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New leaf fall disease in rubber-pathogen characterization and rubber clone resistance evaluation using detached leaf assay

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Abstract. *Darojat MR, Ardie SW, Oktavia F, Sudarsono. 2023. New leaf fall disease in rubber-pathogen characterization and rubber clone resistance evaluation using detached leaf assay. Biodiversitas 24: 1935-1945.* Leaf fall disease (LFD) has become a significant issue for rubber plantations worldwide. Over the last four years, a newly emerging LFD has posed an alarming problem in natural rubber production. The most efficient way to control LFD is to use resistance rubber clones. Therefore, this study aims to characterize the pathogen causing the newly emerging LFD and evaluate rubber clone resistance to the pathogen using detached-leaf assay. The fungal pathogens were isolated from 32 F1 progenies of PB 260 x SP 217 crosses, and the fungi were characterized and identified based on their morphological and molecular characteristics. The results showed that the isolated pathogen causing LFD was *Neopestalotiopsis* sp. Although they were all pathogenic, the arrays of isolated fungi exhibited various degrees of virulence, and P-212 was the most virulent fungal isolate. The resistance evaluation showed that rubber clones, isolates, and rubber clones by isolate interactions had a significant ($p<0.05$) effect on the lesion-symptom diameters. Based on the lesion diameter responses, the IRR 112 and RRIC 100 rubber clones were resistant to *Pestalotiopsis* sp. since they only showed less than 10 mm lesion diameters. The IRR 39 and PB 260 rubber clones were susceptible and showed more than 20 mm lesion diameters. The detached-leaf assay can easily screen rubber clones' responses to the fungi causing LFD. The resistance evaluation results can assist future rubber breeding strategies for the newly emerged LFD-resistant characters.

Keywords: Fungal pathogen, indirect resistance evaluation, ITS sequence, LFD, *Neopestalotiopsis*, rubber clones

INTRODUCTION

The rubber tree (*Hevea brasiliensis* Muell. Arg.) is a perennial plant and the primary source of natural rubber for various industries worldwide. Rubber has contributed significantly to Indonesia's foreign exchange, with the export of natural rubber value reaching $3.01 billion in 2020 (Direktorat Jenderal Perkebunan [Ditjenbun] 2022). However, rubber production declined in the last four years by 795,783 tons. This condition is influenced by climate change, low prices, the high composition of aged trees, and disease outbreaks (Premadasa 2020). Priyadarshan (2017) stated that leaf fall disease (LFD) had become a major limiting factor for rubber plantations. The three main LFDs are caused by fungi, such as *Corynespora cassiicola* (*Corynespora* LFD), *Oidium heveae* (powdery mildew), and *Colletotrichum gloeosporioides* (*Colletotrichum* LFD) (Mazlan et al. 2019). A new LFD has been reported to infect several rubber plantations in Indonesia since 2017 with different symptoms from common LFD. This new LFD was also reported quite extensively attacking several rubber plantations in other countries in Southeast Asia, India, China, and even Colombia. These pathogens infect the mature phases of rubber leaves (phase C and phase D) with symptoms of necrotic spots, yellowing of the leaves, and leaf fall (Febbiyanti and Fairuza 2019; Kusdiana 2021). Infection with the disease reduces the leaf area index and affects latex rubber production (Kusdiana and Saputra 2022).

Identifying the pathogen is essential for diagnosing disease, deciding the control measure, and selecting the disease-resistant rubber tree genotypes. Several reports have found that the isolates influenced the resistance of rubber tree clones to *Corynespora* and *Colletotrichum* LFD (Fernando et al. 2010; Cao et al. 2019). The new LFD was initially thought to result from common LFD pathogen infections such as the *Fussicoccum* sp. (Febbiyanti et al. 2018) or the *Colletotrichum* spp. (Balai Penelitian Sungei Putih 2017; Rodesuchit 2020). However, recent research revealed that this new LFD is associated with *Pestalotiopsis* sp. (Febbiyanti and Fairuza 2019). To date, several species of *Pestalotiopsis* are related to the etiology of new LFD, namely *Neopestalotiopsis cubana* and *N. formicarum* (Pornsuriya et al. 2020), *N. aotearoa* (Li et al. 2021), and *P. microspora* (Kusdiana et al. 2020). Sometimes, the new LFD was associated with a single pathogen and a combination of two pathogens, such as *Colletotrichum* sp. and *Pestalotiopsis* sp (Aliya et al. 2022a), or with another genus (*Calonectria*, Thaochan et al. 2022). Other studies reported that *Pestalotiopsis* sp. has also infected several plant species in Indonesia, such as

Coconut (Eris et al. 2017), Strawberry (Widyaningsih and Triasih 2021), and Jabon (Herliyana et al. 2022).

The new LFD is also chemically controlled through fungicides combined with fertilizer applications. Thaochan et al. (2020) reported that systemic and contact fungicides inhibit and reduce the development of new LFD and induce a defense response in rubber tree leaves. Another controlling method is by misting sulfur in rubber plantation areas. Although these two methods are effective for rapid control, the long-term impact of chemicals on plants and the environment is still unknown. Bio-fungicides can be an alternative method of controlling LFD in rubber trees (Abraham et al. 2013; Pujade-Renaud et al. 2019; Rabiey et al. 2019).

