Diverse Functions of Polypeptide Effectors from *Phytophthora palmivora*, a Pathogen of *Hevea brasiliensis*

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ชื่อวิทยานิพนธ์ หน้าที่หลากหลายของเอฟเฟคเตอร์ชนิดโพลีเปปไทด์จากเชื้อ *Phytophthora palmivora* ซึ่งก่อให้เกิดโรคในยางพารา

ผู้เขียน นางสาวดุษฎี ชินนาพันธ์

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บทคัดย่อ

Phytophthora palmivora เป็น *Oomycete* ที่ก่อให้เกิดโรคใบร่วงและเส้นดำในยางพารา ซึ่งเป็นปัญหาสำคัญของเกษตรกรสวนยาง เพราะเมื่อเกิดโรคแล้ว ทำให้ใบร่วงก่อนเวลาอันควร ทำให้หน้ายางเสียกรีดไม่ได้ นั่นคือ ได้ผลผลิตลดลง ซึ่งมีผลกระทบต่ออุตสาหกรรมยางพาราในระดับประเทศ การใช้สารเคมีสามารถควบคุมเชื้อโรคได้เช่นเดียวกัน แต่มีผลในระยะสั้นเท่านั้น อย่างไรก็ตามเอฟเฟคเตอร์ต่างๆที่ผลิตออกมาจากเชื้อโรค สามารถนำมาใช้ประโยชน์ในการเหนี่ยวนำให้พืชเกิดระบบป้องกันตนเอง โดยเรียกเอฟเฟคเตอร์ชนิดนี้ว่าอิลิซิเตอร์ซึ่งจัดอยู่ในกลุ่มของ avirulent factor ส่วน virulent factor เช่น protease inhibitor สามารถนำมาศึกษากลไกการก่อโรคในพืชเจ้าบ้านได้

ผู้วิจัยตรวจพบอิลิซิเตอร์ชนิดโพลีเปปไทด์ในน้ำเลี้ยงเชื้อ *P. palmivora* สองชนิด คืออิลิซิเตอร์ขนาด 10 กิโลดาลตัน (ซึ่งมีชื่อเฉพาะว่าอิลิซิติน) สามารถทำให้บริสุทธิ์ได้โดยใช้คอลัมน์ DEAE cellulose และอิลิซิเตอร์ชนิดใหม่ขนาด 75 กิโลดาลตันซึ่งทำให้บริสุทธิ์ได้ด้วยคอลัมน์ DEAE cellulose และ hydrophobic ตามลำดับ อิลิซิเตอร์ทั้งสองชนิดเสถียรต่อความร้อนและความเป็นกรด-ด่าง แต่ไม่เสถียรต่อเอนไซม์ ProteaseK และสามารถกระตุ้นให้เกิดการสร้างระบบป้องกันตนเองในเซลล์แขวนลอยยางพารา ได้แก่ การสังเคราะห์สารฟีนอลิก สคอพอลิติน และเอนไซม์เปอร์ออกซิเดส (*o*-dianisidine and scopoletin ไอโซไซม์) อิลิซิเตอร์ทั้งสองกระตุ้นให้มีการสร้างสารฟีนอลิก สคอพอลิติน และ scopoletin เปอร์ออกซิเดส ในเซลล์แขวนลอยยางพาราได้สูงสุดที่ชั่วโมงที่ 64 สำหรับ *o*-dianisidine เปอร์ออกซิเดส สังเคราะห์ได้สูงสุดที่ชั่วโมงที่ 24 นอกจากนี้อิลิซิเตอร์ทั้งสองชนิดยังสามารถกระตุ้นการสร้างสารฟีนอลิก *o*-dianisidine เปอร์ออกซิเดส และเหนี่ยวนำความต้านทานต่อเชื้อ *P. palmivora* ในต้นอ่อนยางพาราได้ แต่อัตราเร็วในการสร้างของสารแต่ละชนิดแตกต่างจากการสร้างในเซลล์แขวนลอย นั่นคือ อิลิซิเตอร์ทั้งสองจะกระตุ้นให้มีการสร้างสารฟีนอลิกได้สูงสุดที่ชั่วโมงที่ 40 หลังจากการพ่นอิลิซิเตอร์บนต้นอ่อนยางพารา ส่วน *o*-dianisidine เปอร์ออกซิเดส สร้างได้สูงสุดที่ชั่วโมงที่ 48 นอกจากนี้ยังพบว่าหลังจากพ่นเชื้อราลงบนต้นอ่อนยางพาราที่ผ่านการกระตุ้นด้วยอิลิซิเตอร์แล้ว สามารถเหนี่ยวนำการต้านทานโรคตลอดจนการผลิต *o*-dianisidine เปอร์ออกซิเดส ได้อย่างรวดเร็ว และมีปริมาณมากกว่าชุดควบคุม จากการทดลองพบว่ายางพาราตอบสนองต่ออิลิ

ซิเตอร์ชนิดใหม่ได้ดีกว่าอิลิซิติน เพราะในการกระตุ้นในเซลล์แขวนลอยต้องใช้อิลิซิตินมากกว่าอิลิซิเตอร์ชนิดใหม่ประมาณ 30 เท่า จึงจะกระตุ้นการสร้างสารต่างๆได้ในระดับที่เท่ากัน และในต้นอ่อนของยางพาราต้องใช้อิลิซิตินมากกว่าอิลิซิเตอร์ชนิดใหม่ประมาณ 2 เท่า

P. palmivora สามารถผลิต virulent factor คือ protease inhibitor (PpEPI10) ชนิด Kazal ซึ่งจัดอยู่ในกลุ่มของ serine protease inhibitor ผู้วิจัยสามารถแยก protease inhibitor ชนิดนี้ได้จากเส้นใยของเชื้อ *P. palmivora* โดยใช้กระบวนการ reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) ยีน *Ppepi*10 ประกอบไปด้วยดีเอ็นเอ 669 คู่เบสซึ่งแปลงรหัสได้เป็นกรดอะมิโน 222 ตัว ซึ่งประกอบด้วย 3 Kazal domains ได้แก่ *Kazal1*, *Kazal2* และ *Kazal3* โดย *rKazal1* และ *rKazal2* สามารถแสดงออกคือผลิตโปรตีนได้ใน *Escherichia coli* BL21 โดยใช้ pFLAG-ATS เป็น vector ส่วน *rKazal3* จะไม่แสดงออกในเวกเตอร์ทั้ง pFLAG-ATS และ pGEX 4T-1 Kazal1 และ Kazal2 ยับยั้ง serine protease ชนิด subtilisin A แต่ไม่ยับยั้ง chymotrypsin และ trypsin ส่วนการทดสอบด้วย zymogram และ co-immunoprecipitation พบว่า Kazal1 และ Kazal2 สามารถจับและยับยั้ง protease ขนาด 95 kDa ในใบยางพาราได้ โดย Kazal1 สามารถยับยั้ง subtilisin A และ protease ขนาด 95 kDa ได้ดีกว่า Kazal2

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Abstract

Phytophthora palmivora is the causative agent of "leaf fall" and "black stripe" in rubber plant, *Hevea brasiliensis*. It infects petioles, causing leaves to fall prematurely and attacks the tapping surface resulting in poor latex production. Chemicals can be used to control the pathogen; however, they work in only a short period. The effectors produced from pathogens can be used for study interaction between plant and pathogen. There are two types of effectors produced from the pathogens, avirulent and virulent molecules. The first one can be used to induce the plant defense reaction which is called elicitor, while the later such as protease inhibitor, can be used to investigate the infection process.

P. palmivora secreted two polypeptide elicitors into its culture filtrate, 10 kDa elicitin and a new 75 kDa elicitor. The elicitin was purified in single step by DEAE cellulose chromatography while the 75 kDa elicitor was obtained by two steps of chromatography: the DEAE cellulose column followed by a hydrophobic column. Both elicitors were stable to heat and a wide range of pH values; however, sensitive to ProteaseK. Both elicitors induced syntheses of total phenolic compounds, scopoletin and peroxidase isozymes (with substrate *o*-dianisidine and scopoletin) in cell suspension of *H. brasiliensis* with similar kinetics. Total phenolic compounds, scopoletin and scopoletin peroxidase were produced and reached to the highest levels at 64 h; however the *o*-dianisidine peroxidase reached the highest point at 24 h. In addition, both elicitors can cause the accumulations of total phenolic compounds, *o*-dianisidine peroxidase and enhanced local resistance against *P. palmivora* on young rubber tree seedlings but the induction rate of each substance was different from that of the cell suspension. That is the phenolic compounds and *o*-dianisidine peroxidase reached the highest levels after treatment for 40 and 48 h, respectively. Furthermore,

during the expression of local resistance, the zoospore of *P. palmivora* caused the young seedlings to synthesize the *o*-dianisidine peroxidase more rapidly and with higher level than the control plants. *H. brasiliensis* is more responsive to the new elicitor than elicitin in triggering defense responses. The new elicitor was active at 30 fold lower concentration than those required for elicitin, when the activation was studied in cell suspension. For induction of phenolic compounds, *o*-dianisidine peroxidase and local resistance of rubber plants against *P. palmivora*, the 75 kDa protein was active at about 2 fold lower concentration comparing to elicitin.

