รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษายีนที่ผลิตอิลิซิตินของเชื้อ *Phytophthora palmivora* ซึ่งเป็นเชื้อก่อโรคในยางพารา และความสัมพันธ์กับความรุนแรงของเชื้อไอโซเลทต่าง ๆ

Elicitin of *Phytophthora palmivora*, a pathogen of rubber plants: cloning, expression and its role in virulence of variant isolates

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มีนาคม 2557

สัญญาเลขที่ MRG5480104

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

บทคัดย่อ

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ชื่อโครงการ : การศึกษายีนที่ผลิตอิลิซิตินของเชื้อ *Phytophthora palmivora* ซึ่งเป็นเชื้อก่อโรคในยางพารา และความสัมพันธ์กับความรุนแรงของเชื้อไอโซเลทต่าง ๆ

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ไฟทอปทอร่า (*Phytophthora* spp.) เป็นเชื้อก่อโรคซึ่งสร้างความเสียหายต่อพืชหลายชนิดรวมถึงระบบนิเวศ ในประเทศไทยเชื้อดังกล่าวก่อให้เกิดโรค และทำลายพืชเศรษฐกิจหลายชนิดรวมทั้งยางพารา สายพันธุ์ที่ทำให้ยางพาราเป็นโรคเส้นดำ ผลเน่าและใบร่วง ได้แก่ *P. palmivora* เชื้อนี้เข้าทำลายทางรอยเปิดกรีดสำหรับเก็บน้ำยาง จึงทำให้ผลผลิตลดลง

การวิจัยนี้มีวัตถุประสงค์เพื่อติดตามการแพร่กระจายของเชื้อ *Phytophthora* spp. และ *P. palmivora* ในพื้นที่ปลูกยางพาราใน 3 จังหวัดทางภาคใต้ของประเทศไทย ได้แก่ สงขลา สตูล และนครศรีธรรมราช ในระหว่างปี พ.ศ. 2554–2555 และคัดแยกเชื้อดังกล่าวจากตัวอย่างพืช โดยการคัดแยกเชื้อด้วยการเลี้ยงบนอาหารเฉพาะ และตรวจสอบยืนยันด้วยวิธี polymerase chain reaction (PCR-based technique) ซึ่งในการวิจัยครั้งนี้ได้พัฒนาวิธี single round semi-nested multiplex PCR สำหรับการตรวจวินิจฉัยเชื้อ *Phytophthora* spp. และ *P. palmivora* พร้อมกันในการทำ PCR เพียงครั้งเดียว ในระยะเวลาดังกล่าว ผู้วิจัยไม่สามารถแยกเชื้อ *Phytophthora* spp. และ *P. palmivora* ได้เลย พบว่าเชื้อราที่แยกได้ส่วนใหญ่ ได้แก่ *Colletotrichum gloeosporioides*, *Fusarium* sp., *Cercophora* sp., *Trichoderma* sp. และ *Podospora* sp.

การศึกษายีนที่ผลิตอิลิซิตินในเชื้อ *P. palmivora* (*Ppal_Eli*) พบว่า ความยาวของลำดับนิวคลีโอไทด์ของทั้งหมด (full length) ของ cDNA ของยีนนี้ มีขนาด 485 คู่เบส โดยมี open reading frame (ORF) ขนาด 357 คู่เบส ซึ่งถอดรหัสได้เป็นสายโพลีเปปไทด์ (Ppal_Eli) ที่มีกรดอะมิโน 118 หน่วย มีมวลโมเลกุล 12208.90 ดาลตัน และมีค่า pI เป็น 4.68 เมื่อเปรียบเทียบลำดับกรดอะมิโนของ Ppal_Eli กับอิลิซิตินอื่น ๆ ที่ผลิตจากเชื้อ *Phytophthora* spp. พบว่ามีความเหมือนอยู่ในช่วง 70–90% ภายในโมเลกุล Ppal_Eli มีลำดับกรดอะมิโนที่เป็น signal peptide ขนาด 20 หน่วย พบอิลิซิตินโดเมน (elicitin domain) จำนวน 1 โดเมน พบพันธะไดซัลไฟด์ Cys3–Cys71, Cys27–Cys56 และ Cy51–Cys95 และมีกรดอะมิโนวาลีน (Valine, Val) ตรงตำแหน่งที่ 13 ซึ่งเป็นลักษณะเฉพาะของอิลิซิตินในกลุ่ม acidic alpha

elicitin นอกจากนี้ จากผลการศึกษาความสัมพันธ์และสายวิวัฒนาการ (phylogenetic tree) ของลำดับนิวคลีโอไทด์และลำดับกรดอะมิโน ทำให้สามารถจัดแบ่งอิลิซิตินที่ผลิตโดยเชื้อ *Phytophthora* spp. ได้ 3 กลุ่ม ได้แก่ Class I Class II และ Class III ซึ่งพบว่าอิลิซิติน Ppal_Eli ที่ศึกษานี้มีความเหมือนกับอิลิซิตินที่จัดอยู่ในกลุ่ม Class I

ในการศึกษาความสัมพันธ์ของยีนอิลิซิตินกับความรุนแรงของเชื้อ *P. palmivora* ได้ทำการแยกไอโซเลทต่าง ๆ จากหัวเชื้อ *P. palmivora* สายพันธุ์ KBNM 9 ที่เก็บไว้ พบว่าสามารถแยกได้จำนวน 4 ไอโซเลท คือ Ppal1 Ppal3 Ppal4 และ Ppal5 ที่มีความแตกต่างกันทั้งในแง่การเจริญเติบโตบนอาหารเลี้ยงเชื้อ (แบ่งได้เป็นกลุ่ม very slow-growing slow-growing และ fast-growing) การผลิตสปอร์แรงเจียม (sporangium production) การปลดปล่อยซูโอสปอร์ (zoospore releasing) เมื่อนำเส้นใย (mycelium) ของ *P. palmivora* ทั้ง 4 ไอโซเลทไปทดสอบวางบนใบยางพารา แล้วติดตามรอยแผล necrosis ที่เกิดบนใบยางหลังวางเชื้อเป็นเวลา 24 ชั่วโมง รวมทั้งการแสดงออกของยีนอิลิซิตินทั้งจากเชื้อที่โตบนอาหาร (*in vitro*) และจากเชื้อที่เจริญเติบโตได้บนใบยางพารา (*in planta*) พบว่า ไอโซเลท Ppal3 และ Ppal4 ทำให้เกิด necrosis ขนาดใหญ่ที่สุด ในขณะที่ Ppal1 และ Ppal5 ทำให้เกิด necrosis ขนาดเล็กใกล้เคียงกัน สอดคล้องกับระดับการแสดงออกของยีนอิลิซิตินแบบ *in planta* ซึ่งแสดงให้เห็นถึงแนวโน้มความสัมพันธ์ของการแสดงออกของยีนอิลิซิตินแบบ *in planta* กับความสามารถของเชื้อในการบุกรุกพืช แต่พบว่าการแสดงออกของยีนอิลิซิตินแบบ *in vitro* มีแนวโน้มที่จะสัมพันธ์กับการผลิตสปอร์แรงเจียมและการปลดปล่อยซูโอสปอร์ของเชื้อ *P. palmivora* อย่างไรก็ตาม ยังไม่สามารถสรุปหน้าที่หรือบทบาทของอิลิซิตินต่อความรุนแรงของ *P. palmivora* ได้อย่างชัดเจน ทั้งนี้ต้องอาศัยผลจากการทดลองเพิ่มเติมเพื่อสนับสนุนหรือยืนยันความสัมพันธ์ของยีนดังกล่าวกับความรุนแรงของเชื้อ เช่น ติดตามการแสดงออกของยีนนี้ในระยะเวลาต่าง ๆ ควบคู่กับการเจริญเติบโตของเชื้อ *P. palmivora* ทั้งในอาหารเลี้ยงเชื้อและในใบยางพารา นอกจากนี้ ควรศึกษาปัจจัยอื่น ๆ ที่เชื้อผลิตขึ้นนอกเหนือจากอิลิซิตินที่จะส่งผลให้เชื้อมีความรุนแรงต่างกันได้ด้วย

คำหลัก : Elicitin, *Hevea brasiliensis*, *Phytophthora palmivora*, Single round semi-nested multiplex PCR, Virulence

Abstract

Project Code : MRG5480104

Project Title : Elicitin of *Phytophthora palmivora*, a pathogen of rubber plants: cloning, expression and its role in virulence of variant isolates

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Project Period : 2 years

Phytophthora spp., the plant pathogenic oomycetes have devastating effects on crops and natural ecosystems worldwide. In Thailand, these pathogens cause many diseases on economic crops. Among *Phytophthora* spp., *P. palmivora* is an important pathogenic species of the rubber plant (*Hevea brasiliensis*) causing black stripe, green pod rot and abnormal leaf fall. It also attacks the tapping surface that results in reduced latex production and large economic losses.

This study aimed to elucidate the distribution of *Phytophthora* spp. and *P. palmivora* in rubber plantations in 3 provinces in southern Thailand, Songhkla, Satun and Nakhon Si Thammarat, between 2011 and 2012. These pathogens were isolated from plant specimens by suing selective media and confirmed by polymerase chain reaction, PCR-based technique. The technique named single round semi-nested multiplex PCR was developed to simultaneously detect and identify *Phytophthora* spp. and *P. palmivora* in a unique reaction. Unfortunately during the survey period, neither *Phytophthora* spp. nor *P. palmivora* could be isolated. Most of the isolated fungi were *Colletotrichum gloeosporioides*, *Fusarium* sp., *Cercophora* sp., *Trichoderma* sp. and *Podospora* sp.

The gene encoding elicitin produced in *P. palmivora* (*Ppal_Eli*) was cloned and characterized. The full length of *Ppal_Eli* cDNA was 485 bp in length. *Ppal_Eli* cDNA contained an open reading frame (ORF) of 357 bp encoding a polypeptide of 118 amino acids with a calculated molecular mass of 12208.90 Da and pI value of 4.68. The comparison of the deduced amino acid sequence of Ppal_Eli protein with elicitins produced in other *Phytophthora* spp. revealed an overall %identity in a range of 70–90%. Ppal_Eli protein consisted 1 predicted signal peptide of 20 amino acids and an elicitin domain. Disulfide tool predicted the cysteine disulfide bonding state and connectivity as follows: Cys3–Cys71, Cys27–Cys56 and Cy51–Cys95, moreover, the N terminal portion of deduced amino acid sequence has Val at position 13 which are typical characteristics of the class acidic alpha elicitins. To study the evolutionary relationship of elicitin, phylogenetic trees were constructed based on both nucleotide sequence and amino acid sequence of the elicitin from *P. palmivora*, and those of elicitin from other *Phytophthora* species. The constructed trees were used to base trees with the 3 major clusters including Class I, Class II and Class III while our interested Pal_Eli were presented in Class I.

In order to elucidate the relationship between elicitin gene and the virulence of *P. palmivora*, different isolates was isolated from cultured *P. palmivora* KBNM 9 lineage. Ppal1, Ppal3, Pal4 and Pal5 was collected and maintained for further studies. These 4 isolates showed many different characters including growth rate on medium, which could be classified into 3 groups of very slow-growing, slow-growing and fast-growing, sporangium production and zoospore releasing. The detached rubber leaves were inoculated with mycelium of all isolates for 24 h and necroses were then observed. The elicitin gene expression in cultured mycelium (*in vitro*) as well as that in the pathogen growing in infected leaf tissues (*in planta*) were also measured. The results showed that *in planta Ppal_Eli* gene expressed in high level in Ppal3 and Ppal4, which induced large necrosis, while it was lower in Ppal1 and Ppal5 corresponding to the smaller necrotic lesions. The evidence suggested the trend of positively relation between invasion potency into host plant and *in planta* elicitin gene expression. Conversely, *in vitro* elicitin gene expression was seemed to be involved in the sporangium production and zoospore releasing. Therefore, based on our data it could not conclude for the precise role or involvement of elicitin in pathogen virulence or virulent pathogenecity. To gain more insight in this controversial issue, the kinetics of elicitin gene expression during pathogen growth in medium or in host plant should be established. In addition, the determinations of any additional factors that may directly influence elicitin activity or pathogen virulence are needed in further studies.

Keywords : Elicitin, *Hevea brasiliensis*, *Phytophthora palmivora*, Single round semi-nested multiplex PCR, Virulence

Executive Summary

1. Research Significance and Background

Hevea brasiliensis (Wild.) Muell.-Arg. or the rubber plant is an economically important crop in Thailand, the latex of which and its products are major Thai export incomes. However, one consequence of frequent tapping is that infection can occur at the wounds of the tapping sites. This problem is common in the southern part of Thailand where the humidity and temperature is high and conducive for pathogen growth. In this area, RRIM600 is the commonly plantation cultivar because of its high-yield of latex, nonetheless, this cultivar is highly susceptible to *Phythophthora* pathogens.

Phytophthora diseases cause widespread economic and environmental losses worldwide. *Phytophthora* leaf disease caused by members of the oomycete genera *Phytophthora* are the most important disease of the rubber tree. *P. palmivora*, *P. botryosa*, *P. heveae*, *P. meadii* and *P. parasitica* are described as pathogens of the rubber tree. In the south of Thailand *P. palmivora* and *P. botryosa* are the most frequently isolated pathogens that cause black stripe, green pod rot and abnormal leaf fall (Butler, 1996; Erwin and Ribeiro, 1996). *P. palmivora* attacks the petioles causing mature leaves to fall prematurely and also attacks the tapping surface resulting in reduced latex production.

P. palmivora is reported to be an important pathogen of several plant crops in Thailand; however, the method to identify and diagnose this pathogen is not yet developed. Additionally the data of the distribution and dispersion of *Phytophthora* spp. and in particular *P. palmivora*, as the causing agent of leaf fall and black stripe of rubber plants has been investigated since 2004 (Sdoodee, 2004), evidence on its distribution recently remains rare. This research therefore aims to study on the distribution of the *Phytophthora* genus and *P. palmivora* in southern Thailand covering the important rubber plantations in Songkhla, Nakhorn Sri Thammarat and Phang Nga province. The PCR-based method using the specific primers for the internal transcribed spacer (ITS) regions of their nuclear ribosomal DNA (rDNA) repeat region towards *Phytophthora* genus and *P. palmivora*, respectively, will be performed for identification of the pathogen. *P. palmovora* isolated from different specimens and different areas will be collected and screened for their virulence on detached rubber leaves. Moreover, the gene encoding a pathogen elicitor, elicitin; a superfamily of 10 kDa extracellular proteins, of *P. palmivora* will be investigated (for its full sequence) and its expression level will also be measured in these same isolates *in vitro* and examined for possible correlations with virulence. Altogether, these findings will indicate the areas containing the virulent strain of *P. palmivora* and this will help to lead to further investigate the appropriate disease management and crop protection.

2. Objectives

Surveying in the rubber plantations located in southern Thailand in order to;

1) Elucidate the distribution of *Phytophthora* spp. and *P. palmivora*.
2) Isolate and collect the different strains of *P. palmivora* as a cultural collection.
3) Address the biological function of elicitin as a virulence factor of *P. palmivora* involved in pathogen infection.
4) Comparative analyze the virulence of the isolated *P. palmivora* strains.

3. Research Methodology

1) Survey and collection of *Phytophthora* spp.

To assess the distribution of *Phytophthora* spp. and *P. palmivora* in southern Thailand, the field survey will be carried on in the rubber plantations located in 3 provinces in which large number of rubber trees is planted. Naturally infected tissues exhibiting *Phytophthora* diseases; black stripe, stem canker and abnormal leaf fall will be used for the screening of different strains of *Phytophthora* spp. Isolation from collected samples will be assayed for the presence of *Phytophthora* spp. and *P. palmivora*.

Small pieces of diseased plant tissue will be plated directly onto a selective media for *Phytophthora* spp., Potato Dextrose Agar (PDA) containing antibiotics, and incubated at room temperature. Hyphal tips will be cut from the edges of growing colonies and subcultured onto a new PDA and clarified V_8 juice agar for further study and storage. Isolates will be separated and divided into groups based on their visual colony morphology and their zoospore morphology under the microscope. The isolates that are considered to be *Phytophthora* spp. by virtue of their morphology considered being or similar to *Phytophthora* spp. will be transferred to grow in PDB for more detailed study.

2) Determination of unknown *Phytophthora* spp. and *P. palmivora*

To verify whether the selected isolates belong to the *Phytophthora* genus, genomic DNA will be extracted from mycelia using the DNeasy Plant Mini Kit (Qiagen) followed by PCR using the primers specific to genus *Phytophthora* (Phy1s/Phy2a) according to Tsai *et al*. (2006). These oligonucleotide primers have been designed based on the sequences of 28S rRNA and ITS1-5.8S rRNA-ITS2 from a variety of *Phytophthora* spp. PCR amplified products will be analyzed by 1.5% agarose gel electrophoresis in 1× TAE. The fragment sizes will be estimated by comparison with a 100 bp DNA ladder (Vivantis). PCR products exhibiting positive result, showing the band with expected size, will be purified and directly sequenced (ABI Biosystems), to confirm that they are the oomycete *Phytophthora* and to identify the species of the isolates. In addition, the obtained PCR product of each isolate will be used as template for the PCR conducted with the species-specific primer pair which has been tested for its specificity to *P. palmivora* (Pal1s/Pal2a) (Tsai *et al*., 2006). Altogether, these will indicate which isolates represent *Phytophthora* spp. and *P. palmivora* and then evaluate the frequency of these pathogens which may cause the diseases found in the selected locations.

Isolates of *P. palmivora* will be used for further study on elicitin gene expression and comparable virulence on rubber leaf.

3) Cloning of full-length cDNA of the elicitin gene of *P. palmivora*

Total RNA will be extracted from the mycelia of *P. palmivora* isolate using the RNeasy Plant Mini Kit (Qiagen) and first-strand cDNAs will be consequently synthesized by the action of the enzyme SuperScript™ III Reverse Transcriptase (Invitrogen). The degenerate primers designed from conserved sequences of the elicitin genes of *Phytophthora* spp. published in the NCBI website (http://www.ncbi.nlm.nih.gov/) and the primer designed from nucleotide sequence of a partial elicitin gene of *P. palmivora* that has been submitted to GenBank (Accession no. AY206986) will be used for RT-PCR. The PCR amplified products will be purified and directly sequenced. This gained nucleotide sequence will then be used for designing the primers for the 5' and 3' rapid amplification of cDNA ends (RACE). The RACE-PCR product will be cloned into the pGEM-T

Easy Vector (Promega) and transformed into *Escherichia coli* TOP10 cells, according to the manufacturer's instructions. Plasmids will be extracted and sequenced. The specific primer for determining the expression level of the elicitin mRNA will be designed from the obtained full sequence, and then the PCR will be carried out to evaluate the virulence comparatively.

Comparative and bioinformatic analyses of the obtained full-length of the elicitin gene will be carried out online at the NCBI website. In addition, the nucleotide sequences, deduced amino acid sequences, and ORF encoded the elicitin gene of *P. palmivora* will be analyzed. The sequence comparison will also be conducted through the NCBI database using the BLAST program (http://www.ncbi.nlm.nih.gov/). The deduced amino acid sequences will also be compared with the N-terminal sequences of palmivorein, a purified elicitin secreted by *P. palmivora* that has been described for its effect on *Hevea* leaves and calli (Chirapongsatonkul *et al*., 2008; Churngchow and Rattarasarn, 2000; 2001).

4) Pathogenicity studies

The virulence of 15 isolates of *P. palmivora* randomly sampling from different specimens and areas will be tested by a zoospore inoculation method. For every tested isolates, 3 detached rubber leaves will be inoculated with the zoospore suspension while sterile distilled water will be replaced as control or with a 5-mm hyphal plug. The size, measured by the average diameter of the necrotic lesions, and characteristic of the lesions will be observed every day, for 5 days, after inoculation. The necrosis size and progress of mycelium during the inoculation period will be concurrently assessed.

5) Determination of the expression level of the elicitin gene of *P. palmivora* isolates

Total RNA will be extracted from the mycelia of each isolate, comparable to those used in the method **4)**, then followed by cDNA synthesis. RT-PCR for the analysis of the elicitin gene of different strains of *P. palmivora* will be performed using cDNA as template and amplified with specific primers described in **3)**. Control reactions to normalize RT-PCR amplification will be run parallel with the specific primers of the β–tubulin gene. The reported elicitin gene expression values will be relativized to β-tubulin.

6) Comparative analysis of elicitin gene expression and virulence of *P. palmivora* isolates

To establish the possible relation of the expression of elicitin gene and the potency to infect the host, rubber plant, the expression level of this gene of each *P. palmivora* isolate will be compared with their virulence as observed in the corresponding pathogenicity test.

4. **Research Plan**

Activities	Months			
	6	**12**	**18**	**24**
1. Field survey and screening of *Phythophthora* spp.				
2. Determination of unknown *Phytophthora* spp. and *P. palmivora*				
3. Evaluation of the distribution of *Phytophthora* spp. and *P. palmivora* in the rubber plantations in southern Thailand				
4. Cloning of full-length cDNA of the elicitin gene of *P. palmivora*				
4. Virulence and pathogenicity studies				
5. Determination of the expression level of the elicitin gene of *P. palmivora* isolates				
6. Comparative analysis of elicitin gene expression and virulence of *P. palmivora* isolates				

5. **Outcome**

Expected publications : - Cloning and expression of an elicitin gene from *Phytophthora palmivora* and its involvement in virulence in *Hevea brasiliensis*

- *In vivo* quantification of elicitin gene expression in *Phytophthora palmivora* during infection and colonization

Expected journals for publication : Physiol. Mol. Plant Pathol. (impact factor ~ 1.4) or Phytopathology (impact factor ~ 2.2) or Mol. Plant Microbe Interact. (impact factor ~ 4.1)

6. **Research Budget**

	Year 1	Year 2	Total
1. Payment - Payment for researcher	120,000	120,000	240,000
2. Survey expense	30,000		30,000
3. Payment for chemical and scientific materials, e.g. - Culture media and antibiotics for *Phytophthora* spp. - DNA and RNA isolation kit - Superscript III and Taq DNA polymerase - 5' and 3' RACE kit - Cloning systems; vector and competent cell - Plasmid/PCR product purification kit	80,000	110,000	190,000
4. Payment for operating expenses, e.g. - DNA Sequencing service	10,000	10,000	20,000
Total	240,000	240,000	480,000

การศึกษายีนที่ผลิตอิลิซิตินของเชื้อ *Phytophthora palmivora* ซึ่งเป็นเชื้อก่อโรคในยางพารา และความสัมพันธ์กับความรุนแรงของเชื้อไอโซเลทต่าง ๆ

Elicitin of *Phytophthora palmivora*, a pathogen of rubber plants: cloning, expression and its role in virulence of variant isolates

Acknowledgements

We are grateful for the generous financial support from a Thailand Research Fund (TRF) Grant for New Researcher (project number MRG5480104) and the Faculty of Science Research Fund, Prince of Songkla University. This project is not accomplished without kindly help, supports and encouragements from Associate Professor Dr.Nunta Churgchow, graduated students and staffs of Department of Biochemistry, Faculty of Science, Prince of Songkla University.

We would like to acknowledge Dr. Brian Hodgson for a revision and valuable comments. The Songkhla Rubber Research Center, Songkhla, the Thailand Institute of Scientific and Technological Research and Ms. Waraporn Prakob, ex-staff of Department of Entomology and Plant Pathology, Faculty of Agriculture, Chiang Mai University are warmly thanked for providing the fungi used throughout this research.

Nion Chirapongsatonkul
(Researcher)

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Introduction

Hevea brasiliensis (Wild.) Muell.-Arg. or the rubber plant is an economically important crop in Thailand, the latex of which and its products are major Thai export incomes. At present, South East Asia especially Thailand is the world's largest producer and exporter of natural rubber, which is a key source of income for many farmers especially in the southern provinces. (FAO, 2013).

However, frequent tapping causes a consequence infection occurred at the wounds of the tapping sites. This problem is common in the southern part of Thailand where the humidity and temperature is high and conducive for pathogen growth. The cultivar that give high-yield of latex RRIM600, the commonly plantation in this area, is highly susceptible to *Phythophthora* pathogens.

Phytophthora diseases cause widespread economic and environmental losses worldwide. *Phytophthora* leaf disease caused by members of the oomycete genera *Phytophthora* are by far the most important disease of the rubber tree. *Phytophthora* spp. described as pathogens of the rubber tree include *P. palmivora*, *P. botryosa*, *P. heveae*, *P. meadii* and *P. parasitica*. In the south of Thailand *P. palmivora* and *P. botryosa* are the most frequently isolated pathogens that cause black stripe, green pod rot and abnormal leaf fall (Butler, 1996; Erwin and Ribeiro, 1996). *P. palmivora* attacks the petioles causing mature leaves to fall prematurely and also attacks the tapping surface resulting in reduced latex production.

