Agr. Nat. Resour. 54 (2020) 180–189

AGRICULTURE AND NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

A novel, virus-like double-stranded RNA in *Phytopythium cucurbitacearum* isolated from para rubber tree (*Hevea brasiliensis*) in eastern Thailand

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Article Info

Article history:
Received 24 May 2019
Revised 13 June 2019
Accepted 5 July 2019
Available online 24 April 2020

Keywords:
dsRNA,
Hymexazol,
Para rubber tree,
Phytopythium cucurbitacearum,
Virus

Abstract

Members of the order Peronosporales, especially *Phytophthora*, *Phytopythium* and *Pythium*, are notorious plant pathogens. A virus associated with the oomycetes from the para rubber tree has not been reported to date. In this report, rubber tree petioles with symptoms of abnormal leaf fall from eastern Thailand were isolated for oomycetes that were then screened for virus-like, double-stranded RNA using cellulose column chromatography. From 79 isolates, only isolate R84 contained three dsRNA segments with approximately 8.0, 3.7 and 2.3 kilobases. This was further confirmed by their ionic-strength-dependent sensitivity to hydrolysis using RNase A. Two-step reverse transcription using available Phytophthora virus-specific primers revealed that the dsRNA segments were not any of the currently reported Phytophthora viruses. Isolate R84 was described as: homothallic and having plerotic oogonia and paragynous antheridia; zoosporangia usually spherical, or occasionally ovoid, pyriform, with or without papilla; zoosporangia germinate by extension of the exit tube, vesicle formation and zoospore discharge. Phylogenetic analysis based on sequences of the internal transcribed spacer rDNA region of the isolate and those from other *Phytopythium* (*Phy.;* previously *Pythium* in clade K) from GenBank showed that the most closely related species was *Phytopythium cucurbitacearum*. Detached leaf assay showed that zoospores could infect both young and mature leaves. The growth rate, sexual and asexual reproduction, hymexazol resistance and leaf infection of isolate R84 suggested that the virus-like dsRNA infection was in a symptomless manner. This was the first report of dsRNA elements in *Phy. cucurbitacearum* and of *Phy. cucurbitacearum* on para rubber trees.

Introduction

Thailand is the world's largest rubber exporter, accounting for 36.2% of world exports (Workman, 2018). To produce a high rubber yield, rubber trees require a warm temperature (25–28°C), well-distributed rainfall (100–150 d throughout the year) with 2,000–4,000 mm annual precipitation and a high humidity (67–82%) (Priyadarshan, 2003). These optimal conditions for latex production are similar to the climate in southern and eastern Thailand. The most-planted rubber clone in Thailand is RRIM 600, which has been recommended by the Rubber Research Institute of Thailand (RRIT) for its high latex yield (Havanapan et al., 2016). However, this rubber clone is susceptible to various rubber diseases, especially *Phytophthora* spp. causing abnormal leaf fall (ALF) and black stripe disease (Havanapan et al., 2016).

The Peronosporales is an order of oomycetes, which are fungus-like organisms or water molds (Erwin and Ribeiro, 1996). The physiology

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https://doi.org/10.34044/j.anres.2020.54.2.09

and habitat of oomycetes are similar to those of filamentous fungi, but they are more related to brown-algae and diatoms (Erwin and Ribeiro, 1996). Regardless, oomycetes are actively studied by mycologists as the three genera, namely *Phytophthora* (*P.*), *Pythium* (*Py.*) and *Phytopythium* (*Phy.*) are recognized among plant pathologists as being devastating plant pathogens (Baten et al., 2014, Kamoun et al., 2015). The three species have been classified in the family Pythiaceae. Later *Phytophthora* was moved to family Peronosporaceae (Riethmuller et al., 2002). Species of the oomycetes that have been reported to cause diseases in para rubber trees are *P. botryosa, P. capsici*, *P. citrophthora*, *P. meadii*, *P. nicotianae*, *P. palmivora* and *Phy. vexans* (previously named *Py. vexans*), with all six of these species of the genus *Phytophthora* being reported worldwide, while *Phy. vexans* has been mentioned only in China (Jayasuriya et al., 2003; Laohasakul et al., 2017; Zeng et al., 2005). The most common species infecting para rubber trees in Thailand are *P. palmivora* and *P. botryosa* (Johnston, 1989). Species in the Peronosporales produce different types of spores to complete their life cycle. In the asexual stage, sporangia or zoosporangia are produced and germinate through the plant's stomata, releasing motile zoospores, which are responsible for primary infection and disease outbreak in the rainy season. In addition, the chlamydospore, an asexual spore, and the oospore, an sexual spore, are responsible for long term survival under stress conditions (Erwin and Ribeiro, 1996). ALF and black stripe diseases caused by *Phytophthora* may reduce the field latex yield by up to 30–50% (Chee, 1969).

A mycovirus is one that infects fungi and can be commonly found in all major taxonomic groups of fungi. Most mycoviruses have the genomes of double-stranded RNA (dsRNA) or positive single-stranded RNA (ssRNA). Almost all mycoviruses live quietly without interfering with their hosts but some show hypovirulence or hypervirulence (Pearson et al., 2009). Introduction of Cryphonectria hypoviruses in field conditions resulted in the successful control of chestnut disease in Europe that initiated the interest in mycovirus research (Dawe and Nuss, 2001).

