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A rapid in vitro leaf inoculation assay to investigate *Phytophthora palmivora*–coconut interactions

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Abstract

Bud rot, caused by *Phytophthora palmivora*, is one of the major lethal diseases of the coconut palm. It is a sporadic disease but can assume epidemic proportions occasionally. There is a high risk involved in carrying out studies of plant–pathogen interactions through field trials, especially in a crop like coconut. We have developed an in vitro leaf assay, which would enable an easy and close monitoring of interactions occurring between *P. palmivora* and coconut during the course of infection by recreating the field infection process in the laboratory. Spindle leaves from adult coconut palms were surface sterilized and inoculated in basal Y3 medium in Petri plates. After a week in culture, the surface of the leaves was pricked and inoculated with zoospore suspension of *P. palmivora* culture. Typical lesion development was noticed on the surface of the coconut leaves 12–24 hr after inoculation of the pathogen. Electrolyte leakage assays and histological studies using scanning electron microscopy, were also undertaken to study the disease progression. The presence of the pathogen in the coconut leaflets was confirmed using PCR and RT-PCR with *Phytophthora*-specific primers. The pathogen could be re-isolated from the infected regions, fulfilling Koch's postulates. The novel in vitro leaf assay developed through this study is rapid, space-effective, precise and inexpensive. It could be utilized for detailed investigations of the qualitative interactions between coconut and *P. palmivora*.

KEYWORDS
bud rot, coconut, in vitro inoculation, leaf assay, *Phytophthora palmivora*

1 | INTRODUCTION

Phytophthora spp. are notorious phytopathogens with a substantial global impact as they affect an extensive array of crops ranging from vegetables, ornamentals, plantation crops to forest trees. Placed under Stramenopiles (syn. Heterokonta; kingdom Chromista, SAR super-group), they are oomycetes which can produce hyphae (Cavalier-Smith, 2018). While some species like *P. ramorum* and *P. sojae* possess a narrow host range, others like *P. cactorum* and *P. palmivora* have a broad host range (Erwin & Ribeiro, 1996). Coconut (*Cocos nucifera* L.), eulogized as the *'Tree of Life'*, forms an essential backbone of the economies of regions along the tropics. Apart from the importance of copra and coconut oil, which is widely used as edible oil and in the manufacture of hair oils, soaps, cosmetics and other industrial products, the husk is a source of fibre which supports a sizeable coir industry. The tender nut supplies coconut water, a popular thirst quencher of health and hygienic value. *P. palmivora* and *P. katsurae* have been mainly associated with diseases of the coconut palm (Dollet et al., 2012). *P. palmivora* is the only species reported till date as a pathogen infecting coconut palms in

India and causes bud rot disease, which causes significant economic loss to the palms in states of Kerala, Karnataka, Tamil Nadu and Andhra Pradesh (Srinivasulu et al., 2008). It is usually a fatal disease of the coconut palm, characterized by the rotting of the terminal bud and surrounding tissues. Even though it affects the palms of all ages, young palms in low lying and moist situations are more susceptible to the disease. The disease is usually sporadic but has been reported to assume epidemic proportions (Srinivasulu & Raghava Rao, 2009). Climatic conditions play a significant role in the disease spread and its severity (Srinivasulu et al., 2008).

Detection of the disease in the early stages and adopting appropriate remedial measures may avert the palm's eventual loss. Integrated disease management strategies are recommended throughout the year, especially in endemic areas, for effectively managing the disease. Severely affected trees need to be removed and burnt. If disease detection is done early, the infected tissue has to be removed and the cut portion protected with Bordeaux paste (Chidananda & Pandurang, 2001; Ramesh & Maruthadurai, 2014).

Crop breeding programmes, aiming for development of disease-resistant cultivars, assume significance in a crop like coconut given the cryptic nature of the *P. palmivora* and its detection at a stage in which successful control measures can be implemented. Whole plant testing is of limited relevance for large-scale screening of bud rot disease in coconut for apparent reasons; also, studies can be restricted only to seedlings in containment/quarantine facilities or assessment in regions with a history of disease outbreaks. A deep understanding of molecular mechanisms underlying plant–pathogen interactions could enable novel approaches for crop protection. One of the techniques frequently used for investigating plant defence responses to pathogens, under laboratory conditions, is the detached leaf assay, which has been utilized in several studies such as soybean (Surin et al., 1993), wheat (Arraiano et al., 2001), alfalfa (Irwin et al., 2003), rice (Jia et al., 2003) and durian (Vawdrey et al., 2005). This laboratory assay possesses several advantages: higher reproducibility, the requirement of less space, labour, plant materials and inoculum, more consistent delivery of inoculum, uniform incubation conditions can be maintained, inoculation site can be localized to specific areas of the leaf, and more accurate quantification of disease is possible (Dhingra & Sinclair, 1995). Besides, contamination can be controlled, more replicates can be maintained, and there is no risk of spreading the phytopathogen to the environment. However, this assay also possesses disadvantages: detached leaves may not be a typical reflection of the variability of whole plants in terms of actual physical condition and age/phyllotaxy of leaves, and some of the host defence responses may be attenuated in detached leaves (Parke et al., 2005).

Histopathological and molecular events occurring during *P. palmivora* infection have not been reported in coconut. In the current study, an in vitro leaf assay was developed through challenge inoculation of *P. palmivora* on the coconut leaf segments cultured in vitro. Tracking of the infection and disease progression was undertaken using histological studies, electrolyte leakage assays, and PCR and RT-PCR with specific primers. This assay would enable detailed molecular investigations on the cascade of events during *P. palmivora* infection in coconut.

2 | METHODOLOGY

2.1 | Isolation and morphological characterization of the pathogen

Samples were collected from bud rot affected coconut palms from Kasaragod and Dakshina Kannada districts of Kerala and Karnataka state, respectively, India, during South-West monsoon season (June-July) in 2017 (Table 1). The infected palms showed typical symptoms such as decaying of the spindle leaf and its bending, commonly associated with bud rot disease (Figure 1a). The palms' infected bud region (Figure 1b) also had a characteristic foul smell and conspicuous irregular water-soaked lesions in the leaf lamina (Figure 1c).

Along with young infected leaves, the bud region was collected in an autoclaved polythene bag and brought to the laboratory in an icebox. Tender leaves with lesions were separated in laminar airflow. Segments of 1 × 1 cm size were surface sterilized by rinsing with a 0.01% mercuric chloride solution followed by washing in sterile distilled water thrice. Direct plating method (Chowdappa et al., 2014) was used to isolate pathogen by placing the leaf segments in carrot agar medium supplemented with rifampicin (125 ppm). The inoculated samples were kept in a growth chamber with a constant temperature (25 ± 2°C) and humidity (95%). The individual isolates obtained were characterized through morphological traits as detailed by Gallegly and Hong (2008). Mating or compatibility type of *P. palmivora* isolate was determined using the method adopted by Chowdappa and Chandramohanan (1997) where the isolates were paired with known *Phytophthora* isolates obtained from ICAR-Indian Institute of Spices Research (ICAR-IISR), Kozhikode, Kerala, India. Individual isolates were inoculated separately on to carrot broth medium and maintained at 25 ± 2°C for the formation of mycelial mats. They were filtered and washed thoroughly with sterile water and dried using sterile filter paper.