P. palmivora could produce protease inhibitor (PpEPI10), an effector that is belong to a virulent factor. PpEPI10 is a Kazal-like extracellular serine protease inhibitor. The *Ppepi10* was isolated from mycelium of *P. palmivora* by reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE). It contained a 669 bp open reading frame, which encoded 222 amino acids and contained three domains of Kazal inhibitors, designated *Kazal1, Kazal2 and Kazal3*. The *rKazal1 and rKazal2* genes expressed in *Escherichia coli BL21* using pFLAG-ATS vector. However, *rKazal3* did not express in either vector pFLAG-ATS or vector pGEX 4T-1. *In vitro* protein expression and enzyme analyses revealed that both rKazal1 and rKazal2 proteins independently inhibited the activity of subtilisin A; however, neither had effect on the standard proteases chymotrypsin and trypsin. Moreover, zymogram and co-immunoprecipitation assays showed that both rKazal1 and rKazal2 interacted and inhibited a 95 kDa protease isolated from *H. brasiliensis* leaf. It was also revealed that the rKazal1 had a higher activity than the rKazal2 to inhibit subtilisin A and the 95 kDa protease.

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List of Abbreviations and Symbols

Avr	=	Avirulence
bp	=	base pair
BSA	=	Bovine serum albumin
°C	=	Degree Celsius
cDNA	=	Complementary DNA
DEAE	=	Diethylaminoethyl
DNA	=	Deoxyribonucleic acid
dNTPs	=	Deoxyribonucleotide triphosphates
E. coli	=	*Escherichia coli*
EDTA	=	Ethylenediaminetetra acetic acid
EPI	=	Extracellular protease inhibitor
fresh wt	=	Fresh weight
g	=	Gram
h	=	Hour
HR	=	Hypersensitive response
kDa	=	Kilodalton
L	=	Litre
LA media	=	Luria Bertani media agar
LB media	=	Luria Bertani media broth
M	=	Molar
m	=	Meter
mg	=	Milligram
min	=	Minute
ml	=	Mililiter
mRNA	=	Messenger ribonucleic acid
nm	=	Nanometer
nmole/g	=	Nanomole per gram
µg	=	Microgram
µl	=	Microliter

List of Abbreviations and Symbols

(continued)

μM	=	Micromolar
OD.	=	Optical density
pH	=	-log hydrogen ion concentration
PCR	=	Polymerase chain reaction
PDA	=	Potato dextrose agar
PDB	=	Potato dextrose broth
R	=	Resistance
RACE	=	Rapid amplified of cDNA ends
RNA	=	Ribonucleic acid
rpm	=	Round per minute
RT-PCR	=	Reverse transcriptase polymerase chain Reaction
SAR	=	Systemic acquired resistance
Scp	=	Scopoletin
SDS	=	Sodium dodecyl sulphate
SDS-PAGE	=	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	=	Second
TEMED	=	N,N,N, N-tetramethylenediamine
Tris-HCl	=	Tris (hydroxy methyl aminomethane) hydrochloride
V_8	=	V_8 agar
v/v	=	Volume/volume
w/v	=	Weight/volume
α	=	Alpha
β	=	Beta
λ	=	Lamda
%	=	Percent

CHAPTER 1
INTRODUCTION

Introduction

Rubber tree (*Hevea brasiliensis*) is the primary source of natural rubber. It is an economically important crop in Thailand because its sap-like extract (known as latex) can be collected to export to other countries. The latex can be obtained by tapping the bark of rubber tree. However, the consequence of frequent tapping causes infection at the raw surface of tapping site.

In the South of Thailand, *Phytophthora* is the important pathogen that infects rubber tree. The *Phytophthora* leaf disease was first found in Malaysia and Thailand in 1966. It attacks the petioles, causing mature leaves to fall prematurely. *Phytophthora* spp. described as pathogens of the rubber tree are *P. palmivora, P. botryosa, P. heveae, P. meadii* and *P. parasitica*. Among these, *P. palmivora* and *P. botryosa* are the most frequently isolated and described as the causal agents of *H. brasiliensis* abnormal leaf fall in Thailand.

P. palmivora is a ubiquitous pathogen with a wide host range (Erwin and Ribeiro, 1996; Holiday, 1980). It causes root, stem, and fruit rot in more than a hundred plant species, including pineapple, papaya, orange, tomato, tobacco and rubber tree. The first evidence of an active defense response in *H. brasiliensis* was reported by Tan and Low (1975).

Proteins and small molecules that alter host-cell structure and function produced from pathogen are called effectors. These alterations either facilitate infection (virulence factors and toxins) or trigger defense responses (avirulence factors and elicitors) or both (Huitema *et al.*, 2004; Kamoun, 2006, 2007). The effector activities were grouped into 4 types: inducing plant defenses that molecules called elicitors, suppression of plant immunity, inhibition and protection against plant hydrolytic enzymes such as protease inhibitor and altering plant behavior, morphology and development.

Naturally, plant has defense mechanism to protect invading of its pathogens. Plant defense mechanisms consist of passive (constitutive defense mechanism) and active responses (induced disease resistance). The passive defense mechanisms are already present prior to contacting with the pathogen, while the active defense mechanisms are activated only after recognition of the pathogen (Guest and Brown, 1997). The passive responses include the physical and chemical barriers such as cuticle, stomatal aperture and phytoanticipins. The active defenses consist of two classes of responses that include the local resistance such as accumulation of phenolic compounds, cell wall reinforcement, hypersensitive cell death and phytoalexin accumulation, the phytoalexin in rubber tree named scopoletin (Scp) which is toxic to leaf fungal pathogens of the rubber tree (Churngchow and Rattarasarn, 2001; Garcia *et al.*, 1995 a, b) while the systemic resistances include production of systemic acquired resistance (SAR) and pathogenesis-related proteins (PR-proteins) such as peroxidase enzyme (POD) and protease.

The active defense responses in plant can be stimulated by substances produced from plant pathogens called elicitors. As a result of host–pathogen or avirulence molecules co-evolution, plants have developed mechanisms to protect themselves from disease. When these induced responses are triggered rapidly during a given plant–pathogen interaction, the plant is resistant to disease. All *Phytophthora* species produce and secrete protein elicitors that called elicitin when cultured in appropriate culture media. Elicitin is a non-glycosylated protein with molecular weight of 10 kDa. Elicitin can stimulate plant responsive activities such as phytoalexin accumulation, synthesis of pathogenesis related-proteins and the occurrence of hypersensitive cell death or cell necrosis (Huet, 1991). Palmivorein is an elicitin from *P. palmivora* causes tissue necrosis on tested tobacco leaves. It also causes severe wilting and necrosis of *Hevea* tissue and leaves (Churngchow and Rattarasarn, 2000; Chirapongsatonkul *et al.*, 2008). *Phytophthora* species produce not only elicitins but also other proteins that have elicitor activity such as a polypeptide of 34 kDa found in the culture media of *P. nicotianae* (Billard *et al.*, 1988) and a 90 kDa protein (PB90) in the culture medium of *P. boehmeriae* (Wang *et al.*, 2003). The PB90 induce the hypersensitive reaction in tobacco leaves and triggere SAR in TMV, *Alternaria alternata*, *P. parasitica* and *Ralstonia solanacearum*. In addition, it cause

hypersensitive necrosis in leaves of the Chinese cabbage and induced SAR in *Collectorium higginsianum* (Wang *et al.*, 2003).

However, plant defense response can be inhibited by some effectors such as protease inhibitor and glucanase inhibitor. *Phytophthora sojae* secretes glucanase inhibitor proteins (GIPs) that specifically inhibit the endoglucanase activity of soybean (Jocelyn *et al.,* 2002). EPI1 and EPI10 of the Kazal family are secreted from *P. infestans*. The two domains EPI1 and the three domains EPI10 protein are shown to inhibit and interact with the pathogenesis-related protein P69B subtilase of tomato (Tian *et al.,* 2004, 2005).

For *P. palmivora*, little is known about other effectors aside from elicitin to stimulate or suppress plant defense in *H. brasiliensis*. Therefore, in this research, I investigated a new protein elicitor and a protease inhibitor from *P. palmivora*.

Review of Literature

1 *Hevea brasiliensis*

Hevea brasiliensis is grouped into the family of *Euphorbiaceae. H. brasiliensis* is called in various common names: rubber tree, para rubber, jebe, siringa, seringueira-branca, etc. However, it is often simply called rubber tree. *H. brasiliensis* is the most economically important member of the genus *Hevea.* It is of major economic and the source of virtually all the world's rubber production. The products of natural rubber are now produced from a few countries in Asia: Malaysia, Indonesia and Thailand. They produce rubber product about 80% of the world total. The other country producers, Sri Lanka, India, Liberia and Nigeria produce only 12% of rubber product of the world total (Grilli, 1980).

After planting, the rubber tree can reach a height of up to 34 m. The white or yellow latex occurs in latex vessels in the bark, mostly outside the phloem. These vessels spiral up the tree in a right-handed helix which forms an angle of about 30 degrees with the horizontal (http://en.wikipedia.org/wiki/Para_rubber_tree). The rubber tree grows well in soils that have a pH of 4.0 to 6.5 and at least 1 m deep. The optimal temperature is 25°C. The minimum temperature is 15°C. Relative humidity should not exceed 70 to 80 % (Nieto and Rodriguez, 1999). The rubber tapping can begin when the trees are 5-6 years old. The incisions of the tree are made orthogonal to the latex vessels, just deep enough to tap the vessels without harming the tree's growth, and the sap is collected in small buckets.