RNA viruses have been reported in several species of the Peronosporales. Phytophthora endornavirus 1 (PEV1) was the first virus found infecting *Phytophthora* sp. from Douglas fir in the USA, which was later seen in *P. ramorum* that causes sudden oak death (Hacker et al., 2005, Kozlakidis et al., 2010). Cai et al. (2009) detected four patterns of dsRNA segments in nine *P. infestans* isolates from the USA and Mexico and named them PiRVs 1–4, respectively. The double-stranded RNA element was reported in four species of the genus *Pythium*, namely *Py. butleri*, *Py. irregulare*, *Py. nunn* and *Py. polare*. No virus-like particle (VLP) was found in *Py. butleri* (Moffitt and Lister, 1975). *Py. irregulare* that causes disease in cucumber was detected containing dsRNA both with or without VLP (Klassen et al., 1991), while all Australian isolates with different dsRNA patterns have VLP (Gillings et al., 1993). Pythium nunn virus 1, isolated from *Py. nunn*, is a virus with bipartite dsRNA genome classified in the genus *Ganmmapartitivirus* in the family *Partitiviridae* (Shiba et al., 2018). *Py. polare* strain OPU1176 has three monopartite dsRNA viruses, namely Pythium polare RNA virus 1 (PpRV1) related to the unclassified arthropod toti-like viruses, Pythium polare RNA virus 2 (PpRV2) related to Beihai barnacle virus 15 and Pythium polare bunya-like RNA virus 1 (PpBRV1) (Sasai et al., 2018). However, a mycovirus of the Pythiaceae associated with the para rubber tree has not been reported to date.

This study aimed to isolate dsRNA elements, expected to be a mycovirus of the Pythiaceae that cause ALF disease in rubber trees from plantations in eastern Thailand, to identify the host, and to study the effects of viral infection on the host.

Materials and Methods

Pythiaceae isolation and storage

Leaves were collected from para rubber trees showing ALF symptoms from 15 plantations in eastern Thailand, 7 plantations in Rayong province, 5 plantations in Chanthaburi province and 3 plantations in Trad province. The leaves were kept in plastic bags at 10–15°C and processed within 48 hr. Petioles were cut to 1 cm in length, surface sterilized by soaking in 0.5% hypochlorite for 45 s, washed twice, blotted dry on sterile gauze, transferred onto 5% V8 agar (5%V8A; The Campbell Soup Co.; USA) selective medium (Jeffers and Martin, 1986) and incubated at 25°C in the dark. Colonies with hyaline coenocytic mycelia or with zoosporangia formation were purified on 5%V8A. For short-term storage, agar discs from the edge of a colony grown on 5%V8A were added into sterilized distilled water and kept at 25°C in the dark. The virus-containing isolate, isolate R84, was deposited at the culture collection of the Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand in the Thailand Bioresource Research Center (TBRC) as MSCU 1025.

Double-stranded RNA detection

The methods for isolation of dsRNA were adapted from Das et al. (2014) using cellulose fiber (C6288; Sigma; USA). Double-stranded RNA banding patterns were determined using electrophoresis on 1% agarose gel in a buffer made up of Tris base, acetic acid and ethylenediaminetetraacetic acid (TAE buffer), stained with ethidium bromide and visualized under ultraviolet (UV) illumination.

Nuclease treatment of dsRNA

The RNA elements were treated with DNase I, RNase A or S1 nuclease. Each sample of 200 ng of dsRNA was treated with 1 µg/mL of RNase A (Bio Basic; Canada) in 0.1× SSC (15 mM NaCl, 1.5 mM sodium citrate) and 2× SSC (300 mM NaCl, 30 mM sodium citrate) for treatments as low salt and high salt conditions, respectively, and then incubated at room temperature for 10 min. S1 nuclease 0.1 U (Promega; USA) or DNase I 0.1 U (Bio Basic; Canada) was added in the 20 µL reaction comprising 500 ng of dsRNA and buffers and then incubated at 37ºC for 30 min. The nuclease-treated reactions were cleaned up as described by Das et al. (2014) and determined using electrophoresis on 1% agarose gel in TAE buffer, stained with ethidium bromide and visualized under UV illumination.

Detection of known Phytophthora viruses

A sample of 10 µg of DNase I-treated total nucleic acid was used as a template for cDNA synthesis. Two-step real-time (RT) polymerase chain reaction (PCR) assay (RT-PCR) was performed to detect genes of the Pythiaceae viruses with genome sequences deposited in GenBank (NCBI) consisting of PEV1, PiRV-1, PiRV-3 and PiRV-4. The β-tubulin gene, with and without reverse transcriptase was used as controls. The reaction contained approximately 500 ng of RNA. Random hexamers were used for the first-strand synthesis using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen; USA). The virus specific primers (Table 1) were used for second-strand synthesis with KOD Hot Start DNA Polymerase (Novagen; USA). The virus specific primers were designed to target the RdRp region by aligning with the published sequences of PEV1 (AJ877914.1), PiRV-1 (NC_013220.1), PiRV-3 (JN603241.1) and PiRV-4 (JN400241.1), with PEV1-FRd/PEV1-RRd primers for PEV1, PiRV1-FRd/PiRV1-RRd primers for PiRV1, PiRV3-FRd/PiRV3-RRd primers for PiRV3 and PiRV4-FRd/PiRV4-RRd primers for PiRV4 and the length of amplicons was in the range 400–800 base pairs (bp). Other primer sets were designed or obtained from previous reports and their products were approximately 900–3,000 bp, targeting the entire open reading frames (ORFs) of the viruses, consisting of PiRV1-F/PiRV1-R primers for PiRV1, PiRV3-F/PiRV3-R primers for PiRV3 and PiRV4-F/PiRV4-R primers for PiRV4 and udp-F2/udp-R2 primers targeting 5' of the UGT gene towards the helicase-like region of PEV1. BT5 and BT-Pyt were forward and reverse primers, respectively, for the β-tubulin gene (Table 1). β-Tubulin partial coding sequences of *Py. vexans* (GU133402.1, GU133454.1, GU931700.1, EU080484.1) and *Py. cucurbitacearum* (KJ595460.1) were used for designing the antisense primer (BT-Pyt) of the β-tubulin gene. The reaction mixture of 25 µL comprised 2 µL of the first-strand synthesis, 1× buffer, 1.5 mM $MgSO_4$, 0.2 mM dNTPs (each), 0.2 µM Sense primer, 0.2 µM Anti-Sense primer and 0.02 U/µL of KOD Hot Start DNA Polymerase (Merck; Germany). The reaction conditions for all primer sets were: 2 min at 94 ºC, 40 cycles of 10 s at 94 ºC, 30 s at the lowest melting temperature of each primer set minus 5 ºC (Table 1) and 60 s at 68 ºC. The results were checked by separating 5 µL of DNA products on 1% agarose gel in Tris, borate and ethylenediaminetetraacetic acid (TBE) buffer.