2.2 | Molecular characterization of the pathogen

To confirm the identity of each *Phytophthora* species, DNA was extracted from the mycelial mats of individual isolates using the DNeasy Plant Mini Kit (Qiagen, USA). The ITS6 (5'-GAAGGTGAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primer pairs (Cooke et al., 2000; White et al., 1990) were used to amplify the complete sequence of 5.8S ribosomal RNA gene, and I and II spacer regions completely and partial sequence of 18S ribosomal RNA gene and 28S ribosomal RNA gene regions of the pathogen. PCR, gel electrophoresis, elution of bands, sequencing and sequence analysis were conducted as described by Prathibha et al. (2018). A phylogenetic tree was

TABLE 1 List of *Phytophthora palmivora* isolates collected in the present study

Sl. No.	District/State	Place of collection	Isolate no.	GPS co-ordinates
1.	Kasaragod/Kerala	Mannipady	TR-PP–1	12°32'12.1"N 74°59'55.5"E
2.	Kasaragod/Kerala	Pachakkad	TR-PP–2	12°32'38.0"N 74°57'45.7"E
3.	Dakshina Kannada/Karnataka	Kidu	TR-PP–3	12°42'25.1"N 75°34'27.6"E
4.	Kasaragod/Kerala	Konnakkad	TR-PP–4	12°21'59.3"N 75°22'35.7"E
5.	Kasaragod/Kerala	Elerithattu	TR-PP–5	12°19'54.3"N 75°18'33.0"E

FIGURE 1 Bud rot infection in coconut palm. (a) Bud rot-infected palm. (b) The bud region of the infected palm showing decaying. (c) Leaf lamina of the infected palm showing typical water-soaked lesions

constructed based on ITS sequence of TR-PP-2 isolate with other *P. palmivora* isolates, including various species of *Phytophthora*, *Pythium*, *Peronospora* and *Fusarium*, sourced from GenBank, with the software MEGA -X version 10.2.0 (Kumar et al., 2018) using the neighbour-joining (NJ) method (Saitou & Nei, 1987) under 1,000 bootstrap replicates. The details of selected species for phylogenetic analysis are listed in Supplementary Table S1.

2.3 | Screening *P. palmivora* isolates for virulence levels

P. palmivora isolates collected from bud rot affected palms were initially screened for their virulence level using the detached leaf technique (Sharadraj & ChandraMohanan, 2014). For this, zoospore suspensions were prepared from the isolates by growing 5-mm mycelial disc in carrot agar and incubated at 25 ± 2°C. After sporulation at the 7th day, sterile distilled water (20 ml) was added and incubated at 15°C for 15 min. Plates were then kept at room temperature till the release of zoospores (for approximately 20 min). The concentration of zoospores was standardized based on the procedure given in Mohamed-Azni et al. (2017). A haemocytometer (Rohem, India) was utilized for the calculation of zoospore concentration. A series of inoculum concentrations, *viz*., 1, 2, 3, 4 and 5 × 10^4/ml, were prepared to determine the zoospore concentration capable of inducing severe infection. Based on this experiment, the zoospore concentration was fixed at 2 × 10^4/ml.

To screen its virulence, spindle leaves, sampled from Chowghat Orange Dwarf (COD), a cultivar susceptible to bud rot, were wiped with sterile water and pricked (four times) on its dorsal side with a sterile needle to facilitate easy entry of the pathogen to the leaf and kept above sterile test tubes lying horizontally on wet cotton in a tray. Ten microlitres of zoospore suspensions (2 × 10^4/ml) of each isolate were individually inoculated in the pricked region, and the inoculated area was covered with a thin layer of wet cotton. The entire set-up was covered with a polythene sheet and kept in a humid chamber at 25 ± 2°C (Rachana & Rajesh, 2019; Sharadraj & ChandraMohanan, 2014). Different isolates were screened for its level of virulence based on the time required for the initiation of infection and the size of the lesion formed after five days of inoculation. The highly virulent isolate of *P. palmivora* obtained from this exercise was used for the rest of the studies.

2.4 | In vitro leaf assay

Spindle leaves, sampled from three-year-old COD palm, were cleaned by swiping with 70% ethanol, and the midrib portion was removed (Figure 2a-f). Leaf lamina was cut into segments (approx. 5 cm) aseptically under laminar airflow chamber (Figure 2g), surfaced sterilized with mercuric chloride (0.01%) for 10 min with constant shaking (Figure 2h) and then washed 4–5 times in sterile water (Figure 2i). These leaf segments were then placed with its dorsal side upwards in Y3 medium (Eeuwens, 1976), supplemented with sucrose (30 g/L), agar (6.5 g/L) and charcoal (0.5 g/L), in Petri plates (Figure 2j). All the cultures were kept at 25 ± 2°C and 95% humidity in a plant growth chamber (Panasonic MLR-352H-PE). After a week in culture, leaf