Many species of *Phytophthora* can now be identified from their unique DNA sequences in the internal transcribed spacer (ITS) regions of their nuclear ribosomal DNA (rDNA) repeat region (Chimento *et al*., 2005; Cooke and Duncan, 1997; Drenth and Irwin, 2001; Winton and Hansen, 2001). Recently, the Polymerase Chain Reaction (PCR) has been used extensively for detection of plant pathogens due to the advantages of sensitivity, speed, and reliability (Martin *et al*., 2000; Ward *et al*., 2004). The PCR-based methods have been utilized worldwide in order to identify and detect *P. palmivora*, for example, from orchids (Tsai *et al*., 2006), important plants in Australia (Drenth and Irwin, 2001), coconut (Chowdappa *et al*., 2003) and citrus (Bowman *et al*., 2007).

Most of the oomycete *Phytophthora* species and *Pythium* species ubiquitously produce and secrete a unique class of highly conserved effector molecules named elicitins. (Panabières *et al*., 1997) Elicitins are a superfamily of 10 kDa extracellular proteins that share a 98-amino-acid domain (Baillieul *et al*., 2003; Ponchet *et al*., 1999; Qutob *et al*., 2003). They are lipid-binding proteins which are thought to aid in sterol uptake from the environment, an absolute requirement for sporulation (Hendrix, 1970). The biological function as a virulent or avirulent factor of elicitins in plant-pathogen interactions remains in conflict since they could induce hypersensitive response (HR, a form of programmed cell death) and other biochemical changes associated with plant defense responses in some host plants such as *Nicotiana* species in the Solanaceae and some radish and rape varieties in the Brassicacae (Blein *et al*., 2002; Kamoun *et al*., 1993, 1997; Pernollet *et al*., 1993; Ponchet *et al*., 1999; Ricci *et al*., 1989; Sasabe *et al*., 2000). The ability of elicitin has been reported to induce necroses and disease-like lesions (Menter *et al*., 2010; Takemoto *et al*., 2005). It has been demonstrated that *P. palmivora* elicitin, palmivorein, induces tissue necrosis on tobacco leaves and its host *H. brasiliensis* (Churngchow and Rattarasarn, 2000; 2001). Palmivorein triggers HR cell death and defense responses including scopoletin (Scp) production and enzyme peroxidase activities in *Hevea* calli in a dose dependent manner while these phenomenon decrease in the treatment of too high elicitin concentration (Chirapongsatonkul *et al*., 2008). However, *Phytophthora* elicitin production has been shown to vary among isolates (Kamoun *et al*., 1994) and the host species (Colas *et al*., 2001; Kamoun *et al*., 1997).

P. palmivora is reported to be an important pathogen of several plant crops in Thailand; however, the method to identify and diagnose this pathogen is not yet developed. Additionally the data of the distribution and dispersion of *Phytophthora* spp. and in particular *P. palmivora*, as the causing agent of leaf fall and black stripe of rubber plants has been investigated since 2004 (Sdoodee, 2004), evidence on its distribution recently remains rare.

This research aimed to study on the distribution of the *Phytophthora* genus and *P. palmivora* in southern Thailand covering the important rubber plantations in Songhkla, Satun and Nakhon Si Thammarat province. The PCR-based method using the specific primers for ITS region towards *Phytophthora* genus and *P. palmivora*, respectively, including our developed single round semi-nested multiplex PCR was performed to identify these target pathogens. Various isolates of *P. palmovora* that exhibit different characters were screened and collected. These *P. palmovora* isolates were used to determine their virulence on detached rubber leaves. Moreover, the full length of gene encoding elicitin of *P. palmivora* (*Ppal_Eli*) was cloned and characterized. The elicitin gene expression levels were also measured *in vitro* and *in planta* in each selected

P. palmovora isolates and inspected for its possible correlation with virulence by comparative observing its potency to induce necrosis and cell death on rubber leaves. Altogether, the obtained results could address the biological function of elicitin that involved in pathogen infection. These findings could lead to further investigate the appropriate disease management and crop protection.

Research Methodology

1) Survey and collection of fungi and *Phytophthora* spp.

To assess the distribution of *Phytophthora* spp. and *P. palmivora* in southern Thailand, the field survey was carried on in the rubber plantations located in 3 provinces; Songhkla, Satun and Nakhon Si Thammarat (marked with asterisks in Fig. 1). Naturally infected tissues exhibiting *Phytophthora* diseases (observed by naked eyes) including black stripe, stem canker and abnormal leaf fall were collected between June, 2011 and February, 2012. These samples were screened for the presence of *Phytophthora* spp. and *P. palmivora*.

Small pieces of diseased plant tissue were plated directly onto a selective medium for *Phytophthora* spp., Potato Dextrose Agar (PDA) containing antibiotics (Ceftazidime Chloramphenicol Rifampicin and Nystatin) or PDA Anti, to prevent the contamination by other organisms and incubated at room temperature. Two or three d later, mycelium tips were cut from the edges of growing colonies and subcultured onto a new PDA and clarified V8 juice agar for further study and storage. Isolates were separated and divided into groups based on their visual colony morphology and their zoospore morphology under the microscope. All isolates were kept for more detailed study.

2) Isolation of DNA and RNA and first strand cDNA synthesis

The genomic DNA from mycelia of each isolate and plant materials (50–100 mg of each sample) was extracted using the DNAzol or the NucleoSpin® Plant II Kit (Macherey-Nagel) according to the supplied protocol from the manufacturer. Total RNA from mycelia and plant tissues was extracted using the RNeasy Plant Mini Kit (Qiagen) according to the supplied protocol from the manufacturers. To obtain only RNA, the contaminated DNA was removed using the DNase I (Thermo Scientific) followed the manufacturers' protocol. The concentration of DNA and total RNA was determined spectrophotometrically.

The first-strand cDNA synthesis was made from 2 µg of RNA using the enzyme SuperScript™ III Reverse Transcriptase (Invitrogen) at 42°C for 1 h, followed by 72°C for 10 min and held at 4°C.

Fig. 1 The sampling sites for sample collection in this research. Asterisks (★) represent provinces where the rubber plantations were located.

3) Determination of unknown isolated fungi

To analyze whether the fungi collected are *Phytophthora* spp. or *P. palmivora*, the morphological and molecular analysis were performed. The mycelium, sporangium and zoospore of each isolate were revealed under microscope. The results of nucleotide sequences of ITS region within rDNA, obtained from PCR with universal primer and DNA from each isolate, indicating of which species isolated were considered. In addition, the single round semi-nested multiplex PCR, described below, was also used to verify whether the interesting isolates were that belonging to the genus *Phytophthora* and *P. palmivora*.

3.1) Identification of unknown fungi

Genomic DNA of all unknown isolates and the universal primer ITS1/ITS4 (Bi *et al*., 2011), the detail as shown in Table 1, was used for PCR amplification of the fungal

18S rDNA in ITS1-5.8S rDNA-ITS2 region. The fragment sizes were estimated by comparison with a 100 bp Plus DNA Ladder (Vivantis). The PCR products were gel-purified with the QIAquick Gel Extraction Kit (Qiagen). Purified PCR products were bidirectional sequenced directly, using the dye-terminator cycle-sequencing reaction (Applied Biosystems).

The obtained nucleotide sequence of each isolate was determined by alignment with the sequences submitted to the NCBI database (http://www.ncbi.nlm.nih.gov/) by nucleotide BLAST analysis in order to identify the species of the isolates.

3.2) The specificity of primers for *Phytophthora* spp. and *P. palmivora* used in the single round semi-nested multiplex PCR

Primer pair covering the *Phytophthora* genus (Phy1s/Phy2a), and those specific to *P. palmivora* (Pal1s/Pal2a) were available from Tsai *et al*. (2006). These oligonucleotide primers have been designed based on the sequences of 28S rRNA and ITS1-5.8S rRNA-ITS2 from a variety of *Phytophthora* spp. collected from the NCBI website and analyzed by multiple sequence alignment using Clustal X (Thompson *et al*., 1994). The nucleotide sequences of primers and expected product sizes are shown in Table 1. The single round semi-nested multiplex PCRs were carried out using the set of 3 primers Phy1s/Phy2a/Pal2a and the DNA extracted from *P. palmivora* (isolate KBNM 9 maintained in our laboratory, a positive strain) at approximately 20 ng was used as the template. These PCRs were performed in 25 µl reaction using EmeraldAmp® GT PCR Master Mix (Takara) and each primer at final concentration of 0.4 µM. No template (negative control, water) was run in each experiment. The PCR reactions were run on a Techne TC-512 Gradient Thermal Cyclers (Techne). The PCR conditions were as follows: denaturation at 94°C for 5 min, followed by 30–35 cycles of 1 min at 95°C, 1 min at 55°C, 1 min at 72°C, and a final extension at 72°C for 10 min. PCR amplified products were analyzed by electrophoresis as previously described.

The specificity of the single round semi-nested multiplex PCR were examined by amplification of the positive and negative controls as well as the DNA extracted from *Penicillium funiculosum* (TISTR 3563)*, Aspergillus niger* (TISTR 3254), *Fusarium solani* (TISTR 3436) and *Colletotrichum gloeosporioides* or *Phytophthora* spp. as templates. To check whether these primers could amplify any genes from the rubber plant, the PCR reaction using the DNA of healthy rubber plant tissues was carried out; in addition, the actin primer pair (actF/actR), amplifying actin gene of *H. brasiliensis* or *HbActin*, was used as internal control. PCR amplified products were analyzed by electrophoresis on

1.5% (w/v) agarose gel with 0.5 µg/ml ethidium bromide in 1× TAE. The fragment sizes were estimated by comparison with a 100 bp Plus DNA Ladder (Vivantis). The PCR products from each primer pair were cut from the gel, purified using QIAquick Gel Extraction Kit (Qiagen), ligated and then transformed into *Escherichia coli* Top10 cells. Purified plasmids were bidirectional sequenced directly, using the dye-terminator cycle-sequencing reaction (Applied Biosystems).

3.3) Determination of *Phytophthora* spp. and *P. palmivora* by single round semi-nested multiplex PCR

The single round semi-nested multiplex PCR procedure was used to amplify the DNA extracted from all unknown fungi isolated from the plant materials collected from the naturally planted rubber trees expressing leaf fall or black stripe. These reactions were done to verify if the isolated fungi are *Phytophthora* spp. and *P. palmivora*. The positive result, one band sized 1000 bp and two bands sized 1000 bp and 650 bp, indicating the species belonging to *Phytophthora* spp. and *P. palmivora*, respectively.

The frequency of these pathogens which may cause the diseases found in the selected locations was evaluated. Isolates of *P. palmivora* were used for further studies on elicitin gene expression and comparable virulence on rubber leaf.

4) Full length of elicitin gene from *P. palmivora*

4.1) Primer designing, amplification and cloning of full length cDNA of the elicitin gene of *P. palmivora*

Total RNA was extracted from mycelium of *P. palmivora* KBNM 9 and first-strand cDNA was synthesis followed the method mentioned above.

For designing the primers of the elicitin gene, the multiple alignment of the elicitin nucleotide sequence from *P. palmivora* (accession number AY206986) and other *Phytophthora* species were performed using ClustalW to align the sequences, and to locate well conserved regions. Primers were designed based on the conserved regions and primers properties were studied using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) and Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome)

Elicitin gene from *P. palmivora* was amplified by PCR using primer F_Elicitin and R_Elicitin which the details are shown in Table 1. The cDNA prepared from *P. palmivora* KBNM 9 was taken for PCR amplification along with EmeraldAmp® GT PCR Master Mix

(Takara) and the primer pair. The PCR conditions were 94°C for 5 min, followed by 40 cycles of 1 min at 95°C, 1 min at 55°C, 1 min at 72°C, and a final extension at 72°C for 10 min. The PCR amplified products were analyzed by electrophoresis. The amplified fragment with expected size was ligated and transformed into *E. coli* Top10 cells. Purified plasmids were and the sequencing results were analyzed through BLAST program. This gained nucleotide sequence was used for designing the primers for the 5' and 3' rapid amplification of cDNA ends (RACE) to archive the full length of the *P. palmivora* elicitin gene. RACE was performed using GeneRacerTM RACE Ready cDNA Kit following the instructions of the manufacturer (Invitrogen). The RACE-PCR products were cloned into vector and transformed into *E. coli*. The plasmids were then purified and sequenced.

4.2) Sequence analysis and homology modeling of the elicitin gene from *P. palmivora*

Comparative and bioinformatic analyses of the obtained full length of the elicitin gene were carried out. In addition, the nucleotide sequences, deduced amino acid sequences and ORF encoded the elicitin gene of *P. palmivora* was also analyzed via BioEdit and ExPaSy (http://web.expasy.org/translate/). The sequence comparison was conducted online through the NCBI database using the BLAST program (http://www.ncbi.nlm.nih.gov/). The complete CDs of elicitin sequence was subjected to protein domain analysis using Interproscan (http://www.ebi.ac.uk/Tools/pfa/iprscan/) while disulfide bonds were found out using Disulfind (http://disulfind.dsi.unifi.it/). The predicted pI and molecular weight (MW) of deduced elicitin protein was figured out by Compute pI/Mw (http://web.expasy.org/compute_pi/). The probable signal sequence was predicted using SignalP (https://www.google.co.th/#q=signalP). The deduced amino acid sequences was compared with the N-terminal sequences of palmivorein, a purified elicitin secreted by *P. palmivora* that has been described for its effect on *Hevea* leaves and calli (Chirapongsatonkul *et al*., 2008; Churngchow and Rattarasarn, 2000; 2001). Prediction of three-dimemtional structure and homology modeling of the elicitin form *P. palmivora* was performed using Phyre^2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/). In order to consider the relationship between the obtained *P. palmivora* elicitin and elicitins from different *Phytophthora* spp., a phylogenetic analysis based on nucleotide and amino acid sequence of 25 elicitins and elicitin like sequences were carried out by the maximum likelihood (ML) method using MEGA6.

5) Culture and zoospore preparation of various isolates of *P. palmivora*

Cultural stock of *P. palmivora* KBNM 9 lineage, maintained in the sterile distilled water since 2008, was transferred to the new PDA plate. Monospore technique in our laboratory was performed to isolate the stains which may varied develop because of long time collection. The various isolated strains were further cultured on PDA.

Each isolate was studied for its morphological characters under microscope. To comparative verify the ability of each isolated strains to produce zoospore, the zoospore suspension of each isolate of *P. palmivora* was prepared following the method of Chirapongsatonkul *et al*. (2008). Briefly, a 0.5 cm diameter plug was cut from the edge of the growing mycelium and transferred to a V_8 agar for 5 days then chilled using sterile water (8–10^{o}C) to trigger zoospores release from the sporangium. Zoospores were collected 30 min later and counted under a microscope.

Table 1 PCR primers used in this study.

Target	**Name**	**Primer sequence (5´ to 3´)**	**Expected size of PCR product (bp)**
ITS region of *Phytophthora* spp.	Phy1s Phy2a	ACTTTCCACGTGAACCGTATCA GCACGAGCCACTCAGGGATG	~1000
ITS region of *P. palmivora*	Pal1s Pal2a	CACGTGAACCGTATCAAAACT CAATCATACCACCACAGCTGA	648 (~650)
Elicitin gene of *P. palmivora*	F_Elicitin R_Elicitin	ATGAACTTCCGCGCTCTGTTCGCCG TGCAAGCCGTCGACGCGCACATGAG	220
	EliPro_F EliPro_R	ACCACGTGCACCACCACCCAG TAGCGACGCACACGTAGCAGA	290
Actin gene of *P. palmivora*	F_act-Pal R_act-Pal	ATGTCGATATCCGCAAGGAC TTCGAGATCCACATCTGCTG	210
Actin of *H. brasiliensis (HbActin)*	actF actR	CACCACCACTGCCGAACGGG ACGGTCTGCAATGCCAGGGA	350
ITS1-5.8S rDNA-ITS2 (Universal primer)	ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	

6) Pathogenecity test and necrosis on the rubber leaves

6.1) Selection of *P. palmivora* strains

Seven isolates from *P. palmivora* KBNM 9 lineage were first tested for their virulences by the following protocol. Three detached rubber leaves were inoculated with the zoospore suspension while sterile distilled water was replaced as control. Zoospore suspension should be used to inoculate plant within 30 min to avoid the encysment could occur. After the abaxial surface of each leaf was placed facing the zoospores for 2 h, all leaves were rinsed with sterile distilled water then placed on moist Whatman paper. The inoculated leaves were kept at 25°C under 12 h of daylight. The necrosis sizes measured by the average diameter of the necrotic lesions and characteristic of the lesions were observed after treatment for 3 days. The necrosis size and progress of mycelium during the inoculation period was concurrently assessed in order to elucidate the virulence of each strain. Afterwards, 4 strains of *P. palmivora* causing the necrosis with difference in size were selected for studying more in the detail of their virulence.

6.2) Treatment procedure

The healthy rubber leaves were cut into 2x2 cm^2. Twenty pieces from 5 individual plants were placed in a Petri dish containing moist Whatman paper. Every piece was stabbed on the abaxial surface with a sterile needle (Nipro needle 21 G (0.8x 40 mm)) to make a wound for 3 mm in length. A 5-mm hyphal plug of each isolate of *P. palmivora*, growing on V_8 agar, was placed on the wound. A mycelium-free V_8 agar was placed on the wounded leaf piece as control. The inoculated leaf pieces were kept at 25°C under 12 h of daylight. After treatment for 24 h, the leaf samples of both mycelium treatment and control were randomly sampling for cell death assay. In addition the leaf pieces and mycelium plug was separately collected and kept at -80°C for RNA extraction and further study for the expression of elicitin gene.

6.3) Analysis for the pathogenecity

In order to elucidate the virulence of each strain, the necrosis, the progress of mycelium and cell death was concurrently assessed.

7) Necrosis assay

The necrosis of treated and control leaves was visually observed during the inoculation period, measured in size and photographed. To improve the vision of necrotic lesion, chlorophyll was removed from the leaf tissue by boiling the leaf pieces in 95% (v/v) ethanol. The necrosis was photographed.

8) Cell death assay

Cell death was monitored in untreated and mycelium inoculated leaves by trypan blue staining a method adapted and performed according to Koch and Slusarenko (1990). At the leaf pieces was placed in 0.1% (v/v) Triton X-100 for 10 min and transferred to the 0.5% (w/v) trypan blue dye for 30 min. The excess and unbound dye was rinsed out with sterile water. The dead cells were monitored as blue color. Cell death was observed visually and photographed.

9) Determination of the expression level *in vitro* and *in palnta* of the elicitin gene of *P. palmivora* isolates

The expression level of elicitin gene was analyzed *in vitro* (in cultured mycelium) and *in planta* (in pathogen growing in host plant) via semi-quantitative PCR. Total RNA was extracted from the mycelia and rubber leaf tissue infected with mycelia of each isolate. The first strand cDNA was synthesized followed the procedure described above. The set of specific primer, of which sequence was shown in Table 1, for determining the expression level of the elicitin mRNA was designed from the obtained full sequence of the elicitin gene. RT-PCR for the analysis of the elicitin gene of different strains of *P. palmivora* was performed using EmeraldAmp® GT PCR Master Mix (Takara), synthesized cDNA as template and amplified with specific primers. The PCR was started with 94°C for 5 min, followed by 25–40 cycles of denaturing, annealing and extension temperature at 95°C for 1 min, 55°C for 1 min and 72°C for 1 min, respectively and a final extension at 72°C for 10 min. The PCR products were separated on 1.5% (w/v) agarose gel electrophoresis Quantification of the PCR products was made in-gel using UVP BioSpectrum AC Chemi HR410 apparatus. Control reactions to normalize RT-PCR amplification was done parallel with the specific primers of *P. palmivora* actin gene (Table 1) designed from the sequence of *P. palmivora* actin gene submitted to GenBank with accession number AY729846. The reported elicitin gene expression values were relativized to internal marker *P. palmivora* actin gene. The relative expression level was analyzed by determining the band intensity, measured by ImageJ software, comparing between elicitin gene and internal marker. In order to analyze the elicitin gene expression in the rubber leaf tissues inoculated with *P. palmivora*, the PCR using the primer of the actin gene (actF/actR) of the rubber plant (*HbActin*) was also used as the control to confirm an equal amount of cDNA in all samples. The primer for using as an internal marker in the rubber plant tissue was designed from the actin (GU270586.1) of *H. brasiliensis* previously used in genomics work on rubber plants and submitted, to GenBank.

10) Comparative analysis of elicitin gene expression and virulence of *P. palmivora* isolates

To establish the possible relation of the expression of elicitin gene and the potency to infect the host, rubber plant, the expression level of this gene of each *P. palmivora* isolate was compared with their virulence as observed in the corresponding pathogenicity test described above.

Results and Discussion

Development of the diagnosis method to simultaneous detection of *Phytophthora* spp. and *P. palmivora*

The primers used throughout this research, Phy1s/Phy2a and Pal1s/Pal2a to identify and detect the genus *Phytophthora* and *P. palmivora*, respectively were according to Tsai *et al*. (2006). Based on the alignment with the sequences submitted to the NCBI database by nucleotide BLAST analysis and ClustalW (BioEdit), the binding locations of each primer within the ITS1-5.8S rDNA-ITS2-28S rDNA region of *Phytophthora* spp. and *P. palmivora* are shown in Fig. 2a and Fig. 2b.

Using the DNA extracted from the mycelia of the positive control *P. palmivora* strain KBNM 9, maintained in our laboratory, as template, the PCRs using Phy1s/Phy2a and Pal1s/Pal2a primers yielded a single band of approximately 1000 bp (Fig. 2c) and 650 bp (Fig. 2d), respectively. The obtained PCR products were purified, cloned and sequenced. The results indicated that the fragments carried the ITS1-5.8S-ITS 2-28S rDNA region of *P. palmivora*. The nucleotide sequences were deposited at the National Center for Biotechnology GenBank (Accession number: JX863409 and HQ446453). The PCR reactions were additionally performed using DNA from 3 *Phytophthora* spp. including *P. palmivora*, *P. botryosa* and *P. infestans* and other fungi that are often found in the environment, *Penicillium funiculosum*, *A. niger*, *F. solani* and *C. gloeosporioides* as templates. The DNA fragments of approximately 1000 bp in length were obtained from all tested *Phytophthora* spp. (Fig. 2c) while no amplification signal was detected when PCR was performed using DNA from other fungal isolates indicating the specificity of the primer to the genus *Phytophthora*. The fragment of the expected length of approximately 650 bp was obtained only when DNA from the positive strain *P. palmivora* KBNM 9 was used indicating the species-specificity (Fig. 2d).