Identification of oomycetes harboring dsRNA elements

Colony patterns and asexual and sexual organs produced by isolate R84 were determined on 5%V8A with or without fungicides and antibiotics, potato dextrose agar (PDA) and black bean agar (Sopee et al., 2012) at 25°C. Sporangia production was induced via a bating technique (involving co-incubation of culture agar plugs with black sesame seeds in sterile natural pond water) and then incubated at 25°C under fluorescent light.

Molecular identification was carried out using ITS1/ITS4 primers (White et al., 1990). The reaction mixture composed of 50 ng of DNA, 1× PCR buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 µM of each primer and 1.25 U of *Taq* polymerase (Apsalagen; Thailand) in 25 µL total volume. Reaction conditions were: 3 min at 94ºC, 30 cycles of 30 s at 94ºC, 30 s at 55ºC and 60 s at 72ºC, and a final incubation for 5 min at 72ºC. The product was subjected to electrophoresis on 1% agarose gel in TBE buffer. The amplicons from ITS1/ITS4 reaction were ligated into a TA cloning vector, RBC TA cloning kit (RBC Bioscience; Taiwan) and transformed into *Escherichia coli* DH5α. Plasmid containing ITS fragments was extracted using a HiYield Plasmid Mini Kit (RBC Bioscience; Taiwan) and sequenced by Macrogen Inc (South Korea). The sequencing results were then subjected to blast analysis with the GenBank database in the National Center for Biotechnology Information (NCBI; USA). Multiple sequences alignment were performed using the Clustal Omega software (Sievers et al., 2011) and a phylogenetic tree was constructed using the Mega X program and the neighbor-joining method with 1,000 bootstrap replications (Kumar et al., 2018).

Table 1 Primers showing sequence, melting temperature (Tm) and reference

Primer	Sequence (5'→3')	Tm (°C)	Reference
BT5	GTATCATGTGCACGTACTCGG	55	Villa et al. (2006)
BT-Pyt	GCCTTGATGTTGTTCGGGAT	55	Current study
PEV1-FRd	GATGGGAACTGGAGACTGCC	58	Current study
PEV1-RRd	ATACCCTTAGCCTGCCACAC	57	Current study
PiRV1-FRd	ACTCTCAGGTGTGGACTG	53	Current study
PiRV1-RRd	CTCAGATTGTCATCGCCAT	52	Current study
PiRV3-FRd	ACAGGCGTCGTTTTTATGGG	55	Current study
PiRV3-RRd	CCGTTCTACCAATATCTCACC	52	Current study
PiRV4-FRd	ACTGAGGTAAAGGCGAAGGC	57	Current study
PiRV4-RRd	CTCAGGCACCGTAGAAGCTC	58	Current study
udp-F2	GTCAGAGCCACTTTCTCGCG	63	Kozlakidis et al. (2010)
udp-R2	CTCCTGCGTAGGTGGAGTAGG	63	Kozlakidis et al. (2010)
PiRV1-F	ATGCAGATAAACGTCGGAGGG	61	Current study
PiRV1-R	TCAAAACATGACTTCAGTCGGG	58	Current study
PiRV3-F	TCTAGCTTTCCAGGTTGCGG	61	Current study
PiRV3-R	ATCCGACAAATGGGTGATACC	58	Current study
PiRV4-F	GTGGAAAGCACGTTTATGCAGG	60	Zhan and Zhu (2016)
PiRV4-R	GTATCTACGCCTCACCCTAC	58	Zhan and Zhu (2016)

Koch's postulates

To determine if the dsRNAs affected the virulence of the host, zoospore suspensions of the dsRNA-containing isolate and a dsRNA-free isolate were inoculated onto detached para rubber leaves. The dsRNA-free strain, *Phy. cucurbitacearum* isolate L69, was isolated from a rubber farm in Rayong province, Thailand (12°54'55.0"N 101°28'52.4"E). Leaves of the RRIM600 variety were collected from an ALF-free organic farm (13°23'19.0"N 101°13'07.8"E) in Chonburi province, Thailand. The leaves were surface sterilized by rinsing with tap water, soaking in 0.5% hypochlorite for 45 s, washing twice with sterile deionized water and then blotting dry on sterile gauze. A sample of 20 mL of zoospore suspension (1×10^4 spores per Petri dish) in sterile deionized water were added to each leaf. The plates were incubated at 25°C under fluorescent light and determined daily for 7 d. On day 7, tissue from the edge of an actively growing lesion was cut and transferred onto 5%V8A. Mycelia from the infected leaves were morphologically identified.

Growth rate and resistance to hymexazol

A 6 mm diameter agar plug of each isolate was removed from the edge of a colony aged 3 d grown on 5%V8A at 25°C. The plug was put on 5%V8A to determine its growth rate or on 5%V8A supplemented with 0 mg/mL, 50 mg/mL, 100 mg/mL, 200 mg/mL, 300 mg/mL or 400 mg/mL hymexazol (3-hydroxy-5-methylisoxazole) to determine fungicide resistance. The plates were incubated at 25°C in the dark and the colony diameter was measured daily for 3 d. The growth rates on 5%V8A and on 5%V8A supplemented with hymexazol of each isolate were calculated from three replicates.