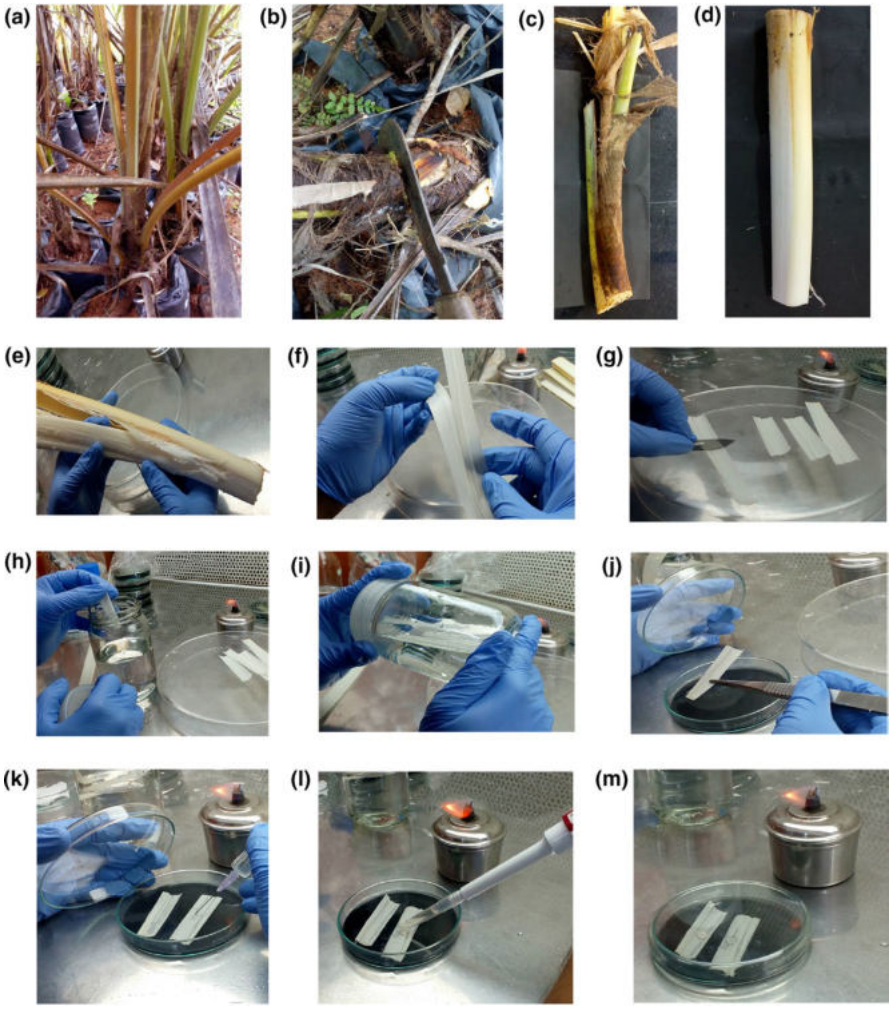

FIGURE 2 In vitro leaf inoculation assay followed. (a) Three-year-old Chowghat Orange Dwarf seedling. (b-e) Collection of bud tissues from seedlings. (f) Separating the leaf tissues. (g) Making tissue bits. (h) Surface sterilization. (i) Subsequent washing. (j) Inoculation of leaf bit on Y3 media. (k) Pinpricking on leaf bit using a sterile syringe. (l) Inoculation of *Phytophthora palmivora* zoospore suspension on leaf bit. (m) Inoculated leaf bit

lamina segments were pricked (five equal pricks) using a sterile needle (Figure 2k) before inoculation with zoospore suspension (10 µl) of the highly virulent isolate (Figure 2l & 2m). Non-inoculated leaf segments with similar pricks served as control. Cultures were observed at regular intervals to record the initiation of infection, its advancement and other attributes such as the size of the lesions. Postinfection, the identity of pathogen was confirmed through morphological and molecular techniques, as described earlier.

2.5 | Leaf viability assay using membrane stability index

The viability of the in vitro cultured leaf segments was assessed using membrane stability index (Sairam et al., 1997) since this parameter is negatively related to senescence or leaf death (Gupta et al., 2012). Detached leaflets were taken for comparison. Approximately 100 mg of leaf segments were taken, immersed in 10 ml distilled water and initial electrical conductivity (μScm^{-1}; Model PP20, Sartorius) was determined (C1) after overnight incubation in room temperature. Final electrical conductivity was determined (C2) after placing the test tubes in boiling water for 30 min. The membrane stability index (MSI) was calculated as:

$$MSI = \{1 - (C1/C2)\} \times 100$$

2.6 | Histopathological studies using scanning electron microscopy

For scanning electron microscopy, leaf bits (1 x 1 cm) with infected portion were placed in aluminium cylinder adhered with a graphite tape and placed in a vacuum chamber. The sample was coated with high vacuum evaporator for six minutes in an ionizing metal JEOL JFC-1100 (Fine Coat® sputter ion, JEOL Ltd). The gold-coated samples were observed in a scanning electron microscope (JEOL-JSM-6490LV) under vacuum to get higher resolution.

2.7 | Disease assays and pathogenicity tests

To satisfy Koch's postulate, pieces of in vitro inoculated leaf bits, showing disease lesions at 48 hpi, were removed from the Petri

plates, surface sterilized with 70% ethanol, plated on to carrot agar medium in Petri plates and maintained at 25 + 2°C and 95% humidity in a plant growth chamber (Panasonic MLR-352H-PE). Subsequent growth of mycelia was recorded. Pathogenicity tests were conducted by detached leaf assay (Sharadraj & ChandraMohanan, 2014), as mentioned in Section 2.3.

2.8 | RNA extraction and expression studies

Infected leaf samples collected for the isolation of RNA, include samples 12 hr postinoculation (12 hpi) and 12 hr uninoculated control (12 hc), 24 hr postinoculation (24 hpi) and 24 hr uninoculated control (24 hc) and 36 hr postinoculation (36 hpi) and 36 hr uninoculated control (36 hc). Total RNA was extracted from these samples using the NucleoSpin® RNA Plant Kit (Macherey Nagel). The quality and the purity of the extracted RNA were assessed by observing the absorbance from$A_{260/280}$ ratio. RNA integrity number was analysed using an Agilent Technologies 2,100 Bioanalyzer with the Agilent RNA chip. cDNA was synthesized using the PrimeScript cDNA Synthesis Kit (Takara). The reaction mixture included 4.0 µl of 5 X PrimeScript buffer, 1 µl of PrimeScript RT enzyme mix, 1 µl of random hexamer primer and 100 ng of RNA. The reactions were incubated at 37^{0}C for 15 min, followed by 85^{0}C for 5 s and then kept at 4^{0}C. *Phytophthora* spp. are known to secrete numerous effectors into their hosts to promote infection, and therefore, the expression of effector genes is up-regulated during the infection process (Wang & Jiao, 2019). Primer pairs were designed for an RxLR effector (POM73696.1) previously reported in *P. palmivora* (Ali et al., 2017). Also, 40S ribosomal gene (G40S) was taken as an internal control for *P. palmivora* (Ali et al., 2017), while eukaryotic translation initiation (CneIF-4γ) from coconut was taken as an internal control for coconut (Rachana & Rajesh, 2019). The details of the primers are given in Table 2.

After cDNA synthesis, RT-PCR was conducted on a thermal cycler (MJ Mini™, Bio-Rad). Each reaction included 1 µl of diluted cDNA (~200ng), 1 µl of forward and reverse primer (0.5 µmol), 10 µl of GeNei™ Red Dye PCR Master Mix (1X) and 6 µl distilled water. The reactions conditions were 94°C for 2 min, followed by 34 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1 Min, and a final extension at 72°C for 10 min. Samples were visualized on 1.5% agarose gel electrophoresis (Blue Marine) in 1X TBE buffer and documented.

2.9 | Statistical analysis

For statistical analysis, the data recorded on the size of the lesions and their relative growth rates due to infection by *P. palmivora*, and the membrane stability index (MSI) in in vitro cultured coconut leaf segments in comparison to detached leaflets, were subjected to one-way analysis of variance (ANOVA) as the experiment was laid out in completely randomized design (CRD). The means obtained were then compared using Duncan's multiple range test (DMRT) using SPSS 15.0.