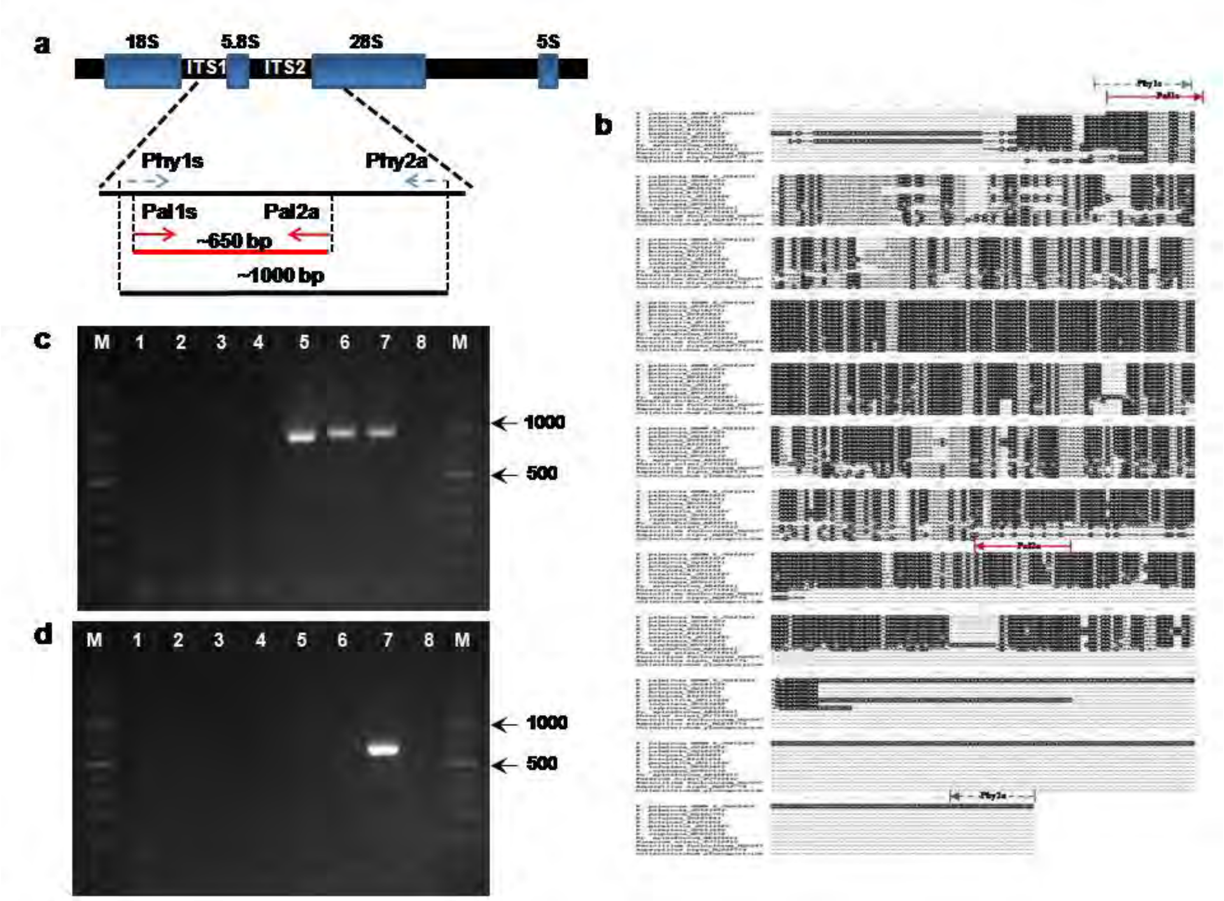

Fig. 2 Test for the primers used for detection the ITS region of *Phytophthora* spp. and *P. palmivora*. The presumed sites that the primers Phy1s, Phy2a, Pal1s and Pal2a match on the ITS rDNA region of *P. palmivora* **(a)** and the sites within the aligned sequences of rDNA region of several fungi and *Phytophthora* spp. **(b)**. Dash arrows (- - -▸and ◂- - -) indicate Phy1s and Phy2a while thick arrows (⟶and ⟵) refer to Pal1s and Pal2a. Specificity test of the primer set; Phy1s/Phy2a **(c)** and Pal1s/Pal2a **(d)**. PCR was performed using DNA from *Phytophthora* spp. and other fungal isolates as the templates. The amplified products were analyzed by electrophoresis on 1.5% agarose gel with 0.5 µg/ml ethidium bromide. Lane 1: *C. gloeosporioides*; 2: *A. niger*; 3: *Penicillium funiculosum*; 4: *F. solani*; 5: *P. botryosa*; 6: *P. infestans*; 7: *P. palmivora*; 8: water control and M: 100 bp Plus DNA Ladder (Vivantis).

To reduce time course for simultaneously detection of *Phytophthora* spp. and *P. palmivora*, the single round nested multiplex PCR using the primer set Phy1s/Phy2a/Pal1s/Pal2a with the equal concentration were performed in the unique PCR reaction. Two bands sizes approximately at 1000 bp and 650 bp were revealed. Since the match site of the primer Phy1s on the ITS region is very close and covered that of Pal1s (Fig. 2a and 2b), Pal1s was then eliminated and the 3 primers Phy1s/Phy2/ Pal2a were instead used to perform the single round semi-nested multiplex PCR. In order to evaluate the specificity of the single round semi-nested multiplex PCR condition, the DNA from 3 various species belonging to the genus *Phytophthora* and other fungi were used as templates. Only one band of 1000 bp was obtained from all the tested *Phytophthora* spp. while there were two bands (1000 and 650 bp) revealed when the template was DNA from *P. palmivora* (Fig. 3).

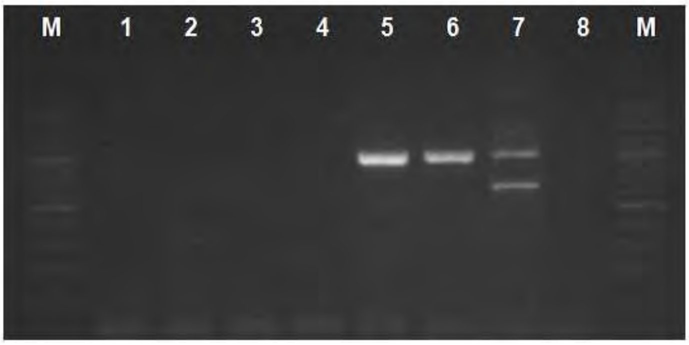

Fig. 3 Specificity of the single round semi-nested multiplex PCR. PCR using DNA from *Phytophthora* spp. and other fungal isolates as the templates and the primer set Phy1s/Phy2a/Pal2a was performed. Lane 1: *C. gloeosporioides*; 2: *A. niger*; 3: *Penicillium funiculosum*; 4: *F. solani*; 5: *P. botryosa*; 6: *P. infestans*; 7: *P. palmivora*; 8: water control. The amplified products were analyzed by electrophoresis on 1.5% agarose gel with 0.5 μg/ml ethidium bromide and M represents 100 bp Plus DNA Ladder (Vivantis).

Screening of *Phytophthora* spp. and *P. palmivora* from the rubber plant materials collected from rubber plantations in southern part of Thailand

Leaves and barks of the rubber plants exhibiting the symptoms of abnormal leaf fall and black stripe were collected from the rubber plantations, in Songhkla, Satun and Nakhon Si Thammarat, and transferred to the Department of Biochemistry, Faculty of Science, Prince of Songkla University. These samples were surface sterilized and screened for the *Phytophthora* spp. and *P. palmivora* by growing the possibly infecting fungi on selective medium (PDA Anti). The mycelia of all collected fungi were white to light cream, however, the level of swell and growing character on selective medium were different (Fig. 4). Each isolate of unknown fungi was coded and their names are shown in Table 2. The fungi were subcultured weekly onto fresh PDA Anti medium for 3 times to test whether they could resist and alive on this medium. After growing on the selective medium for 3–5 d, the mycelium of unknown fungi was then transferred into the new PDA plate to maintain as the culture stock.

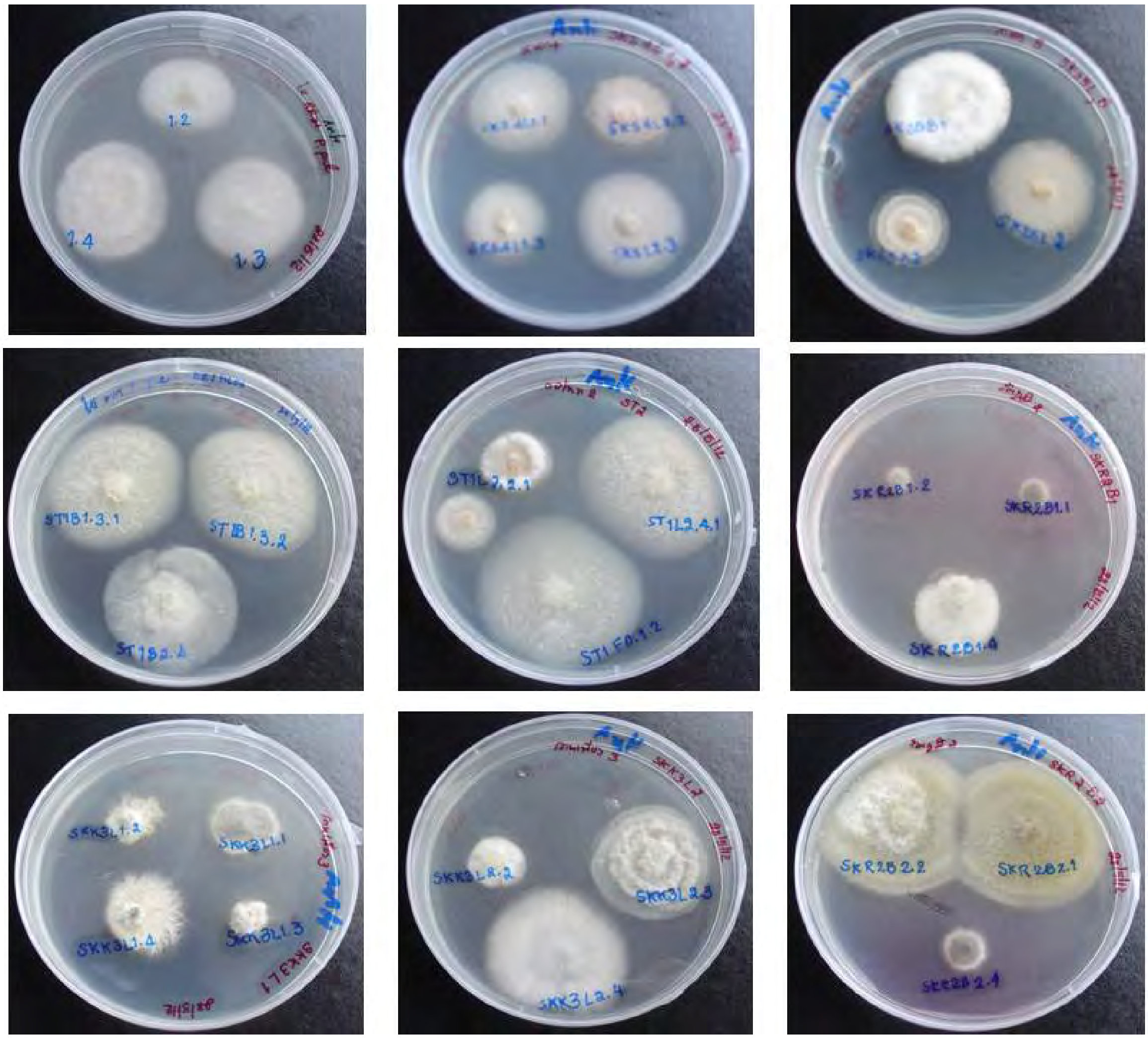

Fig. 4 The mycelium of unknown fungi, isolated from disease symptom expressing materials in southern part of Thailand, growing on selective medium (PDA Anti).

Table 2 The detail and name of unknown fungi isolated from diseased materials.

Province	District	Number of isolate	Name
Songhkla	Rattaphum	5	SKR2B1.1
			SKR2B1.2
			SKR2B1.3
			SKR2B2.1
			SKR2B2.2
	Kuan nieng	7	SKK3L1.1
			SKK3L1.2
			SKK3L1.3
			SKK3L1.4
			SKK3L2.2
			SKK3L2.3
			SKK3L2.4
	Sadao	7	SKS4L1.1
			SKS4L1.3
			SKS4L2.2
			SKS4L2.3
			SKS5B1
			SKS5B2
			SKS5L2
Satun	Khuan kalong	6	ST1B1.3.1
			ST1B1.3.2
			ST1B2.4
			ST1L2.2.1
			ST1L2.4.1
			ST1F0.3.2
Nakhon Si Thammarat	Thung song	3	1.2
			1.3
			1.4

Identification of fungi isolated the from the rubber plant materials collected from rubber plantations in southern part of Thailand

After being transferred to the new PDA plate and cultured for 7 d, the mycelium of each isolate was collected for DNA extraction. The integrity of DNA from unknown fungal mycelia was checked by spectrophotometry and agarose gel electrophoresis (Fig. 5). Our developed single round semi-nested multiplex PCR using the obtained DNA as template was performed to analyze whether that unknown fungus was belong to the genus *Phytophthora* and *P. palmivora*. No band of interest at expected size was visible as shown in Fig. 6. Therefore, these results revealed that any isolates was *Phytophthora* spp. and *P. palmivora*.

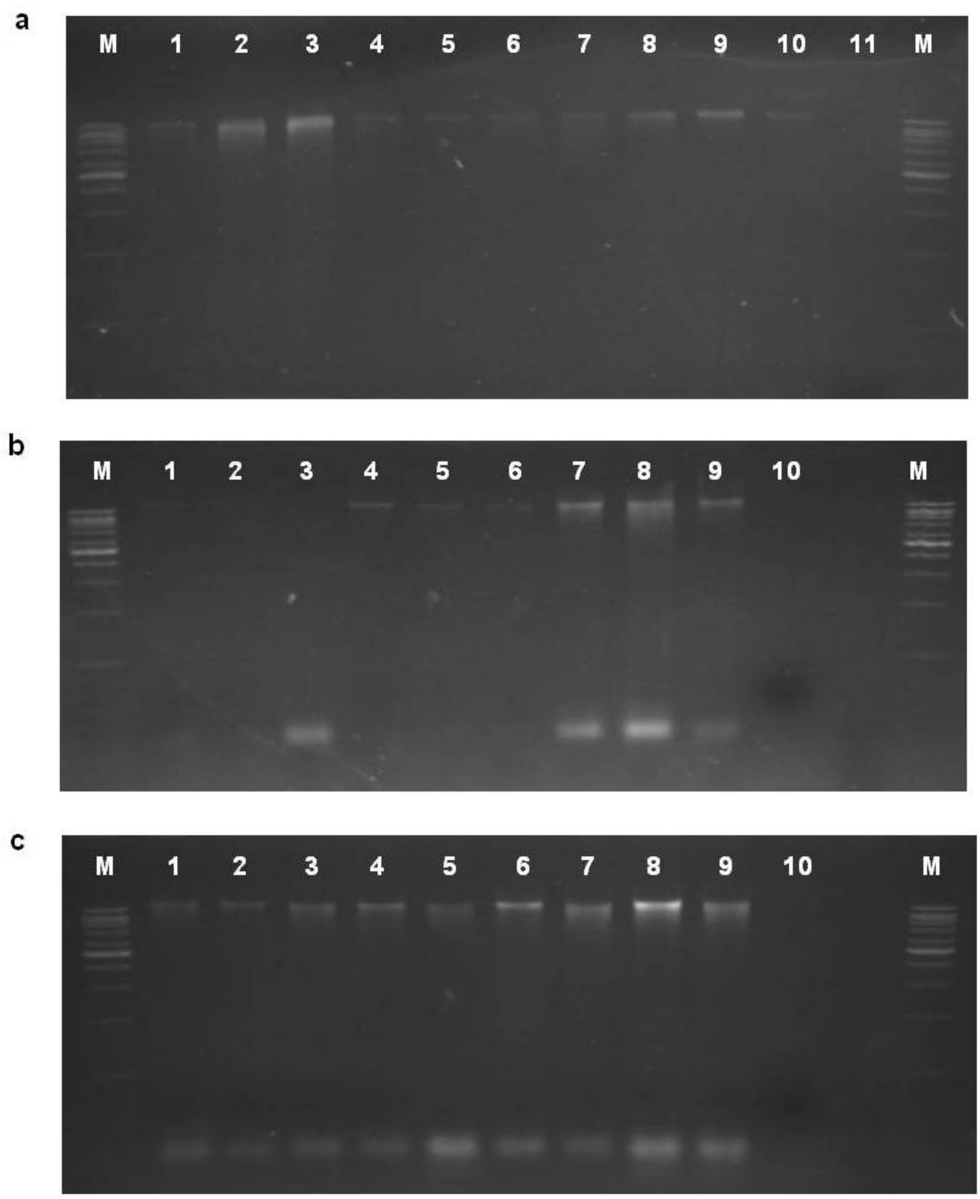

Fig. 5 The DNA extracted from the mycelium of unknown fungal isolates. The DNA were analyzed by electrophoresis on 1.5% agarose gel with 0.5 µg/ml ethidium bromide and M represents 1 kb DNA Marker (Vivantis). **(a)** Lane 1: 1.2; 2: 1.3; 3: 1.4: 4; SKS4L1.1; 5: SKS4L1.3; 6: SKS4L2.2; 7: SKS4L2.3; 8: SKS5B1: 9; SKS5B2: 10; SKS5L2; 11: H_2O. **(b)** Lane 1: ST1B1.3.1; 2: ST1B1.3.2; 3: ST1B2.4; 4: ST1L2.2.1; 5: ST1L2.4.1; 6: ST1F0.3.2; 7: SKR2B1.1; 8: SKR2B1.2; 9: SKR2B1.3; 10: H_2O. **(c)** Lane 1: SKK3L1.1; 2: SKK3L1.2; 3: SKK3L1.3; 4: SKK3L1.4; 5: SKK3L2.2; 6: SKK3L2.3; 7: SKK3L2.4; 8: SKR2B2.1; 9: SKR2B2.2; 10: H_2O.

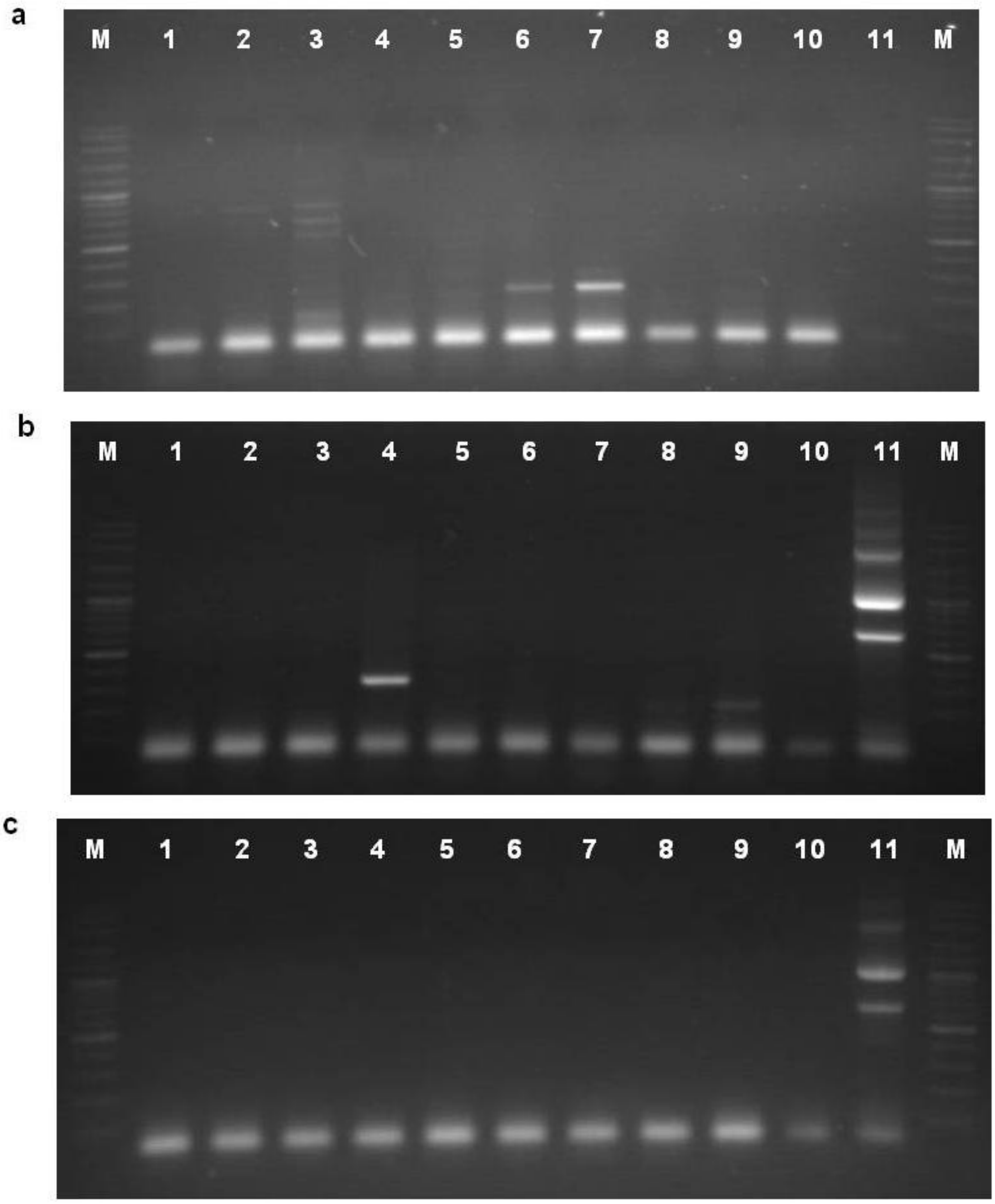

Fig. 6 The single round semi-nested multiplex PCR using DNA of unknown fungal isolates. The DNA were analyzed by electrophoresis on 1.5% agarose gel with 0.5 µg/ml ethidium bromide and M represents 100 bp Plus DNA Ladder (Vivantis). **(a)** Lane 1: 1.2; 2: 1.3; 3: 1.4: 4; SKS4L1.1; 5: SKS4L1.3; 6: SKS4L2.2; 7: SKS4L2.3; 8: SKS5B1: 9; SKS5B2: 10; SKS5L2; 11: H_2O. **(b)** Lane 1: ST1B1.3.1; 2: ST1B1.3.2; 3: ST1B2.4; 4: ST1L2.2.1; 5: ST1L2.4.1; 6: ST1F0.3.2; 7: SKR2B1.1; 8: SKR2B1.2; 9: SKR2B1.3; 10: H_2O. **(c)** Lane 1: SKK3L1.1; 2: SKK3L1.2; 3: SKK3L1.3; 4: SKK3L1.4; 5: SKK3L2.2; 6: SKK3L2.3; 7: SKK3L2.4; 8: SKR2B2.1; 9: SKR2B2.2; 10: H_2O; 11: *P. palmivora* KBNM 9 (positive control).

To verify the genus or species probable of the fungal isolated, the ITS-rDNA region was amplified by using the universal primer ITS1/ITS4 and DNA of each isolate as template. In addition, PCR performing with the DNA of *P. palmivora* KBNM 9 as template was also carried out. The PCR of the ITS regions using DNA of each isolate, amplified by the mentioned universal primer set, yielded a single band sized varied in a range of 500–600 bp (Fig. 7). The product size was approximately 900 bp when using the DNA of *P. palmivora* (data not shown). The PCR products were purified and sequenced. The obtained nucleotide sequences were subjected Blastn of NCBI to identify the genus of our isolated fungi. Based on %Identity of more than 90%, the results revealed the ITS-rDNA region of possible genus of the unknown isolates. The detail of each isolate was summarized in Table 3. However, one isolate could be identified as more than one genus which might be due to the high conserve of the nucleotide sequences within ITS region. More than one genus with close relation within the same family could be revealed in one analyzed nucleotide sequence since the studied ITS region, amplified by our selected primer ITS1/ITS4, may not be the genus- or species-defining site. Overall, most of the fungi isolated here were *Colletotrichum gloeosporioides*, *Fusarium* sp., *Cercophora* sp., *Trichoderma* sp. and *Podospora* sp.

Unfortunately that any of the *Phytophthora* genus and *P. palmivora* could not be isolated from any diseased rubber plant tissues. The plausible explanation for the false lies in the sampling disease expressing materials might not be caused by *Phytophthora* or *P. palmivora* since the symptoms between diverse disease causing pathogens are similar within the same or different genus (Drenth and Guest 2004; Ellis and Boehm 2008).