The manufacture of high-end furniture uses the wood from this tree to produce furniture. Rubber-wood is now valued as an environmentally friendly wood, as it makes use of plantation trees that have already served a useful function. However, the wood is very susceptible to decay and should be used only indoors. It tends to warp notably during drying. However, it is fairly easy to work, and glues well. As the wood is rather soft, it is mostly used in products such as toys and cutting boards, but it can be used in furniture.

The quality and yield of rubber latex are decreased if the diseases of leaf-fall and black stripe develop in *H. brasiliensis* (Fig.1). These diseases can occur after infection by pathogens in *H. brasiliensis*. The species of fungi frequently found

and known to attack *Hevea* trees are *Colletotrichum heveae* (leaf spot), *Fomes lamaensis* (brown root rot), *Gloeosporium heveae* (die-back), *Oidium heveae* (powdery mildew), *Pellicularis salmonicolor* (pink disease), *Polystichus occidentalis* (white spongy rot), *Sphaerella heveae* (rim bright), *Sphaerostilbe repens* (red rot), *Ustulina maxima* (charcoal rot) and *Phytophthora palmivora* (fruit rot, leaf-fall, black stripe and die-back). It is also attacked by bacteria, nematodes, insects and snails. In 1984, several species of *Phytophthora* were identified as causes of black stripe infection in China: for example *P. citrophthora* (Smith & Smith) and *P. cactorum* (Lebert & Cohn Schrodter). The early symptoms of black stripe are not obvious. Series of sunken and slightly discolored areas just above the cut surface (tapping surface), followed by the appearance of vertical fissures in the renewing bark are observed. Dark vertical lines are visible when bark is removed. As the infection progresses, the stripes form broad lesions, and finally spread to the full width of the tapping panel.

Fig.1 Black stripe disease on rubber tree.
(http://www.live-rubber.com/rubber-black- stripe disease)

2 *Phytophthora* spp.

Phytophthora is a genus of plant-pathogen Protista of the *Oomycetes*. It is sometimes referred to as a fungal-like organism. Currently, this genus is assigned to

the order *Pythiales*, phylum *Oomycota* within the group of heteroknot, biflagellate organisms that comprise the Kingdom *Chromista* (Cooke *et al.*, 2000).

The reproduction of *Phytophthoras* can reproduce both sexually and asexually. In many species, sexual structures have never been observed. *Homothallic* species is found as asexual structures. *Heterothallic* species were usually found sexual structures. They have mating strains, designated as A1 and A2. Antheridia introduce gametes into oogonia, either by the oogonium passing through the antheridium (amphigyny) or by the antheridium attaching to the proximal (lower) half of the oogonium (paragyny), and the union producing oospores after mating. Like animals, but not like most true fungi, meiosis is gametic, and somatic nuclei are diploid. Asexual (mitotic) spore types are chlamydospores, and sporangia which produce zoospores. Chlamydospores are usually spherical and pigmented, and may have a thickened cell wall to aid in its role as a survival structure. Sporangia may be retained by the subtending hyphae (non-caducous) or be shed readily by wind or water tension (caducous) acting as dispersal structures. Also, sporangia may release zoospores, which have two unlike flagella which they use to swim towards a host plant (Fig.2, 3) (Erwin, 1983).

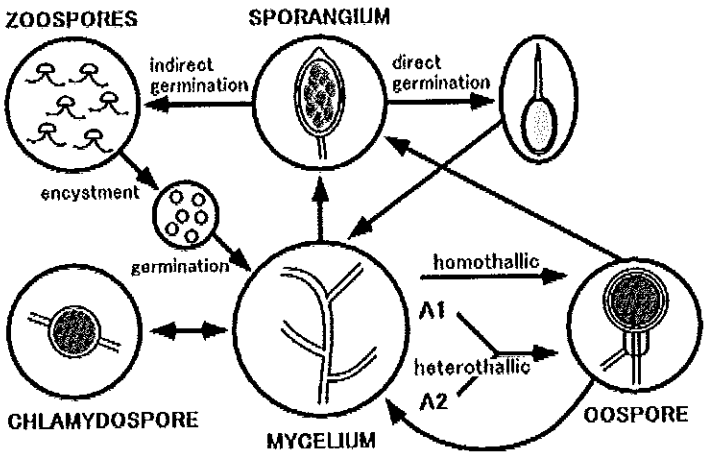


Fig.2 The Life cycle of *Phytophthora*.
(http://en.wikipedia.org/wiki/Phytophthora)

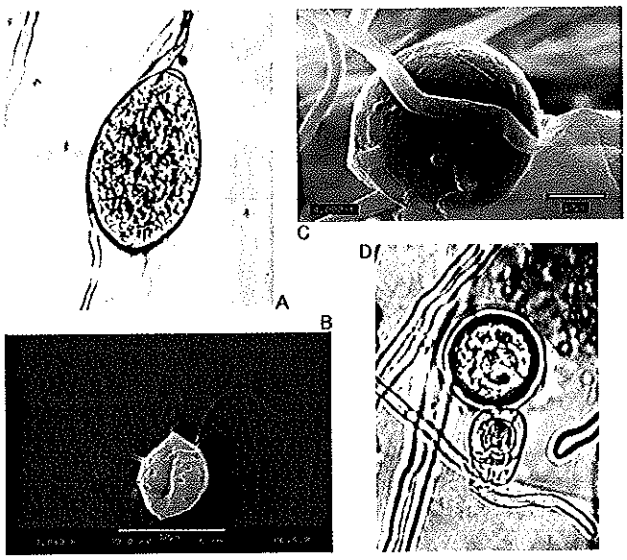


Fig.3 The reproductive structures of *Phytophthora*. The asexual (A) sporangium, (B) zoospore and (C) chlamydospore, and the sexual (D) oospore.

(http://dx.doi.org/10.1371/journal.pbio.0020213)

Phytophthora palmivora can produce abundant sporangia on V-8 agar if it is under continuous fluorescent light. Sporangia are usually produced in clusters sympodially. Sporangia are papillate and ovoid with the widest part close to the base. They are easily washed off and each detached sporangium contains a short pedicel. The sporangia have an average size about 50 X 33 μm and 1.6 times longer than it is wide for a length. Sporangia germinate directly in a nutrient medium by producing germ tubes that develop into mycelia masses. In water, however, zoospores are released from germinating sporangia.

Many species of *Phytophthora* are plant pathogens of considerable economic importance. If the plants develop diseases caused by this genus, it is difficult to control chemically then resistant cultivars are grown as a management strategy. The research about *Phytophthoras* in Asia began since 1990s (Erwin and Riberio, 1966). Important *Phytophthora* diseases are *Phytophthora alni* causes alder root rot, *Phytophthora cactorum* causes rhododendron root rot affecting rhododendrons, azaleas and causes bleeding canker in hardwood trees, *Phytophthora cinnamomi* causes cinnamon root rot affecting woody ornamentals including arborvitae, azalea, Chamaecyparis, dogwood, forsythia, Fraser fir, hemlock, Japanese

holly, juniper, Pieris, rhododendron, Taxus, white pine, and American chestnut, *Phytophthora fragariae* causes red root rot affecting strawberries, *Phytophthora palmivora* causes fruit rot in coconuts, root rot in durian, leaf fall and black stripe in the rubber plant, *Phytophthora ramorum* infects over 60 plant genera and over 100 host species causes Sudden Oak Death, *Phytophthora quercina* causes oak death, *Phytophthora sojae* causes soybean root rot.

Phytophthora spp. produce and secrete extracellular proteins, some of them are elicitors which can trigger plant defense reactions. Most *Phytophthora* spp. secretes 10 kDa proteins which are generally called elicitins (Pernollet *et al.*, 1993). It can invade tobacco stem and leaf necrosis at a distance from the inoculation site (Bonnet, 1985). *Phytophthora* species produce not only elicitins but also other proteins such as a polypeptide of 34 kDa found in the culture media of *Phytophthora nicotianae* that could induce lipoxygenase activity in tobacco plants (Billard *et al.*, 1988) and in culture media of some *Phytophthora* spp, a 32 kDa glycoprotein of cell wall fragment was detected. This protein was reported to cause similar necrosis of tobacco leaf (Baillieul *et al.*, 1996). In addition, a potent elicitor of phytoalexin accumulation in cultured parsley cells, a 42 kDa glycoprotein, was purified from the culture filtrate of *P. megasperma* (Parker *et al.*, 1991). A glycoprotein of molecular mass 46 kDa isolated from *P. nicotianae* was shown to elicit phytoalexin accumulation in tobacco callus (Ricci *et al.*, 1989). A 90 kDa protein (PB90) in the culture medium of *Phytophthora boehmeriae* induced the hypersensitive reaction in tobacco leaves and triggered systemic acquired resistance (SAR) to TMV, *Alternaria alternata*, *P. parasitica* and *Ralstonia solanacearum*. In addition, it caused hypersensitive necrosis in leaves of the Chinese cabbage and induced SAR to *Collectorium higginsianum* (Wang *et al.*, 2003).