3 | RESULTS

3.1 | Initiation of cultures and morphological characterization

Strains isolated from bud rot infected palms and cultured in carrot agar medium, showed similar morphological attributes like greyish white colony, with stellate colony pattern, the colony possessing sharp well-defined leading edges (Figure 3a). The growth rate of the cultures was 1–1.3 cm^2/day, and among the isolates, TR-PP-5 recorded the maximum growth rate (1.3 cm^2/day). The isolates showed network-like mycelia without cross walls (Figure 3b). Also, abundant ovoid to ellipsoid sporangia, with a round base and conspicuous papilla were produced (Figure 3c). The sporangia of the pathogen showed sympodial arrangement and were caducous, breaking away rapidly from sporangiophore. The average size of sporangia was found to be 59.03 × 26.63 µm with 2.21 LB ratio. Globose chlamydospores were found terminal as well as intercalary on mycelia of the isolate (Figure 3d). The average diameter of the chlamydospore was 27 µm. All the isolates found to be A2 mating type. The oogonia were spherical, and the average size was 34 µm (Figure 3e). All the above said morphological features are highly similar to those reported for *P. palmivora*. Challenge inoculation on the detached leaflets indicated that necrotic lesions reached a maximum size (7.11 cm^2) on the fifth day after inoculation (Figure 3f). Among the isolates, the isolate TR-PP-2 showed the highest virulence in detached leaf assay. Hence, this isolate was used for the rest of the study.

TABLE 2 Primers used for amplification of genes of host and pathogen genes via RT-PCR

Sl. No.	Gene name (organism)	Primer name	Primer sequence (5'–3')	Product size (bp)
1.	Eukaryotic initiation factor- 4γ (*CneIF–4γ*) (*C. nucifera*)	CneIF–4γF	CACCAGGAGACTTGGGAACT	110
		CneIF–4γR	CAGCTTACCAACCACTTCACC	
2.	40 S rRNA (*G40S*) (*P. palmivora*)	G40SF	AACTCTGATTGAGGCCTTCG	243
		G40SR	AACTCTGATTGAGGCCTTCG	
3.	RxLR effector (*GRxLR*) (*P. palmivora*)	GRxLRF	CCTTCAAGCGGTAATCCAAA	244
		GRxLRR	ATGACCCTTGTGGTTCTTGC	

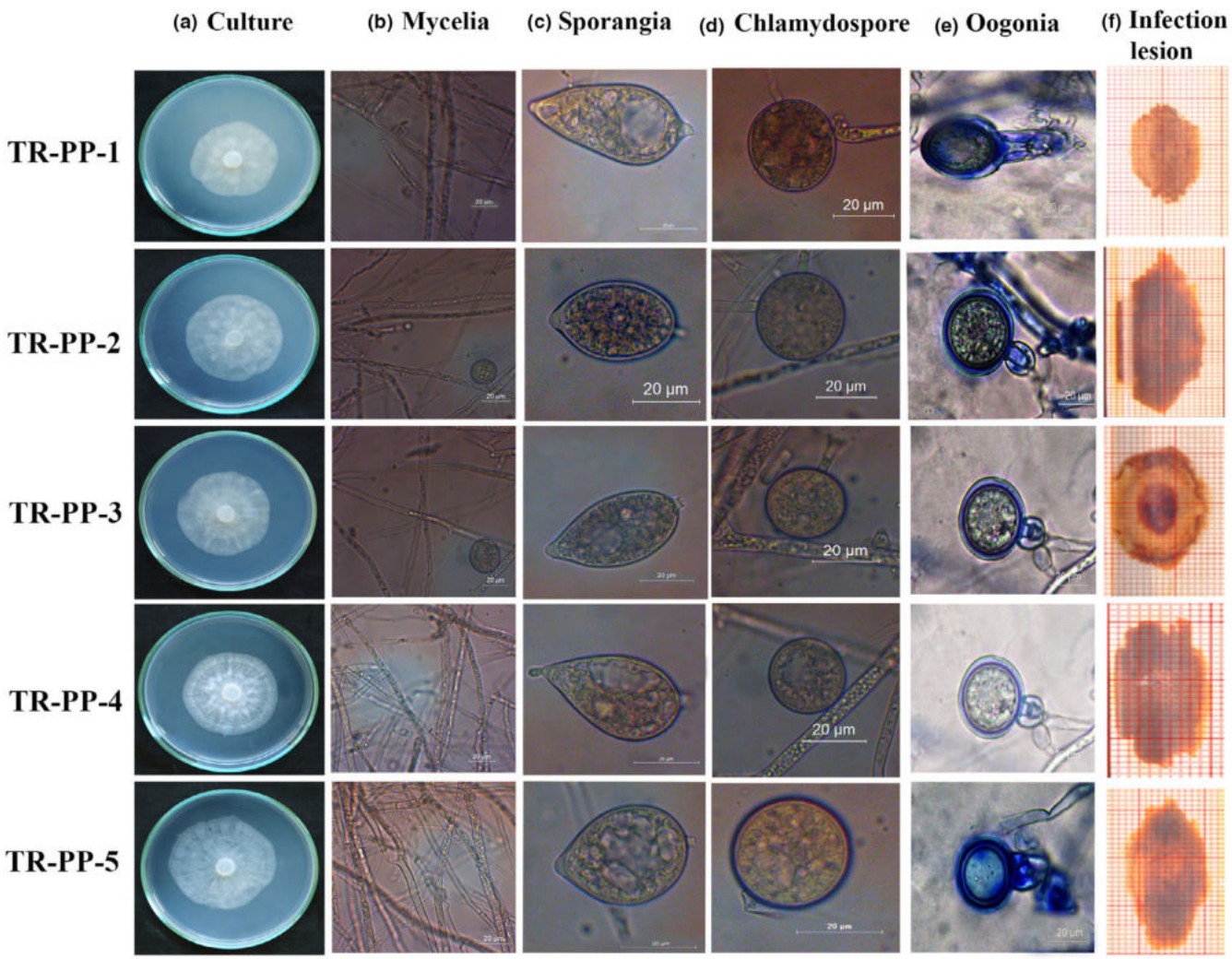

FIGURE 3 (a-e) Colony morphology of different isolates (TR-PP-1 to TR-PP-5). (f) The infection lesions on the detached leaflet of coconut

3.2 | Molecular characterization of the virulent isolate

Molecular characterization of the isolate TR-PP-2 was undertaken using *Phytophthora*-specific internal transcribed spacer (ITS) primers. The expected amplicon of around ~ 900 bp was obtained (Figure 4). The amplicon was sequenced, and a sequence of 846 bp (48.6% G + C) was obtained (GenBank accession no. MK500842.1). The phylogenetic tree constructed, based on NJ method, based on 23 ITS regions of the different *Phytophthora* spp. and other phytopathogens, revealed two main clusters (Figure 5). Within the first main cluster, the isolate TR-PP-2 formed a separate sub-cluster with other *P. palmivora* isolates from coconut, cocoa and oil palm. Also, TR-PP-2 isolate was genetically closest to a *P. palmivora* isolate from coconut from India (GenBank accession no. AM422704). *P. megakarya* represented the closest group to other *P. palmivora* isolates. The second sub-cluster within the first main cluster was formed by *Peronospora* spp. and *Pythium* spp. formed the third sub-cluster. *Fusarium* isolates formed the second main cluster.