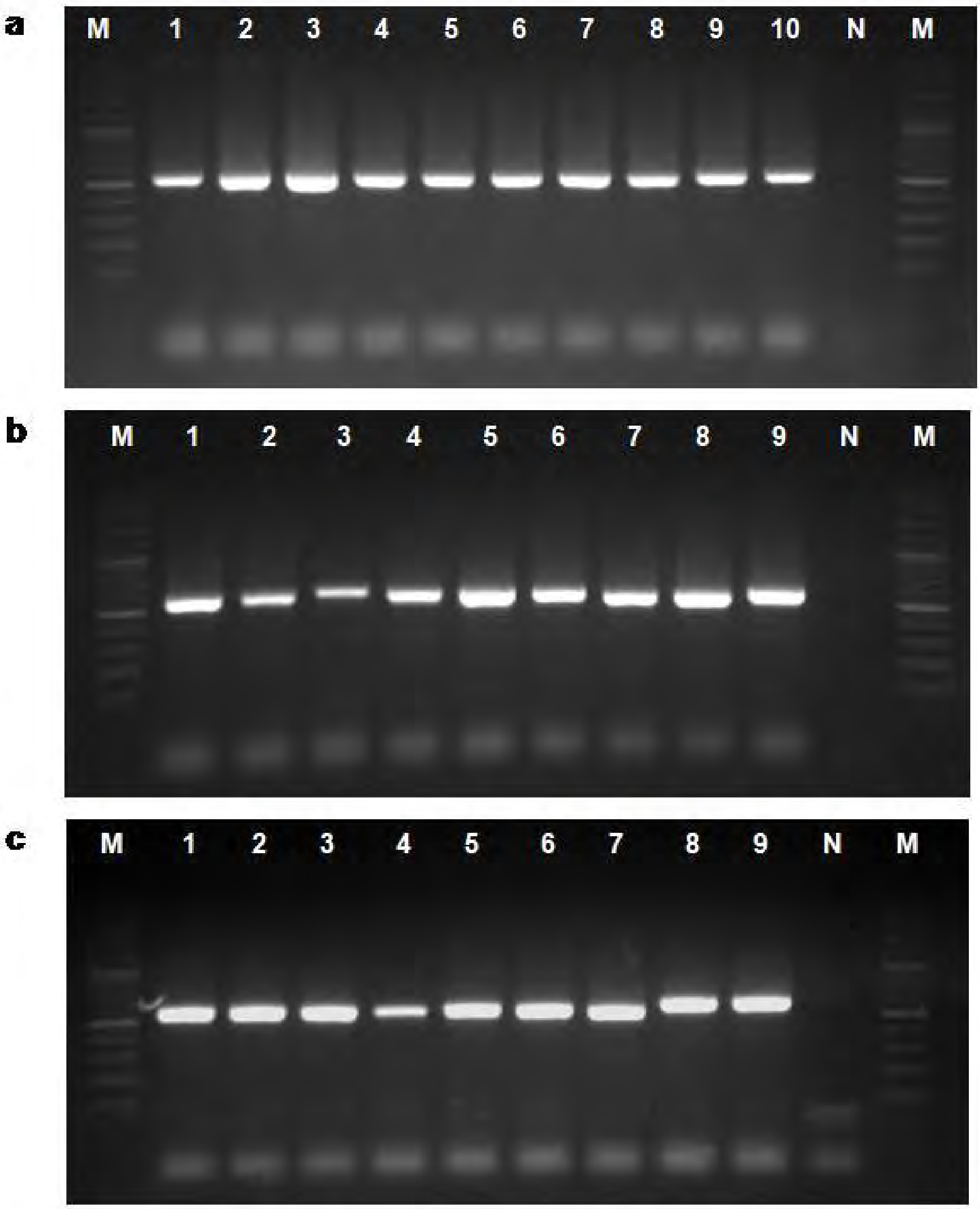

Fig. 7 The PCR products amplified by the universal primer ITS1/ITS4 and using the DNA of unknown fungal isolates as templates. The DNA were analyzed by electrophoresis on 1.5% agarose gel with 0.5 µg/ml ethidium bromide and M represents 100 bp Plus DNA Ladder (Vivantis). **(a)** Lane 1: 1.2; 2: 1.3; 3: 1.4: 4; SKS4L1.1; 5: SKS4L1.3; 6: SKS4L2.2; 7: SKS4L2.3; 8: SKS5B1: 9; SKS5B2: 10; SKS5L2; N: water control. **(b)** Lane 1: ST1B1.3.1; 2: ST1B1.3.2; 3: ST1B2.4; 4: ST1L2.2.1; 5: ST1L2.4.1; 6: ST1F0.3.2; 7: SKR2B1.1; 8: SKR2B1.2; 9: SKR2B1.3; N: water control. **(c)** Lane 1: SKK3L1.1; 2: SKK3L1.2; 3: SKK3L1.3; 4: SKK3L1.4; 5: SKK3L2.2; 6: SKK3L2.3; 7: SKK3L2.4; 8: SKR2B2.1; 9: SKR2B2.2; N: water control.

Table 3 The possible genus of isolated unknown fungi revealed by the ITS-rDNA region amplified by the universal primer ITS1/ITS4.

Province	District	Name of unknown isolate	Possible genus and species (according to ITS-rDNA region)
Songhkla	Rattaphum	SKR2B1.1	*Lasiodiplodia* sp., *L. theobromae*, *L. parva*, *L. rubropurpurea* *Rhizoctonia bataticola* *Botryosphaeria rhodina*
		SKR2B1.2	*Lasiodiplodia* sp., *L. theobromae*, *L. parva*, *L. mahajangana* *Botryosphaeria rhodina*
		SKR2B1.3	*Fusarium* sp., *F. solani* *Nectria ipomoeae*, *N. haematococca*
		SKR2B2.1	*Trichoderma* sp. *T. piluliferum*, *T. harzianum*, *T. aureoviride* *Hypocrea lixii*, *H. nigricans* *Colletotrichum gloeosporioides*
		SKR2B2.2	*Trichoderma* sp., *T. piluliferum*, *T. harzianum*, *T. harzianum*, *T. tomentosum*, *T. atroviride* *Colletotrichum gloeosporioides* *Hypocrea nigricans*, *H. albocornea* , *H. lixii*
	Kuan nieng	SKK3L1.1	*Glomerella cingulata* *Colletotrichum gloeosporioides*, *C. siamense*
		SKK3L1.2	*Phomopsis* sp., *P. longicolla* *Diaporthe* sp., *D. arctii*
		SKK3L1.3	*Phomopsis* sp., *P. phyllanthicola*, *P. phoenicicola, P. liquidambari, P. lagerstroemiae*, *P. eucommii* *Diaporthe helianthi*, *D. ceratozamiae*, *D. eucalyptorum*
		SKK3L1.4	*Phomopsis* sp. *Diaporthe* sp., *D. phaseolorum*, *D. eucalyptorum*
		SKK3L2.2	*Myrothecium* sp., *M. masonii, M. roridum*, *M. lachastrae*, *M. verrucaria* *Hymenopsis* sp. *Pteris ensiformis*
		SKK3L2.3	*Phomopsis* sp., *P. longicolla*, *P. bougainvilleicola* *Diaporthe arctii, D. phaseolorum*,

Province	District	Name of unknown isolate	Possible genus and species (according to ITS-rDNA region)
		SKK3L2.4	*Fusarium oxysporum, F. subglutinans*, *F. proliferatum* *Gibberella intermedia*, *G. moniliformis* *Botryosphaeria dothidea*
	Sadao	SKS4L1.1	*Cercophora coprophila*, *C. samala* *Zopfiella tetraspora* *Podospora austroamericana*, *P. setosa*, *P. anserina*, *P. comata* *Chaetomium longicolleum*
		SKS4L1.3	*Cercophora coprophila*, *C. salama* *Podospora anserina*, *P. comata*, *P. setosa* *Chaetomium longicolleum* *Zopfiella longicaudata*
		SKS4L2.2	*Cercophora coprophila*, *C. salama* *Podospora austroamericana*, *P. anserina*, *P. comata*, *P. setosa*, *Zopfiella tetraspora*
		SKS4L2.3	*Cercophora coprophila*, *C. salama* *Zopfiella tetraspora* *Podospora comata*, *P. austroamericana*, *P. anserina*, *P. setosa* *Apiosordaria backusii* *Zopfiella longicaudata* *Cladorrhinum phialophoroides*
		SKS5B1	*Fusarium* sp., *F. oxysporum*, *F. equiseti*, *F. solani*
		SKS5B2	*Cercophora coprophila*, *C. salama* *Zopfiella tetraspora* *Podospora anserina,P. comata*, *P. austroamericana*, *P. anserina*, *P. setosa*
		SKS5L2	*Cercophora coprophila*, *C. salama* *Zopfiella tetraspora*, *Podospora anserina*, *P. comata*, *P. austroamericana*, *P. setosa*

Province	District	Name of unknown isolate	Possible genus and species (according to ITS-rDNA region)
Satun	Khuan kalong	ST1B1.3.1	*Fusarium* sp., *F. solani* *Nectria haematococca*
		ST1B1.3.2	*Fusarium* sp., *F. solani* *Nectria haematococca*
		ST1B2.4	*Trichoderma* sp., *T. koningiopsis*, *T. gamsii*, *T. ovalisporum*, *T. intricatum* *Hypocrea koningii*
		ST1L2.2.1	*Acremonium* sp., *A. kiliense*, *A. strictum*, *Sarocladium kiliense* *Hypocreales* sp. *Sigmoidea* sp. *Phaeoacremonium tuscanum* *Nectria mauritiicola*
		ST1L2.4.1	*Fusarium* sp., *F. solani* *Nectria haematococca* *Trametes corrugata*
		ST1F0.3.2	*Fusarium* sp., *F. solani* *Nectria haematococca* *Trametes corrugate*
Nakhon Si Thammarat	Thung song	1.2	*Fusarium* sp., *F. equiseti, F. chlamydosporum*
		1.3	Fusarium sp., *F. equiseti*
		1.4	*Fusarium* sp., *F. equiseti, F. oxysporum*, *F. incarnatum*

Molecular cloning and sequence analysis of the cDNA encoding elecitin gene of *P. palmivora*

Cloning of elecitin gene of *P. palmivora* (*Ppal_Eli*)

The elicitin cDNA of *P. palmivora* (*Ppal_Eli*) was cloned by firstly obtained a fragment of the gene through PCR amplification using a pair of primers (F_Elicitin and R_Elicitin) (Table 1), designed from the conserved regions of elicitin gene in other *Phytophthora* spp. Comparative analysis of nucleotide sequences of elicitin gene among several species in the genus *Phytophthora* showed that the nucleotide sequences were highly conserved. The primer F_Elicitin and R_Elicitin were designed corresponding to the highly conserved sequences of elicitin of *P. palmivora* (AY206986), *P. rubi* (DQ012534), *P. ramorum* (DQ680026 and DQ229220), *P. alni* (DQ012517 and DQ012512), *P. capsici* (AY206985), *P. megasperma* (AJ493606), *P. cryptogea* (Z34462) and *P. laterais* (AY659654) submitted in the GenBank database. The product amplified by F_Elicitin/R_Elicitin yield a single band sizes approximately 220 bp (Fig. 8). To confirm our obtained fragment was a part of elicitin gene, the nucleotide sequence was compared with the partial mRNA of *P. palmivora* elicitin previously submitted in GenBank accession number AY206986.

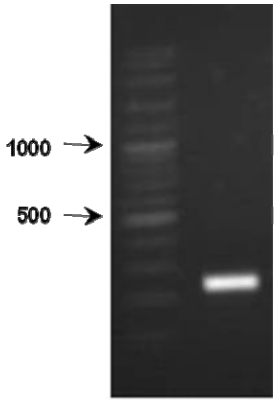

Fig. 8 The elicitin of *P. palmivora* amplified using the primer set F_Elicitin/R_Elicitin. The PCR product of the elicitin cDNA analyzed by electrophoresis on 1.5% agarose gel with 0.5 µg/ml ethidium bromide compared with 100 bp Plus DNA Ladder (Vivantis).

After the *Ppal_Eli* cDNA was obtained, 5' and 3' RACE approach was used to gain the nucleotide sequence of both ends. A full length elicitin cDNA was reconstructed from overlapping sequences. To confirm the correction of obtained sequence, the information from both 5' and 3' ends was used to design a primer set for amplification of a full length *Ppal_Eli* cDNA. The full length of *Ppal_Eli* was 485 bp in length (Fig. 9a) and consisted of a sequence corresponding to a signal peptide. An open reading frame (ORF) of elicitin gene of *P. palmivora* (*Ppal_Eli*) with or without the sequence of signal peptide was later amplified. The full ORF fragment and ORF without signal peptide (including the stop codon) sized approximately 357 bp and 297 bp, respectively as shown in Fig. 9b. All these PCR products were also cloned and sequenced.

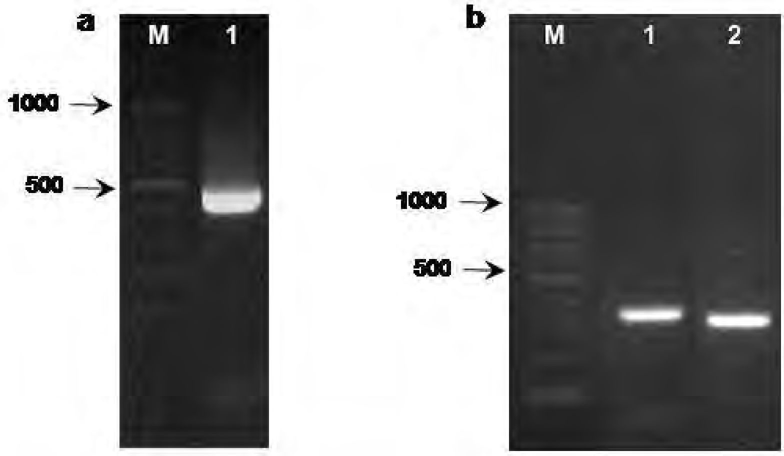

Fig. 9 The elicitin gene of *P. palmivora*. **(a)** Full length of elicitin cDNA (start codon to poly A tail) (Lane 1) compared with 100 bp Plus DNA Ladder (M, Vivantis). **(b)** ORF of elicitin gene Lane 1: the full ORF (start codon to stop codon); Lane 2: ORF coding for the mature protein (after that of signal peptide to stop codon) and Lane M: 100 bp DNA Ladder (Vivantis). The PCR products were analyzed by electrophoresis on 1.5% agarose gel with 0.5 µg/ml ethidium bromide.

Sequence analysis and molecular data of elecitin gene of *P. palmivora* (*Ppal_Eli*)

BLAST analysis of the full length of *Ppal_Eli*, 485 bp, showed perfect match with elicitin sequence from *P. palmivora* (AY206986) present in NCBI database. The sequence was deposited in NBCI and the accession number is KJ530971 Moreover, the Blast analysis of the sequence (accession number KJ530971) revealed a high similarity with the elicitin from other *Phytophthora* spp. for example 90% Identity with acidic elicitin (AE2) from *P. alni* (DQ012517.1) and RAM1B from *P. ramorum* (DQ229219), 89% identity with acidic elicitin A1 from *P. cryptogea* (Z34462) and 87% Identity with alpha elicitin 1 from *P. lateralis* (DQ680028).

The full length cDNA sequence of *Ppal_Eli* revealed the presence of one ORF consisting of 357 bp. The start and stop codons were located at positions 1 and 355, respectively. The coding region of *Ppal_Eli* gene encoded for a protein of 118 deduced amino acids (Fig. 10). The characterization of the ORF amino acid accomplished by its comparison to amino acid sequences of elicitin from other *Phytophthor*a species showing only one signal peptide and an elicitin domain. The calculated molecular mass of Ppal_Eli protein was 12208.90 Da and a pI of 4.68 (Compute pI/MW program available: http://web.expasy.org/compute_pi/).

Using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/), it contained a predicted signal peptide of 20 amino acid from residues 1 to 20 (Met1 to Ala20) and the cleavage site between Ala20 and Thr21. The molecular mass and pI was reduced to 10228.59 Da and 4.23, respectively after signal peptide was removed (Compute pI/MW program available: http://web.expasy.org/compute_pi/). The signal peptide is highly conserved 18–20 amino acids sequence. Elicitins are synthesized as pre-proteins which undergo post translational modifications through the removal of signal peptide (Panabieres *et al*., 1997; Kamoun *et al*., 1997). InterproScan identified one elicitin domain of 95 amino acid length from T21 to A116 (Fig. 11). This elicitin protein lacked protein kinase C-dependent phosphorylation sites found in the C-terminal region of basic elicitins (Ponchet *et al*., 1999).

The complete CDS of the mature protein, without signal peptide, of Ppal_Eli consisted of 98 amino acids. The N-termianl of deduced amino acid sequence was identical with that of palmivorein, a purified elicitin secreted by *P. palmivora* (Churngchow and Rattarasarn, 2000; 2001). Moreover, the N terminal portion of deduced amino acid sequence has Val at position 13 which are consistant with the class acidic alpha elicitins (Huet *et al*., 1994) (Fig. 12). There has been reported that amino acid residue 13 is correlated with necrotic activity of elicitins (Vijesh Kumar *et al*., 2013). The beta elicitins contain a basic Lys residue, which is more necrotic, whereas alpha elicitins contain a hydrophobic Val residue at this position instead (O'Donohue *et al*., 1995).

Disulfide tool predicted the cysteine disulfide bonding state and connectivity as follows: Cys3–Cys71, Cys27–Cys56 and Cy51–Cys95 which is a typical characteristic of elicitin (Fig. 13). The even number of cysteine residues can induce defense response in plants, and disulfide brigdes formed by the cysteine residues are essential for HR induction and avirulence function (Laugé and De Wit, 1998; Von't Slot and Knogge, 2002). The disulfide bridges might enhance stability in the plant apoplast which known to be rich in degradative proteases (Joosten *et al*., 1997).

Like other eliciitns, Ppal_Eli contains 9 Leu, 6 Cys and 3 Met while lacks Trp, His and Arg (Vijesh Kumar *et al*., 2013).

The predicted 3-dimentional (3D) structure, by Phyre^2 (http://www.sbg.bio.ic.ac.uk/ phyre2/html/page.cgi?id=index), of Ppal_Elicitin is shown in Fig. 14. The modeled structure composed of 2 antiparallel sheet and 5 helices which was similar to those of the predicted 3D model of other elicitins.

1 **ATG** AAC TTC CGC GCT CTG TTC GCC GCC ACC GTC GCC GCC CTC GTT
M N F R A L F A A T V A A L V
46 GGC TCC ACC TCC GCC ACC ACG TGC ACC ACC ACC CAG CAG ACT GCC
G S T S A T T C T T T Q Q T A
91 GCG TAC GTC GCG CTC GTG AGC ATT CTC TCG GAT TCA TCT TTT AAC
A Y V A L V S I L S D S S F N
136 CAG TGC TCG ACG GAC TCG GGC TAC TCG ATG CTG ACG GCC ACC TCG
Q C S T D S G Y S M L T A T S
181 CTC CCC ACC ACG GCC CAG TAC AAG CTC ATG TGC GCG TCG ACG GCT
L P T T A Q Y K L M C A S T A
226 TGC AAC ACG ATG ATC ACC AAG ATC GTG TCG CTC AAC CCG CCC GAC
C N T M I T K I V S L N P P D
271 TGT GAG CTG ACG GTA CCA ACG AGC GGC CTG GTA ATC AAC GTCTAC
C E L T V P T S G L V I N V Y
316 TCG TAC GCG AAC GGC TTC TCT GCT ACG TGT GCA TCG CTA **TAA** GCA
S Y A N G F S A T C A S L *
406 TGT ATA CAG CTT CAG CGC TTG CTG TTG TTG TCA CGC TCT TAA ATG
451 AAG GTG AAG GTT CAT GTG TTT AGA AAA AAA AAA AA 485

Fig. 10 Nucleotide sequence and deduced amino acid sequence of the elicitin gene of *P. palmivora* (*Ppal_Eli*). The predicted ORF is from 1 to 354 bp (the start and stop codon are shown in bold letters). Start codon (ATG) and stop codon (TAA) are presented in bold while the signal peptide is presented in box.

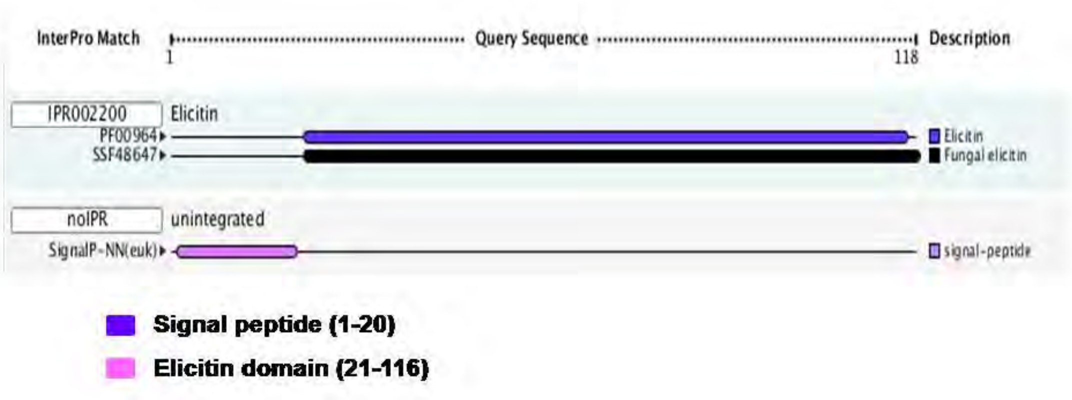

Fig. 11 Complete coding sequence of the putative protein Ppal_Eli, the elicitin of *P. palmivora*, showing a leading signal sequence and a core elicitin domain.

1 TTCTTTQQTAAYVALVSILSDSSFNQCSTDSGYSMLTATSLPTTAQYKLMCASTACNTMI

61 TKIVSLNPPDCELTVPTSGLVINVYSYANGFSATCASL

Fig. 12 Complete CDS of the mature protein (without signal peptide) of Ppal_Eli, the elicitin of *P. palmivora*.

```
                                                              +-----------------------------
                                      +-----------------------|----+
              +-----------------------|-----------------------|----|--------------+
              |                       |                       |    |              |
            .........10........20........30........40........50........60........70........
AA          TTCTTTQQTAAYVALVSILSDSSFNQCSTDSGYSMLTATSLPTTAQYKLMCASTACNTMITKIVSLNPPDCELTVPTSG
DB_state      1                       1                       1    1              1
DB_conf       7                       8                       8    8              8

            ---------------+
                           |
                           |
                           |
            80........90.......
AA          LVINVYSYANGFSATCASL
DB_state                   1
DB_conf                    7

DB_bond      bond(3,71)
DB_bond      bond(27,56)
DB_bond      bond(51,95)
```

Fig. 13 Cysteine disulfide bonding state and connectivity in the elicitin protein from *P. palmivora*, Ppal_Eli.

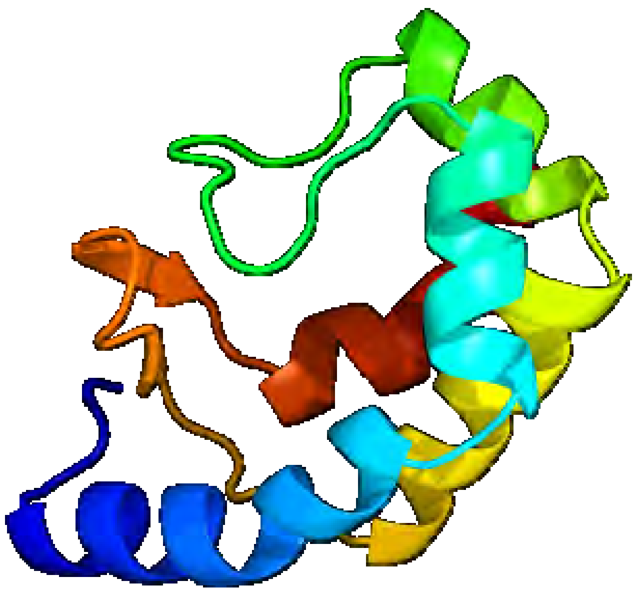

Fig. 14 Predicted 3-dimentional (3D) structure of the elicitin protein from *P. palmivora*, Ppal_Eli.

Phylogenetic analysis of the elicitin Ppal_Eli sequence

To study the evolutionary relationship of elicitin, phylogenetic trees were constructed based on both nucleotide sequence and amino acid sequence of the elicitin from *P. palmivora* Ppal_Eli, and those of elicitin from other *Phytophthora* species. The nucleotide and amino acid sequences of elicitins produced in the closely related genus *Pythium* were used as outgroup. In the phylograms based on both nucleotide and amino acid sequences, maximum likelihood (ML) with 1000 bootstraps (MEGA6) was used for tree construction. The constructed trees were used to base trees with the 3 major clusters including Class I, Class II and Class III. Elicitin like proteins belonging to Pythium groups formed a separate cluster, as outgroup in the phylogenetic tree.

Our interested Pal_Eli were presented in Class I. The nucleotide based constructed tree showed high similarity with alpha (or acidic) elicitin of *P. parasitica* and *P. ramorum* while the amino acid based tree showed closely relation with alpha elicitin of capsicein, cambivorin and ram-1 produced in *P. capsici*, *P. cambivora* and *P. ramorum*, respectively. Both phylogenetic trees therefore indicating that the protein Pal_Eli was grouped in alpha elicitin (Fig. 15 and 16). The clade indication the acidic protein was correlated to the predicted pI of the deduced amino acid. However, the members of Class I could be divided into 2 groups; acidic and basic groups which is clearer in the phylogram of amino acid data. The proteins belonging to Class I appeared on separate cluster in the tree confirming the division of elicitins as acidic and basic (Kumoun *et al*., 1997). The highly acidic elicitins containing a short hydrophobic C-terminal tail were appeared on a distinct branch as class II. The INF2A, INF2B, INF6, INF5 and γ-megaspermin clustered as class III which consists of elicitin domain and O-glycosylated domain.

Since elicitin, especially certain Class I elicitins, are also sterol carrier proteins which bind sterols and lipids and catalyze their transfer between membranes (Mikes *et al*., 1997; Vijesh Kumar *et al*., 2013). Binding of elicitin to ligands appears to be essential for induction of a biological response in plant (Osman *et al*., 2001).Interaction between the protein and ergosterol involves several residues, among which Try residues are the most denoted (Boissy *et al*., 1999). Based on the close location (Fig. 16) as well as 3D modeled structure similarity (Fig. 14) of our Ppal_Elicitin and capsicein protein, we could conclude that there is the ergosterol binding site in Ppal_Elicitin. Vijesh Kumar *et al*. (2013) reported that ergosterol binds to the groove located inside the gravity of capsicein via hydrogen bonding between ergosterol and Tyr47.