Using resistant clones and suitable plantation management is a more effective and strategic alternative for controlling LFD (Narayanan and Mydin 2012). Khan et al. (2020) also showed that disease-resistant plants are the cheapest, safest, and most practical controlling methods. However, the presence of a resistance rubber tree to this new LFD is still unknown. Breeding programs for resistance should be the top priority given the challenges of environmental (climate) change (Miedaner 2016). Moreover, climate change may alter rubber diseases' pathogenicity and geographical distribution, especially LFD. By using resistant clones, the growers have many advantages, including reducing pesticides, being environmentally friendly, and not requiring specialized equipment to control the disease. This study aimed to characterize the pathogen causing the new LFD and evaluate rubber tree clones' resistance to this pathogen.

MATERIALS AND METHODS

The study was conducted in the Experimental and Production Field and the Laboratory of Plant Protection, Indonesian Rubber Research Institute, located in Sembawa Sub-district, Banyuasin District, South Sumatra, Indonesia (-2.934880, 104.535025). In this field station, the new LFD was observed in several areas of rubber tree plantations, including F1 seed collection, bud-wood collection, and immature and mature trees.

Isolation of the fungal pathogen associated with new LFD

Diseased rubber leaves were collected from the PB 260xPB 217 population from two different areas. Each diseased leaf with symptoms of circular spots was selected, stored in a clear plastic bag, and then brought to the laboratory. Two different methods carried out the isolation of the fungal pathogen associated with the new LFD. The new method adopted a single-spore isolation technique. In this single spore method, infected tissues were cut and incubated in high humidity for 7-14 days to induce acervuli. The presence of acervuli was confirmed by microscopic observation, and they were then taken using a sterile needle and placed in a PDA medium.

Meanwhile, method B used a common fungal isolation technique with a surface disinfectant following Kusdiana et al. (2020). Therefore, to isolate the fungal pathogen associated with new LFD, infected tissues were cut (approximately 5 mm) and sterilized by immersing in 1% sodium hypochlorite for 30 sec, followed by 70% ethanol for 30 sec, and then rinsed three times with sterile distilled water. The sample pieces were then dried on a sterile tissue paper and placed in potato dextrose agar (PDA) medium. In addition, the single spore method was used to purify all the isolates following Solarte et al. (2018). The success of isolation was determined using the following formula:

$$Si = (n/N)*100\%,$$

Where: Si: the success of isolation, n: the number of sample leaves, and N: the total number of samples.

Morphological identification

The morphological identification was based on the visual observation of both macroscopic features (colony growth, mycelium texture and color, sporulation, and type of acervuli) and microscopic features (conidia size, color, and length of median cells, length of apical appendages, length of apical and basal cells, and length of basal appendage). Colony color was defined using *A Mycological Color Chart*, and culture characteristics were evaluated based on the method reported by Maharachchikumbura et al. (2014) and Solarte et al. (2018). Microscopic features were observed with a DP20 camera integrated with a BX41 light microscope (Olympus, Japan), and 32 conidial measurements were taken for each isolate.

Molecular identification

Molecular identification was carried out by phylogenetic analysis based on a 5.8S rDNA internal transcribed spacer (ITS). Total DNA isolation and polymerase chain reaction (PCR) amplification were adopted following the method of Maharachchikumbura et al. (2014) and Kusdiana (2021). The ITS region was amplified with ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5) primer pairs (White et al. 1990). The PCR amplicons were then sequenced directly by sending the samples to Macrogen, Singapore, and the consensus sequences were obtained using the BioEdit program (Hall 1999; Sofi et al. 2022). Sequence homology was determined using the BLASTn program in the NCBI Genbank Database (https://blast.ncbi.nlm.nih.gov/). Subsequently, the identified sequences were aligned using the online MAFFT v.6 (Katoh et al. 2019). Phylogenetic analysis was performed by the Maximum Likelihood (ML) method in MEGA X (Kumar et al. 2018) using ITS sequences from the selected reference strains (Maharachchikumbura et al. 2014; Nozawa et al. 2017) from the NCBI GenBank DNA Database. A reference ITS sequence of *Neopestalotiopsis australis* from Australia (NCBI accession no. KM199348) was used as the outgroup to construct a phylogenetic tree using the ML method with the Tamura-3 parameter model, and the robustness was tested by bootstrap analysis using 1,000 replicates.

Pathogenicity test

The pathogenicity of identified fungal isolates was evaluated *in vitro* by a modified detached leaf assay (DLA) method (Ribeiro et al. 2019; Aliya et al. 2022a). Samples of healthy PB 260 rubber clone leaves at 15 days old (phase C) were collected from bud-wood. The sample leaves were cleaned using running water, and the leaf surface was wiped using 70% ethanol and then air-dried. Inoculation procedures were conducted by placing mycelium plugs (5 mm in diameter) on the abaxial side of each leaf. Before inoculation, the infected leaf was injured three times with a sterile needle (two sites per leaf). Next, each fungal isolate was inoculated onto three leaves and repeated three times. Finally, a plug inoculated the control leaves with no mycelia. The petioles were covered with sterile moist cotton, and the leaves were placed in a plastic box. The box was stored in a room at 25±2ºC temperature, and the humidity was maintained at approximately 100%. A separate experiment was conducted with two isolates (GT-1 and P-212) and repeated four times to determine the wounding effect. The pathogenicity was assessed using ImageJ software on the size of lesion diameter at 3, 5, and 7 days post-inoculation (dpi) (Schneider et al. 2012). The emerging acervuli were then isolated from symptomatic leaves to conduct Koch's postulates.

