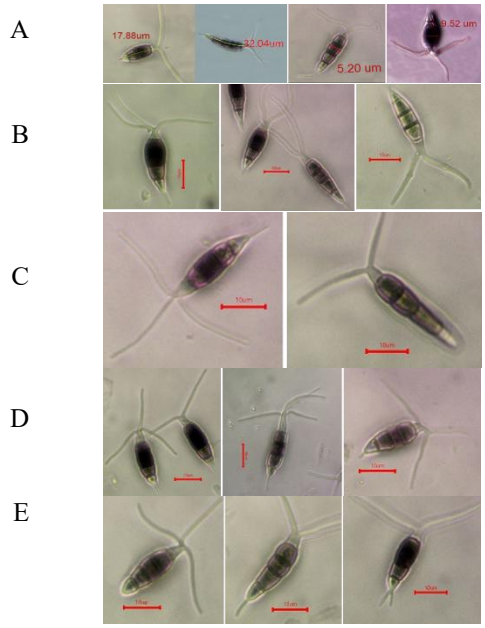

Figure 2. Conidial morphological variability of *P. microspora* isolates

(A) Variation in conidial length and width. (B) Pigmentation diversity of the three median cells. (C) Shape variation of the apical cell (conical and cylindrical). (D) Variation in the number and morphology of apical appendages (2–4, branched or unbranched). (E) Variation in the number of basal appendages (0–2). Scale bar = 10 µm.

Table 3. Conidial dimensions of *P. microspora* isolates cultured on MEA

Isolate	Conidial length (µm)	Conidial width (µm)
PesBL03	23.92^{bcd}	7.98^{bcd}
PesBL06	20.56^{efg}	8.20^{bc}
PesBT03	19.24^{g}	6.84^{ef}
PesBT04	23.34^{bcd}	9.55^{a}
PesDN52	24.58^{bc}	6.87^{ef}
PesDN59	27.34^{a}	7.05^{def}
PesDP01	23.85^{bcd}	6.86^{ef}
PesDP02	23.72^{bcd}	8.73^{ab}
PesLN05	25.16^{d-g}	8.07^{f}
PesLN07	20.84^{fg}	6.69^{b-e}
PesPR20	23.85^{bcd}	8.94^{ab}
PesPR22	21.97^{c-f}	6.46^{f}
PesTB02	22.65^{cde}	7.10^{def}
PesTN01	25.91^{ab}	8.50^{bc}
PesTN02	23.38^{bcd}	8.33^{bc}
PesTNg01	23.20^{cd}	7.52^{c-f}
CV (%)	8.62	10.75
F Value	11.35**	12.88**

Values followed by the same letter within a column are not significantly different at p < 0.01.

3.2. Growth characteristics and morphology of *Colletotrichum siamense* on different culture media

3.2.1. Colony morphology and mycelial growth

Table 4. Colony morphological characteristics of 16 *C. siamense* isolates on different culture media

Morphological trait	Category	Description	Representative isolates*
Mycelial structure	Aerial, dense	Thick mycelia, raised above the agar surface	CoBL03, CoDN52, CoDP01, CoDP02, CoPR20, CoPR22, CoTN01, CoTN02, CoTNg01
	Submerge, thin	Thin mycelia, largely embedded in agar	CoBL06, CoBT04
Concentric- rings	Present	Clearly visible concentric growth rings (medium-dependent)	CoBL06, CoDP01, CoDP02, CoLN05, CoPR20, CoPR22
	Absent/indistinct	Rings absent or weakly expressed	Remaining isolates
Colony pigmentation	Gray	Gray to dark gray colonies	Majority of isolates
	White	White colonies	CoLN05, CoTB02
	Orange/pale pink	Orange to pale pink colonies, often with early sporulation	CoBL06, CoBT04, CoDN52
Sporulation time	Early	Conidia formed ~7 days after inoculation	CoLN05, CoBT03, CoBT04, CoBL06, CoDP02
	Late	Conidia formed ~14 days after inoculation	Remaining isolates

** Colony morphology was assessed on MEA, PDA, and PSA at 25°C. Some morphological traits, particularly mycelial density, concentric ring formation, and pigmentation, varied depending on culture medium.*

All 16 *C. siamense* isolates grew well on MEA, PDA, and PSA at 25°C, but exhibited marked variation in colony morphology, pigmentation, and growth rate across isolates and media. Most isolates

produced dense aerial mycelia that formed circular colonies, whereas others developed sparse mycelia largely embedded within the agar (Table 4).

Concentric ring formation was frequently observed, particularly on PDA and MEA. Colony coloration varied from white and gray to orange or pale pink. Isolates producing orange or pale pink colonies sporulated earlier (5–7 days after inoculation) and formed conspicuous acervuli, whereas white or gray colonies sporulated later (≈14 days) (Figure 3).

Colony diameter measured at 7 days differed significantly among isolates, media, and isolate × medium interactions ($p < 0.01$). PDA and PSA supported greater mean colony diameters than MEA, indicating superior suitability for vegetative growth of *C. siamense* (Table 5).