3 Effectors

In the past, effectors were identified as proteins secreted from pathogens to reach cellular targets at the intercellular interface between the plant and pathogen or inside the host cell (Torto, 2003). However, recently biochemical, genetic, and bioinformatic strategies, often in combinations, has been applied to the identification of effector genes from oomycetes. Then the concept of effector was

exchanged to facilitate understanding. The effectors are all pathogen proteins and small molecules that alter host-cell structure and function. These alterations either facilitate infection (virulence factors and toxins) or trigger defense responses (avirulence factors and elicitors) or both (Huitema *et al.*, 2004; Kamoun, 2006, 2007).

The effectors from plant pathogens frequently have more than one host target (Saskia, 2009). For example, a kinase inhibitor AvrPto, effector from *Pseudomonas syringae*, binds and inhibits the tomato kinase Pto (Xing *et al.*, 2007). In addition, AvrPto inhibits the kinase domains of flagellin sensitive2 (FLS2) and elongation factor Tu receptor (EFR), which are two pathogen recognition receptors (Shan *et al.*, 2008; Xiang *et al.*, 2008). *Pseudomonas syringae* AvrRpt2 is the type III secretion system (T3SS) effector with proteolytic activity against at least five *Arabidopsis* proteins (Chisholm *et al.*, 2005; Takemoto and Jones, 2005).

3.1 Effector activities

3.1.1 Inducing plant defenses by elicitor molecules

The effectors found of present have many arrays of activities. One common activity of effectors from fungal and oomycete pathogens is their ability to induce defense reactions in host plant. The molecules triggering plant defense are called elicitor. Originally the term elicitor was used for molecules capable of inducing only the production of phytoalexins, but now it is used for compounds stimulating any type of plant defenses (Ebel and Cosio, 1994; Hahn, 1996). The elicitors can be both substances of pathogen origin (exogenous elicitors) and compounds released from plants by the action of the pathogen (endogenous elicitors) (Boller, 1995; Ebel and Cosio, 1994). Plants are able to recognize compounds produced or released by the aggressor (so-called elicitors) and employ these to trigger defense signaling (Macros *et al.*, 2003).

All of chemical structures including oligosaccharides, peptides, proteins and lipids can be elicitor if they have activity to induce plant defenses. Therefore the elicitor compounds are consisted of a wide range of chemical structures (Macros *et al.*, 2003). This huge array of elicitor compounds precludes the presence of a common elicitor motif and suggests that plants have the ability to recognize a number of structurally distinct molecules as signals for pathogen defenses. Elicitors

act as signal compounds at low concentrations by providing information for the plant to trigger defenses. However, may be it cannot induce plant defenses if using at higher concentrations. Elicitors can be distinguished from toxin, which may act only at higher concentrations and/or affect the plant detrimentally without activate plant metabolism (Boller, 1995). Elicitors can act as many functions depending on the type of interaction. For example, coronatine can be seen as a toxin, elicitor or a plant hormone. Because coronatine is a phytotoxin produced by certain races of the pathogen *Pseudomonas syringae*. It mimics Jasmonic acid, acting as a plant hormone and disturbing the 'appropriate' response in Arabidopsis (Feys *et al.*, 1994; Kloek *et al.*, 2001). On the other hand, isolated from the pathogen, coronatine does nothing but elicits the expression of defence genes in Arabidopsis (Bohlmann *et al.*, 1998).

Elicitors may be classified into two groups, 'general elicitors' and 'race specific elicitors'. The elicitors are able to trigger defenses both in host and non-host plants called general elicitors. Where as race specific elicitors induce defence responses leading to disease resistance only in specific host cultivars. A race specific elicitor encoded by or produced by the action of an avirulence (*avr*) gene present in a particular race of a pathogen will elicit resistance only in a host plant variety carrying the corresponding resistance (*R*) gene. A complementary pair of genes in a particular pathogen race and a host cultivar is a model of cultivars specific (gene-for-gene) resistance. However, if it absence of either gene product, host plant will often result in disease (Cohn *et al.*, 2001; Hammond-Kosack and Jones, 1997; Luderer and Joosten, 2001). It is opposite of general elicitors. Which signal the presence of potential pathogens to both host and non-host plants (Nürnberger, 1999). However, some of these are only recognized by a restricted number of plants (Shibuya and Minami, 2001).

To induce plant defenses, *P. palmivora* and other *Phytophthora* spp. produce elicitor in a family of structurally related extracellular proteins, known as elicitins that can induce hypersensitive cell death and other biochemical changes associated with defense responses in *Nicotiana* spp. (Kamoun *et al.*, 1997). *Phytophthora* species produce not only elicitins to induce plant defenses but also other proteins that have elicitor activity such as A 32-kDa glycoprotein isolated from *Phytophthora megasperma* also displays a similar necrotic action on tobacco leaves

(Baillieul *et al.*, 1995). Harpins isolated from *Pseudomonas* species also induce necrotic zones when infiltrated into tobacco leaves in a similar way to those produced by the proteins isolated from *Phytophthora* spp. (Arlat *et al.*, 1994). A 90 kDa protein (PB90) in the culture medium of *Phytophthora boehmeriae* induced the hypersensitive reaction in tobacco leaves and triggered systemic acquired resistance (SAR) to TMV, *Alternaria alternata*, *Phytophthora parasitica* and *Ralstonia solanacearum*. In addition, it caused hypersensitive necrosis in leaves of the Chinese cabbage and induced SAR to *Collectorium higginsianum* (Wang *et al.*, 2003).

3.1.2 Suppression of plant immunity

Suppression of plant innate immunity is the important activity of effector (Saskia, 2009). Almost plant pathogenic bacteria, pathogenic gram-negative, utilize a specialize machinery type III secretion system (T3SS) as suppression effectors to deliver proteins inside host cells (Abramovitch *et al.*, 2006; Block *et al.*, 2008). Several T3SS effectors contribute to virulence by suppressing basal defenses induced by conserved pathogen epitopes named pathogen-associated molecular patterns (PAMPs) (Hauck *et al.*, 2003; Kim *et al.*, 2005). Some T3SS effectors suppress hypersensitive cell death elicited by various avirulence (Avr) proteins (Abramovitch *et al.*, 2003; Jamir *et al.*, 2004; Kim, H. S. *et al.*, 2005, Tsiamis *et al.*, 2000).

3.1.3 Inhibition and protection against plant hydrolytic enzymes

The other activity of effector is inhibition and protection against plant hydrolytic enzymes, such as proteases, glucanases, and chitinases (Misas-Villamil and van der Hoorn, 2008). *Cladosporium fulvum* secretes Avr2 protein into the apoplast of tomato leaves to bind and inhibit extracellular tomato cysteine protease Rcr3. In the presence of the extracellular leucine-rich repeat receptor-like Cf-2 protein, interaction between Avr2 protein and tomato cysteine protease Rcr3 can trigger a hypersensitive response (HR) in tomato leaves (Henrietta, 2005). *Phytophthora infestans* secretes serine protease inhibitors, EPI1 and EPI10, which are multi-domain serine protease inhibitors of the Kazal family that bind and inhibit the pathogenesis-related (PR) protein P69B, a subtilisin-like serine protease of tomato that is thought to function in defense (Tian *et al.*, 2004, 2005). *P. infestans* also secretes cysteine protease inhibitors, such as extracellular cystatin protease inhibitor (EPIC) domain EPIC2B,

which inhibits a novel papain-like extracellular cysteine protease (Tian *et al.*, 2007). Moreover, *Phytophthora* spp. are also known to secrete glucanase inhibitors that inhibit the host apoplastic enzyme endo-β-1,3 glucanase (Damasceno *et al.*, 2008; Rose *et al.*, 2002).

3.1.4 Altering plant behavior, morphology and development

Some effectors of fungal and oomycete pathogens alter plant behavior, morphology and development. Expression of *Xanthomonas citri pthA* in citrus cells is sufficient to cause macroscopic hyperplastic lesions analogous to the canker symptoms caused by the pathogen (Duan *et al.*, 1999). In *H. brasiliensis*, infection occurs on untapped bark resulting in a wound called "canker". These canker lesions are thought to facilitate pathogens release from infected tissue and to enhance pathogen dissemination. The coronatine, which was shown by Melotto *et al.* (2006) to trigger stomatal reopening in *Arabidopsis* and thereby facilitate bacterial entry inside the plant apoplast. *Xanthomonas* effectors are known to induce cellular division and enlargement in susceptible host plants (Duan *et al.*, 1999; Kay *et al.*, 2007).

3.2 Effector classification

The effectors can be divided into two classes based on their target sites in the host: apoplastic effectors and cytoplasmic effectors. Apoplastic effectors are the molecules secreted into the plant extracellular space, where they interact with extracellular targets and surface receptors. It is different completely from cytoplasmic effectors. That is cytoplasmic effectors are translocated inside the plant cell presumably through specialized structures like infection vesicles that attack inside living host cells (Fig.4) (Kamoun, 2006).