Evaluation of rubber tree clones

The procedures of inoculations were conducted using the previously described pathogenicity method. Nine rubber tree clones (PB 260, RRIC 100, GT 1, PR 261, BPM 24, IRR 5, IRR 39, IRR 112, and IRR 220) were evaluated using two isolates, GT-1 and P-212. The experiment was conducted in a completely randomized design (CRD) with three replications and was repeated twice. Disease incidence (%) was calculated based on the percentage of leaves affected by the pathogens by observing the presence or absence of lesions seven days post-inoculation. The diameter of the lesion (mm) was measured by scanning the leaves with a scanner (Canon E510) at 400 dpi and measuring it using ImageJ software (Schneider et al. 2012). Disease severity and clonal resistance were measured following Kusdiana et al. (2020) with modifications presented in Table 1.

Data analysis

Morphological features and pathogenicity tests were analyzed using one-way ANOVA ($p<0.05$) followed by Duncan's Multiple Range Test (DMRT) ($p<0.05$). Clonal resistance evaluation was conducted to determine factors related to clone resistance, such as clone, isolate, and the clone by isolate interactions. The lesion diameter data were analyzed using two-way ANOVA followed by the Scott-Knott test ($p<0.05$). Data analysis used XLSTAT v.2019 (Addinsoft 2019) program and RStudio version 2022.02.3 Build 492.

RESULTS AND DISCUSSION

Field observation and symptoms of new LFD

The new leaf fall disease (LFD) was observed at all stages of rubber tree development, such as in the nurseries, immature plants, mature plants, F1 seedling collections, and bud-wood clone collections. A photograph of the canopy condition of mature rubber trees in the field is presented in Figure 1A. Observations showed that the pathogen attacked leaves at the mature phase (C phase), and symptoms were visible in the mature leaf phase (D phase). The disease generally produces necrotic lesions with irregular round shape lesions, and more than one spot was found per leaf (Figure 1B). The lesions were 1-2 cm dark brown to light brown or light grey. The occurrence of acervulus on rubber leaf is shown in Figure 1C.

Isolation of fungal pathogens associated with new LFD

Eight fungal isolates (P-091, P-109, P-126, P-137, P-174, P-178, P-212-P231) were collected from symptomatic rubber tree leaves. Direct isolation from acervuli had a higher percentage of success than isolation using method B (Table 2). Five fungal isolates were obtained by method A and three isolates by method B. The contamination from *Colletotrichum* and *Corynespora* has caused a lower success rate of method B in isolating the fungal pathogen associated with new LFD than method A. Subsequently, four pure culture isolates (P-126, P-137, P-212, and P-231) were selected for further morphological and molecular identification. The other isolates (P-091, P-109, P-174, P-178) did not produce conidia and were not included in the subsequent analysis.

Table 1. The rating scales for measuring disease resistance to fungal pathogens (modified from Kusdiana et al. 2020)

Score	Lesion diameter (mm)	Resistance responses
0	No symptoms	Highly Resistance (HR)
1	1-5	Highly Resistance (HR)
3	6-10	Resistance (RE)
5	11-20	Moderate (MO)
7	20-30	Susceptible (SU)
9	> 30	Highly Susceptible (HS)

Table 2. Isolation results of fungal isolates associated with new leaf fall disease (LFD) in rubber using two different methods

Methods	Locations	Number of isolates	Percentages
A	F1 Seedling collections	5 (P-109, P-126, P-137, P-178, and P-231)	33
B	F1 Seedling collections	2 (P-091, P-174)	18
	Small-Scale Clone Trial	1 (P-212)	

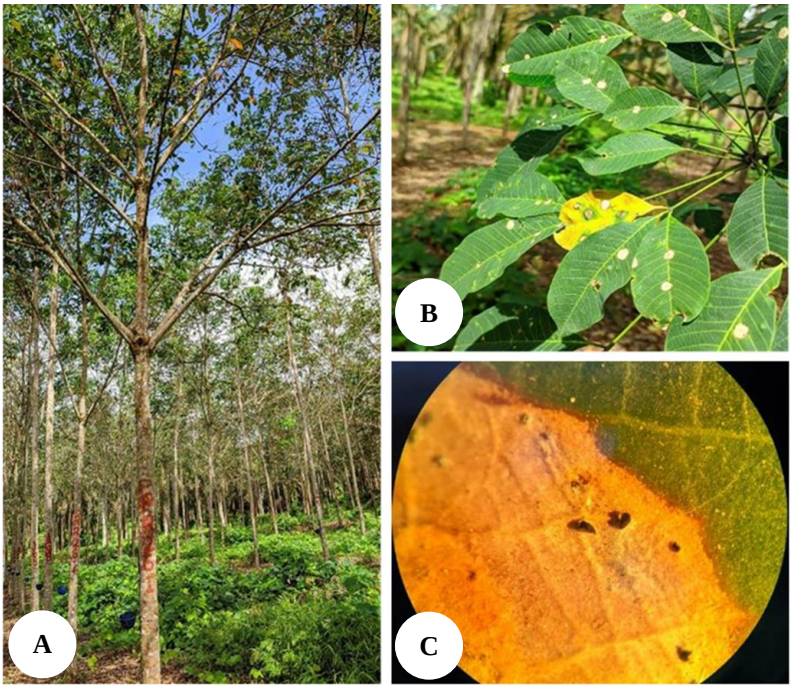

Figure 1. Typical symptoms of new leaf fall disease (LFD) in rubber plants: A. Canopy condition of mature rubber trees in the field; B. More than one necrotic lesion occurring on rubber leaf; C. Appearance of acervuli on rubber leaf necrotic lesions