Table 5. Colony diameter (mm) of *C. siamense* isolates grown on different culture media at 25°C (6 days after inoculation)

Isolate	Culture medium			Mean
	MEA	PDA	PSA	(Isolate)
CoBL03	75.8^{f}	78.6^{ef}	86.7^{a-d}	80.4^{D}
CoBL06	82.3^{de}	86.1^{a-d}	88.3^{abc}	85.6^{C}
CoBT03	86.5^{a-d}	88.9^{ab}	90.0^{a}	88.5^{AB}
CoBT04	79.2^{ef}	88.3^{abc}	87.8^{abc}	85.1^{C}
CoDN52	87.3^{abc}	89.9^{a}	89.0^{ab}	88.7^{AB}
CoDN59	87.8^{abc}	88.8^{ab}	90.0^{a}	88.9^{AB}
CoDP01	90.0^{a}	90.0^{a}	90.0^{a}	90.0^{A}
CoDP02	88.6^{abc}	88.5^{abc}	83.7^{cd}	86.9^{BC}
CoLN05	69.5^{g}	78.2^{ef}	88.4^{abc}	78.7^{D}
CoLN07	89.6^{a}	90.0^{a}	85.2^{a-d}	88.3^{AB}
CoPR20	90.0^{a}	90.0^{a}	90.0^{a}	90.0^{A}
CoPR22	90.0^{a}	90.0^{a}	88.0^{abc}	89.3^{AB}
CoTB02	84.1^{bcd}	89.3^{a}	86.8^{a-d}	86.7^{BC}
CoTN01	89.7^{a}	87.5^{abc}	89.7^{a}	89.0^{AB}
CoTN02	86.8^{a-d}	89.3^{a}	84.8^{a-d}	87.0^{BC}
CoTNg01	89.8^{a}	86.0^{a-d}	88.8^{ab}	88.2^{AB}
Mean (Medium)	85.4^{B}	87.5^{A}	87.9^{A}	

*CV = 2.2%; $F_{isolate}$ = 24.8**; F_{medium} = 22.1**; $F_{isolate*medium}$ = 8.8**. Means followed by the same letter are not significantly different at $p < 0.01$ for isolate, culture medium, and the isolate × medium interaction.*

3.2.2. Conidial morphology

All isolates produced unicellular, hyaline conidia on PDA. Conidial length ranged from 13.1 to 15.4 µm and width from 3.8 to 4.7 µm. Although size variation was subtle visually, statistical analysis confirmed highly significant inter-isolate differences ($p < 0.01$) (Table 6).

Conidia were predominantly cylindrical with rounded ends, though some isolates also produced asymmetrical conidia with one rounded and one slightly tapered end (Figure 4).

Table 6. Conidial dimensions of *C. siamense* isolates cultured on PDA

Isolate	Conidial length (µm)	Conidial width (µm)
CoBL03	14.3^{a-e}	4.1^{bcd}
CoBL06	13.9^{b-e}	4.5^{abc}
CoBT03	14.5^{abc}	4.2^{bcd}
CoBT04	13.6^{cde}	3.8^{d}
CoDN52	14.2^{a-e}	4.1^{bcd}
CoDN59	14.5^{abc}	4.5^{abc}
CoDP01	15.0^{ab}	4.4^{abc}
CoDP02	13.6^{cde}	4.0^{cd}
CoLN05	13.1^{e}	4.4^{abc}
CoLN07	14.5^{abc}	4.4^{abc}
CoPR20	14.3^{a-e}	4.2^{a-d}
CoPR22	13.4^{cde}	4.6^{ab}
CoTB02	15.4^{a}	4.7^{a}
CoTN01	13.1^{de}	4.2^{a-d}
CoTN02	13.4^{cde}	3.9^{d}
CoTNg01	14.4^{a-d}	3.9^{d}
CV (%)	6.9	8.5
F value	4.7**	5.0**

Values followed by the same letter within a column are not significantly different at $p < 0.01$.

Figure 3. Representative colony morphology of *C. siamense* on different culture media

(A): Inter-isolate variation on PDA, showing dense gray (CoDP02), dense white (CoTB02), and thin orange (CoBL06) colonies. (B): Medium-dependent morphological variation of isolate CoBT03 grown on MEA, PDA, and PSA. (C): Presence and absence of concentric rings in isolate CoLN05 on different media.

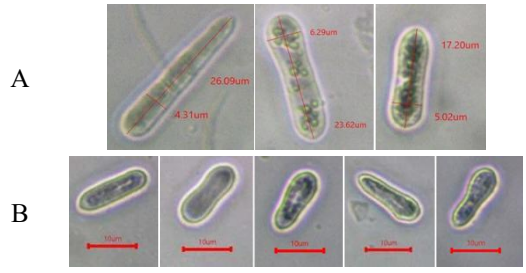

Figure 4. Conidial morphology of representative *C. siamense* isolates

(A): Variation in conidial size among isolates, represented by CoPR20 (small), CoDN52 (medium), and CoDN59 (large). (B): Representative conidial shapes observed across isolates, including cylindrical conidia with rounded ends, cylindrical conidia with one end slightly tapered, and slightly curved conidia. Scale bar = 10 µm.

3.3. Effects of temperature on mycelial growth of *P. microspora* and *C. siamense*

The effects of temperature on mycelial growth of *P. microspora* and *C. siamense* were evaluated at four incubation temperatures (20, 25, 30, and 35°C). For *P. microspora*, most isolates (14 out of 16) exhibited maximum colony diameters at 25°C, indicating that this temperature is optimal for mycelial growth. In contrast, all *Pestalotiopsis* isolates completely ceased growth at 35°C, demonstrating a strong inhibitory effect of high temperature. However, mycelial growth resumed when the cultures were returned to room temperature, suggesting that 35°C exerted a fungistatic rather than fungicidal effect. Although 25°C was optimal for most isolates, two isolates (PesPR20 and PesTNg01) showed better growth at 30°C, indicating isolate-specific thermal adaptation. No isolate exhibited optimal growth at 20°C, confirming that this temperature was suboptimal for *P. microspora*.

Based on mean colony diameter at 25°C, *Pestalotiopsis* isolates were classified into three growth groups: strong, moderate, and weak. Analysis of variance revealed that isolate, temperature, and their interaction significantly affected mycelial growth ($p < 0.01$; data not shown). Considerable phenotypic variability was also observed among isolates grown under the same temperature conditions, including differences in colony pigmentation.