Statistical analysis

Data were analyzed using one-way analysis of variance facilitated by IBM SPSS 22.0 software (IBM; USA). Means were compared using Duncan's multiple range test with multiple comparison corrections and critical value at $p < 0.05$.

Results

Detection of dsRNA virus-like elements in isolate R84

In total, 79 isolates were obtained of Pythiaceae from para rubber leaves showing ALF symptoms, based on sampling from 15 plantations in eastern Thailand. However, only isolate R84 from Chanthaburi province (12°39'27.2"N 101°56'15.7"E) contained three dsRNA segments (approximately 2320 bp, 3750 bp and 8000 bp in length) as shown in Fig. 1. The largest band was more abundant, while bands of the smaller sizes were always fainter, even after multiple subculturing or storage of the agar plugs in water.

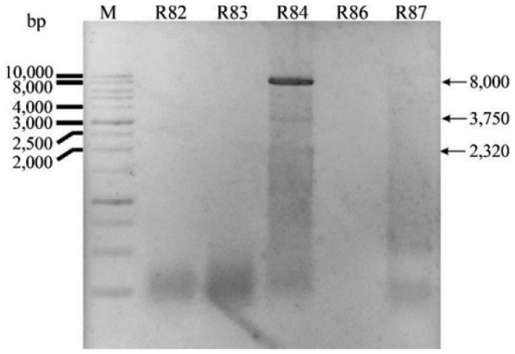

Fig. 1 Cellulose column chromatography purified dsRNA of isolate R84 separated on 1% agarose gel, where M = 1Kb DNA Ladder RTU (GeneDireX; Taiwan) and R82, R83, R86 and R87 are virus free isolates

Analysis of dsRNAs in isolate R84

To confirm the type of nucleic acid in isolate R84, nucleic acid purified by cellulose column chromatography was treated with DNase I, RNase A and S1 nuclease. DNase I digests ssDNA and dsDNA. The results showed that nucleic acid purified using cellulose column chromatography was resistant to DNase I (results not shown). RNase A digests different structures of nucleic acid under different conditions. It cleaves ssRNA, dsRNA and RNA-DNA hybrid in low salt buffer (0–100 mM NaCl), while it cleaves only ssRNA in high salt buffer (0.3 M NaCl). S1 nuclease hydrolyses ssRNA and ssDNA. Fig. 2A and 2B showed that the nucleic acid resisted S1 nuclease and RNase A in high salt buffer digestion which confirmed the dsRNA nature of the viral genome. In Fig. 2A, the dsRNA band of the viral genome of length 2,320 bp disappeared and the dsRNA band of the viral genome of length 3,750 bp was very faint. This could have been the reason that dsRNA reactions were precipitated to remove salt before separating on agarose gel.

The sizes of the three fragments of isolate R84 were in the range 2,980–13,880 bp of known Phytophthora viruses, namely Phytophthora endornavirus 1 (PEV1) and Phytophthora infestans RNA virus 1–4 (PiRV1–4) (Pearson et al., 2009). To determine whether the dsRNA elements in isolate R84 were the same mycoviruses previously reported in Pythiaceae, two-step RT-PCR was performed. Four Pythiaceae viruses with nucleotide sequences available in GenBank were used for primer design in this study consisting of PiRV-1, PiRV-3, PiRV-4 and PEV1. Primers for the first two viruses were targeted to the entire ORF of RdRP gene of the viruses, while primers for the last two viruses were from Zhan et al. (2016) targeting the entire ORF of the PiRV-4 genome and from Kozlakidis et al. (2010) targeting the UGT gene of PEV1. The predicted amplicon lengths were approximately 940 bp for PEV1 and approximately 3,000 bp for all PiRVs. DNase I-treated total RNA was used as a template. The quality of the RNA was confirmed with the presence of PCR product from β-tubulin primers. The results in Fig. 3A showed that there was no band in any of the Pythiaceae virus reactions. However, the absence of PCR products from the first primer sets could mean that the RT-PCR was not effective enough to amplify a large fragment.

An RT-PCR experiment was performed later with primers targeting the RdRP region with the fragment size closer to the size of the β-tubulin control. The expected sizes of products were approximately 590 bp, 480 bp, 720 bp and 520 bp for PEV1, PiRV-1, PiRV-3 and PiRV-4, respectively. There was no band with the predicted size of the viral targets present in any primer set (Fig. 3B). The non-specific bands found in PiRV-4 were between 300–400 bp and 650–850 bp and were different from expected sizes. Self-priming tests were performed to confirm that the non-specific bands of the PiRV-4 reaction were not from the viral genome (results not shown). The results suggested that the dsRNA segments hidden in isolate R84 were not any of the reported Phytophthora viruses.