Morphological characteristics of fungal pathogens associated with new LFD

All fungal isolates had white to creamy colony color, smooth to lobate edges, concentric rings to petal-form colony growth pattern, and cotton-like texture (Figure 2A). The acervulus colors were black and appeared 20-30 days after culture. Each fungal isolate produced acervuli in different quantities. All the isolates had grown to 50-63 mm in diameter after four days of incubation at 25 ± 2ºC on PDA. The P-231 isolate colony showed the highest growth rate of about 16.82 mm/day. All isolates produced similar conidial shapes but varied in size (Figure 2B). Conidia were fusoid straight to slightly curved, with five cells (one apical cell, three median cells, and one basal cell), 2-3 apical, and single basal appendages. Conidia length and width of all isolates ranged from 19.5-22.2 µm and 6.5-8.2 µm. All isolates had median cells with versicolorous type (the upper two cells were darker than the lowest median cell) with a size ranging from 13.1-15.1 µm (Figure 2C). Apical and basal cells were relatively uniform, 3.1-3.5 µm and 3.4-4.1 µm in length.

Phylogenetic analysis of fungal pathogens associated with new LFD

The BLAST analysis using query sequences of the fungal ITS fragment (553 562 bp) showed that they shared 99.6%-100% sequence identity to several published accessions in the NCBI GenBank DNA Database (Table 3). The ITS sequences of all fungal isolates (P-126, P-137, P-212, and P-231) showed a high sequence identity to the *Neopestalotiopsis saprophytica*. Phylogenetic analysis of the four isolates was performed using the Maximum Likelihood (ML)-based on ITS sequences of fungal isolates and 13 reference sequences. The analysis showed that evaluated species were divided into three clades: I.A. *Pestalotiopsis*, I.B. *Pseudopestalotiopsis*, and II. *Neopestalotiopsis* (Figure 3). Therefore, evaluated fungal isolates associated with new LFD in the present study belonged to the genus *Neopestalotiopsis* and were identified as *Neopestalotiopsis saprophytica* (Figure 3).

The pathogenicity of fungal pathogens associated with new LFD

Results of the pathogenicity test showed that *Neopestalotiopsis* sp. caused lesions on detached rubber leaves. The isolates produce the same lesion but significantly different lesion diameters. The P-126 and P-212 isolates were the most virulent, producing 15.7 mm and 16.9 mm diameter lesions on infected leaves. The wounded treatment showed a significantly higher incidence of lesions (100%) than without wounding (only 50%). The incubation period and the fungal virulence affected the lesion incidence of inoculation without wounding. The P-212 was the most virulent isolate, producing lesions three days after inoculation (dpi), while GT-1 (the control) isolates produced lesions at 13 dpi (Figure 4).

Evaluation of rubber tree clone resistance to fungal isolates

The resistance of rubber tree clones was evaluated based on the lesion diameter generated by fungal pathogens on the inoculated leaves. All rubber tree clones displayed symptomatic lesions one-day post inoculation (dpi). Isolates, clones, and isolate-by-clone interactions significantly affected the lesion diameter at 5 and 7 dpi. Isolate P-212, on average, generated a higher lesion diameter than isolate GT-1 (Figure 5). These results confirmed that isolate P-212 was more pathogenic than isolate GT-1. In the present study, rubber tree clones responded differently to various isolates, indicating that the isolates influenced the new LFD resistance in rubber trees.