For *C. siamense*, mycelial growth was strongly influenced by temperature, with most isolates growing optimally at 25–30°C. Mean colony diameters at 25°C and 30°C were statistically similar and significantly higher than those at 20°C and 35°C. Growth was markedly reduced at 35°C, indicating thermal inhibition. At 30°C, *Colletotrichum* isolates were clearly differentiated into strong, moderate, and weak growth groups based on colony diameter. General linear model analysis confirmed that isolate, temperature, and their interaction significantly affected mycelial growth of *C. siamense*. ($p < 0.01$; data not shown).

3.4. Spore germination and infection structure formation

The observations presented below describe the infection process of representative isolates selected from each species and are intended to illustrate typical developmental patterns rather than quantify variation among isolates.

3.4.1. *Pestalotiopsis microspora*

Under in vitro conditions, conidial germination of isolate PesDP01 commenced after approximately 16 h, typically initiating from the fourth cell from the apical end. Germ tubes elongated and branched progressively by 20–24 h.

Under in planta conditions, germinated spores and developing germ tubes were observed on detached rubber leaves 2 days after inoculation. Abnormal host cell structures adjacent to germinating spores were evident. By 5 days post-inoculation, extensive epidermal necrosis and hyphal proliferation were observed (Figure 5).

3.4.2. *Colletotrichum siamense*

In sterile distilled water, conidia of CoDP01 germinated within 12 h and rapidly formed appressoria. By 20–24 h, extensive hyphal branching and secondary appressoria formation were observed.

On rubber leaves, germinated spores and well-developed appressoria were detected 2 days after inoculation. By 5 days post-inoculation, distinct necrotic lesions and dense fungal colonization were evident, accompanied by acervulus formation and secondary conidiation (Figure 6).

A

B

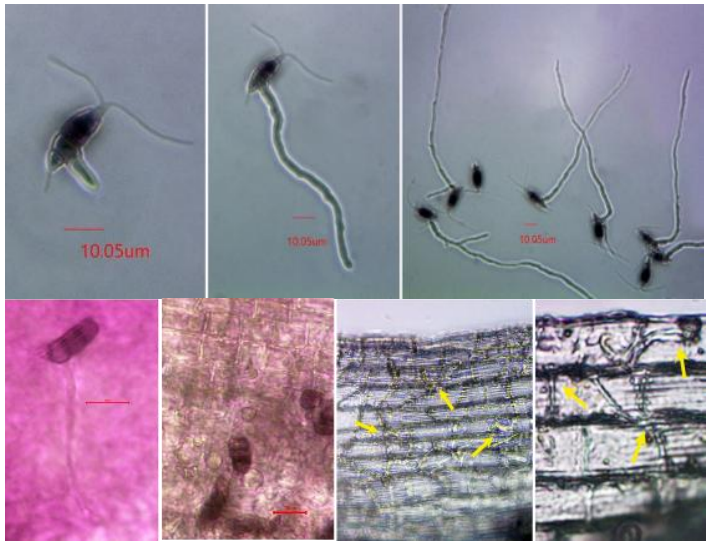

Figure 5. Germination and early infection process of *P. microspora* (isolate PesDP01)

(A): In vitro conidial germination in distilled water at 16 h, 20 h, and 24 h after incubation (left to right). (B): In planta germination and early infection in young rubber leaves: initial germination on the leaf surface (B1), formation of round or irregular structures in adjacent host tissues (B2), intercellular hyphal growth within epidermal tissues (B3, 40X), and direct penetration through epidermal cells (B4, 100X). Scale bar = 10 µm.

A

B

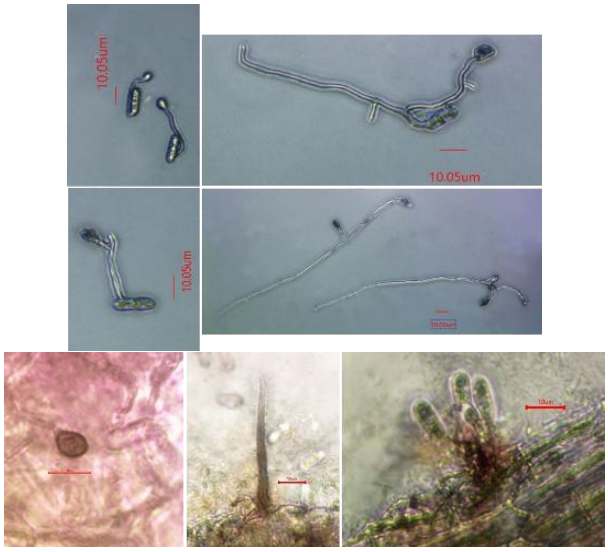

Figure 6. Germination and early infection process of *C. siamense* (isolate CoDP01)

(A): In vitro conidial germination in distilled water at 12 h (A1), 16 h (A2), 20 h (A3), and 24 h (A4) after incubation. (B): In planta germination and early infection in young rubber leaves: conidial germination and appressorium formation on the leaf surface, host penetration by appressoria (B1), development of setae on infected leaf tissues (B2), and formation of conidia on infected epidermal cells (B3). Scale bars = 10 µm.

3.5. Molecular identification and phylogenetic analysis

3.5.1. *Pestalotiopsis microspora*

PCR amplification of the rDNA-ITS region yielded a single ~550 bp fragment from all isolates. Sequence analysis revealed six polymorphic sites, allowing classification into three genetic groups. Pairwise similarity among representative isolates was 99.45%. BLAST analysis showed all isolates shared ≥ 99% identity with *Pestalotiopsis microspora* reference sequences, confirming species identity (Figures 7 and 8).