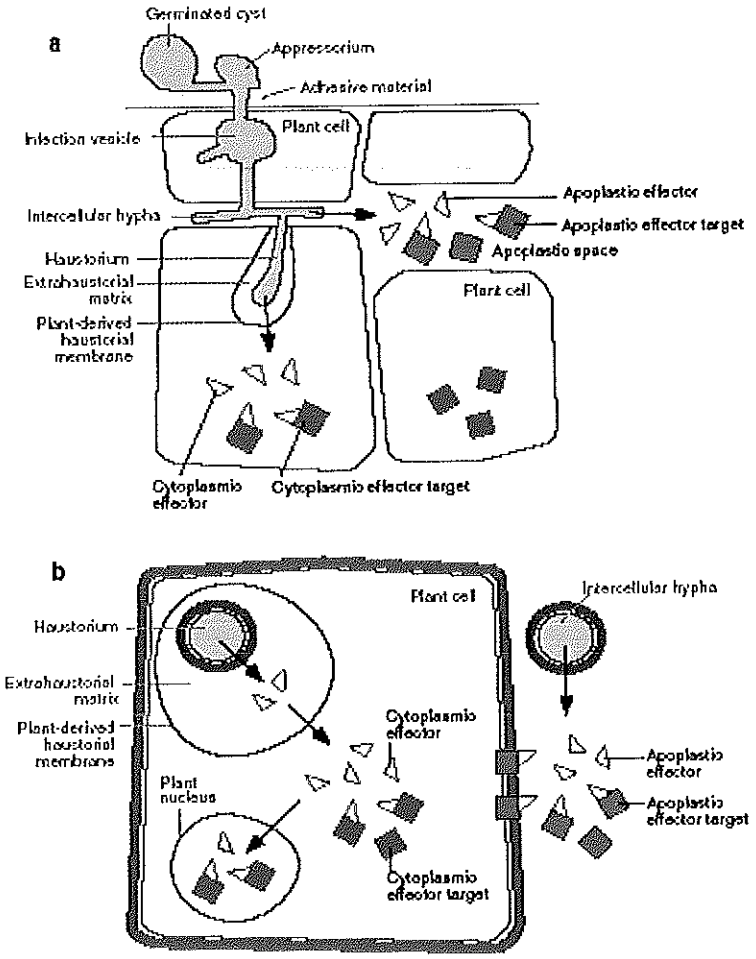


Fig.4 (a) Apoplastic effectors were secreted into the plant extracellular space by plant pathogenic oomycetes and (b) cytoplasmic effectors inside the plant cell (Kamoun, 2006).

3.2.1 Apoplastic effectors

The major biochemical activities of apoplastic effectors have one more activity. It can induce plant defense mechanisms by using elicitors or contribute to counter-defense by inhibiting host enzymes, such as proteases and glucanases that accumulate in response to pathogen infection (Rose *et al.*, 2002; Tian *et al.*, 2004, 2005). Then the group of apoplastic effectors is arranged into two groups. Small cysteine-rich proteins are group of inducing plant defenses and enzyme inhibitors are group of counter-defense in plant.

3.2.1.1 Small Cysteine-Rich Proteins

The basic structural features of this group most notably secretion and the disulfide bridges formed by the pairs of cysteines, are essential for defense induction and avirulence function (Kamoun, 2006). In general, small cysteine residues are secreted from some eukaryotic. Because almost *Avr* gene effectors from some eukaryotic encode small (<150 amino acids) secreted proteins with an even number of cysteine residues, which can induce defense responses when infiltrated into plant tissues (Lauge, 1998; van't Slot, 2002). However, a number of oomycete effectors are small of cysteine-rich proteins. The samples of effector in this group are *Phytophthora* elicitins, *Cladosporium fulvum Avr2*, *Avr4*, and *Avr9* and *Rhynchosporium secalis nip1.*

Elicitin

Elicitin is one member of elicitors. It is highly conserved small extracellular proteins that secreted by species of the genus *Phytophthora*. Elicitin is a non-glycosylated protein with molecular weight of 10 kDa. Its molar structure consists of 98 amino acid residues with six invariant cysteines and three disulfide bonds, 50% of which are in α-helix form (α-elicitin is acidic, having pl about 4.5 and the amino acid residue at position 13 is valine located at the outer surface of the molecule which affects the interaction between elicitin and its target receptor) and few or none in β-pleated sheet (β-elicitin is basic protein, its pl is 8.5) (Nespoulous *et al.*, 1992; Huet and Pernollet, 1993). Whose positions span the entire protein, and a central region rich in hydrophobic amino acids. Leucine, alanine, serine, and threonine represent about 50% of the amino acids in elicitins, while histidine, tryptophan, and arginine are absent (Lloyd, 1995).

All *Phytophthora* species produce and secrete protein called elicitin (Huet and Pernollet, 1993). Elicitins isolated from culture medium of each *Phytophthora* species have the same numbers of amino acids but differ only in amino acid sequences. Purification usually involves some combination of hydrophobic interaction chromatography, ion-exchange chromatography, reversed-phase high-pressure liquid chromatography, and gel filtration (Lloyd, 1995).

Palmivorein is a member of elicitin family purified from the culture filtrate of *Phytophthora palmivora* isolated from the rubber tree. It can be obtained by

ammonium sulfate precipitation and further purified using ion-exchange and gel filtration. It is classified as α-elicitin according to its acidic pI and the valine residue at position 13. The *P. palmivora* elicitin causes tissue necrosis on tested tobacco leaves. It also causes severe wilting and necrosis of *Hevea* tissue and leaves (Churngchow and Rattarasarn, 2000).

Elicitin is classified as biotic elicitor. It stimulates hypersensitive response in tobacco cell suspension by increasing pH. It also increases the synthesis of ethylene and capsidiol, a phytoalexin. The necrosis and the accumulation of pathogenesis-related proteins, PR1a, are also observed when tobacco leaves and trees are tested with elicitin (Huet *et al.*, 1991). The biological activities of elicitins can be inhibited by protease enzyme. In addition to stimulating cellular response in tobacco plant, elicitins can induce systemic acquired resistance to *P. parasitica var. nicotianae* which cause black shank disease (Ricci *et al.*, 1989) and to *Xanthomonas*, the causative pathogen of radish (Kamoun *et al.*, 1993).

Elicitins are grouped to act as avirulence factors. Because of a strong correlation between low or no virulence of *Phytophthora* isolates on tobacco and the elicitin secretion by the isolate into culture fluids, and, of course, the necrotic effects of purified elicitins, which mimic the outcome of the interaction of most nontobacco isolates with tobacco. Two studies surveyed 100 isolates of 15 species of *Phytophthora* with respect to elicitin production in culture fluids and, for selected isolates, pathogenicity on tobacco (Kamoun, 1994; Ricci, 1992) can conclude that the isolates collected from species other than tobacco, 23 of 25 (92%) produced elicitin in culture, and those tested were not pathogenic on tobacco. Only 6 of 21 (29%) tobacco isolates (*Phytophthora parasitica* var. *nicotianae*) produced elicitins and, although pathogenic, these showed lesser virulence than the nonproducing isolates. Elicitins purified from two of the tobacco isolates and from *Phytophthora cryptogea* (from chrysanthemum) were each able to induce necrosis in tobacco at the same threshold concentrations. The production levels of elicitin in cultures of the two tobacco isolates were similar to those of the nontobacco isolates. It is also reported that 118 tobacco isolates of *P. parasitica* that produce elicitin in culture was generally less virulent than those that did not produce elicitin (Lloyd, 1995).

3.2.1.2 Enzyme inhibitors

The one activity of effectors from pathogens is inhibition and protection against plant hydrolytic enzymes, such as proteases, glucanases, and chitinases, that all called plant pathogenesis-related (PR) proteins. The PR proteins in plant can protect infection from its pathogens. However, pathogens produce enzyme inhibitors to protection against the activities of these PR proteins. Plant pathogenic oomycetes, such as *Phytophthora*, have also evolved mechanisms to escape the enzymatic activity of PR proteins (Kamoun, 2006) by secreting inhibitory proteins that target host protease and glucanase.

a) Protease inhibitors (PI)

Protease inhibitors are important tools of nature for regulating the proteolytic activity of their target proteases (Bode and Humer, 1992). Protease inhibitors function as specific substrates for the proteases they inhibit. However, unlike normal substrates, which are cleaved by proteases and released quickly, the PI-protease complex is stable, and proteolysis of the inhibitor is limited and extremely slows (Laskowski, 1985; Garcia *et al.*, 1987). The specificity and efficacy of inhibition are determined by the degree of stereochemical complementation between the protease active site and a short inhibitory loop that extends out from the main body of the inhibitor molecule (Bode and Huber, 1992). By definition, protease inhibitors appear to function by impairing the proteolytic activity of target proteins; in the case of host-microbe interactions, the function of host plant proteases are blocked by the specific recognition and activity of their cognate pathogen inhibitors. In fungi, protease inhibitors may be classified in one of 6 types: serine protease inhibitors (Ser protease inhibitor), cysteine protease inhibitors (CPIs), threonine protease inhibitors , aspartic protease inhibitors, glutamic protease inhibitors and metalloprotease inhibitors. Usually in oomycetes pathogens produce Ser protease inhibitor and cysteine protease inhibitors to inhibit protease activity.

Ser protease inhibitors, the protein inhibitors directed against serine proteases can be divided into at least 16 different families based on sequence similarity, topological similarity, and mechanism of binding (Bode and Huber, 1992). The most families of serine protease inhibitors studied are Serpins, Kunitz, and Kazal families (Meredith *et al.*, 2002).