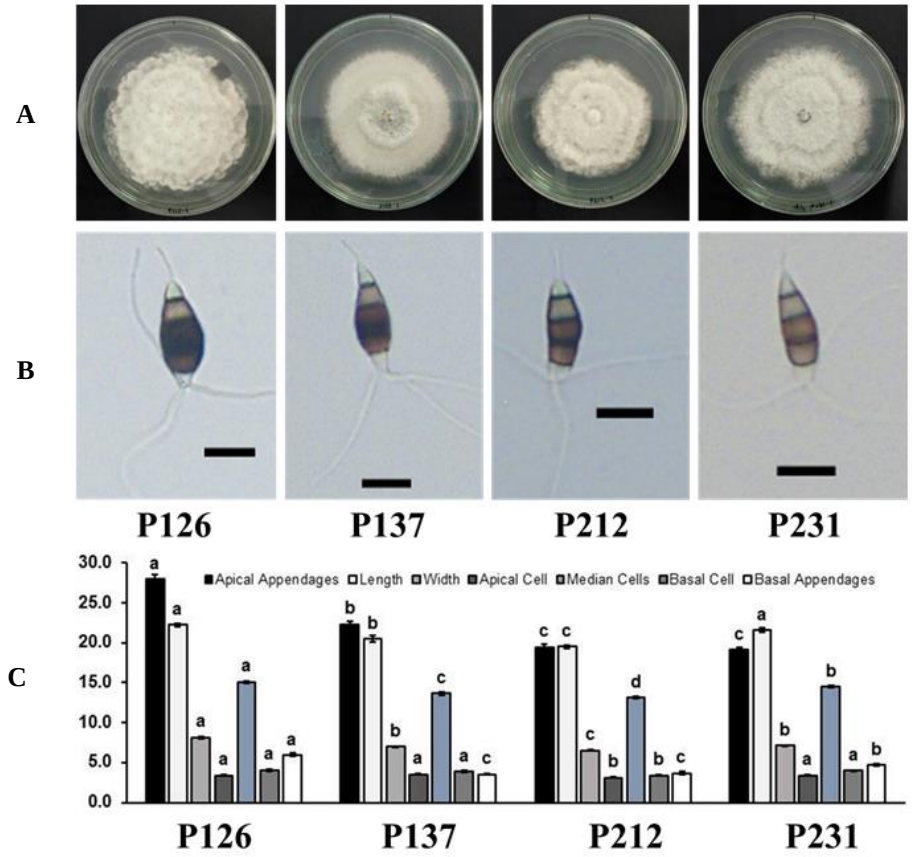

Figure 2. Morphological traits of the four fungal isolates associated with new leaf fall disease (LFD) in rubber. A. Colony characteristics of the isolated fungi on potato dextrose agar (PDA) medium four days after initial culture; B-C. Conidial morphology and measure. Scale bar: 10 µm

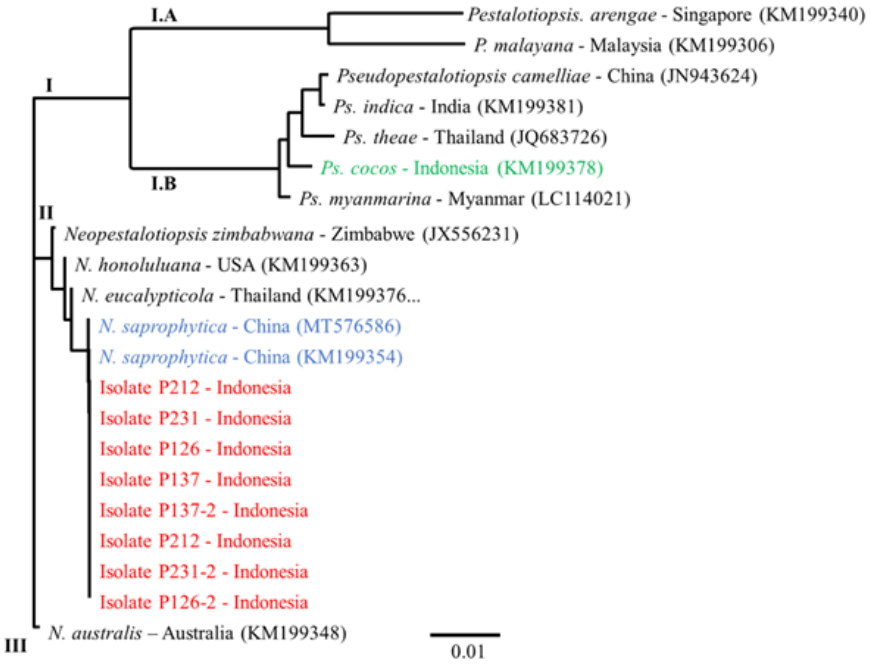

Figure 3. Phylogenetic tree of *Pseudopestalotiopsis* sp., *Pestalotiopsis* sp., *Neopestalotiopsis* sp., and the fungal isolates associated with new leaf fall disease (LFD) from the infected leaf of rubber trees based on ITS sequence using ML method. The red-colored accessions are the four fungal isolates associated with LFD in Indonesia. The *Neopestalotiopsis australis* (KM199348) was used as the *outgroup*

Clone RRIC 100 and IRR 112, on average, had the lowest lesion diameter if inoculated with either GT-1 isolate (7.47 mm) or P-212 isolate (7.62 mm), respectively (Figure 5). The average growth rate of the lesions in those two rubber clones ranged from 1.00-1.36 mm/day for seven days of observation. In contrast, clones IRR 39 and PB 260 had the highest lesion diameter if inoculated with fungal isolate P-212 (21.44 mm and 22.47 mm). This result indicated that clones IRR 39 and PB 260 were susceptible to the evaluated fungal pathogens. In addition, these two clones also had the highest lesion growth rate of 3.04-3.09 mm day^{-1}. Therefore, the evaluation categorized the clones into three groups based on the lesion diameter characteristic: resistant, moderately resistant, and susceptible (Table 4). The RRIC 100 and IRR 112 clones were considered resistant, PB 260 and IRR 39 susceptible, and the remaining moderately resistant.