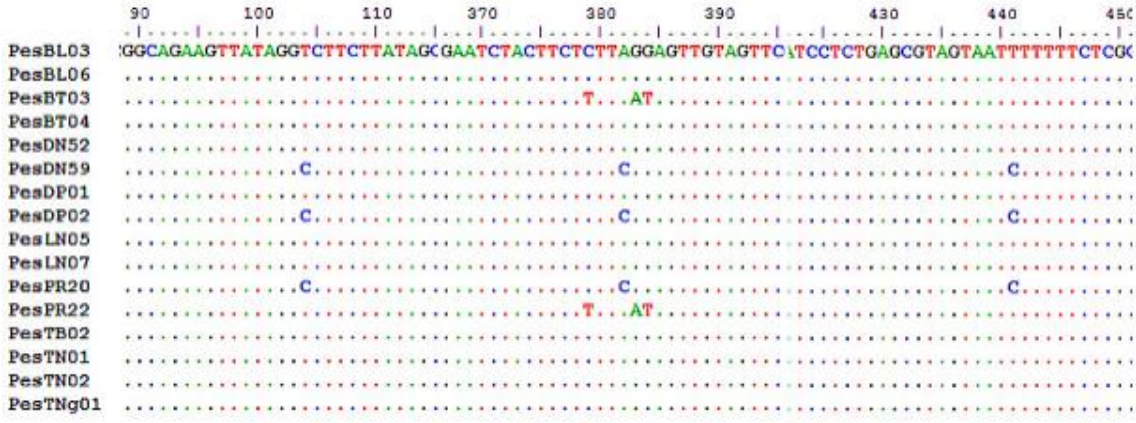

Figure 7. Nucleotide polymorphisms at multiple positions within the rDNA–ITS region of 16 *P. microspora* isolates

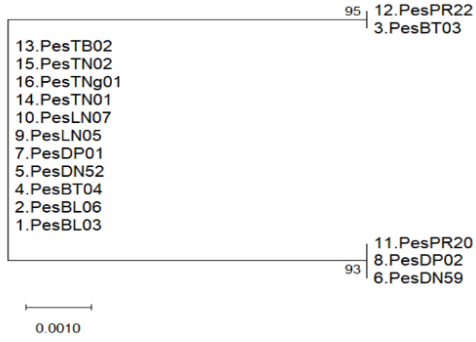

Figure 8. Phylogenetic tree generated using the Neighbor-Joining method based on rDNA-ITS sequences of 16 *P. microspora* isolates using MEGA10 program with bootstrap value for 1,000 replicates

3.5.2. *Colletotrichum siamense*

The ITS sequences obtained from the 16 Colletotrichum isolates ranged from 580 to 584 bp in length and exhibited limited sequence polymorphism, allowing the isolates to be separated into two closely related haplotypic groups (Figures 9 and 10). Despite this minor genetic variation, BLASTn analysis consistently showed that all isolates shared the highest sequence similarity (99–100%) with reference isolates of *C. siamense* available in the GenBank database. The molecular results were consistent with the morphological characteristics observed for the isolates and with previous reports identifying *C. siamense* as a component of the CLSD pathogen complex in Viet Nam. Accordingly, all isolates were provisionally assigned to *C. siamense*. However, because the ITS region provides limited phylogenetic resolution within the *C. gloeosporioides* species complex, species-level identification based solely on ITS sequences should be interpreted cautiously. Confirmation of species identity will require future multi-locus phylogenetic analyses using additional loci, including ACT, TUB2, GAPDH, CHS-1, and ApMat.

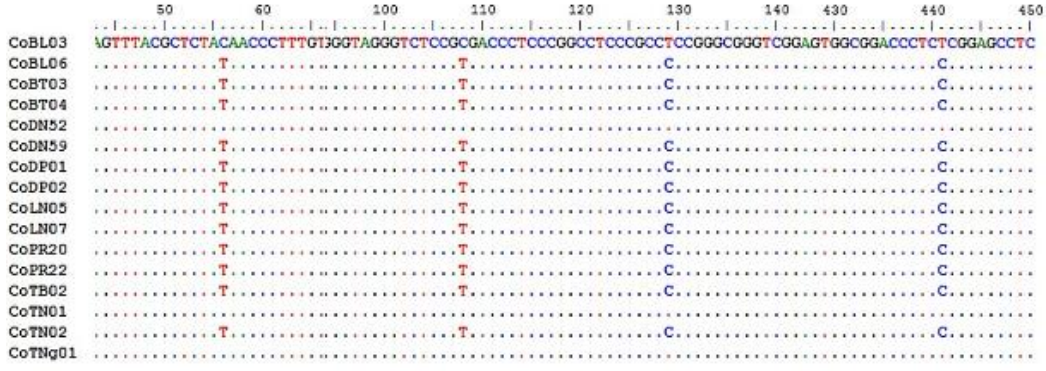

Figure 9. Nucleotide polymorphisms at multiple positions within the rDNA–ITS region of 16 *C. siamense* isolates

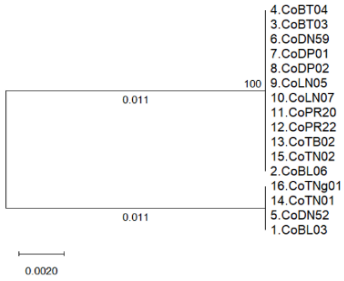

Figure 10. Phylogenetic tree generated using the Neighbor-Joining method based on rDNA-ITS sequences of 16 *C. siamense* isolates using MEGA10 program with bootstrap value for 1,000 replicates

3.6. Pathogenicity assessment

3.6.1. Pathogenicity on rubber clone RRIV 124 (laboratory conditions)

Significant differences in disease severity were detected among *P. microspora* isolates, *C. siamense* isolates, and mixed inocula. *C. siamense* and mixed inocula consistently caused more severe disease than *P. microspora* alone. Mixed inoculations frequently resulted in maximum DSI (DSI = 100%; Figure 11).