Optimal inhibitory Serpins target chymotrypsin-like serine proteases. Such as, poxvirus secretes several Serpins to inhibit inflammation and apoptosis and function in determination of viral host range (Turner, 2001). However, some Serpins can inhibit other classes of protease. Then these Serpins are termed "cross class inhibitors". The squamous cell carcinoma antigen 1 (SCCA-1), the avian serpin myeloid and erythroid nuclear termination stage specific protein (MENT) are examples of cross class inhibitory serpins. All of these inhibitors have been shown to inhibit papain-like cysteine proteases (Schick *et al.*, 1998; McGowan *et al.*, 2006; Ong *et al.*, 2007). Moreover, the viral serpin cytokine response modifier A (crmA) is also a cross class inhibitory. It is a suppressor of the inflammatory response through inhibition of interleukin-1 (IL-1) and IL-18 processing by the cysteine protease caspase-1 (Ray *et al.*, 1992).

The features of proteins from the Kunitz family enclose 170 to 200 amino acid residues and one or two intra-chain disulfide bonds. The best conserved region is found in their N-terminal sections. A Kunitz family serine protease inhibitor can be isolated from the hookworm *Ancylostoma ceylanicaum*. It has activity against chymotrypsin, pancreatic elastase, neutrophil elastase and trypsin, and may contribute to the ability of the parasite to evade the immune system and provide protection during its residence within small intestine (Milstone *et al.*, 2000).

The Kazal family is one of the Ser protease inhibitors. This family is named following L. Kazal, who discovered the pancreatic secretory trypsin inhibitors, PSTI, that are present in all vertebrates (Stephen *et al.*, 2001). The structure of the Kazal repeat includes a large quantity of extended chain, 2 short alpha-helices and a 3 strands anti-parallel beta sheet and highly conserved of amino acid residues including the cysteine backbone, tyrosine, and asparagine residues (Tian *et al.*, 2004). Recently, it has been found in many organisms. Such as found in mammals (pancreatic secretory trypsin inhibitors, mammalian seminal acrosin inhibitors) (Kazal *et al.*, 1948), birds (avian ovomucoids, chicken ovoinhibitor) (Laskowski and Kato, 1980), leeches (leech-derived tryptase inhibitor) (Stubbs *et at.*, 1997), and insects (rhodniin) (van de Locht *et al.*, 1995) and used extensively *in vitro* to study the interactions of serine proteases with their substrates. *Neospora caninum* secretes a single domain Kazal inhibitor that called NcPI-S. It has high activity to inhibit subtilisin but little or

no activity against elastase or chymotrypsin (Meredith *et al.,* 2004). The two-domain EPI1 and the three-domain EPI10 proteins inhibit and interact with the pathogenesis-related protein P69B subtilase of tomato (Tian *et al.*, 2004, 2005). Generally, inhibitors of the Kazal-type exhibit two or more inhibitory domains which may be specific for different proteinases (Kreutzmann *et al.*, 2004). For example, seven Kazal-type domains of ovoinhibitor occurring in avian egg white have several targets of protease, at least five reactive sites: two for trypsin, two for chymotrypsin and one for porcine pancreatic elastase (Saxena and Tayyab, 1997). Another remarkable member of the Kazal-type inhibitors is the thrombin inhibitor rhodniin from the assassin bug *Rhodnius prolixus*. It is composed of two Kazal-type domains of which only one acts in a manner typical for canonical inhibitors. In contrast, the second domain binds to the fibrinogen recognition exosite of thrombin by electrostatic interaction (A. van de Locht *et al.,* 1995). However, it is possible that the individual protein inhibitors inhibit only protease belonging to a single mechanistic class (Laskowski and Kato, 1980) such as the two-domain EPI1 and the three-domain EPI10 proteins from *P. infestans* specifically inhibit only subtilisin A (Tian *et al.*, 2004, 2005) that are thought to function in counterdefense (Kamoun, 2006). They inhibit and interact with the PR-protein P69B, a subtilisin-like serine protease of tomato that is thought to function in defense (Tian *et al.*, 2004, 2005). The *epi1*, *epi10*, and *P69B* genes are concurrently expressed and up-regulated during infection of tomato by *P. infestans*. Recent findings indicate that EPI1 protects several secreted proteins in *P. infestans* from degradation by P69B thereby directly contributing to virulence (Kamoun, 2006).

Cysteine protease inhibitors (CPIs) differ from serine proteases in that they are defined by the presence of a nucleophilic cysteine residue, rather than a serine residue, in their catalytic site. The cysteine protease inhibitors from mammals are divided into three families: Stefin family, Cystatin family and Kininogen family (Otto and Schirmeister, 1997). Common to all CPIs is their enormous stability at high temperatures (up to 100 °C) (Lenney *et al.*, 1979) and at extreme pH (pH 2-12) as well as their specificity for cysteine proteases (Barrett *et al.*, 1986).

The proteins of family Stefin have a molecular weight of about 11 kDa and they lack disulfide bridges and carbohydrate residues. Like biosynthesis on free

ribosomes and lack of a signal peptide, these are also characteristics of intracellular proteins (Barrett *et al.*, 1986, 1987; Turk and Bode, 1991; Abrahamson *et al.*, 1994).

The Cystatin family has low molecular from 12 to 13 kDa weight with 110-120 amino acids. It is not much bigger than the stefins and also has no carbohydrate residues but do have two disulfide loops at the C terminal (Barrett *et al.*, 1986, 1987; Turk and Bode, 1991; Abrahamson *et al.*, 1994).

Kininogen family form the high molecular weight group about 120 kDa and low molecular group about 50-80 kDa. The Kininogens contain three sequences which each correspond to the polypeptide chains of Cystatins. These three domains, together with the 10 amino acids of kinin (any of various structurally related polypeptides) form the N-terminal heavy chain from which a signal peptide of up to 18 amino acids is cleaved after translation. This part of the protein is identical in both Kininogens while the C-terminal light chains differ significantly in both sequence and size. In addition to the two disulfide loops in each segment, domains 2 and 3 each have a disulfide bridge at their N terminals and a ninth disulfide bond links segment 1 with the light chain. Each molecule has three carbohydrate residues. These characteristics of the Kininogens (carbohydrate residues, signal peptide, disulfide bridges) are typical for extracellular proteins (Barrett *et al.*, 1986, 1987; Turk and Bode, 1991).

However, the family of cysteine protease inhibitors in fungi and oomycetes pathogens is now not conclusive classified. But it can be found in these pathogens. For example, *Cladosporium fulvum* secretes Avr2 protein into the apoplast of tomato leaves to bind and inhibit extracellular tomato cysteine protease Rcr3. In the presence of the extracellular leucine-rich repeat receptor-like Cf-2 protein, interaction between Avr2 protein and tomato cysteine protease Rcr3 can trigger a hypersensitive response (HR) in tomato leaves (Henrietta, 2005). *P. infestans* also secretes cysteine protease inhibitors EPIC2B, which interacts with and inhibits a novel papain-like extracellular cysteine protease as well as other apoplastic cysteine proteases of tomato (Tian *et al.*, 2007).

b) Glucanase inhibitors

Glucanase inhibitor proteins (GIPs) are thought to function as counter defensive molecules that inhibit the degradation of β-1,3/1,6 glucans in the pathogen cell wall and/or the release of defense-eliciting oligosaccharides by host β-1,3 endoglucanases. A characteristic plant response to microbial attack is the production of endo-β-1,3-glucanases, which are thought to play an important role in plant defense, either directly, through the degradation of β-1,3/1,6-glucans in the pathogen cell wall, or indirectly, by releasing oligosaccharide elicitors that induce additional plant defenses. However, during invasion of their plant hosts, species of the oomycete genus *Phytophthora* secrete glucanase inhibitor proteins into the plant apoplast, which bind and inhibit the activity of plant extracellular endo-beta-1,3-glucanases (EGases).

To date, glucanase inhibitors from oomycete pathogens were found in *Phytophthora sojae*. Soybean endo-β-1,3-glucanases and corresponding glucanase inhibitor proteins secreted by the oomycete root pathogen *P. sojae* are an attractive system for studying the co-evolution of enzyme-inhibitor systems. *P. sojae* GIPs inhibit up to 85% of soybean endoglucanase activity and appear to be highly specific for particular EGases (Ham *et al.,* 1997). For example, GIP1 completely inhibits soybean EGaseA, which acts as a high-affinity ligand for GIP1, but it does not bind or inhibit the isozyme EGaseB, tobacco PR-2 (an EGase that is structurally similar to EGaseB), or an endogenous *P. sojae* EGase (Ham *et al.,* 1997). EGaseA and EGaseB further differ in that EGaseA is constitutively produced, releases elicitors of additional defense reactions when soybean is challenged by *P. sojae*, and was shown experimentally to increase resistance to *P. sojae*, whereas EGaseB is induced upon pathogen attack and is not known to increase resistance (Yoshikawa *et al.,* 1990; Rose *et al.,* 2002). Moreover, the glucanase inhibitors GIP2 secreted proteins from *P. sojae* is also inhibit the soybean endo-β-1,3 glucanase EGaseA (Rose *et al.,* 2002). These inhibitor proteins share significant structural similarity with the trypsin class of serine proteases, but bear mutated catalytic residues and are proteolytically nonfunctional. There is some degree of specificity in inhibition because GIP1 does not inhibit another soybean endoglucanase, EGaseB (Kamoun, 2006).