Table 4. Responses of rubber tree clones to the fungal isolate associated with the new leaf fall disease (LFD)

Rubber clones	Lesion diameters	Average lesion growth (mm day^{-1})	Resistant responses
RRIC 100, IRR 112	< 10 mm	1.00-1.36	Resistant
IRR 220, PR 261, GT 1, IRR 5, BPM 24	11-20 mm	1.50-2.22	Moderate
IRR 39, PB 260	> 20 mm	3.04-3.09	Susceptible

Table 3. The BLAST analysis used ITS sequences of four fungal isolates associated with new leaf fall disease (LFD) in rubber as the query sequence

New LFD isolate codes	Reference species from NCBI DNA database	The NCBI accession no.	Query coverage	Sequence identity (%)	Plant host
P-126	*Neopestalotiopsis saprophytica*	MT576586	99%	99.82	*Psidium guajava*
	Neopestalotiopsis sp.	LC427180	99%	100	Loquat
P-137	*N. saprophytica*	MT576586	99%	100	*P. guajava*
	Neopestalotiopsis sp.	LC412067	99%	100	*P. japonica*
P-212	*Neopestalotiopsis* sp.	MG599268	99%	100	Oil palm
	N. saprophytica	MT576586	99%	99.82	*P. guajava*
P-231	*Neopestalotiopsis* sp.	MG599268	99%	99.64	Oil palm
	N. saprophytica	MT576586	99%	99.46	*P. guajava*

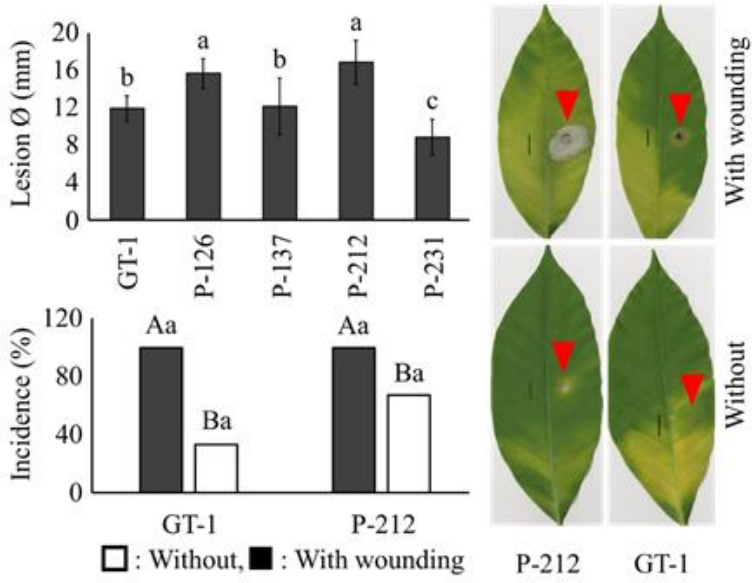

Figure 4. Pathogenicity test of four fungal isolates associated with new leaf-fall disease (LFD) on leaves of rubber tree clone PB 260. (a) The pathogenicity was determined by measuring necrotic lesions on infected rubber tree leaves seven days after inoculation. (b) Lesion incidence (%) among leaves inoculated with two different methods (i.e., with and without wounded leaf) at 13 days post-inoculation. (c) induced symptoms on PB 260 clone leaves inoculated with two fungal isolates and two methods. Different uppercase and lowercase letters showed significant differences among means for inoculation methods and isolates by Duncan's Multiple Range tests ($p<0.05$). Arrows showed the generated lesions from inoculation treatments. Scale bar = 10 mm

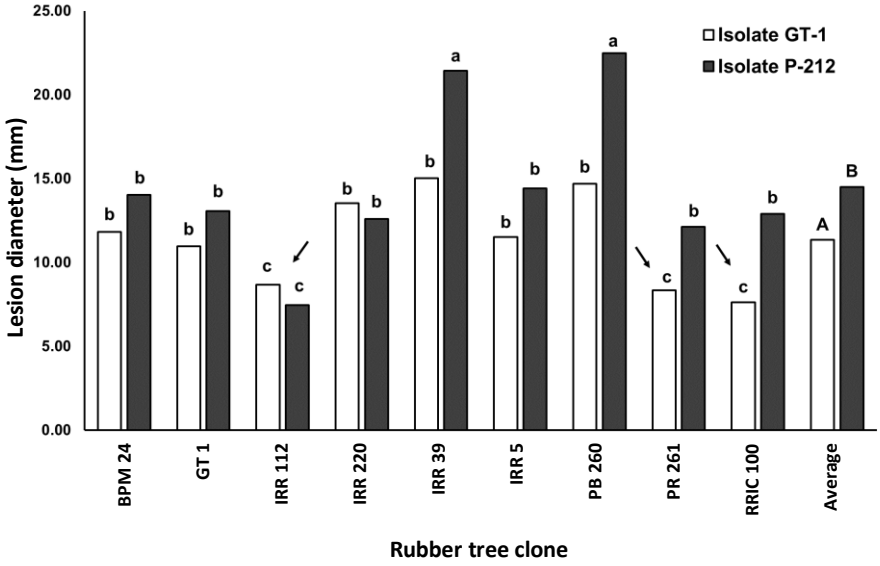

Figure 5. The lesion diameter among nine rubber tree clones inoculated with two fungal isolates associated with the new leaf fall disease (LFD). Different letters showed significant differences ($p<0.05$) by the Scott-Knott test. Arrows denote the lowest values for each isolate