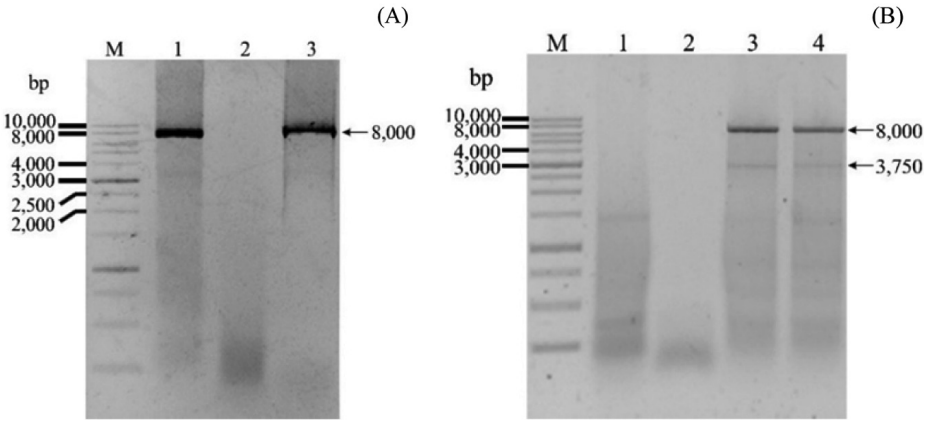

Fig. 2 RNase A and S1 nuclease treatment of cellulose column chromatography purified nucleic acid of isolate R84, where M = 1Kb DNA Ladder RTU (GeneDireX; Taiwan): (A) nucleic acid treated with RNase A, where Lane 1 = no treatment control; Lane 2 = RNase A treatment under low salt condition; Lane 3 = treatment under high salt condition; (B) nucleic acid treated with S1 nuclease, where Lane 1 = total RNA of *Phytopthora botryosa*; Lane 2 = total RNA of *P. botryosa* with S1 nuclease treatment; Lane 3 = cellulose column chromatography purified nucleic acid of isolate R84 without S1 nuclease; Lane 4 = cellulose column chromatography purified nucleic acid of isolate R84 treated with S1 nuclease

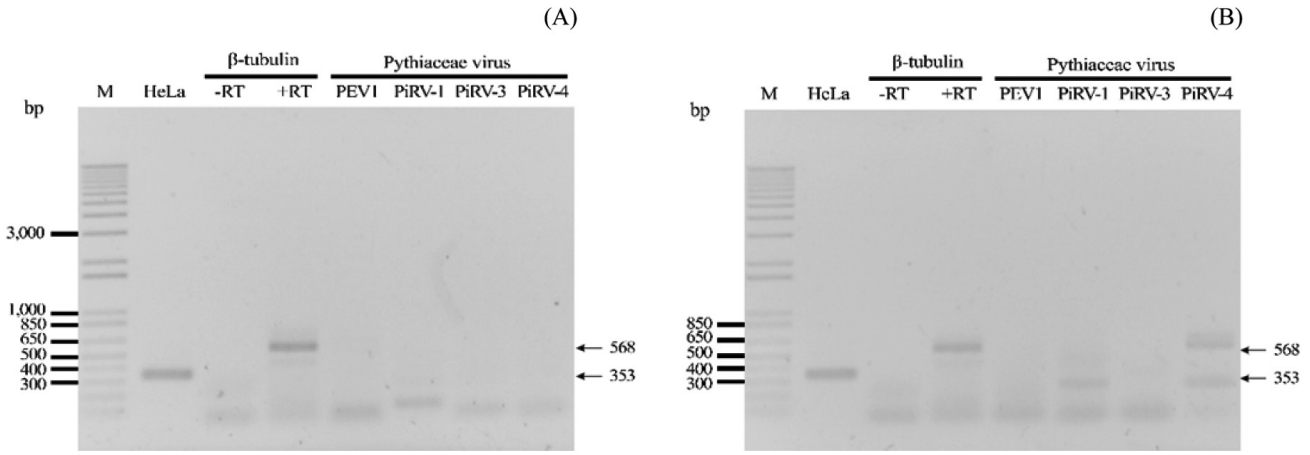

Fig. 3 Detection of reported Phytophthora viruses from total RNA of isolate R84, where M = 1Kb Plus DNA Ladder (Invitrogen, USA): (A) two-step real-time polymerase chain reaction (RT-PCR) using primer sets for the large amplicons (UGT-helicase region of PEV1 and the entire ORF of RdRP gene of PiRVs); (B) two-step RT-PCR using primers targeting partial RdRP region of PEV1 and PiRVs

Identification of isolate R84

The morphological characteristics of isolate R84 were observed on various agar media. Isolate R84 displayed variability in colony patterns on different media as shown in Fig. 4. Sporangia of isolate R84 were only produced in water. Reproductive structures were not detected on various agar media even after 1 mth of observation on 5%V8A or black bean agar which are recommended for sporulation (Jeffers and Martin, 1986, Sopee et al., 2012). Sporangia produced by isolate R84 were varied in shape and papillation. The majority of zoosporangia were globose without a papillum (Fig. 5A). Other shapes were infrequently found such as ovoid and pyriform, with or without a papillum (Fig. 5B, 5C and 5E–5G). Zoospore discharge via vesicle formation could be seen (Fig. 5E and 5F). Isolate R84 was noted as a homothallic species because sexual structures were found in the pure culture, showing a smooth-walled oogonium with a paragynous antheridium (Fig. 5D).

DNA of isolate R84 was amplified using conventional PCR with the fungal and oomycete universal primers, ITS1 and ITS4, with the binding site located in the conserved non-coding region between 18S, 5.8S and 28S of ribosomal RNA genes (White et al., 1990). The sequencing result of the PCR product amplified from the ITS1/ITS4 primers of isolate R84 was 904 bp in length (Accession No. MH243441) and had high similarity to a region between the 18S rRNA gene, ITS1, the 5.8S rRNA gene and ITS2 and the 28S rRNA gene of *Py. cucurbitacearum* isolate 1241Pc (HQ237483.1; identity, 902/904 (99%); query cover, 100%; e-value, 0.0). Evolutionary analyses of isolate R84 with *Phytopythium* (formerly *Pythium* clade K), *Pythium* clade A–J and *Phytophthora* were performed based on the nucleotide sequence of ITS1/ITS4 products. The phylogram in Fig. 6 showed that isolate R84 was closely related to *Py. cucurbitacearum* isolate CBS748.96 (AY598667) and was clustered in the same group with *Phy. vexans* which was more closely related to the genus *Phytophthora* than *Pythium*.