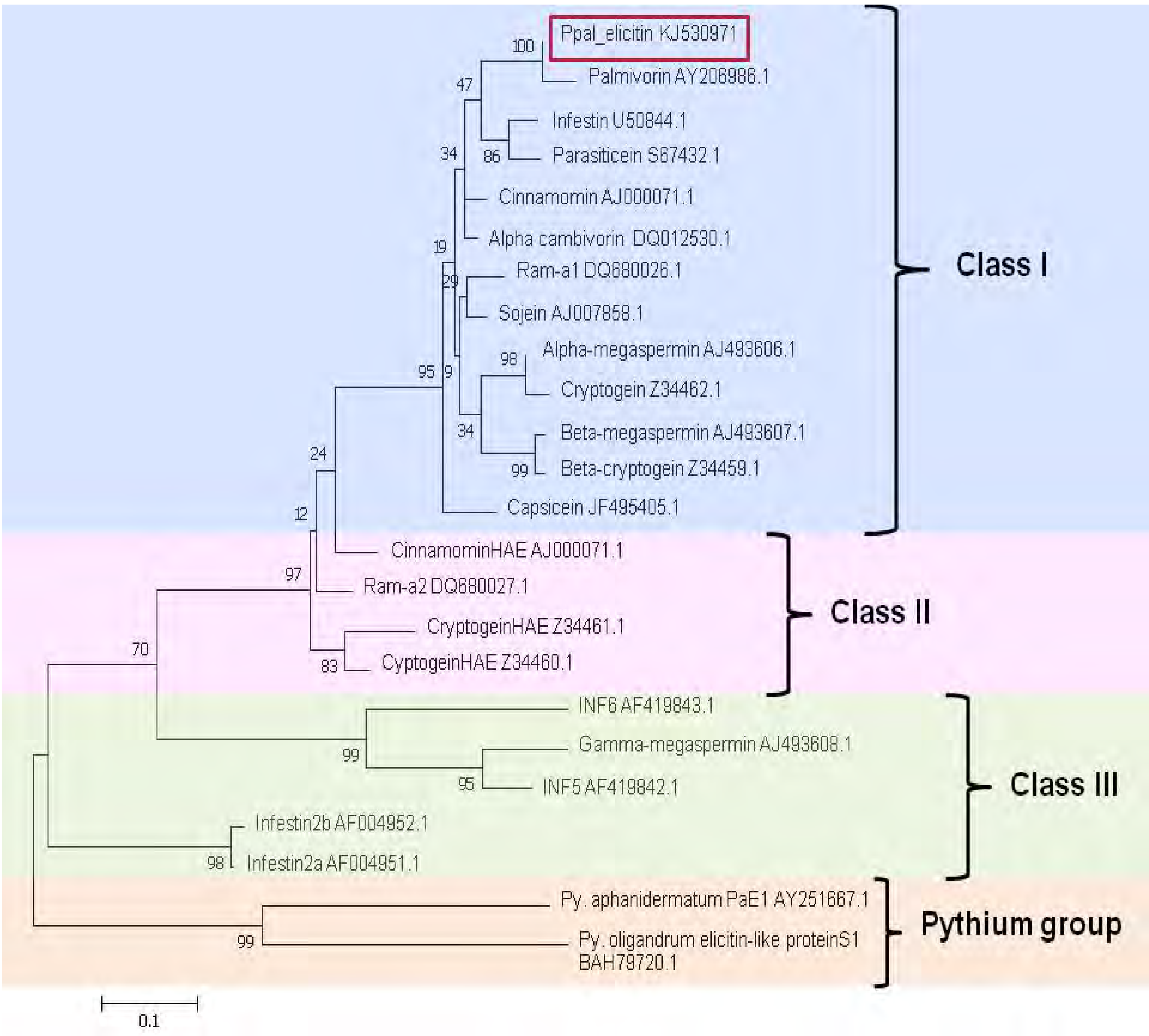

Fig. 15 Phylogenentic analysis based on nucleotide sequences of eliciitin cDNA from *P. palmivora*, *Ppal_Eli* with elicitin gene from other *Phytophthora* spp. The elicitins-like gene in the closely related genus *Pythium* were used as outgroup. The phylogenetic tree was constructed via maximum likelihood (ML) with 1000 bootstraps, K2P and BioNJ algorithm (MEGA6).

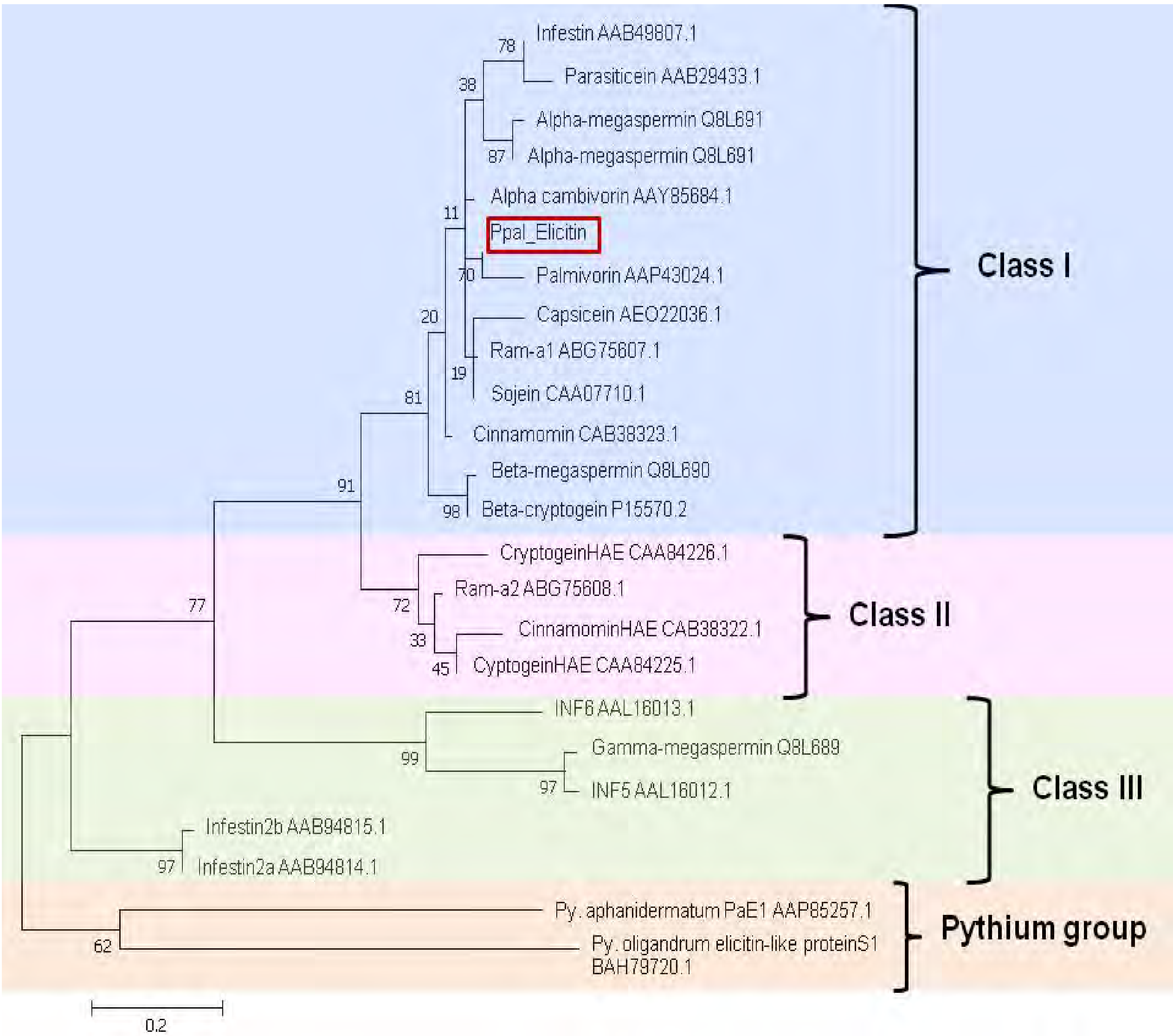

Fig. 16 Phylogenentic analysis based on the deduced amino acid sequences of eliciitin from *P. palmivora*, Ppal_Eli with elicitin from other *Phytophthora* spp. The amino acid sequences of elicitins produced in the closely related genus *Pythium* were used as outgroup. The phylogenetic tree was constructed via maximum likelihood (ML) with 1000 bootstraps, KTT and BioNJ algorithm (MEGA6).

Virulence and pathogenecity of different isolates of *P. palmivora*

Subcultured from the KBNM 9 lineage after maintained in the sterile distilled water since 2008, we noted 3 general colony morphologies; fast-growing, slow-growing and very slow-growing. Subculture weekly from individual KBNM 9 isolate gave rise to 3 type morphologies, although the factors responsible for this variability are unknown. Four isolates including one exhibiting very slow-growing (Ppal3), those 2 for slow growing (Ppal1 and Ppal5) and another one for fast-growing (Ppal4) were selected for further studies. The mycelia of all isolates was white, however, there were some characters varied between isolates such as the pattern growing on agar and the sporangium morphology observed under microscope. The mycelia of each isolate growing on PDA for 10 d are shown in Fig. 17.

However, the rate of growing as well as the mycelia present on V_8 agar was similar (data not shown). For more detail, we found that not only mycelium character, sporangium production and zoospore releasing was also different, that are summarized in Table 4. The zoospore released from each isolated was tested for its potent to infect rubber leaves; the different in infection level was obviously observed (data not shown).

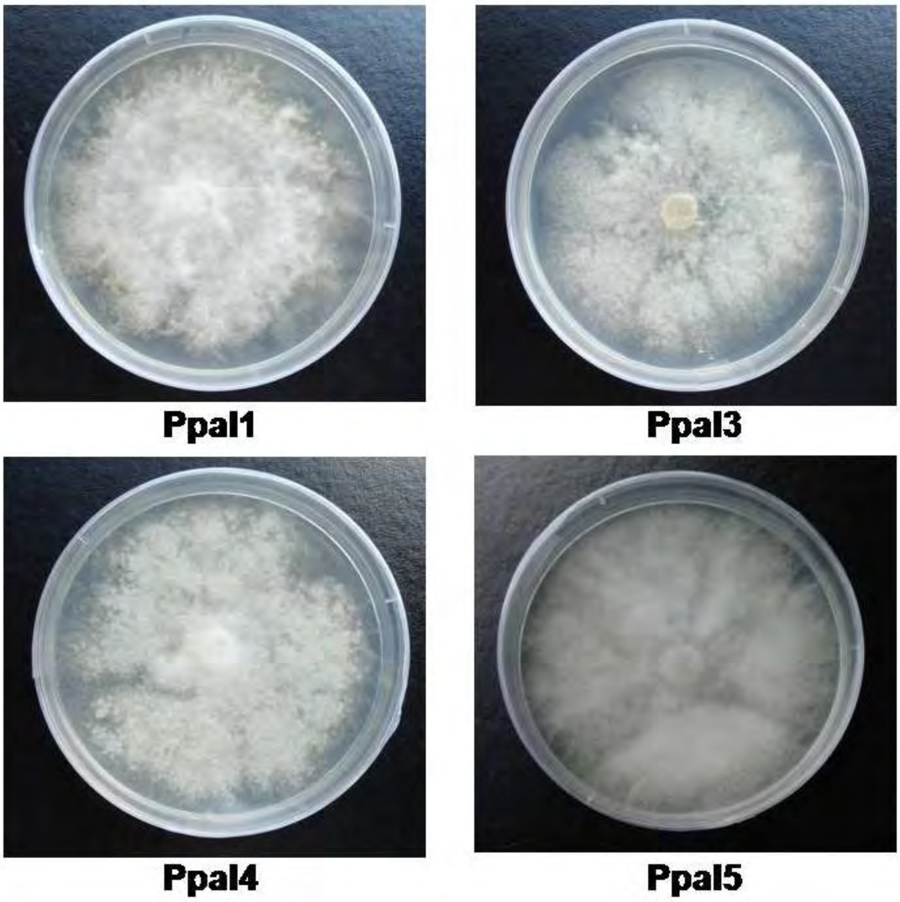

Fig. 17 Mycelium of various isolates obtained from *P. palmivora* KBNM 9 lineage growing on PDA for 10 d.

Table 4 The detail of various isolates obtained from *P. palmivora* KBNM 9 lineage.

Isolate	Sporangium production	Zoospore releasing	Growing diameter on PDA (cm)		Classification group
			4 d	7 d	
Ppal1	++++	++++	3	6	Slow-growing
Ppal3	+++	++	3	4.5	Very slow growing
Ppal4	+	+	4.5	7.5	Fast-growing
Ppal5	++	++	4	6	Slow-growing

The rubber leaf pieces, sized 2x2 cm^2, were stabbed (at the center) to make a wound on the abaxial surface following by inoculated with the mycelium plug, on V_8 agar, of each isolate of *P. palmivora* (Papl1, Papl3, Papl4 or Papl5) on the wound. A mycelium-free V_8 agar was placed on the wounded leaf pieces as mock control while only wounding was set as control. The different in necrotic size between various isolates was observed (Table 5 and Panel a of Fig. 18 and Fig. 19). Ppal1 caused smallest necrotic lesion sizes, average at 0.40 $\pm$ 0.27 cm in diameter while Ppal5 which was also in group of slow-growing showed a bit larger necrotic lesion with diameter of 0.59 $\pm$ 0.19 cm. Fast growing group, Ppal4 caused 0.88 $\pm$ 0.18 cm in diameter of necrosis. Interestingly Ppal3, grouped as very slow-growing on the medium, induced largest lesion size at approximately 0.98 $\pm$ 0.47 cm. To improve the vision of necrotic lesion, chlorophyll was removed from the leaf tissue by boiling the leaf pieces in 95% (v/v) ethanol. We observed that the necrosis lesion appeared were corresponded to those of the measured diameter size mentioned previously (Table 5 and Panel b of Fig. 18). Cell death was also monitored in untreated and mycelium inoculated leaves by trypan blue staining and the dead cells were monitored as blue color. As expected, Ppal3 induced expanded largest cell death more than those caused by Ppal4 followed by Ppal5 and Ppal1, respectively (Panel b of Fig. 19).

According to the necrotic lesion and cell death together, among the tested strains of *P. palmivora* the results indicating that Ppal3 expressed the most virulence followed by Ppal4 while Ppal5 and Ppal1 showed the similar virulent level. However, the relationship between the rate of growing on the medium, sporangium production, zoospore releasing and virulence was still unclear.

Table 5 Necrotic lesion size on rubber leaf pieces treated with mycelium plug of various isolates of *P. palmivora*.

Isolate	Classification group	Diameter of necrotic lesion size (cm)
Ppal1	Slow-growing	0.40 $\pm$ 0.27
Ppal3	Very slow growing	0.98 $\pm$ 0.47
Ppal4	Fast-growing	0.88 $\pm$ 0.18
Ppal5	Slow-growing	0.59 $\pm$ 0.19

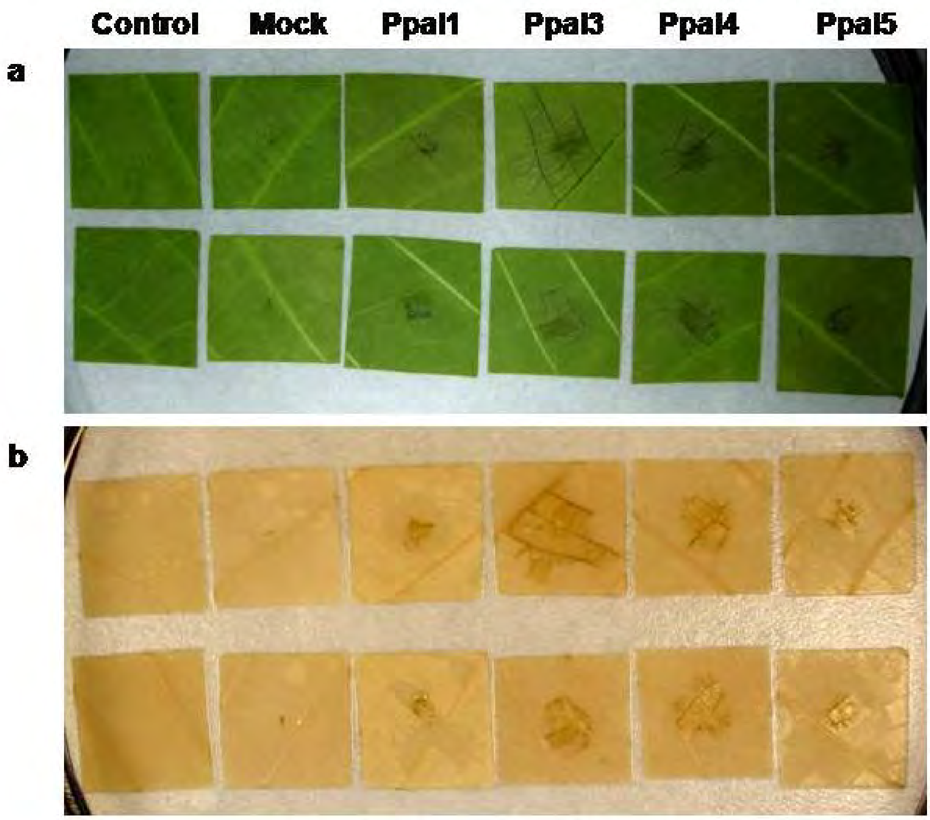

Fig. 18 Pathogenecity (necrosis and cleared necrotic leaion) of various isolates obtained from *P. palmivora* KBNM 9 lineage. **(a)** Necrosis and **(b)** cleared necrotic lesion, obtained after removing of the chlorophyll, on rubber leaf pieces inoculated with the mycelium plug of various isolates for 24 h. Only wounding was set as control whereas the mycelium-free V_8 agar was used as mock control.

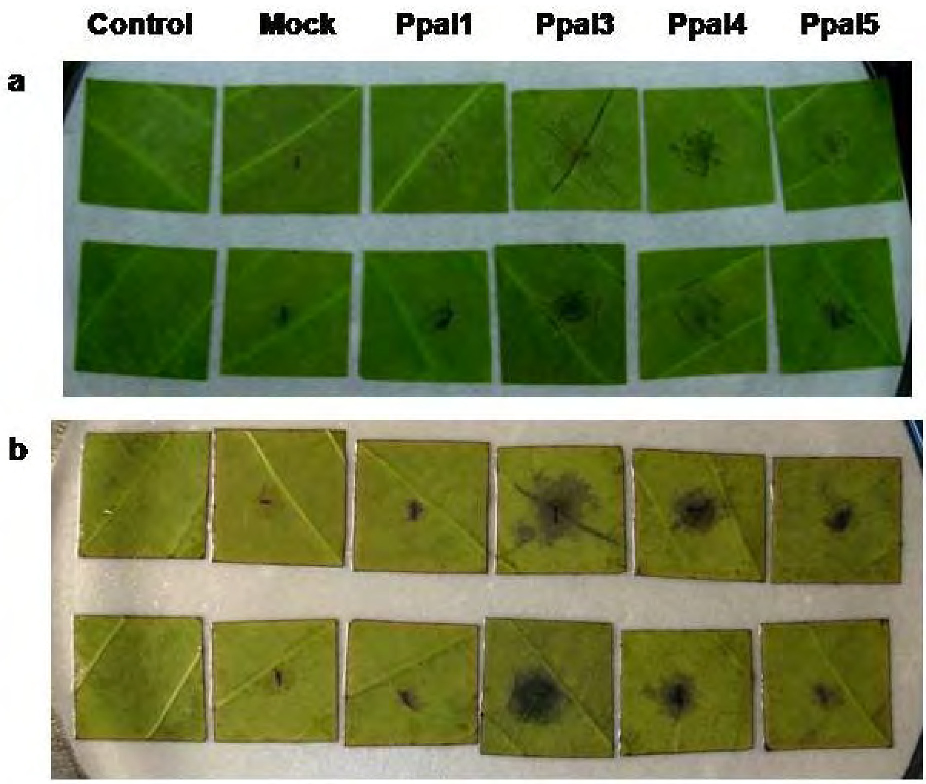

Fig. 19 Pathogenecity of various isolates obtained from *P. palmivora* KBNM 9 lineage detected by necrosis and cell death observation on rubber leaf pieces inoculated with the mycelium plug of various isolates obtained from *P. palmivora* KBNM 9 lineage for 24 h. **(a)** Necrosis observed by naked eye and photographed and **(b)** cell death observed after staining with trypan blue. Only wounding was set as control whereas the mycelium-free V_8 agar was used as mock control.

The *in vitro* and *in palnta* expression level of elicitin gene (*Ppal_Eli*) in various *P. palmivora* isolates

The expression level of elicitin *Ppal_Eli* gene was analyzed via semi-quantitative RT-PCR. Total RNA was extracted from the mycelia and rubber leaf tissue infected with mycelia of each isolate for *in vitro* and *in planta* analysis, respectively.

The *in vitro* elicitin gene expression was shown in Fig. 20. The highest relative expression level was detected in Ppal1 followed by Ppal3 and Ppal4 while it was lowest in Ppal5.

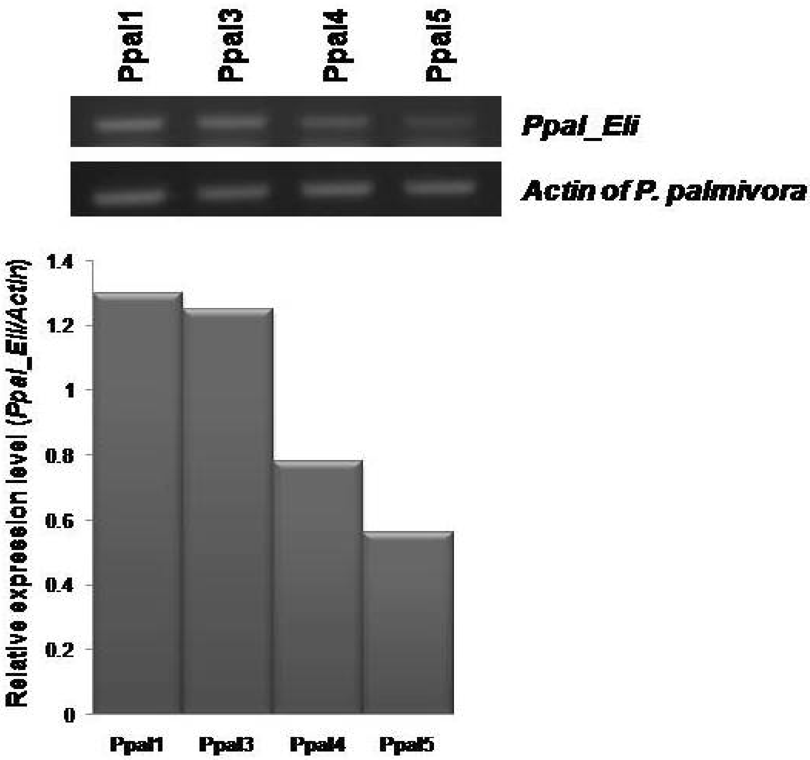

Fig. 20 *In vitro* expression level of *Ppal_Eli* elicitin gene. The elicitin gene was analyzed in the mycelium of Ppal1, Ppal3, Ppal4 or Ppal5 cultured in V_8 medium for 7 d. The elicitin expression level was revealed normalized by the expression level of internal marker *P. palmivora* actin gene.

Since the treated- and untreated-leaf samples were collected 24 h after mycelium inoculation, the occurrence of infected and growing pathogen in the leaves was consequently small as the faint band of *P. palmivora* actin gene was observed. Moreover, the level of *Ppal_Eli* elicitin gene expression seemed to be very low since it could be detected when PCR was performed with great amount of cDNA template and using elevated cycle number, 40 cycles. The expression values of reported elicitin gene expression were calculated from its band intensity normalized to that of the internal marker actin gene as shown in Fig. 21. The result showed that *in planta* elicitin expression level was highest in the Ppal4 treatment while Ppal3 expressed lower level of elicitin gene. However, very small amount of elicitin gene was revealed in the leaves treated with Ppal1 and Ppal5.