3.2.2 Cytoplasmic effectors

Cytoplasmic effectors are translocated into the plant cell, where they target different from apoplastic effectors. Several apoplastic effectors contribute to counter-defense by inhibiting host enzymes, such as proteases and glucanases that accumulate in response to pathogen infection (Rose *et al.*, 2002; Tian *et al.*, 2004, 2005). By contrast, the biochemical activities of cytoplasmic effectors remain poorly understood (Morgan and Kamoun, 2007). Oomycete cytoplasmic effectors have been discovered through their avirulence function, that is, their ability to trigger hypersensitive cell death on host genotypes with corresponding disease resistance (*R*) genes (Allen *et al.*, 2004; Armstrong *et al.*, 2005; Rehmany *et al.*, 2005; Shan *et al.*, 2004).

4 Plant defense mechanisms

Plant defense mechanisms consist of passive (constitutive defense mechanism) and active responses (induced disease resistance). The passive defense mechanisms are already present prior to contacting with the pathogen, including the natural physical and chemical barriers such as cuticle, stomatal aperture and phytoanticipins. While the active defense mechanisms are activated only after recognition of the pathogen (Guest and Brown, 1997).

Active defense mechanisms occur after plants are attacked by potentially pathogenic microorganisms then the signals are produced that induce responses in the plant. This process involves the interaction between pathogen associated molecules (elicitors) and putative plant receptors. This recognition is followed by a signal transduction cascade resulting in defense gene activation and the expression of active defense response in the plant host. Specific recognition between the plant host and the fungal pathogen determines the outcome of the interaction (Keen, 1990; Rosario *et al.*, 1994). The active defense mechanisms consist of two classes of responses that include the local resistance such as hypersensitive response (HR), phenolic compounds, phytoalexin accumulation and cell wall reinforcement, and the systemic resistance such as production of pathogenesis-related proteins (PR-proteins) and systemic acquired resistance (SAR).

4.1 Local resistance

4.1.1 Hypersensitive response (HR)

Hypersensitive response is a form of programmed cell death (PCD) representing one of the mechanisms of plant defense against pathogens that occur in an incompatible host-pathogens relationship. It is a complex, early defense response that causes necrosis and cell death to restrict the growth of a pathogen within a few hours of pathogen contact (Agrios, 1988). The HR can be phenotypically diverse, ranging from HR in a single cell to spreading necrotic areas accompanying limited pathogen colonization (Holub *et al.*, 1994). In a resistant plant the infected cells and those surrounding it turn brown, with the characteristics of a burn-like lesion after cells die (Fig.5). This reduces the amount of nutrients available to the pathogen inside the dead tissue and limits the growth of the pathogen and its further spread. Hypersensitive cell death is a multifaceted defense mechanism, active against viruses, fungi and bacteria (Klement, 1982).

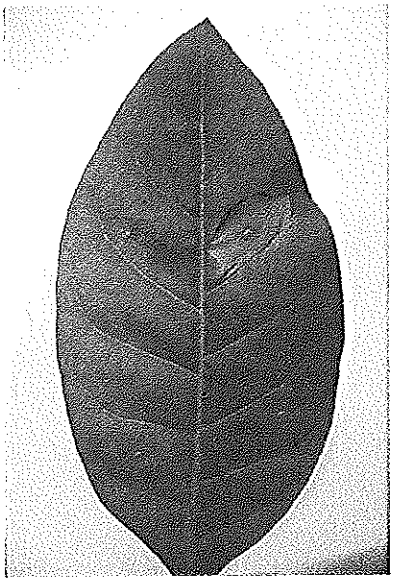


Fig.5 Hypersensitive cell death in tobacco plants (*Nicotiana tabacum*).

Major players involved in the activation of the HR are reactive oxygen species (ROS), nitric oxide (NO), calcium and proton pumps, mitogen-activated protein kinases (MAPKs), and salicylic acid (SA). It is believed that the initial

recognition of the pathogen by a plant receptor (part of the gene-for-gene response) activates a signal transduction pathway that involves the translocation of Ca^{2+} and protons across the plasma membrane into the cytosol, protein phosphorylation/dephosphorylation, activation of enzymes that generate ROS such as NADPH-oxidase and peroxidases, and accumulation of NO and SA. From challenged, HR developing cells, some particular diffusible molecules known as stress phytohormones (SA, jasmonic acid (JA) and ethylene), may play an important signalling function in establishing resistance both locally (local resistance) and systemically (systemic resistance). When attacked by incompatible pathogens, plants respond by activating a variety of defence responses, including ROS-generating enzyme complex (Bolwell, 1999). The increase of cellular concentration of ROS is a key event in plant and animal PCD and occurs as a result of many stresses (Laloi *et al.,* 2004; Neill *et al.*, 2002), but an oxidative burst is an essential prerequisite for induction of plant hypersensitive cell death (Levine *et al.,* 1994; Lamb and Dixon, 1997; Jabs, 1999). Under normal conditions, ROS are cleared from the cell by the action of superoxide dismutase (SOD), catalase, or glutathione peroxidase. Under stress conditions, ROS enhance the lipid catabolism resulting in peroxidation of polyunsaturated fatty acids in the cell membranes that in turn leads to structural decomposition, change in permeability, and induces damage by alterations of essential proteins, and DNA (Elena *et al.,* 2005). Upon pathogen recognition, one of the earliest reactions is opening of specific ion channels and the formation of superoxide and H_2O_2 (Kasparovsky *et al.*, 2003). It has been shown that H_2O_2 participates in the cross-linking of cell wall structural proteins and functions as a local trigger of PCD in pathogen challenged cells. In addition, H_2O_2 acts as a diffusible signal (Hung *et al.,* 2005) and induces genes encoding cellular protectants in adjacent cells when soybean cells have been elicited by glucan elicitor isolated from mycelial walls of the fungal pathogen *P. megasperma* (Levine *et al.*, 1994).

P. palmivora and other *Phytophthora* spp. produce a family of structurally related extracellular proteins, known as elicitin, that can induce hypersensitive cell death and other biochemical changes associated with defense responses in *Nicotiana* spp. (Kamoun *et al.*, 1993, 1997; Ponchet *et al.,* 1999; Ricci *et al.*, 1989; Sasabe *et al.*, 2000; Chirapongsatonkul *et al.*, 2008). A 32 kDa glycoprotein

isolated from *P. megasperma* also displays a similar necrotic action on tobacco leaves (Baillieul *et al.*, 1995). PCD can be also induced in plants by toxins produced by a number of harpins pathogens from *Pseudomonas syringae, Erwinia amylovora, Xanthomonas campestris*, the fungal toxin victorin, xylanase from *Trichoderma viride* (He, 1996; Grant and Mansfield, 1999; Lam *et. al.*, 1999), *Alternaria alternata* AAL toxin, the fungal toxin Fumonisin B1 (FUM) from *Fusarium moniliforme* (Wang *et al.*, 1996), fungal toxin cryptogein from *P. cryptogea* (Hirasawa *et al.*, 2005). Also, plant viruses such as tobacco mosaic virus (TMV) are reported to elicit PCD (del Pozo and Lam, 2003).

4.1.2 Phenolic compounds

Normally, plants have the ability to synthesize secondary metabolites, which may be in the form of simple phenols and phenolic acids, quinones, flavones, alkaloids, etc; collectively referred to as phenolic compounds. The structure of phenolics composes of one or more benzene rings with one or more hydroxyl groups that may be variously elaborated with methyl, methoxyl, amino or glycosyl groups (Fig.6) (Mohamed *et al.*, 2007). There are produced large increases in phenolic synthesis in plants after attack by plant pathogens or inducing by elicitors (De Ascensao *et al.*, 2003; Matern *et al.*, 1995). Phenolics are induced and accumulated early and rapid at the infection site resulting in the effective isolation of the pathogen (Chérif *et al.*, 1991, 1992).