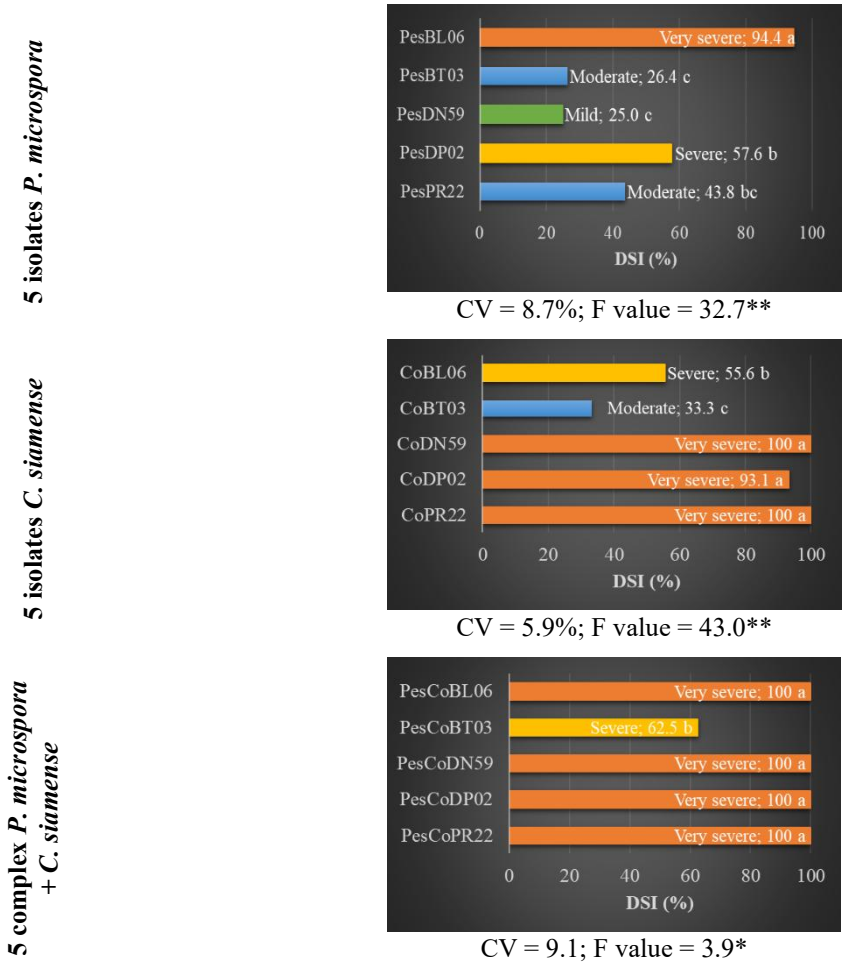

Figure 11. Disease severity assessment of *P. microspora* and *C. siamense* inoculum sources on detached leaves of rubber clone RRIV 124 at 6 days after inoculation (DAI). Means followed by the same letter are not significantly different

3.6.2. Pathogenicity on six rubber clones

Under laboratory conditions, all six clones were highly susceptible to *C. siamense* and mixed inocula, whereas susceptibility to *P. microspora* varied, with RRIV 124 showing the highest sensitivity (Tables 7, 8, 9 and Figure 12).

Table 7. Disease severity of three *P. microspora* isolates on detached leaves of six rubber clones at 6 DAI

Clone	Isolate DSI (%) / Severity level			Mean
	PesBL06	PesDP02	PesPR22	(Clone)
RRIV 209	38.2^{bcd} ++	52.1^{bcd} +++	30.0^{cd} ++	40.1^{B} ++
RRIV 106	66.7^{b} +++	27.1^{cd} ++	49.3^{bcd} ++	47.7^{B} ++
RRIV 114	52.1^{bcd} +++	26.4^{cd} ++	30.6^{cd} ++	36.3^{B} ++
RRIV 103	37.5^{bcd} ++	39.6^{bcd} ++	27.1^{cd} ++	34.7^{B} ++
RRIV 1	60.4^{bc} +++	25.7^{cd} ++	44.4^{bcd} ++	43.5^{B} ++
RRIV 124	57.6^{bcd} +++	100^{a} ++	25.0^{d} +	60.9^{A} +++
Mean (Isolate)	52.1^{A} +++	45.1^{A} ++	34.4^{B} ++	

*CV = 31.1%; $F_{isolate}$ = 10.7**; F_{clone} = 10.9**; $F_{isolate*clone}$ = 13.1**. Means followed by the same letter are not significantly different for the factors isolate, rubber clone, and their interaction (isolate × clone) at p < 0.01. Severity level: +: mild, ++: moderate, +++: severe, and ++++: very severe.*

Table 8. Disease severity of three *C. siamense* isolates on detached leaves of six rubber clones at 6 DAI

Clone	Isolate DSI (%) / Severity level			Mean
	CoBL06	CoDP02	CoPR22	(Clone)
RRIV 209	100 ++++	100 ++++	54.9 +++	85.0 ++++
RRIV 106	100 ++++	100 ++++	68.1 +++	89.4 ++++
RRIV 114	99.3 ++++	95.1 ++++	39.6 ++	78.0 ++++
RRIV 103	99.3 ++++	97.2 ++++	55.6 +++	84.0 ++++
RRIV 1	100 ++++	100 ++++	68.1 +++	89.4 ++++
RRIV 124	100 ++++	100 ++++	41.0 ++	80.3 ++++
Mean (Isolate)	99.8^{A} ++++	98.7^{A} ++++	54.5^{B} +++	

*CV = 18.1%; $F_{isolate}$ = 96.7**; F_{clone} = 1.9^{NS}; $F_{isolate*clone}$ = 0.7^{NS}. Means followed by the same letter are not significantly different at p < 0.01 for the isolate factor. The clone effect and the isolate × clone interaction were not significant (NS). Severity level: +: mild, ++: moderate, +++: severe, and ++++: very severe.*