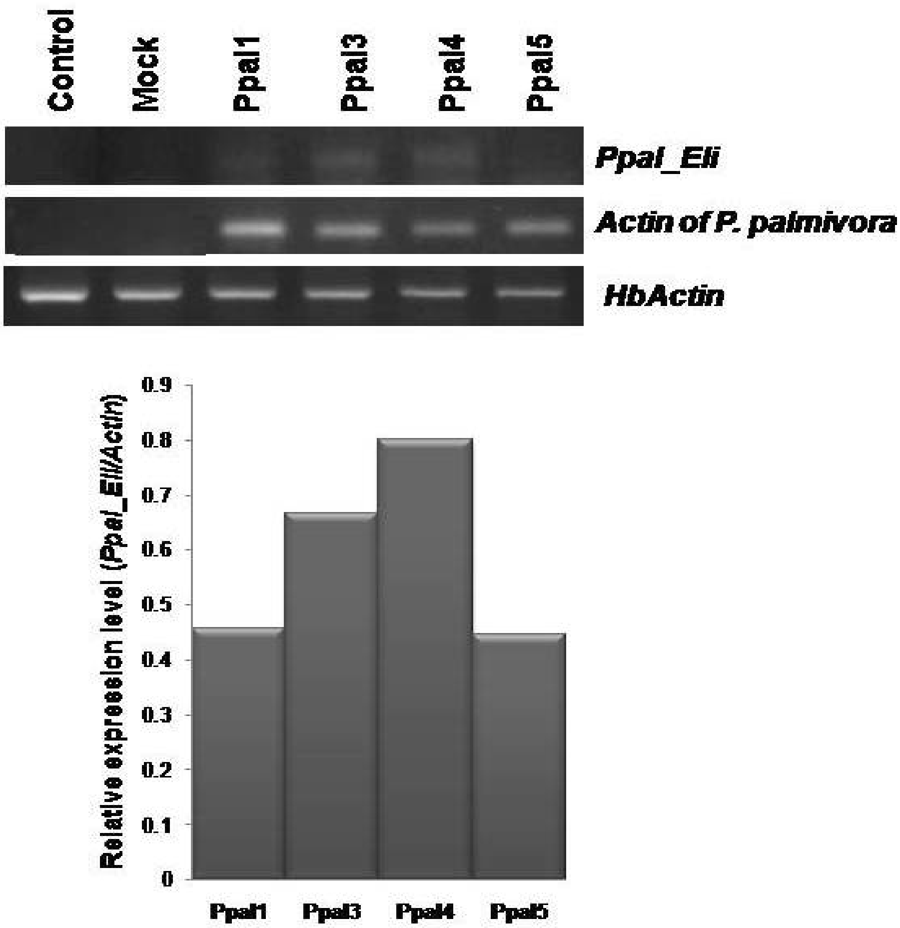

Fig. 21 *In planta* expression level of *Ppal_Eli* elicitin gene in the rubber leaf tissues treated or untreated with the mycelium of various isolates of *P. palmivora*. The rubber leaf pieces were untreated, mock and treated with mycelium plug of Ppal1, Ppal3, Ppal4 or Ppal5 for 24 h. The elicitin expression level was revealed normalized by the expression level of internal marker *P. palmivora* actin gene. The actin gene of *H. brasiliensis* (*HbActin*) was used to verify the equal amount of template.

Comparative analysis of elicitin gene expression and virulence of various isolates of *P. palmivora*

To establish the possible correlation between the expression of elicitin gene (*Ppal_Eli*) and the potency to infect the rubber host plant, the *in vitro* and *in planta* expression level of this gene in each *P. palmivora* isolate was compared with their virulence as observed in the corresponding necrosis mentioned above together with the important characters (Table 6).

Table 6 Character, necrosis induction potency and elicitin gene expression level in various isolates of *P. palmivora*.

Isolate	Group	Sporangium production	Zoospore releasing	necrosis size (cm)	Relative elicitin gene expression (*in vitro*)	Relative elicitin gene expression (*in panta*)
Ppal_1	Slow-growing	++++	++++	0.40 $\pm$ 0.27	1.3	0.46
Ppal_3	Very slow growing	+++	++	0.98 $\pm$ 0.47	1.25	0.67
Ppal_4	Fast-growing	+	+	0.88 $\pm$ 0.18	0.78	0.80
Ppal_5	Slow-growing	++	++	0.59 $\pm$ 0.19	0.56	0.44

The results in this study revealed that the involvement of elicitin and virulent pathogenecity is still unclear. The trend of positively related was denoted, since *Ppal_Eli* gene expressed *in planta* in high level in isolate Ppal3 and Ppal4, which induced expanded necrosis while the elicitin gene expression was lower in Ppal1 and Ppal5 corresponding to the small necrotic lesions they induced. We could not observe any relationship between *in vitro* elicitin gene expression and virulence; conversely, it was seemed to be involved in the sporangium production and zoospore releasing as in Ppal1 and Ppal3. Therefore, based on our data it could not conclude for the role or involvement of elicitin in pathogen virulence.

Further studies are needed to determine any additional factors that may directly influence its activity or the kinetics of this gene expression during it growth in medium or in host plant. Since elicitin gene expression in both *P. parasitica* (Colas *et al*., 2001) and *P. infestans* (Kamoun *et a*l., 1997) was down-regulated during plant infection. Furthermore, Horta *et al*. (2008) observed a decline in elicitin expression in 15 day-old cultures, suggesting that the elicitin expression is maximized during activate mycelium growth.

Many reports have suggested that the mechanistic basis of any elicitin-associated pathogenecity in *Phytophthora* is until recently controversial, as it has been observed that they can act either as virulence or avirulence factors. For example, it has been suggested that elicitins are non-specific toxins capable of inducing necrosis in any plant species (Huet *et al*., 1994; Pernollet *et al*., 1993), perhaps associated with lipid membrane disruption (Nespoulos *et al*., 1999). While other authors have shown that necrosis is restricted to specific plant species or cultivars (Kamoun *et al*., 1997). A number of studies have suggested that elicitins are not required for *Phytophthora* virulence. For example, Ricci *et al*. (1992) found that virulent isolates of *P. parasitica* do not produce the elicitin in culture and Kamoun *et al*. (1998) showed that silencing of the elicitin (INF1) gene enhanced virulence of *P. infestans* in *Nicotiana benthamiana*. Fleischmann *et al*. (2005) found that alpha elicitin gene expression peaks at the onset of host necrosis and the onset of sporulation, they thus speculate the role of alpha elicitins is connected with the process of sporulation and/or pathogen survival under saprophytic conditions. Conversely,

It is possible that pathogenesis involves 2 components: (1) a slow or incomplete HR-like response by the host that, although it cannot limit pathogen colonization, leads to cell death and/or physiological impairment, and (2) the role of elicitins on *Phytophthora* growth and colonization *in planta*. In regard to the latter, elicitins appear to be necessary for the acquisition of plant sterols by *Phytophthora* spp., which are necessary for spore production and hyphal growth (Horta *et al*., 2008; Ponchet *et al*., 1999). More work is clearly needed to identify the full role and mechanism(s), of the certain family in *Phytophthora* spp. pathogenecity and disease development.

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ :

1.1 Manuscript เรื่อง Single Round Semi-Nested Multiplex PCR: A Developed Strategy for Rapid and Simultaneous Detection of Rubber Tree Pathogens *Phytophthora* spp. and *P. palmivora* (submitted) อยู่ในระหว่างการพิจารณาจากวารสาร Journal of Phytopathology

2. การนำผลงานวิจัยไปใช้ประโยชน์ : -
3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

3.1 บทความเรื่อง วิธีการตรวจวินิจฉัยเชื้อไฟทอปทอร่า (*Phytophthora* spp.) ในยางพารา ซึ่งจะตีพิมพ์ในหนังสือชุดความรู้ ม.อ. ด้านยางพารา อยู่ในระหว่างดำเนินการตีพิมพ์

3.2 การเสนอผลงานในที่ประชุมวิชาการ (แบบโปสเตอร์) เรื่อง Rapid Diagnosis of Rubber Tree Pathogen, *Phytophthora* spp. and *P. palmivora*, by the Single Round Semi-Nested Multiplex PCR ในการประชุมนักวิจัยรุ่นใหม่ พบ เมธีวิจัยอาวุโส สกว. วันที่ 16-18 ตุลาคม 2556 ณ โรงแรมเดอะรีเจ้นท์ ชะอำบีช รีสอร์ท หัวหิน ชะอำ จังหวัดเพชรบุรี

Appendix

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Single Round Semi-Nested Multiplex PCR: A Developed Strategy for Rapid and Simultaneous Detection of Rubber Tree Pathogens Phytophthora spp. and P. palmivora

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Keywords:	molecular diagnosis, Phytophthora spp., P. palmivora, single round semi-nested multiplex PCR, rubber plant

Simultaneous detection of Phytophthora spp. and P. palmivora, Department of Biochemistry, Faculty of Science, Prince of Songkla University, Hat-Yai, Songkhla, Thailand

Single Round Semi-Nested Multiplex PCR: A Developed Strategy for Rapid and Simultaneous Detection of Rubber Tree Pathogens *Phytophthora* spp. and *P. palmivora*

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Keywords: molecular diagnosis, *Phytophthora* spp., *P. palmivora*, single round semi-nested multiplex PCR, rubber plant

Abstract

Phytophthora spp. cause devastating effects on crops and natural ecosystems worldwide. In Thailand, *P. palmivora* is an important pathogenic species of the rubber plant (*Hevea brasiliensis*) reducing latex production. This study aimed to develop a PCR-based method to rapidly and simultaneously detect the genus *Phytophthora* and *P. palmivora*. Two selected primer pairs, Phy1s/Phy2a and Pal1s/Pal2a, were tested for their specificities. The nucleotide sequences revealed that the amplified products of 1000 bp and 650 bp represented the ITS1-5.8S-ITS 2-28S region of the genus *Phythophthora* and *P. palmivora*, respectively. A single round nested multiplex PCR with 4 primers, Phy1s/Phy2a/Pal1s/Pal2a, and a single round semi-nested multiplex PCR using 3 primers, Phy1s/Phy2a/Pal2a, were established. By using the various amounts of DNA from a control strain *P. palmivora* KBNM 9 as well as the vector containing ITS region as templates; the sensitivity of these methods was studied compared to the simple PCR method. The single round semi-nested multiplex PCR was further focused because of its sensitivity, more rapidity, less contamination risk and cost. This method was tested to detect *P. palmivora* in infected leaf tissue and soil. It could be further applied to rapidly diagnose *Phytophthora* spp. and *P. palmivora* in naturally diseased plant materials, soil and water. This diagnostic method may be valuable for the detection and management efforts with diseases of other crops.

Introduction

Hevea brasiliensis (Wild.) Muell.-Arg. or the rubber plant is an economically important crop in Thailand; its product latex is a major Thai export. At present, South East Asia especially Thailand, Indonesia and Malaysia are the main sources of natural rubber. One consequence of the frequent tapping to collect the latex is that infections occur at the tapping wound sites. This problem is common in the southern part of Thailand where the humidity and temperature is high and conducive for pathogen growth and disease development. In Thailand, RRIM600 is the commonly used plantation cultivar because of its high-yield of latex but this cultivar is highly susceptible to *Phythophthora* pathogens.

Phytophthora leaf disease, caused by members of the oomycete *Phytophthora* spp., is by far the most important disease of the rubber tree. *Phytophthora* spp. described as pathogens of the rubber tree include *P. palmivora*, *P. botryosa*, *P. heveae*, *P. meadii* and *P. parasitica*. In the south of Thailand *P. palmivora* and *P. botryosa* are the most frequently isolated pathogens that cause black stripe, green pod rot and abnormal leaf fall (Butler 1996; Erwin and Ribeiro 1996). *P. palmivora* attacks the petioles causing mature leaves to fall prematurely and also attacks the tapping surface resulting in reduced latex production.

A rapid and accurate microbial identification method is essential for an inspection and survey program that will allow for an early detection of any pathogen. Conventionally, diagnosis of the *Phytophthora* disease has been performed by isolation of pure cultures of *Phytophthora* from diseased plants follow by identification based on morphological characterization. A definitive diagnosis for the plant pathogen using these traditional tests requires substantial experience in species differentiation within the genus *Phytophthora*. Moreover, there are difficulties in using phenotypic taxonomic characteristics in that there is overlap between some species, and significant variations regularly occur among isolates of the same species (Erwin and Ribeiro 1996; Appiah et al. 2004; Cooke et al. 2007). Nowadays molecular methods allow more accurate species identification and detection of the pathogens.

Many species of *Phytophthora* can now be identified using their unique DNA sequences in the internal transcribed spacer (ITS) regions of their nuclear ribosomal DNA (rDNA) repeat region (Cooke and Duncan 1997; Drenth and Irwin 2001; Winton and Hansen 2001; Chimento et al. 2005). Recently, the polymerase chain reaction (PCR) has been used extensively for detection of plant pathogens (Martin et al. 2000; 2004; Ward et al. 2004).

There are several advantages of PCR based detection methods over the traditional diagnosis methods; for example, microorganisms do not need to be cultured, it is more reliable,sensitive and rapid (Cooke et al. 2007). The PCR-based methods include conventional PCR and quantitative real-time PCR. These methods have been reported as being effective for a number of *Phytophthora* species (Drenth and Irwin 2001; Winton and Hansen 2001; Ioos et al. 2005; Hayden et al. 2006; Schena et al. 2006; Lees et al. 2012). Nested PCR, a two-step system in which the first round PCR product is subjected to a second PCR amplification, is widely used to detect fungal plant pathogens due to its greater specificity (Ippolito et al. 2002). The sensitivity of this technique also allows the detection of the target pathogen in minute amounts of infected material (Renker et al. 2003; Alaniz et al. 2009). However this method is time consuming, expensive and labor intensive as it requires separate PCR reactions and additionally increases the risks of false positive due to cross contamination (Cooke et al. 2007). Multiplex PCR approach is an alternative tool to detect several pathogens simultaneously and reduce the time spent, costs and contamination risk. Multiplex PCR utilizes a single reaction with several primer pairs, each generating an amplicon that can be separated and visualized through electrophoresis (Chamberlain et al. 1988). This technique has been used extensively in plant pathology as it permits the identification of more than one target in a unique reaction (Winton and Hensen 2001: Schena et al. 2006: Boureau et al. 2013).

PCR-based methods have been utilized worldwide in order to identify and detect *P. palmivora* such as in orchids (Tsai et al. 2006), important plants in Australia (Drenth and Irwin 2001), coconut (Chowdappa et al. 2003) and citrus (Bowman et al. 2007). However, rapid identification and diagnosis of the major *Phytophthora* species in particular *P. palmivora* that cause leaf fall and black stripe of rubber tree is limited. The objective of this study was to develop a PCR based assay to detect *Phytophthora* spp. and specifically *P.*

palmivora. This was attempted by the development of the single round semi-nested multiplex PCR method to assist in the early diagnosis of rubber disease caused by *Phytophthora* spp. and to simultaneously verify that the causative species was *P. palmivora* in one reaction.

Materials and Methods

Phytophthora spp. and fungal culture and growth conditions

Two isolates of *Phytophthora* spp., *P. palmivora* named KBNM 9 which had been previously isolated from rubber trees showing disease symptoms (Churngchow and Rattarasarn 2000) and *P. botryosa*, were provided from the Songkhla Rubber Research Center, Songkhla, Thailand. *Penicillium funiculosum* (TISTR 3563), *Aspergillus niger* (TISTR 3254), *Fusarium solani* (TISTR 3436) and *Colletotrichum gloeosporioides* were obtained from the Thailand Institute of Scientific and Technological Research, Thailand. For the isolation of DNA, each isolate was grown in potato dextrose broth (PDB, Difco Laboratories, USA) at 25°C for 7 – 10 d. The mycelia were harvested and frozen at -80°C until used.

Preparation of zoospores of *P. palmivora*

Zoospore suspension of *P. palmivora* was prepared following the method of Chirapongsatonkul et al. (2008). Plugs cut from the edge of the culture were transferred to a V_8 agar plate. Five d later, the growing mycelium was chilled using sterile water (8 – 10°C) for 15 min and zoospores were collected 30 min later. The amount of zoospore was counted using haemocytometer. The concentration of zoospore suspension was adjusted to $5x10^5$ zoospores/ml with sterile distilled water. The suspensions were used within 30 min before encystment and germination could occur.

Plant materials and soil samples

Detached healthy rubber leaves from 3 individual plants were placed in a Petri dish containing 10 ml of the zoospore suspension or sterile distilled water as the control. After the abaxial surface of each leaf was placed facing the zoospores for 1 h, all leaves were rinsed with sterile distilled water then placed on moist Whatman paper. The inoculated leaves were kept at 25°C under 12 h of daylight. Both the zoospore treated- and control leaves were collected at 12 and 24 h and kept at -80°C for DNA extraction.

Twenty, 6 week-old rubber seedlings were divided into 2 groups, untreated control and zoospore-treated group. The seedlings were sprayed with the zoospore suspension or sterile distilled water. All seedlings were kept in a moist controlled plastic bag, with a relative humidity of 70 – 80%, at 25°C under 12 h of daylight to support the pathogen growth. The leaves were collected and pooled from 5 plants 12 and 24 h after treatment and kept at -80°C for DNA extraction.

Commercial soil (Din Lum Duan, Thailand), bought from the garden supplies shop, was sterilized by autoclaving before being divided for the 2 treatments. For the *P. palmivora* treatment, 10 plugs cut from the edge of the growing mycelium after being cultured on PDA for 7 d were inoculated in 25 g of sterilized soil. Both treated- and control soils were kept in moist controlled plastic bag for a week. Agar plugs were removed from the infested soil prior to storing the soil at -80°C for DNA extraction.

Isolation of DNA

DNA of the mycelia of the fungi listed above, plant tissues and soil (50 – 100 mg of each sample) was extracted and purified using the NucleoSpin® Plant II Kit (Macherey-Nagel, Germany) according to the supplied protocol from the manufacturer. DNA of *P. infestans* was kindly provided by Ms. Waraporn Prakob, ex-staff of Department of Entomology and

Plant Pathology, Faculty of Agriculture, Chiang Mai University. The concentration of DNA was determined spectrophotometrically.

Primers for *Phytophthora* spp. and *P. palmivora*

Two sets of primers, specific to the *Phytophthora* genus (Phy1s/Phy2a) and to *P. palmivora* (Pal1s/Pal2a), used throughout this study were based on the report of Tsai et al. (2006). The nucleotide sequences of primers and expected product sizes are shown in Table 1. The primer used as an internal marker in the rubber plant tissue was designed from the *actin* gene (GU270586.1) of *H. brasiliensis*.

Primer specificity

The primer pair covering the *Phytophthora* genus (Phy1s/Phy2a), and those specific to *P. palmivora* (Pal1s/Pal2a) were tested for their specificities by using DNA templates from some fungi or *Phytophthora* spp. as mentioned previously. To check whether these primers could amplify other non-target genes from the rubber plant, a PCR reaction using the DNA from healthy rubber plant tissues was carried out. The actin primer pair (actF/actR), amplifying actin gene of rubber plant, was used as internal control. PCRs were performed in a 25 µl reaction mixture containing EmeraldAmp® GT PCR Master Mix (Takara Bio Inc., Japan), DNA template, forward and reverse primers at the final concentration of 0.4 µM each. The PCR reactions were run on a Techne TC-512 Gradient Thermal Cyclers (Bibby Scientific Limited, UK). The PCR conditions were as follows: denaturation at 94°C for 5 min, followed by 30 – 35 cycles of 1 min at 95°C, 1 min at 55°C, 1 min at 72°C, and a final extension at 72°C for 10 min. PCR amplified products were analyzed by electrophoresis on 1.5% (w/v) agarose gel with 0.5 µg/ml ethidium bromide in 1× TAE. The fragment sizes were estimated by comparison with a 100 bp Plus DNA Ladder (Vivantis Technologies Sdn.

Bhd, Malaysia). The PCR products from each primer pair were cut from the gel, purified using QIAquick Gel Extraction Kit (Qiagen, Singapore), ligated and transformed into *Escherichia coli* Top10 cells. Purified plasmids were bidirectional sequenced directly, using the dye-terminator cycle-sequencing reaction (Applied Biosystems, USA).

Single round nested multiplex PCR and single round semi-nested multiplex PCR

The single round nested and semi-nested multiplex PCRs were carried out using the set of primers Phy1s/Phy2a/Pal1s/Pal2a and Phy1s/Phy2a/Pal2a, respectively and the DNA extracted from *P. palmivora* at approximately 20 ng was used as the template. These PCRs were performed in 25 µl reaction using EmeraldAmp® GT PCR Master Mix under the described PCR procedures. Each primer was used at a final concentration of 0.4 µM. The primer concentration was optimized to increase the efficiency of the single round semi-nested multiplex PCR amplification efficiency by adding 0.4 – 0.05 µM for Pal2a while Phy1s and Phy2a were fixed at 0.4 µM in 25 µl PCR reaction mix using the same PCR condition. No template (negative control, water) was run in each experiment. PCR amplified products were analyzed by electrophoresis as previously described.

The specificity of the single round nested multiplex PCR and the single round semi-nested multiplex PCR were examined by amplification of the positive and negative controls.

Sensitivity test

The sensitivity of the single round semi-nested multiplex PCR was determined by comparing between the simple PCR and the single round nested multiplex PCR by using various amounts of target DNA at 40, 8 and 4 ng, 800, 400 and 40 pg. Ten-fold serial dilutions (1 to 10^{-6}) of cloned vectors containing ITS region of *P. palmivora* were also used as templates. The PCR conditions for all reactions were as previously described.

Detection of *P. palmivora* in the infected rubber leaves and soil

The single round semi-nested multiplex PCR protocol was tested to amplify the DNA samples extracted from the zoospore treated- and untreated detached rubber leaves, rubber seedlings and soil. The DNA extracted from plant materials collected from the naturally planted rubber trees expressing leaf fall or black stripe were also used as templates. These samples were included to ensure that the assay can detect *Phytophthora* spp. and *P. palmivora* in infected plant tissues, as well as in soil.

Results

Primer design and specificity

The primers used throughout this research, Phy1s/Phy2a and Pal1s/Pal2a to identify and detect *Phytophthora* spp. and *P. palmivora*, respectively were according to Tsai et al. (2006). Based on the alignment with the sequences submitted to the NCBI database by nucleotide BLAST analysis and ClustalW (BioEdit), the binding locations of each primer within the ITS1-5.8S rDNA-ITS2-28S rDNA region of *Phytophthora* spp. and *P. palmivora* are shown in Fig. 1a.

The DNA extracted from the mycelia of the positive control *P. palmivora* strain KBNM 9 was used as template to test for the specificity of selected primers. The PCRs using Phy1s/Phy2a and Pal1s/Pal2a primers yielded a single band of approximately 1000 bp (Fig. 1b) and 650 bp (Fig. 1c), respectively. The obtained PCR products were purified, cloned and sequenced. The results indicated that the fragments carried the ITS1-5.8S-ITS 2-28S rDNA region of *P. palmivora*. The nucleotide sequences were deposited at the National Center for Biotechnology GenBank (Accession Number: JX863409 and HQ446453). The PCR reactions were additionally performed using DNA from 3 *Phytophthora* spp. including *P. palmivora*, *P. botryosa* and *P. infestans* and other fungi, *Penicillium funiculosum, A. niger*, *F. solani* and *C. gloeosporioides* as templates. The analysis of the amplified products showed that DNA fragments of approximately 1000 bp in length were obtained from all tested *Phytophthora* spp. (Fig. 1b). No amplification signal was detected when PCR was performed using DNA from other fungal isolates indicating the specificity of the primer to the genus *Phytophthora*. The fragment of the expected length of approximately 650 bp was obtained only when DNA from *P. palmivora* KBNM 9 was used indicating the species-specificity (Fig. 1c).

The single round nested multiplex and single round semi-nested multiplex PCR

By mean of primers used in this study, our multiplex PCR was similar to the nested PCR but there was no need to perform the first and secondary amplification separately as in normal nested PCR, it was thus named single round nested multiplex PCR. In this condition, the primer set Phy1s/Phy2a/Pal1s/Pal2a with the equal concentration were used in the unique PCR reaction. Two bands sizes approximately 1000 bp and 650 bp were revealed, however, the smaller band (sized 650 bp) seemed to be better amplified when using 20 ng of DNA from *P. palmivora* mycelia as template (Fig. 2a Lane 1). Since the match site of the primer Phy1s on the ITS region is very close and covered that of Pal1s, Pal1s was then eliminated and the 3 primers Phy1s, Phy2a and Pal2a were used to perform the single round semi-nested multiplex PCR. Moreover, to obtain the clear 2 bands with equal intensity, the single round semi-nested multiplex PCRs were carried out by varying the concentrations of each primer when the DNA template was fixed (Fig. 2a). The suitable final concentration of the primers were 0.4 μM, 0.4 μM and 0.075 μM for Phy1s, Phy2a and Pal 2a, respectively (Fig. 2a Lane 5).

To evaluate the specificity of the single round semi-nested multiplex PCR condition, the reaction was performed using the mentioned optimal condition and DNA from 3 various species belonging to the genus *Phytophthora* and other fungi as templates. Only one band of 1000 bp was obtained from all the tested *Phytophthora* spp. while there were two bands (1000 and 650 bp) revealed when the template was DNA from *P. palmivora* (Fig. 2b).