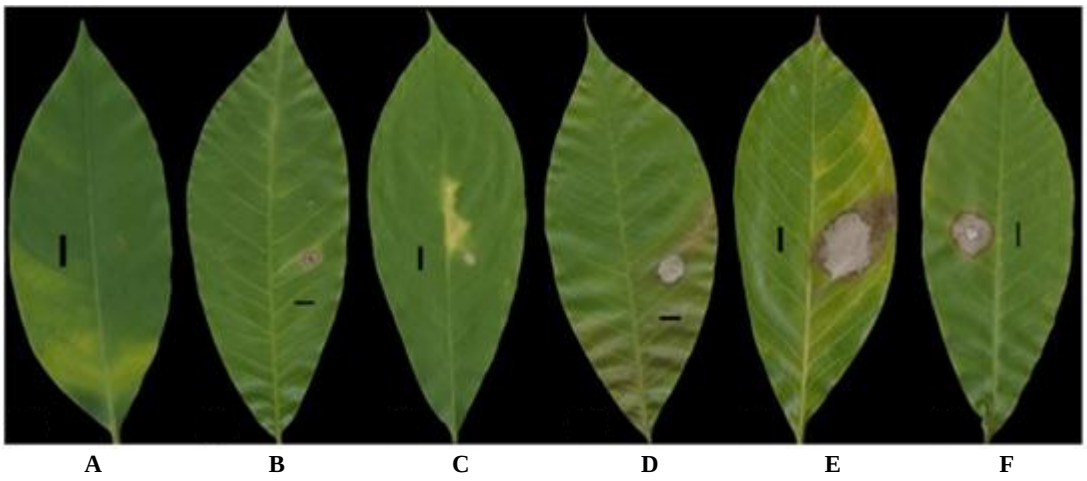

Figure 6. Responses of rubber tree clones to fungal isolate P-212. A. Control, B. Rubber clone IRR 112, C. RRIC 100, D. BPM 24, E. IRR 39, F. PB 260. The (B) and (C) are representative of resistant responses, (D) moderate, (E-F) susceptible clones. Scale bar = 10 mm

Discussion

Pestalotiopsis leaf fall disease is a new leaf fall disease that was first discovered in North Sumatra in mid-2017. Since then, the disease has spread to other provinces in Indonesia and other rubber-producing countries, including Malaysia, Thailand, India, and Sri Lanka (Aliya et al. 2022b). This disease has infected various rubber tree clones and all stages of rubber tree development (Febbiyanti and Fairuza 2019). Field observation has shown that severe infection rubber leaves become discolored and fall off. The severity of the disease could reach 80% (Kusdiana and Saputra 2022). Hence, the disease greatly affected the canopy's condition and decreased the rubber tree's latex production.Five*Pestalotiopsis* species are associated with rubber new LFD reported in Indonesian rubber plantations and other natural rubber-producing countries, including *Neopestalotiopsis cubana*, *N. formicarum*, *N. aotearoa*, *Pestalotiopsis microspora*, and *P. jesteri* (Kusdiana et al. 2020; Pornsuriya et al. 2020; Li et al. 2021; Aliya et al. 2022a).

Four *N. saprophytica* isolates from infected rubber tree leaves were identified in this study. This is the first report on the isolation and characterization of *N. saprophytica* that can potentially cause rubber LFD. A single spore isolation method mainly obtained the isolates from the acervuli. Fei et al. (2019) reported that a single spore isolation method could be carried out on pathogenic fungi producing aerial conidia with practically no contamination. On the other hand, this study showed that *Colletotrichum* sp. and *Corynespora* sp. could be fungal contaminants in

method B. These pathogens are responsible for leaf fall diseases that conclusively attack rubber tree plantations (Manju et al. 2014; Aliya et al. 2022b). At the same time, *Pestalotiopsis* produces spore mass structures (acervuli or pycnidia) in the epidermal tissues of the host at the end of its life cycle (Maharachchikumbura et al. 2011). However, not all infected leaves had necrotic lesions accompanied by the appearance of mature acervuli. This study observed that incubation for 7-14 days in moist conditions could induce the emergence of conidia from acervuli. Several reports revealed that *Pestalotiopsis* spores could be induced by incubating infected leaves for several days or months in highly moist conditions (Kobayashi et al. 2001; Suwannarach et al. 2015).

Morphological colony and conidial characters are typical and the most widely used in the initial identification of *Pestalotiopsis* (Maharachchikumbura et al. 2011). All isolates in the present study had a white colony, fusiform conidia, and versicolor median cells, indicating that isolates were *Neopestalotiopsis*. These characteristics were similar to those observed in the previous studies observing LFD in other plants, such as LFD in Royal Palm and *Erythropalum scandens* (Yang et al. 2021; Ismail et al. 2022). Temperature affected the mycelial growth of the *Pestalotiopsis,* with optimum growth ranging from 24 to 28ºC (Keith et al. 2006; Baggio et al. 2021) and failed to grow at temperatures below 20ºC or above 30ºC (Fovo et al. 2017; Bhuiyan et al. 2021; Li et al. 2021). The conidial characters in this study are similar to those reported by Febbiyanti and Fairuzah (2019) but showed that the conidia have a more significant, more extensive conidial morphology than those reported by Kusdiana et al. (2020). In addition, conidial characteristics were more stable and reliable than colony characteristics as identification means (Maharachchikumbura et al. 2012).