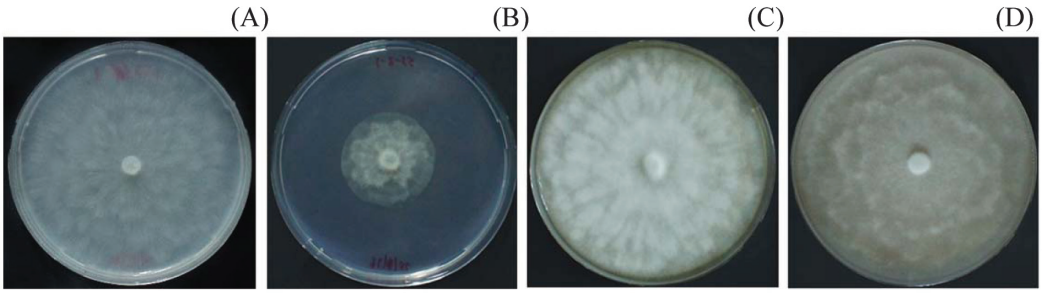

Fig. 4 Colony morphology of isolate R84 on various media: (A) stellate pattern on 5%V8A; (B) petallate pattern on 5%V8A supplemented with antibiotics and fungicides; (C) dense cotton-stellate pattern on potato dextrose agar; (D) petalloid-stellate pattern on black bean agar.

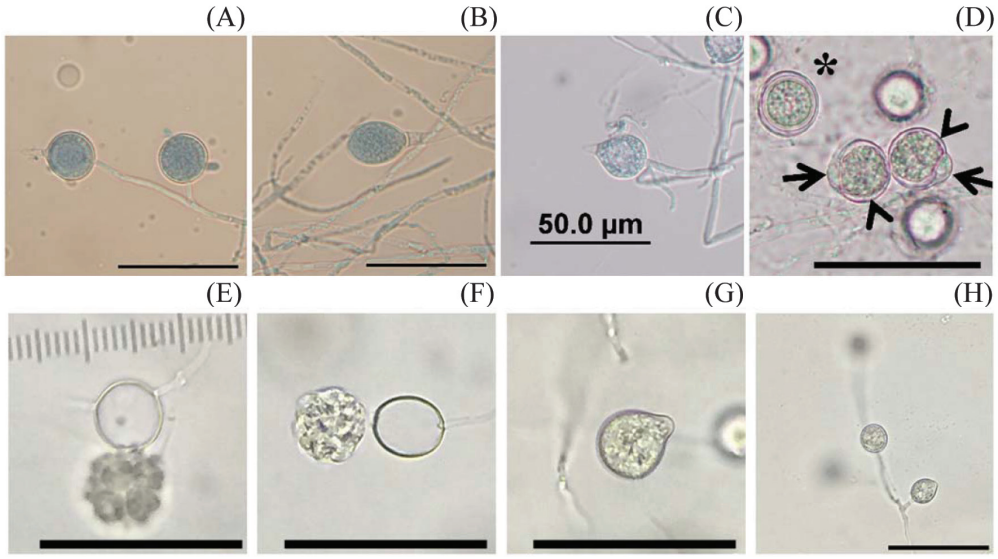

Fig. 5 Asexual structures and sexual structures of isolate R84 induced using baiting technique after 4 d incubation at 25°C, showing sporangia of isolate R84: (A) nonpapillate globose; (B) nonpapillate ovoid; (C and G) papillate pyriform; (E) discharged globose intercalary; (F) discharged ovoid terminal; (H) formed on simple sympodial sporangiophore; (D) sexual structures of isolate R84, with oogonia (arrowheads) with paragynous antheridia (arrows) and mature plerotic oogonium with a thick-walled oospore (*) and scale bar = 50 µm

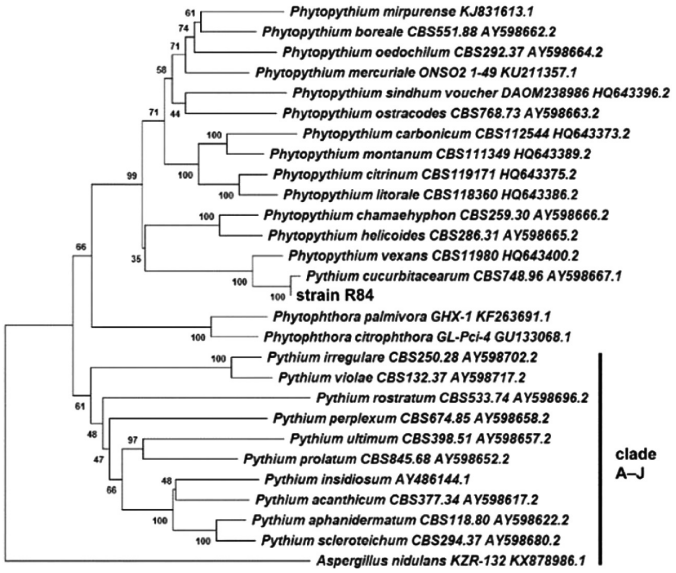

Fig. 6 Phylogram based on the internal transcribed spacer rDNA region of isolate R84, *Pythium* clade A-J, *Phytopythium* spp., *Phytophthora* spp. and *Aspergillus nidulans*, as an out group

Koch's postulates of isolate R84 on detached para rubber leaf

Many isolates with morphological characteristics similar to isolate R84 were consistently isolated along with other *Phytophthora* spp. from necrotic petioles. Strain L69 isolated from Rayong province, Thailand (12°54'55.0"N 101°28'52.4"E) was molecularly identified as *Phytopythium cucurbitacearum* using ITS region sequencing and was devoid of dsRNA elements (results not shown). Suspension of 1×10^4 zoospores was added to surface-sterilized para rubber leaf and incubated for 7 d. On young leaves, lesions were observed 1.5 d after inoculation, while control leaves remained symptomless (Fig. 7). On mature leaves, lesions could be seen on day 4 from both isolates (results not shown). The pathogens were re-isolated from infected leaves and morphologically identified, thus fulfilling Koch's postulates.