3.3 | In vitro leaf assay

The morphological and molecular level characterization confirmed the causal agent as *P. palmivora* and challenge inoculation study verified its virulence. When the TR-PP-2 isolate was inoculated in the pricked portion of the spindle leaf segment cultured in Eeuwens Y3 medium, mild necrotic lesions were first observed by 24 hpi. The lesion became more extensive and more conspicuous with an irregular pattern 48 hpi. No significant changes were observed on the leaf surface of the control samples. The necrotic lesion reached an average of 228 mm^2 on the fourth day after inoculation (Figures 6 and 7).

Viability of the leaf segments cultured on in vitro medium was revealed through membrane stability index (MSI) assay. Preliminary

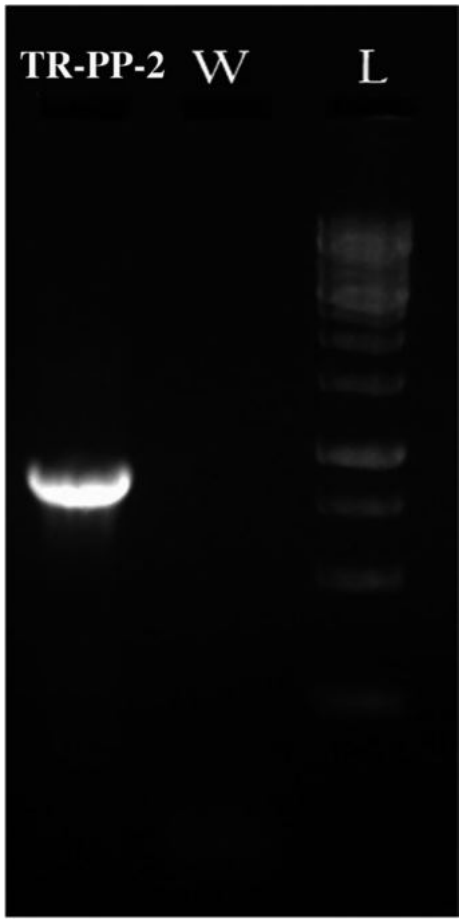

FIGURE 4 Molecular characterization of the isolate TR-PP-2. Gel image of the amplicon (TR-PP-2: *Phytophthora palmivora* DNA, W: -ve control and 1Kb L: 1Kb Ladder)

observations indicate that MSI did not decrease significantly in the leaf segments even at 20 days of in vitro culturing in the medium, whereas it rapidly decreased in detached leaflets (Figure 8).

3.4 | Assessment of infection by scanning electron microscopy

Scanning electron micrographs of the leaf segments from this in vitro leaf assay gave further evidence on the pathogen interaction. It revealed the development of hyphae along the epidermal cell surface with an appressoria like hyphae and its penetration along the walls of the trichome. Fungal hypha seems to emerge through stomata to produce sporangial structures at the surface of the epidermal layer. In the later stages of the infection, the sporangial formation and network like hyphae on the surface seem to proliferate to form lesions (Figure 9).

3.5 | Pathogenicity studies

Re-isolation of the pathogen from the lesions in the leaflets (48 hpi), of in vitro leaf assay, fulfilled Koch's postulates. When cultured in carrot agar medium, the pathogen exhibited similar morphological characteristics like greyish white colony, with stellate colony pattern, with sharp well-defined leading edges. Detached leaf inoculation experiments, with zoospores derived from the mycelia, resulted in typical lesions in the leaves, with initial symptoms being observed at 24 hpi.

3.6 | RT-PCR analysis

RT-PCR analysis revealed amplification of coconut housekeeping gene (CneIF-4γ) in both uninoculated and inoculated leaf samples at all the three time points (12, 24 and 36 hpi). The expression of *P. palmivora* housekeeping gene (G40S) could be observed only in inoculated leaf samples at all the three time points (12, 24 and 36 hpi) and not in uninoculated samples. Expression of *P. palmivora* RxLR effector gene (*GRxLR*) was observed only in inoculated leaf samples at 24 and 36 hpi (Figure 10).

4 | DISCUSSION

Phytophthora palmivora infects approximately 170 different plant species in the tropics. The management of this destructive phytopathogen has been dependent on various cultural and chemical techniques and resistance breeding. Standard screening protocols for crops, for assessing resistant/susceptible plantlets against phytopathogens, include spraying plants with conidial suspensions of pathogen isolates (Xu & Ko, 1998) under greenhouse or field condition. However, these strategies are challenging to adopt in a crop like coconut. Interaction studies of this pathogen with coconut have been challenging due to the lack of an easy, reliable and practical assay method for extensive screening. The palm structure and the long life cycle limit the feasibility of whole plant pathogenicity tests in coconut. Therefore, in this study, we have attempted to mimic the infection progression, occurring in actual field conditions, under in vitro conditions in the laboratory.

We have initially screened multiple isolates of *P. palmivora* collected from various regions of Kerala and Karnataka States, India, during the peak monsoon season, for their virulence using the detached leaf technique. We have undertaken primary identification of these isolates utilizing morphological characteristics. All the isolates exhibited morphological traits similar to those reported previously for *P. palmivora* (Erwin & Ribeiro, 1996; Martin et al., 2012; Martínez et al., 2010). The mycelia are network-like and without any cross walls, while the sporangia are ovoid and ellipsoid, possessing a round base and conspicuous papilla. Among the four isolates, TR-PP-2 was identified as the most virulent among the isolates based on the lesion size (Figure 4). The most virulent isolate's identity was further validated by identifying the ITS sequence data via BLASTN and phylogenetic analysis. Phylogenetic analysis, undertaken using ITS sequence data, clustered the *P. palmivora* TR-PP-2 isolate with other *P. palmivora* isolates and *P. megakarya* (Figure 5); both these species have been