Table 9. Disease severity of three *P. microspora* + *C. siamense* isolates on detached leaves of six rubber clones at 6 DAI

Clone	Isolate DSI (%) / Severity level			Mean (Clone)
	PesCoBL06	PesCoDP02	PesCoPR22	
RRIV 209	100 ++++	100 ++++	94.4 ++++	98.1 ++++
RRIV 106	100 ++++	100 ++++	100 ++++	100 ++++
RRIV 114	100 ++++	100 ++++	82.6 ++++	94.2 ++++
RRIV 103	100 ++++	100 ++++	86.1 ++++	95.4 ++++
RRIV 1	100 ++++	100 ++++	95.1 ++++	98.4 ++++
RRIV 124	100 ++++	100 ++++	93.8 ++++	97.9 ++++
Mean (Isolate)	100^{A} ++++	100^{A} ++++	92.0^{B} ++++	

*CV = 14.5%; $F_{isolate}$ = 6.8**; F_{clone} = 0.45^{NS}; $F_{isolate*clone}$ = 0.45^{NS}. Means followed by the same letter are not significantly different at p < 0.01 for the isolate factor. The clone effect and the isolate × clone interaction were not significant (NS). Severity level: +: mild, ++: moderate, +++: severe, and ++++: very severe.*

Under greenhouse conditions, lesion size was markedly reduced for all treatments. Nevertheless, relative pathogenicity patterns among isolates and clones remained consistent, with RRIV 124 consistently exhibiting greater susceptibility (Table 10; Table 11; Table 12; Figure 13).

Table 10. Lesion area (mm²) caused by three isolates of *P. microspora* on six rubber clones under greenhouse conditions

Clone	Isolate			Mean (Clone)
	PesBL06	PesDP02	PesPR22	
RRIV 209	2.7	2.7	2.4	2.6^{B}
RRIV 106	2.9	2.6	2.5	2.7^{AB}
RRIV 114	2.5	3.0	2.5	2.7^{AB}
RRIV 103	2.5	2.3	2.5	2.4^{B}
RRIV 1	2.5	2.8	2.4	2.5^{B}
RRIV 124	2.8	3.1	2.9	2.9^{A}
Mean (Isolate)	2.7	2.7	2.5	

CV = 10.6%; $F_{isolate}$ = 2.54^{NS}; F_{clone} = 3.15; $F_{isolate*clone}$ = 1.25^{NS}. Means followed by the same letter are not significantly different at p < 0.05 for the clone factor. The isolate effect and the isolate × clone interaction were not significant (NS).*

Pathogen re-isolation from three representative symptomatic leaves per treatment successfully recovered colonies morphologically identical to the original isolates of *P. microspora* and *C. siamense*. No corresponding fungi were recovered from the sterile water controls, thereby confirming pathogen identity and fulfilling Koch's postulates.

Table 11. Lesion area (mm²) caused by three isolates of *C. siamense* on six rubber clones under greenhouse conditions

Clone	Isolate			Mean (Clone)
	CoBL06	CoDP02	CoPR22	
RRIV 209	8.7	9.2	8.0	8.7
RRIV 106	9.1	8.9	7.5	8.5
RRIV 114	9.3	8.7	8.6	8.9
RRIV 103	8.6	8.8	7.6	8.3
RRIV 1	9.8	9.2	9.2	9.4
RRIV 124	8.7	9.3	7.7	8.6
Mean (Isolate)	9.0^{A}	9.0^{A}	8.1^{B}	

CV = 12.3%; $F_{isolate}$ = 4.18; F_{clone} = 1.13^{NS}; $F_{isolate*clone}$ = 0.38^{NS}. Means followed by the same letter are not significantly different at p < 0.05 for the isolate factor. The clone effect and the isolate × clone interaction were not significant (NS).*

Table 12. Lesion area (mm²) caused by three isolates of *P. microspora* + *C. siamense* on six rubber clones under greenhouse conditions

Clone	Isolate			Mean (Clone)
	PesCoBL06	PesCoDP02	PesCoPR22	
RRIV 209	8.8^{a-f}	9.1^{a-f}	9.6^{a-d}	9.2^{B}
RRIV 106	10.3^{ab}	9.5^{a-e}	8.1^{b-f}	9.3^{B}
RRIV 114	10.2^{abc}	7.2^{ef}	9.8^{a-d}	9.1^{B}
RRIV 103	7.7^{def}	8.2^{b-f}	7.0^{f}	7.6^{B}
RRIV 1	7.9^{c-f}	9.5^{a-e}	7.8^{def}	8.4^{B}
RRIV 124	10.7^{a}	11.1^{a}	10.9^{a}	10.9^{A}
Mean (Isolate)	9.2	9.1	8.9	

*CV = 13.4%; $F_{isolate}$ = 0.5^{NS}; F_{clone} = 7.3**; $F_{isolate*clone}$ = 2.1*. Means followed by the same letter are not significantly different at p < 0.05 for the clone factor and the isolate × clone interaction. The isolated effect was not significant (NS).*

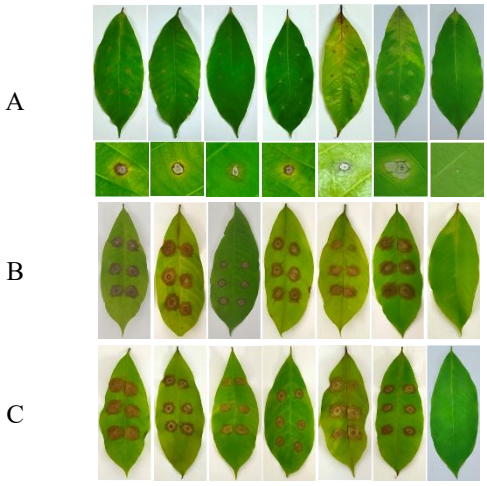

Figure 12. Disease severity of six rubber clones following inoculation with *P. microspora* (PesDP02), *C. siamense* (CoBL06), and the combined inoculation of *P. microspora* + *C. siamense* (CoBL06) on detached leaves at 6 days post-inoculation