Sensitivity test of the single round semi-nested multiplex PCR

The sensitivity of our developed single round semi-nested multiplex PCR was determined compared with those of the conventional PCR for the detection of *Phytophthora* spp. and *P. palmivora* as well as the single round nested multiplex PCR. The results revealed that all methods could detect *P. palmivora* in a dose-dependent manner comparable to the concentrations of the DNA template (Fig. 3). The faint bands, representing the lowest amount of DNA detected, were observed in both simple PCRs at 40 pg of DNA template (Fig. 3a Lane 6 and 3b Lane 6). For the single round nested multiplex PCR, the smaller bands (sized 650 bp representing the species-specific *P. palmivora*) were denser and in addition when the DNA templates were lower than 4 ng, the 1000 bp band (representing the genus *Phytophthora*) was hardly observed (Fig. 3c Lane 4 – 6). The single round semi-nested multiplex PCR showed equal intensity of bands sized 650 bp and 1000 bp in the template ranged between 40 ng and 4 ng (Fig. 3d, Lane 1 – 3). With low DNA amount, the smaller band became denser than those of the larger one (Fig. 3d, Lane 4 and 5). According to the results, the comparative sensitivities of the single round semi-nested multiplex PCR and the simple PCR revealed that both methods showed the same detection limit of 40 pg of template tested.

The sensitivity was tested by using the 10-fold serial-dilutions of the vector contained ITS region of *P. palmivora*. The positive signal was detected at 1 to 10^{-3} dilutions by the single round semi-nested multiplex PCR (Fig. 3f) which was similar to those from the simple PCR methods (data not shown). It seemed that only one band of 650 bp was observed in the low amount of template for the single round nested multiplex PCR (Fig. 3e) comparable with the result when using various DNA amounts as templates.

Detection of *P. palmivora* in the infected rubber leaves and soil by the single round semi-nested multiplex PCR

To verify whether the single round semi-nested multiplex PCR protocol can detect *P. palmivora* in the infected materials, the DNAs extracted from the zoospore treated- and untreated rubber plants and soil were used as templates. The PCR using the primer of the *actin* gene (actF/actR) of the rubber plant was used as the control to confirm an equal amount of DNA in all samples. There was the band sized approximately 650 bp detected in the 12 h zoospore treated leaves while clear 2 bands were present in the detached leaves and soil treated with zoospore for 24 h (Fig. 4a). However, there was only faint band sized 650 bp visualized in the samples collected from the 12 h and 24 h zoospore treated seedlings (Fig. 4b). No visible band in the control untreated plant samples and soil DNA amplified with Phy1s/Phy2a/Pal2a in the single round semi-nested multiplex PCR.

Discussion

This report describes the development and application of a single round semi-nested multiplex PCR assay to detect simultaneously *Phytophthora* spp. and *P. palmivora*, the important pathogens of rubber trees. This technique is simple, rapid, efficient, and cost-effective and additionally greatly reduces the risk of contamination by performing the entire test in a single tube. This assay allows detection of multiple pathogens in a one reaction, which helps manage the reagent costs, time consuming and labor requirements.

Because the primer sets to specifically detect *Phytophthora* spp. and *P. palmivora* are available, the primers used throughout this research were according to Tsai et al. (2006). The primer sets were first tested for the specificity by the conventional PCR method using the DNA of *P. palmivora* as the template. The bands sized 1000 bp amplified by Phy1s/Phy2a (Fig. 1b) and sized 650 bp (Fig. 1c) amplified by Pal1s/Pal2a representing the genus *Phytophthora* and *P. palmivora*, respectively. Analysis of the obtained PCR products by Blastn of NCBI indicated that the fragments carried the ITS1-5.8S-ITS2-28S rDNA region of *P. palmivora*; in addition, the best hits of all sequences were ribosomal ITS sequences obtained from the same species by other laboratories (data not shown). No amplification product was detected when PCR was performed using DNA from other tested fungal isolates indicating the specificity of the used primer set towards their respective targets. Moreover, no PCR band amplified by both Phy1s/Phy2a and Pal1s/Pal2a when using the DNA from rubber plant as template suggesting that these primers could not amplify any gene in the tested host genome (data not shown).

The single round nested multiplex PCR was developed. There would typically be 2 bands produced, sizes at 1000 bp and 650 bp when the primer sets Phy1s/Phy2a and Pal1s/Pal2a were used together in the unique PCR reaction. The result showed that the smaller band (sized 650 bp) seemed to be better amplified (Fig. 2a Lane 1). This phenomenon may be due to the PCR product amplified by Phy1s/Phy2a (1000 bp) could also be the template for Pal1s/Pal2a. Another possible reason is that the template bound with these two primer sets are coverring in the same region, the primer Pal1s/Pal2a could probably better compete and bind to the template. Since the match site of the primer Phy1s on the ITS region is very close and covered that of Pal1s, the single round semi-nested multiplex PCR which eliminated the primer Pal1s was further focused to reduce the cost. The primer Phy1s could therefore involve in the amplification of both 2 bands while Phy2a and Pal2a produced either only the 1000 bp and 650 bp band, respectively. To obtain the clear 2 bands with equal intensity in the single round semi-nested multiplex PCR, we found that the final concentration of primer Pal2a should be lower than those of Phy1s and Phy2a.

However, some reseachers have mentioned to avoid the use of nested primers in multiplex PCRs since there may contribute either false-postive results due to carryover contamination or false-negative results due to the reaction failure (Kwok and Higuchi, 1989; Vangrysperre and Clercq, 1996; Elnifro et al., 2000). The results showed the specificity of our single round semi-nested multiplex PCR procedure since there was no false-positive and false-negative as no band, only 1 and 2 bands occurred when templates were those of other fungi, *Phytophthora* spp. and *P. palmivora*, respectively (Fig. 2b). This result confirmed the specificity of the developed PCR procedure and thus supported its application for simultaneous detection of the target pathogens.

The comparative sensitivity of our developed single round semi-nested multiplex PCR was determined compared with the conventional PCR and the single round nested multiplex PCR. The PCRs were performed using various amounts of DNA extracted from the mycelia of *P. palmivora* (40 ng – 4 pg) and the 10-fold serial-dilutions of the vector contained ITS region of *P. palmivora* (1 to 10^{-6} dilutions) as templates. The results revealed that all methods could detect the presence of *P. palmivora* in a dose-dependent manner (Fig. 3). The sensitivity of the single round semi-nested multiplex PCR was similar to those of the simple conventional PCR. However, when the amounts of templates were low in the single round nested and semi-nested multiplex PCRs, the smaller bands (sized 650 bp representing the species-specific *P. palmivora*) were denser than those of the 1000 bp bands (Fig. 3c and 3d). This may be due to some of the larger band could be used as the template to produce the smaller one as discussed earlier, the opportunity to produce the smaller band was thus higher so its level was consequently increased.

The developed single round semi-nested multiplex PCR protocol was determined whether it can detect *P. palmivora* in plant materials and soil. The positive results were only obtained in the inoculated samples, however, there was only faint band sized 650 bp visualized in the some samples as shown in Fig. 4. This evidence was comparable with the sensitivity analysis discussed earlier. Only smaller band could be observed when there was small amount of *P. palmivora*. Even only 650 bp band detected, it implied that there was the ITS region of *P. palmivora* representing the genus *Phytophthora* and the species *P. palmivora* in the DNA template. In addition, the observed bands implying the amount of pathogen were corresponding with the progression of the pathogen growth as could be seen by the necrotic symptoms and lesions. In our experiments, the detached leaves showed more necroses than those of the seedlings. The single round semi-nested multiplex PCR procedure can therefore be used to detect *Phytophthora* spp. and *P. palmivora* in infected plant tissue and soil. The DNA extracted from plant materials including leaves, barks and fruits collected from the naturally planted rubber trees expressing leaf fall or black stripe were also tested with our semi-nested multiplex PCR procedure. Unfortunately, it could not detect the occurrence of the *Phytophthora* genus and *P. palmivora* in any infected plant tissues.Isolation of *Phytophthora* spp. by conventional culture methods from the same rubber samples also revealed negative results (data not shown). The plausible explanation for the false lies in the disease expressing materials tested here might not be caused by *Phytophthora* spp. This may be due to diverse pathogens within the same or different genus could induce the similar disease symptoms (Drenth and Guest, 2004; Ellis and Boehm, 2008).

In conclusion, the developed single round semi-nested multiplex PCR was the novel strategy providing the rapid and simultaneous detection and identification of *Phytophthora* spp. and *P. palmivora*. It is useful to be applied to identify and detect *P. palmivora* in naturally diseased plant materials, soil and water. Nonetheless, this method may still have a limitation towards its sensitivity limit especially in cases where the number of pathogens might be low, as in soil, water or trees that have been dead for several years. Those conditions may cause the difficulty to reveal the presence of pathogens. Future adaptation of the technique such as real-time PCR or multiplex real-time PCR will allow quantitative measurements of *P. palmivora*, and may increase the sensitivity of the assay. Further, this diagnostic test may prove valuable for detection and management efforts with the diseases caused by *Phytophthora* spp. and *P. palmivora*.

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Figure captions

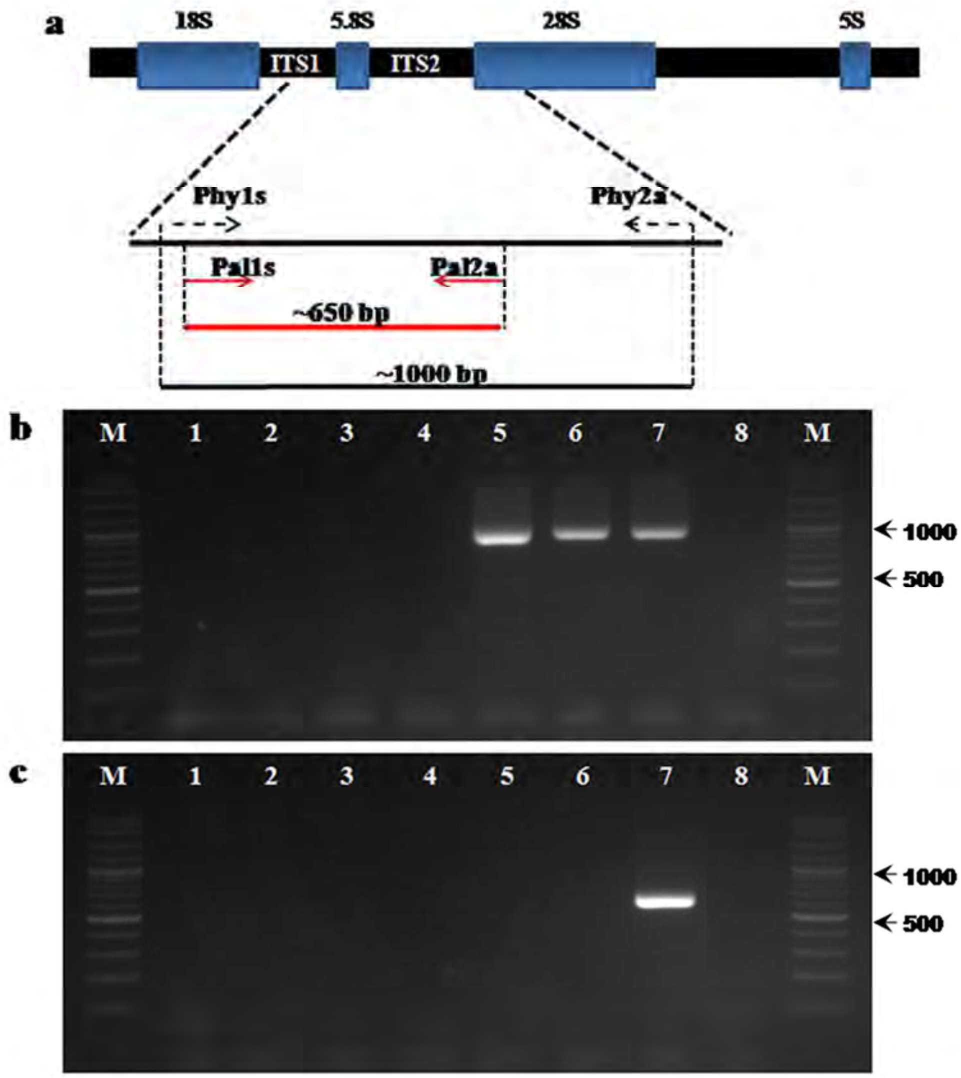
Fig. 1 The sites that the primers Phy1s, Phy2a, Pal1s and Pal2a match on the rDNA of *P. palmivora* and the predicted PCR product sizes obtained by amplifying with these primer sets **(a)**. Dash arrows (- - -► and ◄- - -) indicate Phy1s and Phy2a while thick arrows (⟶ and ⟵) refer to Pal1s and Pal2a. Specificity test of the primer set; Phy1s/Phy2a **(b)** and Pal1s/Pal2a **(c)**. PCR was performed using DNA from *Phytophthora* spp. and other fungal isolates as the templates. The amplified products were analyzed by electrophoresis on 1.5% agarose gel with 0.5 µg/ml ethidium bromide. Lane 1: *C. gloeosporioides*; 2: *A. niger*; 3: *Penicillium funiculosum*; 4: *F. solani*; 5: *P. botryosa*; 6: *P. infestans*; 7: *P. palmivora*; 8: water control and M represents 100 bp Plus DNA Ladder.

Fig. 2 Optimization of single round multiplex PCR. Nested multiplex and semi-nested multiplex PCR were performed using DNA from *P. palmivora* KBNM 9 as the template **(a)**. Lane 1: Single round nested multiplex PCR containing Phy1s/Phy2a/Pal1s/Pal2a at the concentration of 0.4 µM each; Lane 2 – 6: single round semi-nested multiplex PCR using Phy1s/Phy2a/Pal2a as the primer set, the concentrations of Phy1s and Phy2a were fixed at 0.4 µM each while the concentration of Pal2a was varied; Lane 2: 0.4 µM; 3: 0.2 µM; 4: 0.1 µM; 5: 0.075 µM; 6: 0.05 µM and 7: water control. Specificity of the single round semi-nested multiplex PCR **(b)**. PCR using DNA from *Phytophthora* spp. and other fungal isolates as the templates and the primer set Phy1s/Phy2a/Pal2a was performed. Lane 1: *C. gloeosporioides*; 2: *A. niger*; 3: *Penicillium funiculosum*; 4: *F. solani*; 5: *P. botryosa*; 6: *P. infestans*; 7: *P. palmivora*; 8: water control. The amplified products were analyzed by electrophoresis on 1.5% agarose gel with 0.5 µg/ml ethidium bromide and M represents 100 bp Plus DNA Ladder.

Fig. 3 Sensitivity analysis for *P. palmivora* detection. The simple PCR method using diluted DNA from *P. palmivora* as templates and primer Phy1s/Phy2a **(a)** and primer Pal1s/Pal2a **(b)**. The single round nested multiplex PCR using diluted DNA of *P. palmivora* as templates and primer set Phy1s/Phy2a/Pal1a/Pal2a **(c)** and the single round semi-nested multiplex PCR using Phy1s/Phy2a/Pal2a as the primer set **(d)**. Lane 1: 40 ng; 2: 8 ng; 3: 4 ng; 4: 800 pg; 5: 400 pg; 6: 40 pg; 7: 4 pg; 8: water control and M: 100 bp Plus DNA Ladder. The single round nested multiplex PCR **(e)** and the single round semi-nested multiplex PCR **(f)** using 10 fold-serial-dilutions of vector contained ITS region of *P. palmivora* as templates. Lane 1: 1; 2: 10^{-1}; 3: 10^{-2}; 4: 10^{-3}; 5: 10^{-4}; 6: 10^{-5}; 7: 10^{-6} copies; 8: water control. The amplified products were analyzed by electrophoresis on 1.5% agarose gel with 0.5 µg/ml ethidium bromide.

Fig. 4 Detection of *P. palmivora* by the single round semi-nested PCR. The DNA extracted from zoospores treated- and untreated rubber leaves and soil **(a)**. The PCR was performed using Phy1s/Phy2a/Pal2a as the primer set (upper panel) and the primer of the *actin* gene of rubber plant (lower panel). Lane 1: *P. palmivora*; 2: 12 h untreated leaves; 3: 12 h treated leaves; 4: 24 h untreated leaves; 5: 24 h treated leaves; 6: untreated soil; 7: treated soil; 8: water; M: 100 bp Plus DNA Ladder. The DNA extracted from zoospores treated- and untreated rubber seedlings **(b)**. Lane 1: 12 h untreated leaves; 2: 24 h untreated leaves; 3: 12 h treated leaves; 4: 24 h treated leaves; 5: water. The amplified products were analyzed by electrophoresis on 1.5% agarose gel with 0.5 µg/ml ethidium bromide.

Table 1 PCR primers used in this study.



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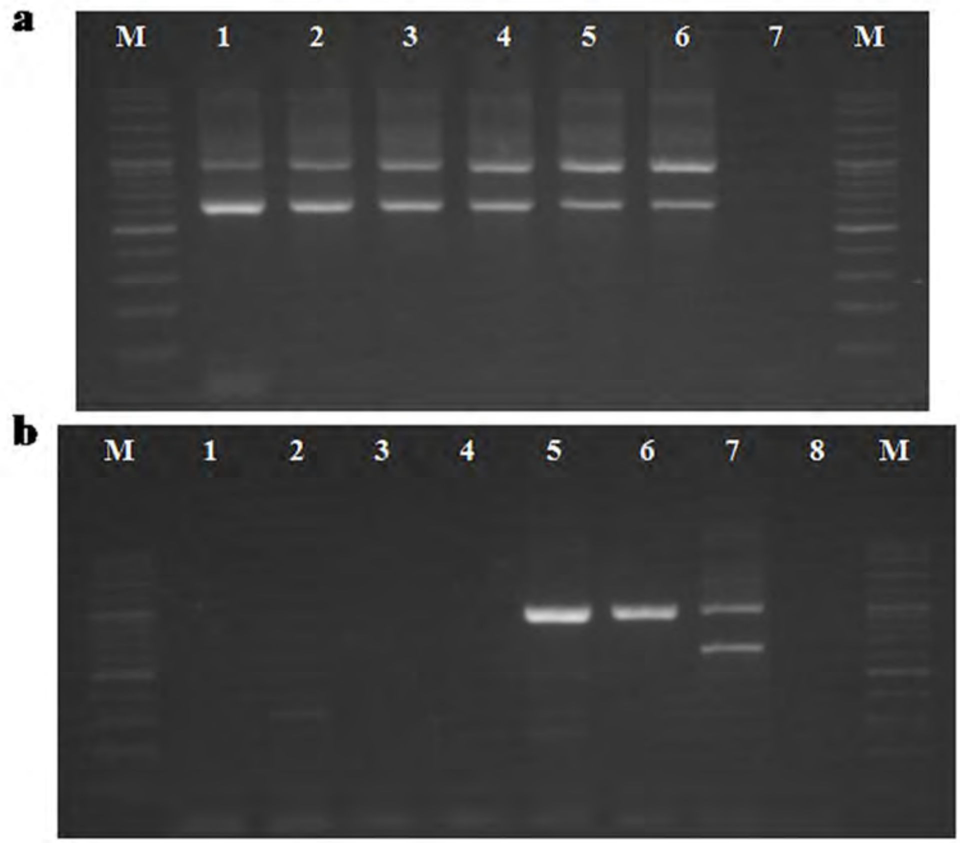

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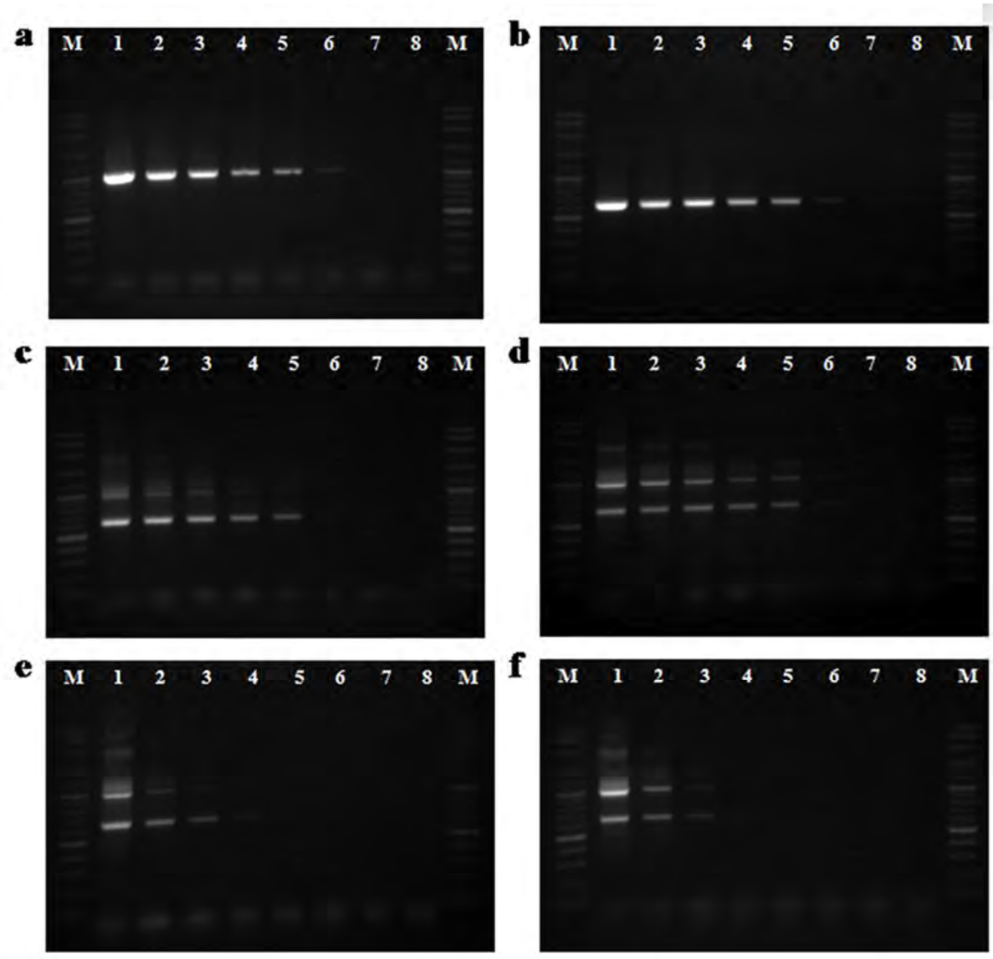

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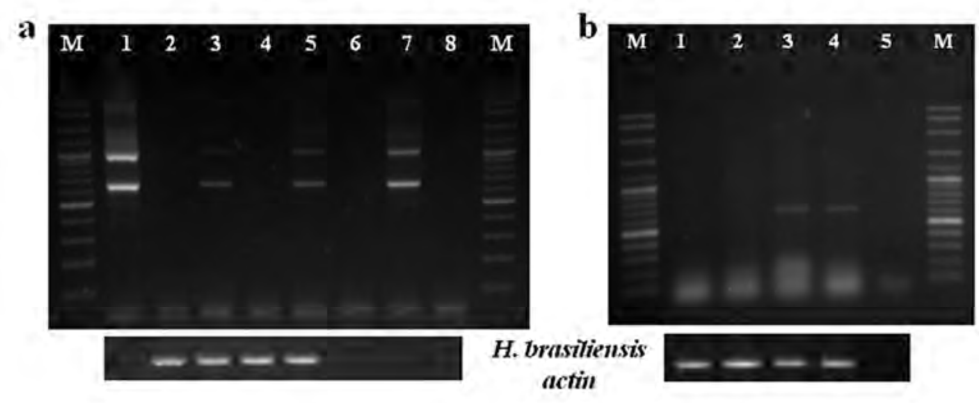
173x168mm (300 x 300 DPI)



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129x53mm (300 x 300 DPI)

Target	Name	Primer sequence (5' to 3')	Location	Reference
Phytophthora spp.	Phy1s	ACTTTCCACGTGAACCGTATCA	ITS1	Tsai et al. (2006)
	Phy2a	GCACGAGCCACTCAGGGATG	28S	Tsai et al. (2006)
P. palmivora	Pal1s	CACGTGAACCGTATCAAAACT	ITS1	Tsai et al. (2006)
	Pal2a	CAATCATACCACCACAGCTGA	ITS2	Tsai et al. (2006)
Actin of *H. brasiliensis*	actF	CACCACCACTGCCGAACGGG	Actin	This study
	actR	ACGGTCTGCAATGCCAGGGA		This study

Table 1 PCR primers used in this study.
196x84mm (96 x 96 DPI)

วิธีการตรวจวินิจฉัยเชื้อไฟทอปทอร่า (*Phytophthora* spp.) ในยางพารา

นิอร จิรพงศธรกุล[1*] กิตติชนม์ อุเทนะพันธุ์[2] และ นันทา เชิงเชาว์[3]