Phylogenetic analysis using the ITS sequences confirmed that all isolates were identified as *Neopestalotiopsis saprophytica*. The ITS gene is a universal fungal identification marker (Schoch et al. 2012). Although the ITS gene in the *Pestalotiopsis* has a low variation between species, this marker is widely used for *Pestalotiopsis* identification (Maharachchikumbura et al. 2012; Maharachchikumbura et al. 2014; Liu et al. 2017; Diogo et al. 2021). The ITS gene has also been successfully used for *Pestalotiopsis* identification which causes leaf fall disease in rubber trees (Kusdiana et al. 2020; Pornsuriya et al. 2020; Li et al. 2021; Aliya et al. 2022a). The results of this study indicated that the ITS gene could be used to identify *Pestalotiopsis.* Previous studies showed that a single ITS gene could be used to identify *Pestalotiopsis microspora* in *H. brasiliensis* (Kusdiana et al. 2020), Oil Palm (Ismail et al. 2017), and *Adenium obesum* (Jesus et al. 2022). The combination of ITS, *β-tubulin,* and *tef1* gene regions was reported to be a better taxonomic marker for *Pestalotiopsis* than a single gene (Maharachchikumbura et al. 2016; Chen et al. 2020; Das et al. 2021; Aliya et al. 2022a; Fiorenza et al. 2022; Nozawa et al. 2022).

Pestalotiopsis has long been known as a weak, opportunistic, and secondary pathogen in certain plants (Maharachchikumbura et al. 2011). This genus has been identified as one of the endophytic fungi found on the leaves and stems of rubber trees (Gazis and Chaverri 2010; Rocha et al. 2011; Vaz et al. 2018; Araújo et al. 2020; de Oliveira Amaral et al. 2022). Hrycan et al. (2020) reported that fungal endophytes might act as latent pathogens that become active and cause disease under certain conditions. Previous studies reported that unwounded inoculations did not generate lesions at five dpi (Pornsurya et al. 2020; Kusdiana et al. 2020; Aliya et al. 2022a). The incubation periods might be too short and need more days for pathogens to infect the leaves. In this study, the virulence of the isolate, the wounding, and the humidity condition affect the lesions' intensity. Several studies reported that wounds might be required to accelerate the development of *Pestalotiopsis* disease symptoms in Guava (Keith and Zee 2010) and *Neolitsea villosa* (Ariyawansa and Hyde 2018). Unwounded leaves with high humidity can produce symptomatic lesions on the leaves of *Eucalyptus* plants (Belisário et al. 2020). In field observation, Rivano et al. (2016) reported that climatic factors, including relative humidity, rainfall, minimum temperature, and dew point temperature, determine the risk and development of SALB disease in rubber trees.

Rubber tree clones displayed various lesion diameters, responding to *Neopestalotiopsis* and *P. microspora* as the comparing isolates. Several studies reported that the isolates influenced the resistance of rubber tree clones to leaf fall disease (Atan et al. 2011; Ngobisa et al. 2015; Antonio et al. 2021). Rubber tree resistance to leaf fall disease is strongly influenced by genetic factors and is thought of as horizontal resistance (Simmonds 1990; Tan and Tan 1996; Roy et al. 2022). Fang et al. (2016) revealed that rubber trees had distinct defense mechanisms against leaf-fall diseases between developed and mature leaves. However, the mechanism of rubber tree resistance to *Pestalotiopsis* is still not widely known. Initial studies showed that *Pestalotiopsis* infected all rubber tree clones in the rubber plantation (Febbiyanti and Fairuzah 2019). Kusdiana et al. (2020) reported that the severity of *Pestalotiopsis* LFD ranged from 50.00-79.89%, with lesion diameter reaching 14 mm in the greenhouse evaluation. Clone IRR 112 and RRIC 100 were reported to be resistant clones when evaluated in the greenhouse, with disease severity below 20% (Damiri et al. 2022). Therefore, this study also revealed that IRR 112 and RRIC 100 generated the lowest lesion diameter and were grouped as resistant clones (Figure 6).

Screening of rubber tree clones is an effective way to control rubber leaf fall diseases. The breeding programs have been conducted to evaluate the genotypes or clones resistant to leaf fall diseases, especially *Pestalotiopsis* LFD. The initial process of plant defense is physical barriers with morphological (cuticle) and anatomical changes in cell wall structure through lignin, suberin, and callose deposits (Krishnan et al. 2018). Pathogen infections can stimulate tissues to produce chemicals such as scopoletin, hydrocyanic acid, and leucine (Guyot and Le Guen 2018; Roy et al. 2019). Several reports revealed that rubber tree resistance to leaf fall diseases was controlled by quantitative loci (Le Guen et al. 2013; Fang et al. 2016;

Oktavia 2016; Tran et al. 2016; Roy et al. 2022). Expression of pathogenesis-related (PR) protein genes can be used to indicate rubber tree resistance to leaf fall disease (Afandi et al. 2022).

In conclusion, four fungal isolates associated with new leaf fall disease (LFD) were successfully isolated from rubber leaves. The fungal isolates derived from single conidial culture (P-126, P-137, P-212, and P-231) were pathogenic to rubber leaves. Colony morphologies and ITS sequences of P-126, P-137, P-212, and P-231 fungal isolates indicate that they were all *Neopestalotiopsis* sp. However, all were pathogenic and exhibited various degrees of virulence. Among all four isolates, P-212 was the most virulent fungal isolate.

On the other hand, based on the lesion diameter responses, IRR 112 and RRIC 100 rubber clones were resistant to the isolated *Neopestalotiopsis* sp. since they only showed less than 10 mm lesion diameter. The IRR 39 and PB 260 rubber clones were susceptible and showed more than 20 mm lesion diameter. The described detached-leaf assay may be easily used to screen rubber clones' responses to the fungi causing LFD. The results of the resistance evaluation may be used to develop breeding strategies for the newly emerged LFD-resistant clones of rubber in the future.

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