(A): Disease severity of six rubber clones following inoculation with Pestalotiopsis microspora *(PesDP02). (B): Disease severity of six rubber clones following inoculation with* Colletotrichum siamense *(CoBL06). (C): Disease severity of six rubber clones following inoculation with the mixed inoculum of* Pestalotiopsis microspora *and* Colletotrichum siamense *(PesCoBL06).*

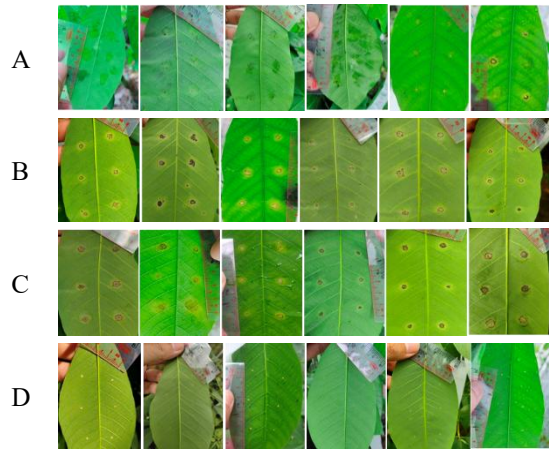

Figure 13. Disease severity caused by *P. microspora* and *C. siamense* on six rubber clones under greenhouse conditions at 7 DAI

(A): Disease symptoms caused by P. microspora *(isolate PesDP02) on six rubber clones, RRIV 209, RRIV 106, RRIV 114, RRIV 103, RRIV 1, and RRIV 124 (from left to right). (B): Disease symptoms caused by* C. siamense *(isolate CoDP02) on six rubber clones, RRIV 209, RRIV 106, RRIV 114, RRIV 103, RRIV 1, and RRIV 124 (from left to right). (C): Disease symptoms caused by* P. microspora *(isolate PesDP02)* + C. siamense *(isolate CoDP02) on six rubber clones, RRIV 209, RRIV 106, RRIV 114, RRIV 103, RRIV 1, and RRIV 124 (from left to right). (D): Control treatment inoculated with sterile distilled water, showing no disease symptoms on six rubber clones, RRIV 209, RRIV 106, RRIV 114, RRIV 103, RRIV 1, and RRIV 124 (from left to right).*

3.7. Discussions

Recent studies indicate that CLSD of rubber is associated with a complex of fungal species, among which *C. siamense* and *P. microspora* are most frequently reported. While *P. microspora* was initially identified as the primary causal agent of Pestalotiopsis leaf fall disease in Indonesia (Kusdiana, 2020; Alchemi, 2022), subsequent investigations in Thailand, Sri Lanka, India, and other rubber-growing regions have increasingly highlighted the important role of *Colletotrichum* spp., particularly *C. siamense*, in disease development (Arom, 2020; Fernando, 2022; Bindu, 2022).

The present study supports these observations. Pathogenicity assays consistently demonstrated that *C. siamense* produced larger lesions and higher disease severity than *P. microspora* under both laboratory and greenhouse conditions, indicating a greater capacity for host colonization and symptom development. In contrast, *P. microspora* generally exhibited lower virulence, suggesting that its contribution to CLSD may vary depending on environmental conditions and host susceptibility. These findings are consistent with reports that some Pestalotiopsis species can persist as latent endophytes or opportunistic pathogens before causing disease under favorable conditions.

Differences in disease severity among rubber clones indicate variation in host response to CLSD-associated fungi. Although several clones developed lower disease severity than RRIV 124, the magnitude of these differences was relatively small under the experimental conditions employed. Consequently, pathogen identity appeared to exert a stronger influence on disease development than host genotype. Nevertheless, clones exhibiting lower disease severity may represent valuable genetic resources for future resistance screening and breeding programs.

Disease severity observed in detached-leaf assays was substantially higher than that recorded under greenhouse conditions. Detached leaves are physiologically isolated from the whole plant and were maintained under near-saturated humidity, conditions that strongly favor conidial germination, appressorium formation, and lesion expansion. In addition, artificial wounding may facilitate pathogen penetration by bypassing natural defense barriers. Therefore, detached-leaf assays provide a sensitive tool for comparing pathogen aggressiveness and host responses, but results should be interpreted cautiously when extrapolating to greenhouse or field conditions.

Mixed inoculations consistently produced greater disease severity than inoculation with *P. microspora* alone and generally resulted in disease levels comparable to those caused by *C. siamense*. These results suggest that co-infection by multiple fungal species may contribute to disease development. However, because the present study was not designed to distinguish additive from synergistic interactions, the observed increase in disease severity should be interpreted as an effect of co-inoculation rather than definitive evidence of synergism.

Several limitations should be acknowledged. First, observations of spore germination and appressorium formation were conducted using only one representative isolate of each species. Although the selected isolates displayed typical morphological characteristics and relatively high pathogenicity, substantial variability was observed among isolates, and the infection processes described here may not fully represent the diversity within each species. Second, species identification was based solely on rDNA-ITS sequences. While all isolates showed high sequence similarity to reference isolates of *P. microspora* and *C. siamense* and exhibited corresponding morphological characteristics, ITS alone provides limited resolution within the *C. gloeosporioides* species complex. Therefore, the identification of *C. siamense* should be regarded as provisional until confirmed through multi-locus phylogenetic analyses using additional markers such as ACT, TUB2, GAPDH, CHS-1, and ApMat.