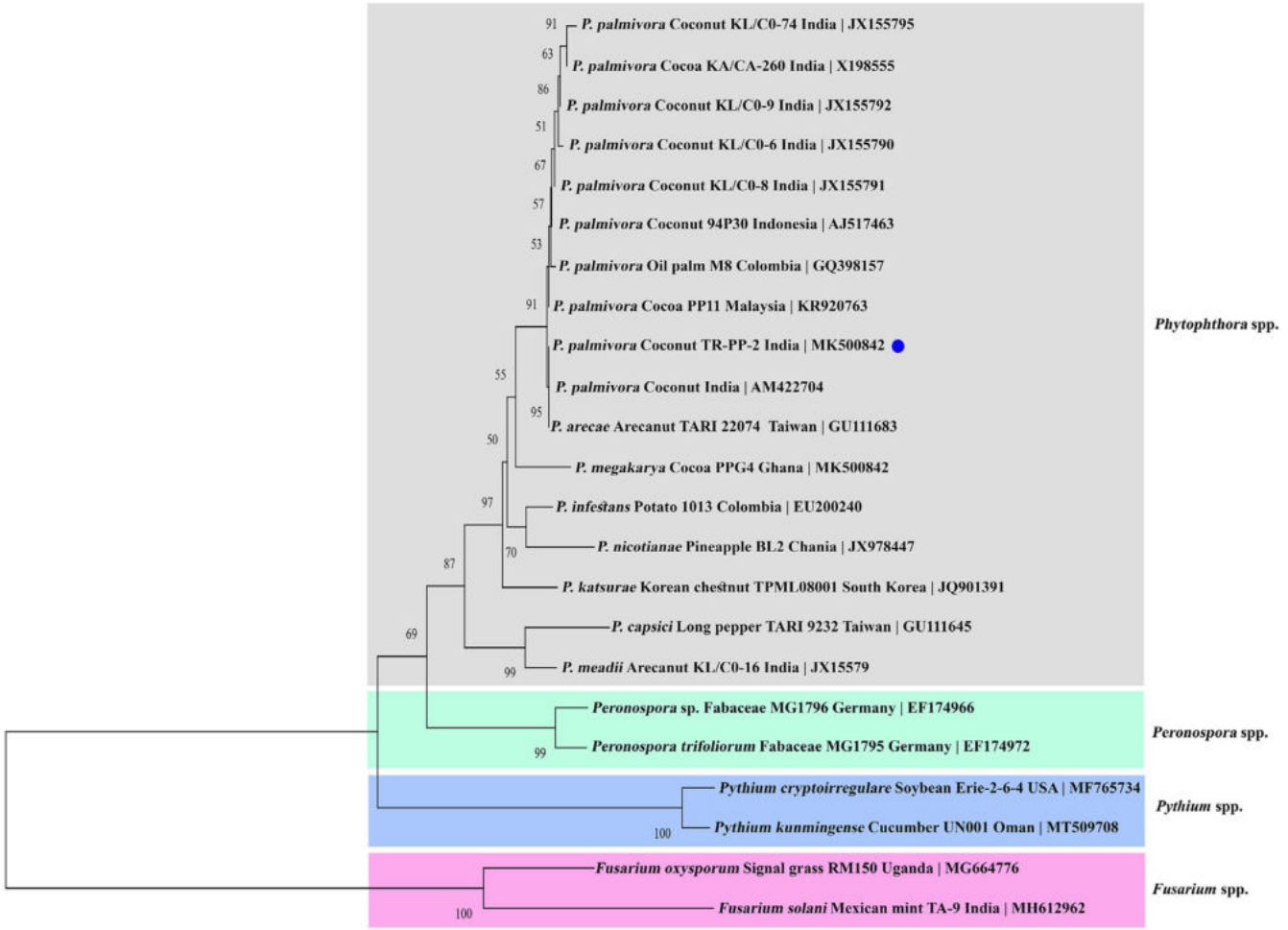

FIGURE 5 Phylogenetic tree, constructed from 23 ITS sequence data, depicting the evolutionary relationships of *Phytophthora palmivora* isolates. Numbers at nodes indicate the bootstrap values in percentage (%)

placed together in Clade 4 based on comprehensive phylogenetic studies undertaken by Yang et al. (2017).

The TR-PP-2 isolate was further used for standardizing the in vitro leaf assay. This isolate caused necrotic lesions on all treated samples, and lesions were first observed 12 hr postinoculation, after which it increased in size (Figure 5). Under field conditions, as the infection progresses, sunken leaf spots are visible on leaves, the spot margins being irregular in shape and are water-soaked (Srinivasulu et al., 2008). The symptom development and its characteristics under in vitro leaf assay were consistent with the symptoms occurring under natural conditions in the field.

The membrane stability index (MSI) of in vitro cultured leaf segments did not change significantly for up to 20 days in the media suggesting that the medium used (Y3) keeps the leaf healthy and turgid till the symptoms develop and most importantly this method did not affect the virulence of the pathogen *P. palmivora* (Figure 7). The consistent MSI indicates the viability as this parameter is negatively related to senescence or leaf death (Gupta et al., 2012). However, the MSI of the detached leaflets showed a rapid decrease indicating loss of viability of leaves. This indicates that actual molecular mechanisms underlying pathogen–host interactions cannot be studied in the detached leaves.

Further, the detection of the pathogen could be performed successfully, and the symptom of the spread of the mycelia and secondary growth assessment was substantiated by SEM (Figure 8). The growth of the lesion size over time and the scanning electron micrographs suggest that the detached in vitro leaf assay is reliable and the interaction between the pathogen and the host proceeds normally akin to field conditions. Also, the conditions of incubation have minimal effect on disease development. The pathogenicity of isolates was investigated by detached leaf assay. Zoospores, derived from mycelia from isolated regions of in vitro infected leaves, could further infect the leaves in detached leaf assays, fulfilling Koch's postulates.

Phytophthora spp. secrete several effector proteins into plants, to suppress the basal plant immunity and promote infection (Wang & Jiao, 2019). To confirm the interaction at the molecular level, we used RT-PCR to detect the secretion or presence of effectors in the inoculated leaves. *P. palmivora* effector gene, *GRxLR*, was detected at 24 hpi and 36 hpi (Figure 10). The internal control for *P. palmivora*