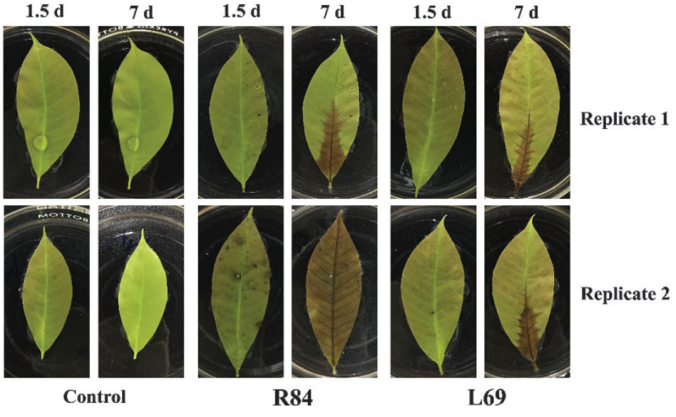

Fig. 7 Young para rubber leaves infected with zoospores of isolate R84 and isolate L69 over time

Growth rate and hymexazol resistance of Phy. cucurbitacearum

Growth on 5%V8A and hymexazol resistance were investigated to determine whether the presence of the virus altered the physiology of isolate R84. Since attempts to generate a virus-free strain from isolate R84 were not successful, six isolates of *Phy. cucurbitacearum* from different para rubber tree plantations were used. The results showed that the growth rate and hymexazol sensitivity of isolate R84 were not significantly different from other *Phy. cucurbitacearum* isolates (Figs. 8 and 9).

Discussion

Mycoviruses have been reported from across fungal phyla; however, so only a few viruses have been studied in the family Pythiaceae. Most members of this family are notorious plant pathogens with worldwide distribution and a broad host range (Erwin and Ribeiro, 1996). This inspired the current study to look for a virus in para rubber tree pathogenic oomycetes in eastern Thailand. From 15 para rubber plantations in Rayong province, Chanthaburi province, and Trad province, only 1 in 79 isolates (1.25%) had virus-like dsRNA. Cai et al. (2009) found virus in 9 isolates of *P. infestans* from 22 isolates (41%) screened. In addition, 14 of 39 isolates (36%) of *Py. polare* from the Arctic have virus-like dsRNA (Sasai et al., 2018). This suggested that the virus is not ubiquitous among para rubber-tree-pathogenic oomycetes.

Most reported mycoviruses have dsRNA genomes, a few have ssRNA genomes and only two isolates have been reported with the DNA genome (Dawe and Kuhn, 1983, Yu et al., 2010). The genome of dsRNA mycoviruses is present in monopartite or multipartite dsRNA (Shiba et al., 2018). Infection of a single fungal host with multiple dsRNA viruses has also been reported (Sasai et al., 2018). Many RNA mycoviruses were capsidless (Klassen et al., 1991; Moffitt and Lister, 1975). The current report showed that *Phy. cucurbitacearum* isolate

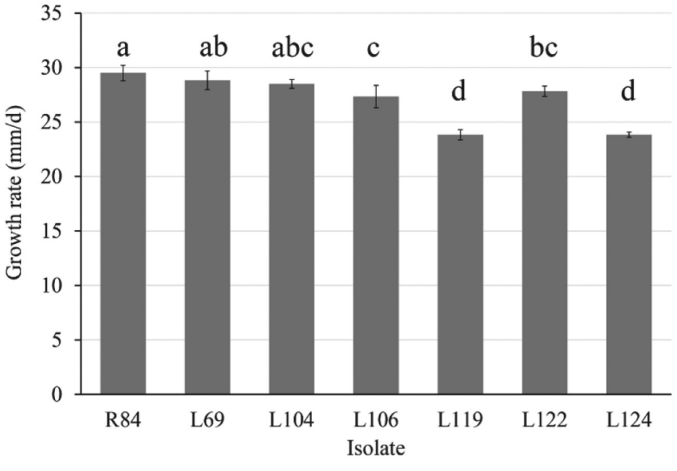

Fig. 8 Growth rates of *Phytopythium cucurbitacearum* isolated from para rubber leaves with abnormal leaf fall on 5%V8A at 25°C and different lowercase letters above a column indicate significant ($p < 0.05$) differences and error bar = ±SD