Overall, the results indicate that CLSD in Viet Nam is associated with a pathogen complex involving *C. siamense* and *P. microspora*, with *C. siamense* appearing to be the dominant pathogenic component under the conditions examined. These findings contribute to a better understanding of CLSD etiology and provide a scientific basis for future studies on disease epidemiology, host resistance, and integrated disease management.

4. CONCLUSIONS

This study provides a comprehensive characterization of fungal populations associated with CLSD of rubber in Viet Nam through morphological, physiological, molecular, and pathogenicity analyses. Considerable variation in colony morphology, sporulation, and growth

characteristics was observed among isolates of both *P. microspora* and *Colletotrichum* spp., indicating substantial phenotypic diversity within the CLSD pathogen complex.

Both fungal groups exhibited distinct biological responses to culture media and temperature. *P. microspora* generally showed optimal growth at approximately 25°C, whereas *Colletotrichum* isolates displayed vigorous growth over a broader temperature range (25 - 30°C), suggesting greater ecological adaptability under tropical conditions.

ITS sequence analyses revealed that all isolates shared high similarity with reference sequences of *P. microspora* and *C. siamense*. However, because ITS alone provides limited resolution within the *C. gloeosporioides* species complex, the identity of the Colletotrichum isolates should be regarded as provisional pending confirmation by multi-locus phylogenetic analyses.

Pathogenicity assays consistently demonstrated that isolates identified as *C. siamense* produced more severe disease symptoms than *P. microspora* under both detached-leaf and greenhouse conditions. Co-inoculation treatments resulted in increased disease severity compared with inoculation by *P. microspora* alone, indicating that multiple fungal species may contribute to disease development. However, the present study was not designed to distinguish additive from synergistic interactions among pathogens.

Differences in disease responses among rubber clones were observed, particularly under controlled laboratory conditions, with clone RRIV 124 exhibiting relatively high susceptibility. Nevertheless, clone effects were less pronounced under greenhouse conditions, suggesting that environmental factors and whole-plant defense mechanisms may substantially influence disease expression.

Overall, the results support the hypothesis that CLSD in Viet Nam is associated with a fungal disease complex involving *P. microspora* and *Colletotrichum* spp., in which isolates identified as *C. siamense* appear to play a major role in symptom development. These findings provide a scientific basis for future studies on pathogen diversity, resistance screening, and the development of integrated management strategies for CLSD in rubber plantations.

5. RECOMMENDATIONS

The outcomes of this study provide a practical foundation for disease management and decision-making in rubber production systems. Disease monitoring, fungicide evaluation, and resistance screening programs should prioritize *C. siamense* as the principal target pathogen. Integrated disease management strategies combining fungicide rotation, cultural practices, and the deployment of less susceptible clones should be developed and implemented at plantation scale.

At the industry and policy levels, routine surveillance of CLSD should be incorporated into national rubber plantation monitoring programs, particularly in regions with climatic conditions favorable to *C. siamense*. The susceptibility of widely planted clones, such as RRIV 124, should be carefully considered in future planting recommendations and breeding strategies to reduce disease risk and ensure sustainable rubber production.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Alchemi, P. J. K. (2022, December 14–15). *Pestalotiopsis leaf disease: Identification, epidemiology studies, and selection of new clone* [Conference presentation]. IRRDB Workshop on Pestalotiopsis Leaf Disease, Kuala Lumpur, Malaysia.

Arom, R. (2020, December 4). *The new leaf fall disease in Thailand* [Conference presentation]. IRRDB Online Workshop.

Bindu, R. (2022, December 14–15). *Current status of leaf disease in India* [Conference presentation]. IRRDB Workshop on Pestalotiopsis Leaf Disease, Kuala Lumpur, Malaysia.

Fernando, S. (2022, December 11–17). *Current status of the circular leaf spot disease in Sri Lanka and the research programme* [Conference presentation]. IRRDB Workshop on Pestalotiopsis Leaf Disease, Kuala Lumpur, Malaysia.

Hieu, N. D., Uyen, N. T. K., & Trang, N. T. T. (2026). Diversity, pathogenicity, fungicide sensitivity, and field management of the circular leaf spot pathogen complex on rubber trees in Vietnam. *Journal of Rubber Research*, *28*, 719–736. https://doi.org/10.1007/s42464-025-00332-3

Hieu, N. D., Uyen, N. T. K., Trang, N. T. T., Mai, N. N., Vinh, N. P., Luan, D. N., & Nghia, N. A. (2023).

Morphological characteristics of Pestalotiopsis spp. and Colletotrichum spp. associated with circular leaf spot disease newly detected on rubber trees in Dong Nai and Binh Phuoc provinces. In *Proceedings of the National Conference on Plant Diseases in Vietnam 2023* (pp. 196 - 201). Agricultural Publishing House, Hanoi, Vietnam (in Vietnamese).

Kuruvilla, J. C. (2023, February 20–22). *Pestalotiopsis and other epidemic diseases of rubber and their management strategies* [Conference presentation]. IRRDB International Rubber Conference, Kuala Lumpur, Malaysia.

Kusdiana, A. P. J. (2020). Diagnosis penyakit gugur daun karet (*Hevea brasiliensis* Muell. Arg.). *Jurnal Penelitian Karet*, *38*(2), 165–178. https://doi.org/10.22302/ppk.jpk.v38i2.730

Tajuddin, I., & Ibadallah, B. (2019, November 5). *A rubber plantation's experience to manage a new serious leaf disease* [Conference presentation]. The First Meeting of ANRPC Technical Committee of Plant Protection, Kuala Lumpur, Malaysia.

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR protocols: A guide to methods and applications* (pp. 315–322). Academic Press.