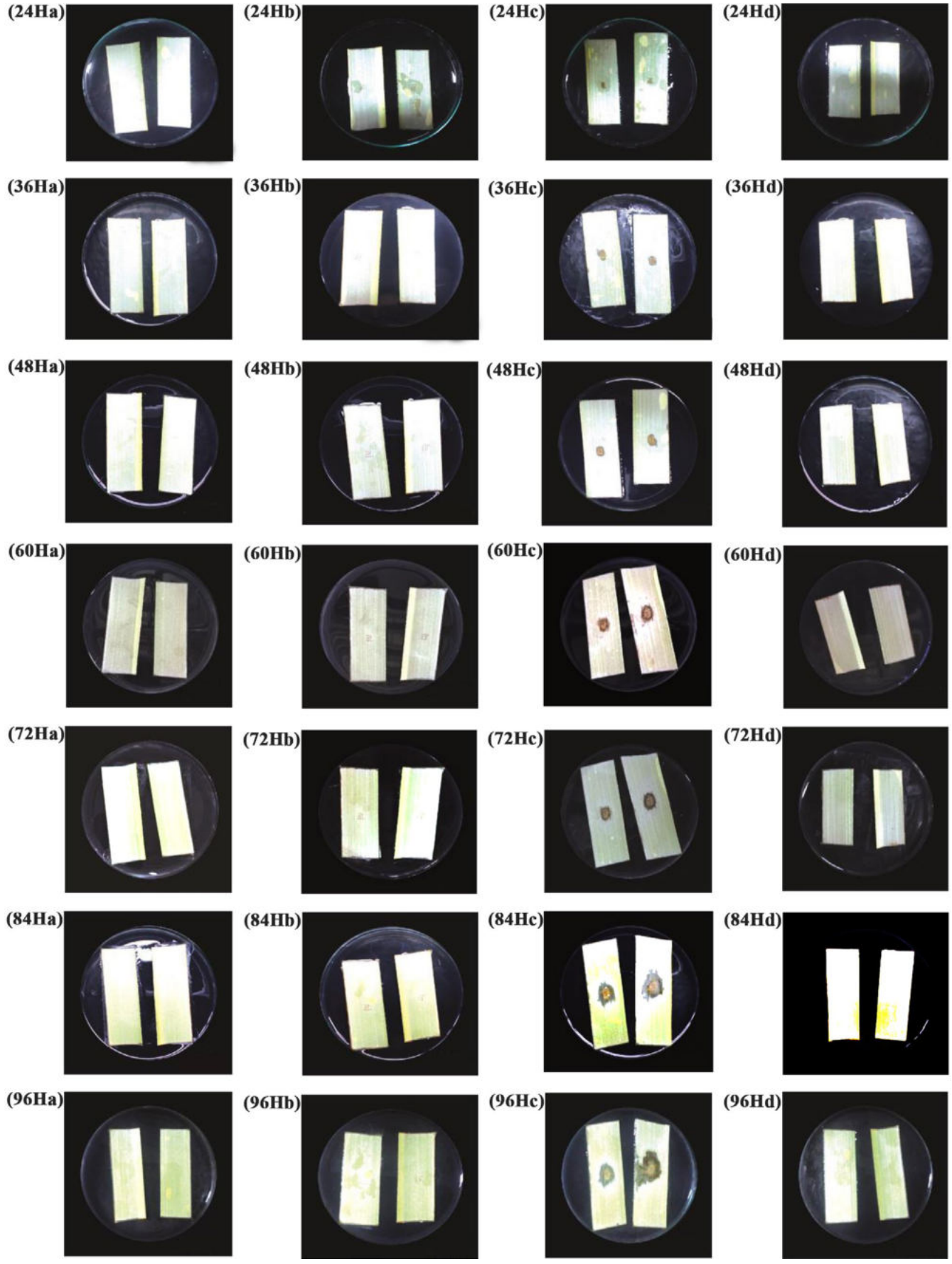

FIGURE 6 In vitro inoculation of *Phytophthora palmivora* on coconut spindle leaf. Ha: un-pricked and uninoculated; Hb: pricked and uninoculated, Hc: pricked and inoculated and Hd: un-pricked and inoculated. Observations were taken at 24, 36, 48, 60, 72, 84 and 96 hr postinoculation (hpi)

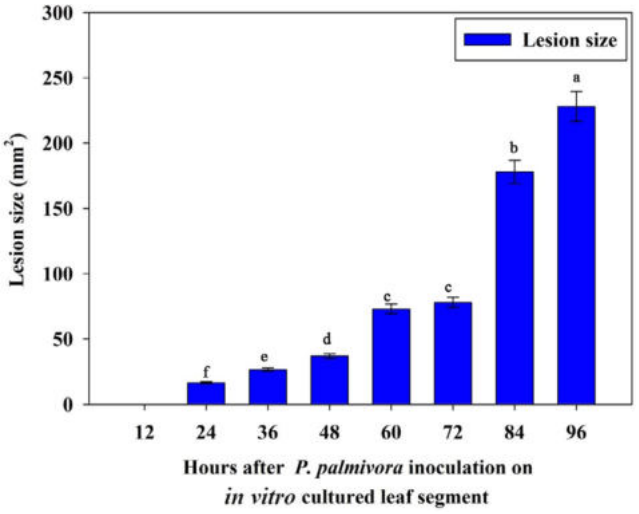

FIGURE 7 Size of the lesions and their relative growth rate caused by the *P. palmivora* on coconut leaf segments cultured in vitro. Observations were taken at 24, 36, 48, 60, 72, 84 and 96 hr after inoculation

G40S was detectable at 12 hpi (Figure 10). The detection of effector in the in vitro leaf assay suggests the feasibility of using this method to map and study finer aspects of host-pathogen interactions, especially identification of specific effectors.

In our study, we observed that wounding was required for producing localized lesions using the in vitro leaf assay in coconut, which corroborates the previous report of Sharadraj and ChandraMohanan (2014) who had reported the same observation using detached leaflets. One reason could be the presence of epicuticular wax (Escalante et al., 2002; Riedel et al., 2009) and phenols in mature coconut leaflets (Jay et al., 1989) could form barriers to infection. Under natural conditions in the field, *P. palmivora* is known only to attack the single terminal bud or 'cabbage' of coconut, which consists of several folded embryonic leaves, but is devoid of sufficient epicuticular wax and phenols. Sarria et al. (2016) and Mohamed-Azni et al. (2019) had reported the development of typical infection in un-wounded, immature leaflets of oil palm inoculated with zoospores of *P. palmivora*. Also, the study by Mohamed-Azni et al. (2019) revealed differences in cross pathogenicity of *P. palmivora* isolates from Malaysia, on different crops. While infection was observed, either directly or via

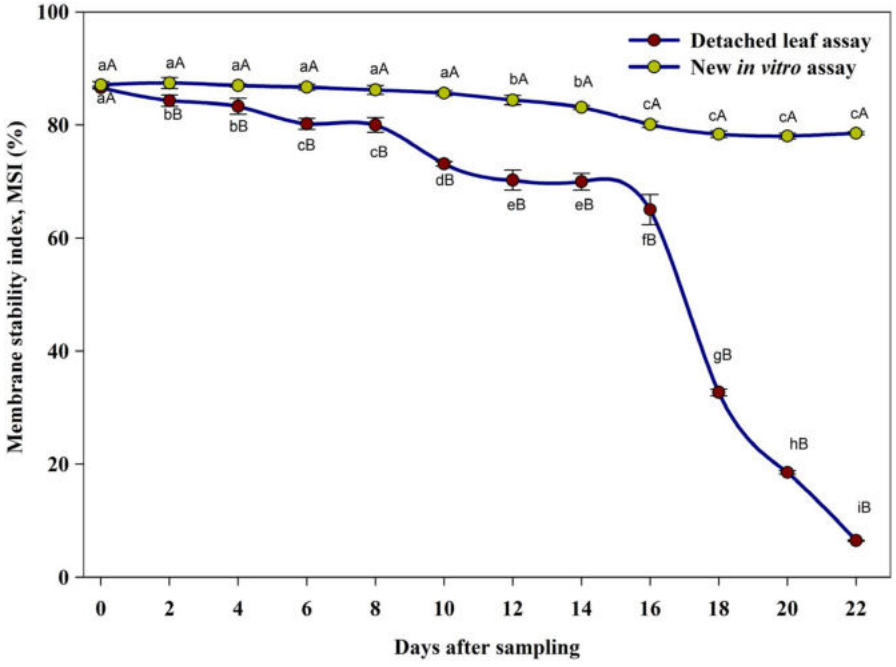

FIGURE 8 A comparison of membrane stability index (MSI) in in vitro cultured leaf segments up to 20 days