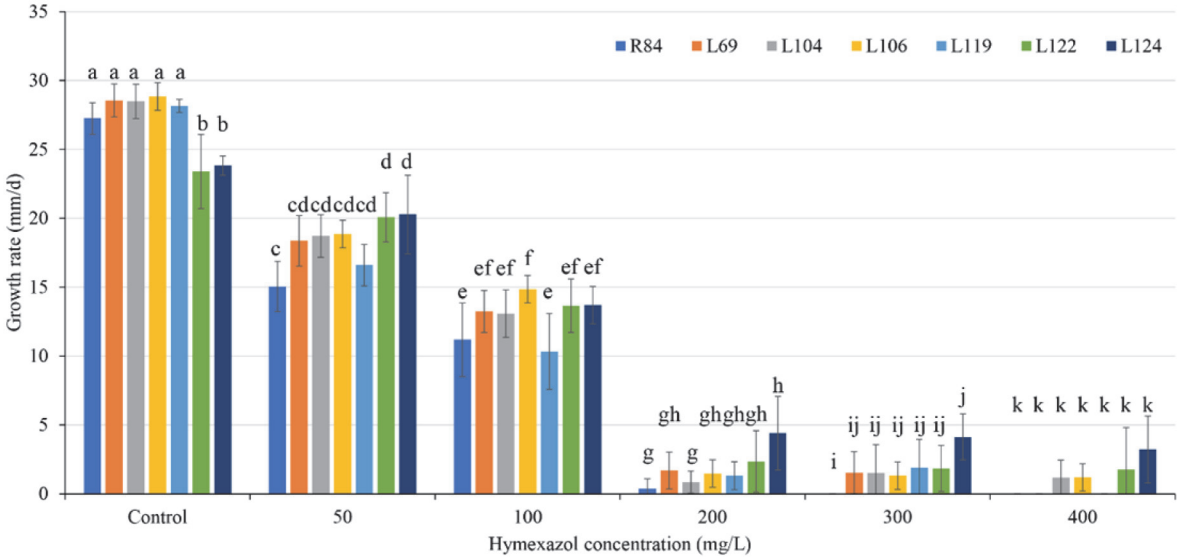

Fig. 9 Growth rates of different *Phytopythium cucurbitacearum* strains isolated from para rubber leaves with abnormal leaf fall on 5%V8A supplemented with different concentrations of hymexazol at 25°C and different lowercase letters above a column indicate significant ($p < 0.05$) differences and error bar = ±SD

R84 had three dsRNA segments. In addition, no virion particles were observed by transmission electron microscopic observation (results not shown). As a result, dsRNA in isolate R84 could be the true genome or the replicative form and could be composed of more than one type of virus. Genome sequencing and identification of coding genes would clarify these uncertainties.

To determine whether viral infection affects the host, an isogenic strain is usually created; however, the current study was unable to generate a virus-cured strain. Six *Phy. cucurbitacearum* isolates were used in experiments to reduce intraspecific variability. The fact that isolate R84 and six other virus-free isolates were indistinguishable in colony morphology, growth rate, ability to produce reproductive structures and hymexazol resistance of *Phy. cucurbitacearum* suggested that the virus of isolate R84 is cryptic. Up to 50 mg/L hymexazol is usually added into *Phytophthora* selective media to inhibit fungi, and many *Pythium* spp. (Jeffers and Martin, 1986); however, seven isolates of *Phy. cucurbitacearum* in the current study were not totally inhibited by 50 µg/ml of hymexazol. Santoso et al. (2015) used PDA supplemented with 50 mg/L hymexazol to isolate pathogens causing tree-decline disease from durian and recovered *Phytophthora* and *Phytopythium* spp. including *Phy. vexans* and *Phy. cucurbitacearum*.

Phy. cucurbitacearum, previously named *Py. cucurbitacearum*, was first reported in 1941, causing damping-off disease in cucumber in Japan (Takimoto, 1941). It had been reported associated with point gourd in India, common bean in Rwanda, leaves submerged in sea water near mangrove habitat in China, and durian in Indonesia (Chaudhuri, 1975, Ho et al., 2012, Nzungize et al., 2011, Santoso et al., 2015), suggesting world-wide distribution. *Py. cucurbitacearum* was mentioned as closely related to *Py. vexans* by sharing various identical characteristics (Santoso et al., 2015). The sporangial morphology of the clade K is varied; globose, ovoid, obpyriform or pyriform, and may be terminal, intercalary or filamentous inflated sporangia, which are only found in *Py. indigoferae* (Baten et al., 2014, Levesque and de Cock, 2004). Levesque and de Cock (2004) also noted that the sporangia of many species in clade K have a papillum. Similarly, sporangia illustrated in the current report and by Santoso et al. (2015) showed that some of the sporangia were papillate. The mode of zoospore discharge in *Pythium* and *Phytopythium* is by moving the undifferentiated mass of protoplasm which is differentiated later into zoospores through a formed discharge tube and vesicle at the tip of the sporangium. For sexual structures, *Py. cucurbitacearum* was described as homothallic, produced smooth oogonia with laterally attached bell-shaped antheridia (paragynous) (Baten et al., 2014, Levesque and de Cock, 2004). Zoospore discharge and the sexual structures of isolate R84 showed similarity to those of previous reports. Levesque and de Cock (2004) grouped *Py. vexans* and *Py.cucurbitacearum* in *Pythium* clade K along with *Py. boreale*, *Py. ostracodes*, *Py. oedochilum*, *Py. chamaehyphon*, *Py. helicoides* and *Py. indigoferae* based on the similarity of ITS sequences. The distinct group of *Pythium* clade K was confirmed in a phylogenetic tree reconstructed by Villa et al. (2006). The clade was later established as a new genus named *Phytopythium* by Bala et al. (2010). De Cock et al. (2015) described nomenclature changing from *Pythium* clade K to the genus *Phytopythium*.

The current report is the first of virus-like dsRNA in *Phy. cucurbitacearum* causing disease in the para rubber tree. In addition, *Phy. cucurbitacearum* has never been reported in Thailand. It was found associated with durian tree-decline in Indonesia (Santoso et al., 2015). In eastern Thailand, where para rubber tree plantations intermingle with durian farms, the presence of *Phy. cucurbitacearum* in the three studied provinces of eastern Thailand could negatively affect the durian industry in the region and farmers there should be informed of this new pathogen.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors thank Assist. Professor Dr Pongtharin Lotrakul for his enlightening suggestions. This research was supported by a New Researcher Scholarship of CSTS, MOST from the Coordinating Center for Thai Government Science and Technology Scholarship Students (CSTS), National Science and Technology Development Agency (NSTDA) and by the Asahi Glass Foundation, Japan. The first author was supported by a Scholarship from the Graduate School, Chulalongkorn University (CU) to commemorate the 72nd anniversary of his Majesty King Bhumibala Aduladeja and by a Research Assistant Scholarship from the CU Graduate School.

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