ยางพารา (*Hevea brasiliensis* Mull-Arg.) เป็นพืชที่มีความสำคัญมากต่อเศรษฐกิจของประเทศไทยโดยส่งผลต่อชีวิตความเป็นอยู่ของเกษตรกรและบุคลากรในอุตสาหกรรมต่อเนื่อง ประเทศไทยเป็นผู้ส่งออกผลิตภัณฑ์ยางพารารายใหญ่อันดับ 1 ของโลก คือ ประมาณ 3 ล้านตัน/ปี คิดเป็น 40 % ของการส่งออกยางทั้งหมดของโลก ด้วยเหตุนี้ทำให้มีการศึกษาวิจัยเกี่ยวกับยางพาราหลายสาขาเพื่อตอบสนองอุตสาหกรรมยางพาราในด้านต่าง ๆ ทั้งที่เกี่ยวข้องกับการวิจัยและพัฒนาผลผลิตจากน้ำยางพารา การเพิ่มคุณภาพน้ำยางพารา การคัดเลือกและพัฒนาสายพันธุ์ยางพาราเพื่อให้มีความทนทานสูงและผลิตน้ำยางปริมาณมาก รวมถึงโรคและปัจจัยที่ส่งผลกระทบต่อต้นยางและการผลิตน้ำยางพารา ซึ่งมีความสำคัญอย่างมากต่อการพัฒนาอุตสาหกรรมยางพาราของไทยอย่างยั่งยืน

ผลผลิตยางพาราส่วนใหญ่ของไทยมาจากแหล่งผลิตทางภาคใต้ แต่เนื่องจากสภาพอากาศในภาคใต้มีความชื้นสูงและฝนตกชุกตลอดปี ทำให้การปลูกยางพาราในภาคใต้ประสบปัญหาการเกิดโรคระบาดของเชื้อ Oomycete ในกลุ่มไฟทอปทอร่า (*Phytophthora* spp.) โดยสายพันธุ์ที่ทำให้เกิดโรคในยางพารามีอยู่หลายชนิด แต่ในประเทศไทยพบว่าสายพันธุ์ที่ทำให้เกิดโรคในยางพารา คือ *P. palmivora* และ *P. botryosa* (Erwin และ Ribeiro 1996) โดยเฉพาะ *P. palmivora* ซึ่งเป็นเชื้อที่มีการแพร่ระบาดอย่างกว้างขวาง โดยก่อให้เกิดโรคใบร่วงและเส้นดำในยางทุกสายพันธุ์ ความรุนแรงของเชื้อไฟทอปทอร่าขึ้นอยู่กับพันธุ์ยางที่ปลูกและสภาวะแวดล้อม ซึ่งโรคใบร่วงส่งผลกระทบโดยตรงต่อต้นยาง ทำให้ต้นยางอ่อนแอ ผลผลิตน้ำยางพาราลดลงอย่างชัดเจน หากติดเชื้อรุนแรงจะทำให้ต้นยางพาราตายได้ ในขณะที่โรคเส้นดำถือเป็นโรคทางลำต้นที่มีความสำคัญมาก เนื่องจากทำลายหน้ากรีดซึ่งเป็นบริเวณที่เก็บเกี่ยวผลผลิต จึงไม่สามารถกรีดยางซ้ำบนหน้าที่เป็นเปลือกงอกใหม่ได้ ทำให้ระยะเวลาในการให้ผลผลิตสั้นลง มีผลกระทบต่อเกษตรกรและภาคการผลิตยางพาราของประเทศไทย

จากการสำรวจของศูนย์วิจัยยางสงขลา รายงานว่าแปลงขยายพันธุ์กิ่งยางและแปลงเพาะยางชำถุงจากพันธุ์ RRIM600 มีความอ่อนแอมากต่อเชื้อ *Phytophthora* spp. ซึ่งยางพันธุ์นี้เป็นพันธุ์ที่นิยมปลูกมากเนื่องจากให้ผลผลิตต่อหน่วยต้นยางในปริมาณสูง ทำให้อาจก่อให้เกิดความเสียหายทางเศรษฐกิจต่อแปลงขยายพันธุ์กิ่งตายางและแปลงเพาะยางชำถุงเป็นอย่างมาก การศึกษานี้มีวัตถุประสงค์เพื่อพัฒนาวิธีการตรวจวินิจฉัยเชื้อในกลุ่มไฟทอปทอร่า (*Phytophthora* spp.) โดยใช้วิธี Polymerase Chain Reaction (PCR) เพิ่มจำนวนดีเอ็นเอ (DNA) บริเวณ internal transcribed spacer (ITS) และ ribosomal DNA (rDNA) เนื่องจากบริเวณดังกล่าวมีความจำเพาะ

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สาขาประมง คณะเกษตรศาสตร์ มหาวิทยาลัยเทคโนโลยีราชมงคลศรีวิชัย อ.ทุ่งสง จ.นครศรีธรรมราช
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สูงในสิ่งมีชีวิตชนิดหนึ่ง ๆ และจะแตกต่างไปในสิ่งมีชีวิตต่างชนิดกัน วิธี PCR นี้มีความแม่นยำและว่องไวสูง เนื่องจากสามารถตรวจพบเชื้อในปริมาณน้อย ๆ ได้ และยังตรวจสอบยืนยันผลได้อย่างรวดเร็วใช้เวลาไม่เกิน 24 ชั่วโมง ซึ่งการศึกษานี้จะใช้เชื้อบริสุทธิ์รวมถึงตัวอย่างพืชที่ทำให้ติดเชื้อในห้องปฏิบัติการเป็นแม่แบบทดสอบ เพื่อหาสภาวะในการตรวจวินิจฉัยที่เหมาะสม ข้อมูลที่ได้จะเป็นวิธีสำหรับตรวจหาการระบาดและการปนเปื้อนของเชื้อดังกล่าวในบริเวณปลูกรวมถึงต้นพันธุ์ยาง ข้อมูลการตรวจพบและการแพร่กระจายของเชื้อไฟทอปทอร่าจะนำมาใช้สำหรับวางแผนเพื่อการป้องกันโรคในยางพาราต่อไป

1. วิธีการวิจัย

1.) สกัดดีเอ็นเอ (DNA) จากเชื้อไฟทอปทอร่าสายพันธุ์ต่าง ๆ ได้แก่ *P. palmivora* *P. botryosa* และ *P. infestans* และเชื้อราอื่น ๆ ได้แก่ *Penicillium funiculosum* *Aspergilus niger* *Fusarium solani* และ *Colletotrichum gloeosporioides* เพื่อใช้เป็นแม่แบบ (template) ในการตรวจสอบความจำเพาะของวิธีการตรวจวินิจฉัยด้วยวิธี PCR โดยสารเริ่ม (primer) ที่ใช้สำหรับตรวจวินิจฉัยเชื้อสกุลไฟทอปทอร่าได้จากการอ้างอิงข้อมูลการวิจัยของ Tsai และคณะ (2006) แล้วทำการเพิ่มปริมาณดีเอ็นเอด้วยเครื่อง Thermal cycler โดยตั้งโปรแกรม PCR ดังนี้

โปรแกรมที่ 1	Initial denaturation	ที่ 95 °C	เป็นเวลา 5 นาที	1 รอบ
โปรแกรมที่ 2	Denaturation	ที่ 95 °C	เป็นเวลา 1 นาที	30 รอบ
	Annealing	ที่ 55 °C	เป็นเวลา 1 นาที	
	Extension	ที่ 72 °C	เป็นเวลา 1 นาที	
โปรแกรมที่ 3	Extension	ที่ 72 °C	เป็นเวลา 10 นาที	1 รอบ
โปรแกรมที่ 4	Hold	ที่ 4 °C		

แล้วตรวจสอบผลที่ได้จากการทำ PCR ด้วยวิธี Agarose Gel Electrophoresis โดยพิจารณาจากขนาดของแถบดีเอ็นเอที่เพิ่มจำนวนได้

2.) ตรวจสอบสภาพไว (Sensitivity) ของสภาวะการทำ PCR ข้างต้น โดยใช้ดีเอ็นเอของเชื้อ *P. palmivora* เจือจางให้มีปริมาณต่าง ๆ (40 ng 8 ng 4 ng 800 pg 400 pg 40 pg และ 4 pg) เป็นแม่แบบ ทำ PCR แล้วตรวจสอบผลด้วยวิธี Agarose Gel Electrophoresis

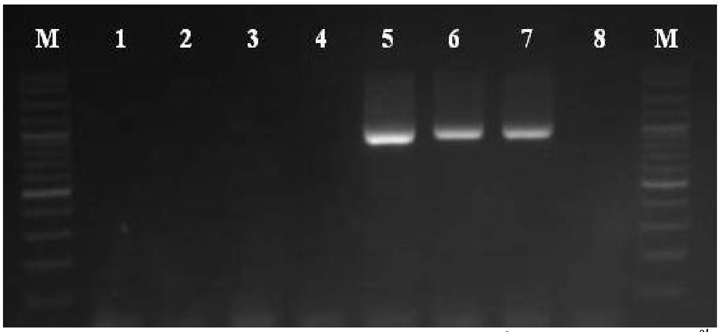
3.) ตรวจสอบว่าสภาวะการทำ PCR ข้างต้นสามารถตรวจพบเชื้อไฟทอปทอร่าที่มีในตัวอย่างใบยางพาราและดินที่บ่มด้วยเชื้อ *P. palmivora* ที่เวลาต่าง ๆ ได้ โดยทำการสกัดดีเอ็นเอจากตัวอย่างใบที่บ่มด้วยเชื้อเป็นเวลา 6 12 18 และ 24 ชั่วโมง และใบที่ไม่บ่มเชื้อเป็นชุดควบคุม ในขณะที่ตัวอย่างดิน จะนำดินมาฆ่าเชื้อก่อนแล้วบ่มด้วยเส้นใยของ *P. palmivora* เป็นเวลา 1 และ 2 สัปดาห์ และใช้ดินที่ปราศจากเชื้อเป็นชุดควบคุม

หลังจากนั้นจึงสกัดดีเอ็นเอจากตัวอย่างดินทั้งหมด แล้วนำมาทำ PCR และตรวจสอบผลด้วยวิธี Agarose Gel Electrophoresis

2. ผลการวิจัย

2.1 ความจำเพาะของไพรเมอร์และวิธี PCR ในการตรวจวินิจฉัยเชื้อไฟทอปทอร่า

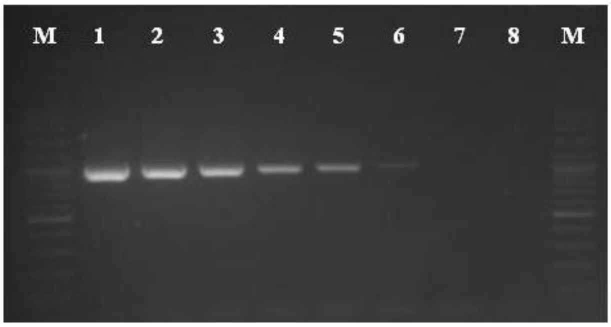
วิธี PCR และไพรเมอร์ที่ใช้มีความจำเพาะต่อเชื้อในกลุ่มไฟทอปทอร่า โดยพบว่าจะมีเพียง 1 แถบดีเอ็นเอขนาดประมาณ 1000 คู่เบสเมื่อใช้แม่แบบเป็นดีเอ็นเอของเชื้อในกลุ่มไฟทอปทอร่า คือ *P. palmivora* *P. botryosa* และ *P. infestans* เท่านั้น และไม่พบแถบดังกล่าวเมื่อใช้ดีเอ็นเอจากเชื้อราอื่น ๆ เป็นแม่แบบ ดังแสดงในรูปที่ 1 นอกจากนี้ได้ทำการส่งตัวอย่างแถบที่ได้จากการเพิ่มจำนวนด้วยวิธี PCR ไปหาลำดับนิวคลีโอไทด์ เพื่อยืนยันว่าเป็นดีเอ็นเอของเชื้อไฟทอปทอร่า (Chirapongsatonkul *et al*. 2010)



รูปที่ 1 ผลการทดสอบความจำเพาะของสารเริ่ม (Primer) และวิธี PCR เมื่อใช้ดีเอ็นเอจากเชื้อไฟทอปทอร่าและเชื้อราอื่น ๆ เป็นแม่แบบ ช่องที่ 1: *C. gloeosporioides*; 2: *A. niger*; 3: *Penicillium funiculosum*; 4: *F. solani*; 5: *P. botryose*; 6: *P. infestans*; 7: *P. palmivora*; 8: น้ำกลั่น และ M: ดีเอ็นเอมาตรฐาน (100 bp Plus DNA Ladder).

2.2 สภาพไว (Sensitivity) ของวิธี PCR ในการตรวจวินิจฉัยเชื้อไฟทอปทอร่า

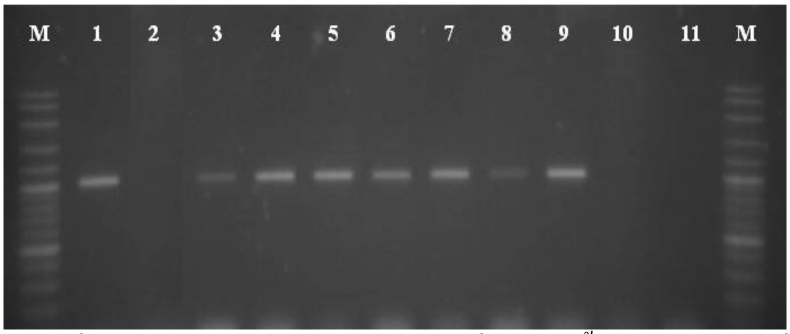
เมื่อใช้ดีเอ็นเอของเชื้อตัวอย่างในกลุ่มไฟทอปทอร่า คือ *P. palmivora* ปริมาณต่าง ๆ ได้แก่ 40 ng 8 ng 4 ng 800 pg 400 pg 40 pg และ 4 pg เป็นแม่แบบในการทำ PCR พบว่าแถบดีเอ็นเอจะจางลงตามปริมาณดีเอ็นเอแม่แบบ (รูปที่ 2) และปริมาณดีเอ็นเอต่ำสุดที่วิธีนี้ตรวจสอบได้ คือ 40 pg (ช่องที่ 6) ซึ่งถือว่าน้อยมาก โดยจะเห็นแถบดีเอ็นเอบาง ๆ



รูปที่ 2 ผลการทดสอบสภาพไว (Sensitivity) ของวิธีการตรวจวินิจฉัยด้วยวิธี PCR เมื่อใช้ดีเอ็นเอจากเชื้อ *P. palmivora* ปริมาณต่าง ๆ เป็นแม่แบบ ช่องที่ 1: 40 ng; 2: 8 ng; 3: 4 ng; 4: 800 pg; 5: 400 pg; 6: 40 pg; 7: 4 pg; 8: น้ำกลั่น และ M: ดีเอ็นเอมาตรฐาน (100 bp Plus DNA Ladder).

2.3 การตรวจวินิจฉัยเชื้อไฟทอปทอร่าในตัวอย่างใบยางพาราและดิน

หลังจากทดสอบความจำเพาะและสภาพไวของวิธี PCR ในการตรวจวินิจฉัยเชื้อไฟทอปทอร่าแล้ว จะทำการทดสอบว่าวิธีดังกล่าวสามารถตรวจติดตามการมีอยู่ของเชื้อดังกล่าวในตัวอย่างธรรมชาติหรือไม่ ในการทดลองนี้เลือกใบยางพาราและดินเป็นตัวอย่างสำหรับทดสอบ โดยบ่มเชื้อ *P. palmivora* บนใบและในดิน แล้วเก็บตัวอย่างที่เวลาต่าง ๆ มาสกัดดีเอ็นเอเพื่อใช้เป็นแม่แบบในการทำ PCR โดยจะเปรียบเทียบกับเมื่อใช้ดีเอ็นเอที่สกัดจากเชื้อ *P. palmivora* มาเป็นชุด positive control ผลการทดลอง (รูปที่ 3) พบว่า ในตัวอย่างใบที่ไม่ติดเชื้อหรือไม่ได้บ่มเชื้อ จะไม่มีแถบดีเอ็นเอ (ช่องที่ 2) แต่จะปรากฏแถบดีเอ็นเอในตัวอย่างใบที่บ่มด้วยเชื้อ *P. palmivora* เป็นเวลา 6 12 18 และ 24 ชั่วโมง (ช่องที่ 3-6 ตามลำดับ) เช่นเดียวกับผลการทำ PCR เมื่อใช้ดีเอ็นเอที่สกัดจากดินที่บ่มด้วยเชื้อ *P. palmivora* (ช่องที่ 8-9) แต่ไม่พบแถบใด ๆ เมื่อใช้ดีเอ็นเอจากดินปราศจากเชื้อ (ช่องที่ 10) โดยแถบดีเอ็นเอที่ปรากฏทั้งหมดจะตรงกับแถบดีเอ็นเอของเชื้อ *P. palmivora* (ช่องที่ 1 และ 7) นอกจากนี้ยังสังเกตพบว่าความเข้มของแถบที่ปรากฏจะสัมพันธ์กับปริมาณดีเอ็นเอแม่แบบ สอดคล้องกับผลการทดสอบสภาพไวข้างต้น (รูปที่ 2)



รูปที่ 3 ผลการทำ PCR เมื่อใช้ดีเอ็นเอจากใบยางพาราและดินที่บ่มด้วยเชื้อ *P. palmivora* ที่เวลาต่าง ๆ ช่องที่ 1 และ 7: ดีเอ็นเอจากเชื้อ *P. palmivora* (ปริมาณ 400 pg) สำหรับเป็น positive control; 2: ใบยางพาราที่ไม่บ่มเชื้อ; 3: ใบยางพาราที่บ่มด้วยเชื้อที่ 6 ชั่วโมง; 4: ใบยางพาราที่บ่มด้วยเชื้อที่ 12 ชั่วโมง; 5: ใบยางพาราที่บ่มด้วยเชื้อที่ 18 ชั่วโมง; 6: ใบยางพาราที่บ่มด้วยเชื้อที่ 24 ชั่วโมง; 8: ดินที่บ่มด้วยเชื้อเป็นเวลา 1 สัปดาห์; 9: ดินที่บ่มด้วยเชื้อเป็นเวลา 2 สัปดาห์; 10: ดินที่ไม่บ่มเชื้อ; 11: น้ำกลั่น และ M: ดีเอ็นเอมาตรฐาน (100 bp Plus DNA Ladder).

3. แนวทางการใช้ประโยชน์

วิธี PCR ที่ทำการศึกษานี้เหมาะสำหรับใช้ตรวจวินิจฉัยเชื้อไฟทอปทอร่าได้อย่างแม่นยำและรวดเร็ว เนื่องจากวิธีนี้มีความจำเพาะต่อเชื้อในกลุ่มไฟทอปทอร่า และยังมีสภาพไวสูง คือ ตรวจพบเชื้อได้ในปริมาณที่

น้อยมาก (40 ng) ซึ่งถือว่าว่องไวกว่าวิธีการตรวจวินิจฉัยอื่น ๆ โดยเฉพาะการเพาะแยกเชื้อบนอาหารเลี้ยงเชื้อที่จำเป็นต้องมีปริมาณเชื้อมากพอ นอกจากนี้วิธี PCR ยังให้ผลการตรวจสอบได้ในระยะเวลาสั้น รวดเร็วกว่าวิธีการเพาะเชื้อมาก และจากการทดลองพบว่าหากมีเชื้อดังกล่าวในตัวอย่างพืชหรือดินจริง ๆ ก็ตรวจพบได้ด้วยวิธีนี้ ดังนั้น สามารถนำวิธี PCR ไปใช้ตรวจตัวอย่างที่เก็บมาจากธรรมชาติจริง ๆ หรือใช้สำหรับตรวจหาการระบาดและการปนเปื้อนของเชื้อดังกล่าวในบริเวณปลูกรวมถึงต้นพันธุ์ยาง เพื่อใช้ข้อมูลสำหรับวางแผนการป้องกันโรคในยางพาราต่อไป

นอกจากนี้ผู้วิจัยกำลังพัฒนาวิธีการตรวจวินิจฉัยที่สามารถระบุในเวลาเดียวกันว่าเป็นเชื้อไฟทอปทอร่าหรือไม่ และแยกชนิด (Species) ของเชื้อไฟทอปทอร่านั้นว่าเป็นสปีชีส์อะไร เพื่อให้ได้ข้อมูลเชิงลึกว่าพืชหรือบริเวณปลูกนั้น ๆ มีการแพร่ระบาดของเชื้อชนิดไหน

เอกสารอ้างอิง

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บทคัดย่อ
การเสนอผลงานแบบโปสเตอร์

การประชุมนักวิจัยรุ่นใหม่ พบ เมธีวิจัยอาวุโส สกว. ครั้งที่ 13

วันที่ 16-18 ตุลาคม 2556
โรงแรมเดอะรีเจ้นท์ ชะอำบีช รีสอร์ท หัวหิน ชะอำ
จังหวัดเพชรบุรี

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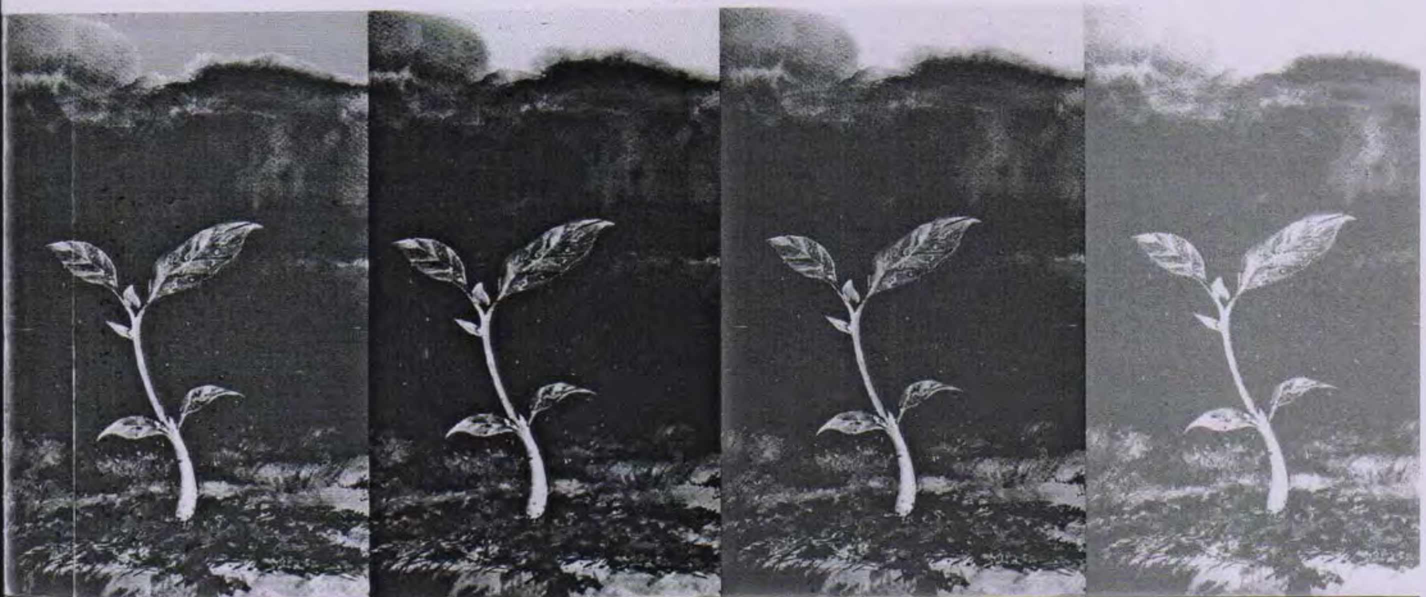

Rapid Diagnosis of a Rubber Tree Pathogen, *Phytophthora spp.* and *P. palmivora*, by Single Round Semi-nested Multiplex PCR

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BIO : Biological Science

Abstract
Phytophthora spp. cause devastating effects on crops and natural ecosystems worldwide. In Thailand, *P. palmivora* is an important pathogenic species of the rubber plant (*Hevea brasiliensis*) reducing latex production. This study aimed to develop a PCR-based method to rapidly detect the presence of the genus *Phytophthora* and *P. palmivora* simultaneously. Two selected primer pairs, Phy1s/Phy2a and Pal1s/Pal2a that amplify the ITS I-5.8S-ITS II-28S region of the genus *Phytophthora* and *P. palmivora*, respectively, were tested for their specificities by using the DNA template of *P. palmivora* compared with other *Phytophthora* species, other fungi and rubber leaf tissue. A single round nested multiplex PCR with 4 primers, Phy1s/Phy2a/Pal1s/Pal2a, and a single round semi-nested multiplex PCR with a set of 3 primers, Phy1s/Phy2a/Pal2a, were established. By using various amounts of DNA of a control strain *P. palmivora* KBNM 9 as templates; the sensitivity of these methods was studied compared to the simple PCR method. The single round semi-nested multiplex PCR was focused because of its sensitivity, more rapidity, less contamination risk and cost. This method was tested to detect the presence of *P. palmivora* in infected leaf tissues and soil. It could be further applied to rapid diagnose the genus *Phytophthora* and *P. palmivora* in naturally diseased plant materials, soil and water. This diagnosis method should therefore be valuable for the detection and management efforts with plant diseases.

Keywords: molecular diagnosis, nested multiplex PCR, *Phytophthora* spp., *P. palmivora*, semi-nested multiplex PCR, single round semi-nested multiplex PCR, rubber plant

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