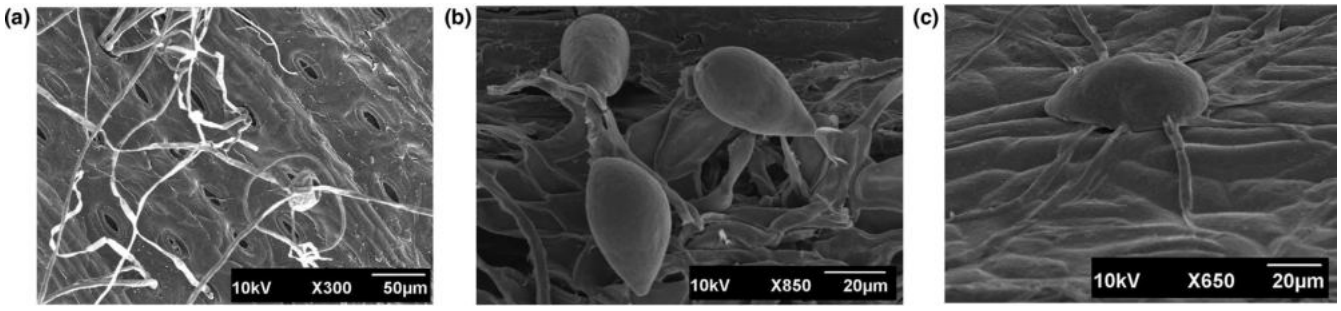

FIGURE 9 Scanning electron micrographs of the leaf segments inoculated with *P. palmivora* using the in vitro method. (a) Mycelial growth through stomata, (b) Sporangia attached in trichome of the leaf and (c) Self-emerging sporangia

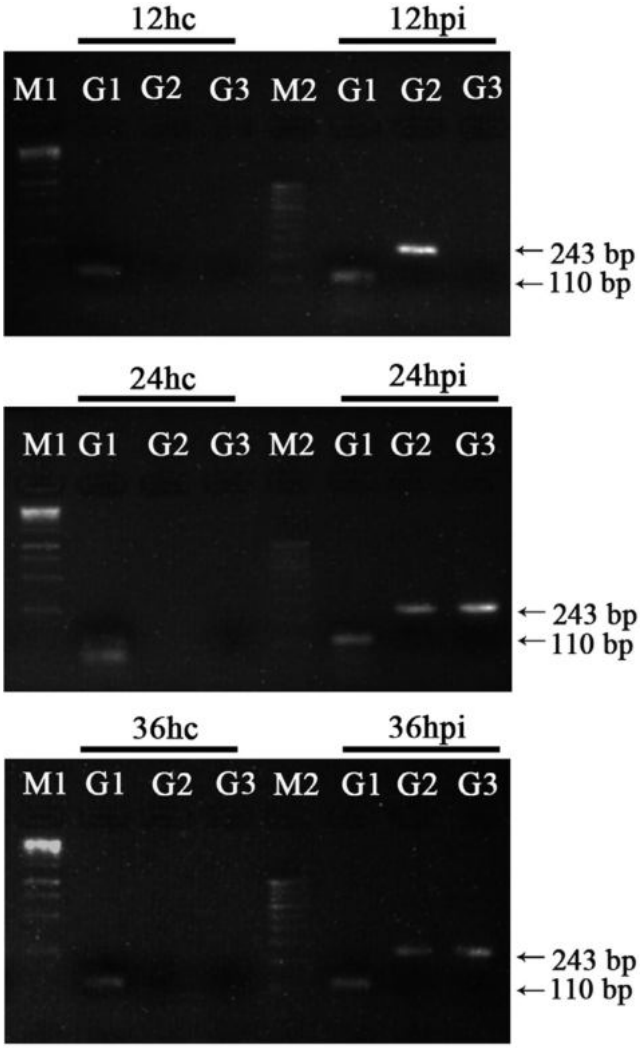

FIGURE 10 RT-PCR products after electrophoresis; M1: 1 Kb DNA ladder; M2: 100 bp DNA ladder; G1: eukaryotic translation initiation (CneIF-4γ) in coconut; G2: 40S ribosomal gene (G40S) of *Phytophthora palmivora*; and G3: *P. palmivora* RxLR effector gene (*GRxLR*)

wounding, on cocoa, durian and rubber seedlings, it did not result in severe bud rot symptoms in oil palm. Freeman and Beattie (2008) reported that disease progression is prevented if the plant's external barriers were not damaged.

The in vitro leaf assay developed in this study requires less human resources and time for screening coconut accessions worldwide for their resistance/susceptibility to *P. palmivora*. The molecular basis underlying the pathogenesis in *P. palmivora*–coconut interaction remains unclear. This assay would be a very useful tool for the detailed examination of the repertoire of potential pathogenic molecules possessing the capacity to induce disease symptoms. This assay would also facilitate studies of the kinetics of the regulatory partners in the disease biology in coconut. The causative elements that regulate programmed cell death, and more specifically, interacting molecules in coconut can also be addressed by using the in vitro infection assay experiments.

5 | CONCLUSION

P. palmivora–coconut interaction is less studied partly due to the logistics and lack of suitable assay method. In this study, we have developed a rapid, efficient, reliable and economical method to study the pathogenicity of *P. palmivora*, which could be performed under controlled conditions and can be used to determine the effectors from *P. palmivora*. The advantage of in vitro leaf screening methods is that despite providing favourable disease expression conditions if the cultivar is identified as resistant to disease, the cultivar can further be considered for breeding programmes. Also, this method reduces the risk of the pathogen spreading to the surrounding. This method is a useful tool for epidemiological studies and beneficial for laboratories and research centres with limited plant growth facilities and minimal budget.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

Peer Review

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in NCBI at https://www.ncbi.nlm.nih.gov/nuccore/MK500842.1/

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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