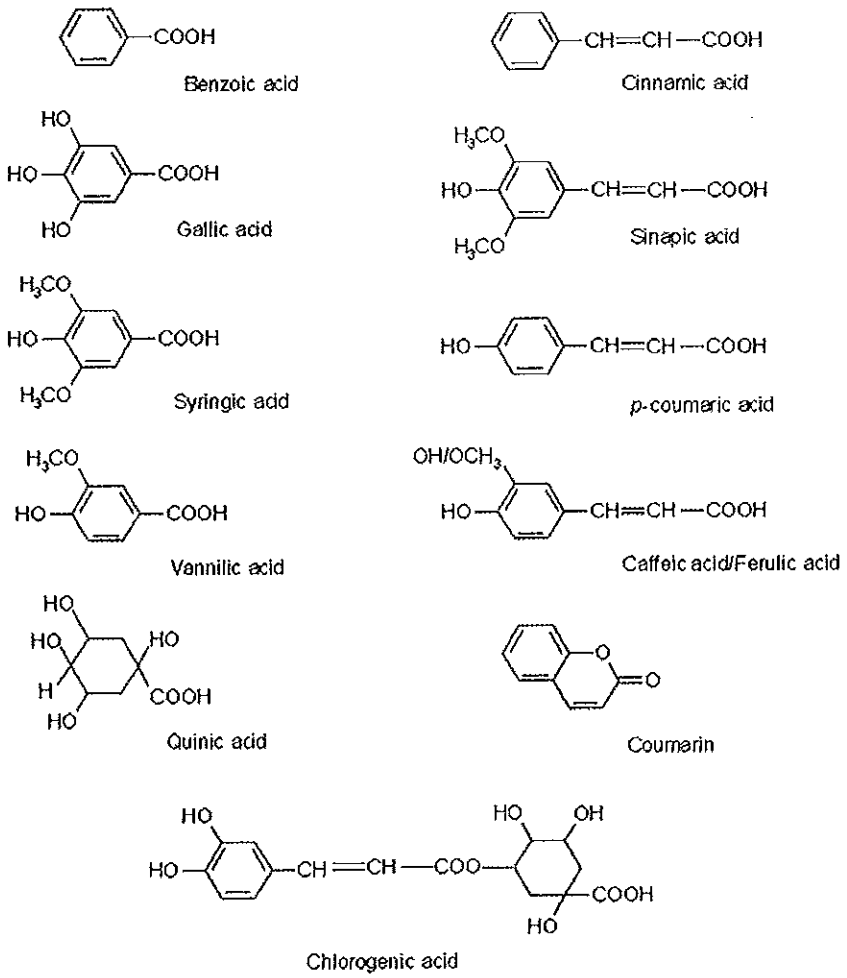


Fig.6 Structures of common phenolic compounds.
(Mohamed *et al.*, 2007)

These substances are designed as plant defense mechanisms against predation by insects, herbivores and microorganisms (Beckman, 2000). They act as intermediate substrate of phenylpropanoid pathway to produce other plant defense responses such as phytoalexins, papillae, as well as the occlusion of plant vessels and synthesis of lignin, a polymer of aromatic phenolics. Results from many studies suggest that esterification of phenols to cell wall materials and the accumulation and deposition of phenols in cell walls is usually serves as an increase in resistance to fungal hydrolytic enzymes as well as a physical barrier against fungal penetration (Mohamed *et al.*, 2007)

4.1.3 Phytoalexin production

Phytoalexins are low molecular weight antibiotics synthesized by plants following infection or stimulation by elicitors. The response is localized to the sites of infection and surrounding areas (Darvill *et al.,* 1984). They inhibit the growth of fungi and bacteria in vitro therefore, it is logical to consider them as possible plant-defense compounds against diseases caused by fungi and bacteria. Phytoalexin molecule is composed of carbon, hydrogen and oxygen. All phytoalexins are lipophilic compounds. The glyceolli, capsidio, orchinol, wyerone acid and scopoletin shown in Fig.7 are samples of diverse types of phytoalexins (Peter and Barbara, 1978).

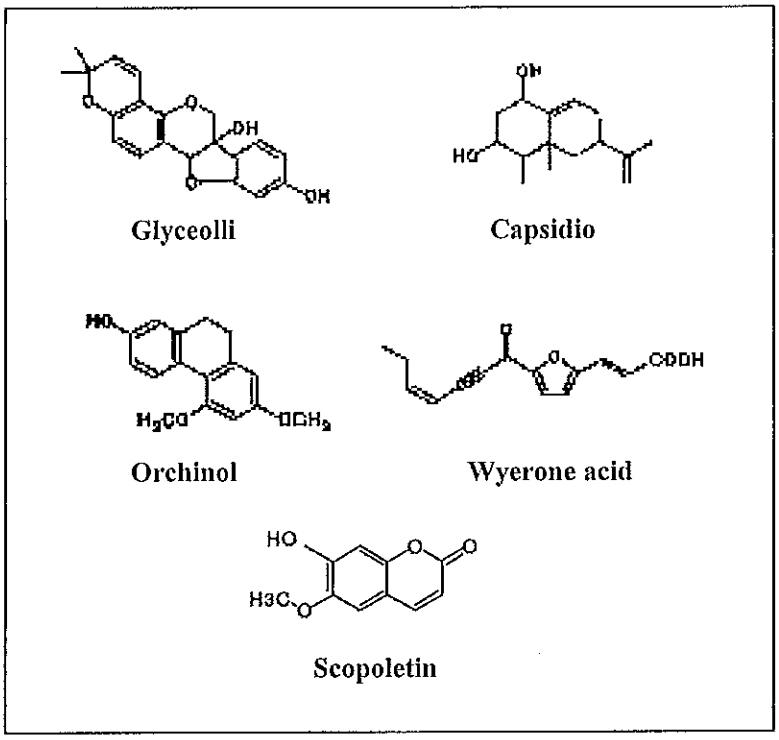


Fig.7 Structurally diverse phytoalexins.
(Peter and Barbara, 1978)

Its toxicity is non selective and the chemical affinity of most phytoalexins for lipids suggests that they accumulate in cell membranes. However, the phytoalexins mechanism to stop cells growth is not understood. Some research

suggests that phytoalexins alter plasma membranes (Oku *et al.*, 1976; Vanetten and Bateman, 1971) and inhibit oxidative phosphorylation (Oku *et al.*, 1976).

Each plant family produces different members of phytoalexins but they are structurally related (Ingham and Harborne, 1976). Over 350 phytoalexins have been found in over 100 plant species from 30 families of dicotyledons and monocotyledons, for examples, phaseolin from bean pod; ipomeamarone, chlorogenic acid, umbelliiferone and scopoletin from sweet potato; orchinol, hircinol and loroglosol from orchids (Kuć, 1995). It is possible that different part of plant can synthesize different phytoalexin. The closely-related plants in general synthesize similar structured phytoalexin.

Phytoalexin biosynthesis occurs after a diversion of primary metabolic precursors into novel secondary metabolic pathways. The molecules that signal plants to begin the process of phytoalexin synthesis are called elicitors. Elicitors of biotic origin may be involved in the interaction of plants and potential pathogens. In natural conditions, the stimulus is provided by the presence of the micro-organism and its perception by the host initiates the chain of events leading to phytoalexin synthesis. Phytoalexi often arises from the induction of enzymes, such as phenylalanine ammonia lyase (PAL), that control key branch points in the biosynthetic pathways (Dixon and Paiva, 1995). The function of phenylalanine ammonia lyase is metabolite L-phenylalanine to trans-cinnamic acid. Then trans-cinnamic acid transform to precursor para-coumaric acid to produce phytoalexin (Fig.8)

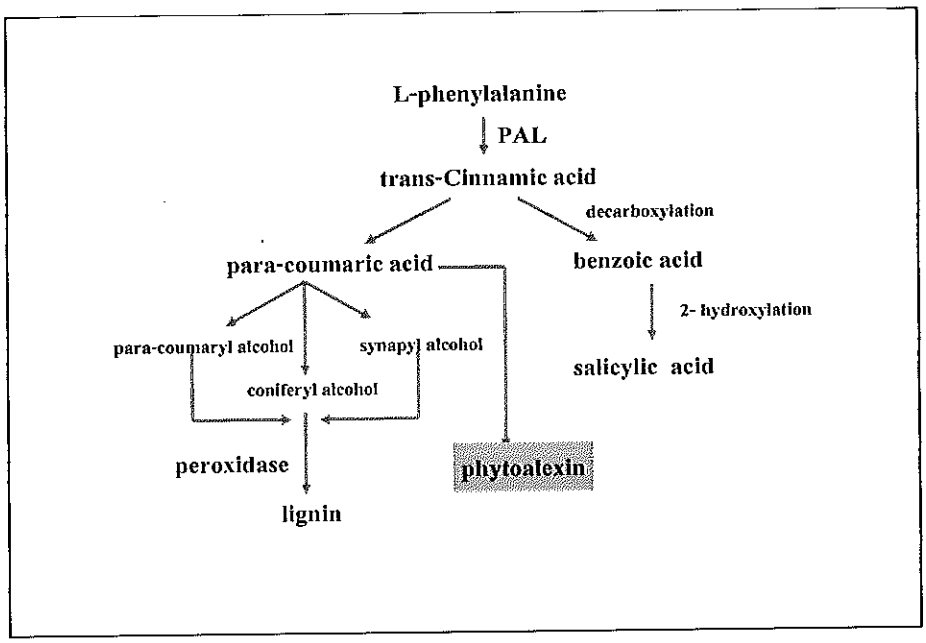


Fig.8 Phytoalexin systhesis from substrate L-phenylalanine.
(Dixon and Paiva, 1995)

The first evidence of an active defense response in *H. brasiliensis* was reported by Tan and Low (Tan and Low, 1975). They detected a blue fluorescent compound in leaf tissue after infection with *Colletotrichum gloeosporioides.* Later, this compound was induced in *H. brasiliensis* through infection by *Microcyclus ulei* and was identified as scopoletin (Scp), a hydroxycoumarin (Giesemann *et al.*, 1986). Scp is considered to be a phytoalexin which is toxic to leaf fungal pathogens of the rubber tree (Churngchow and Rattarasarn, 2001; Garcia *et al.*, 1995 a, b). The biosynthesis of scopoletin comes from phenylpropanoid pathway (Fig.9).

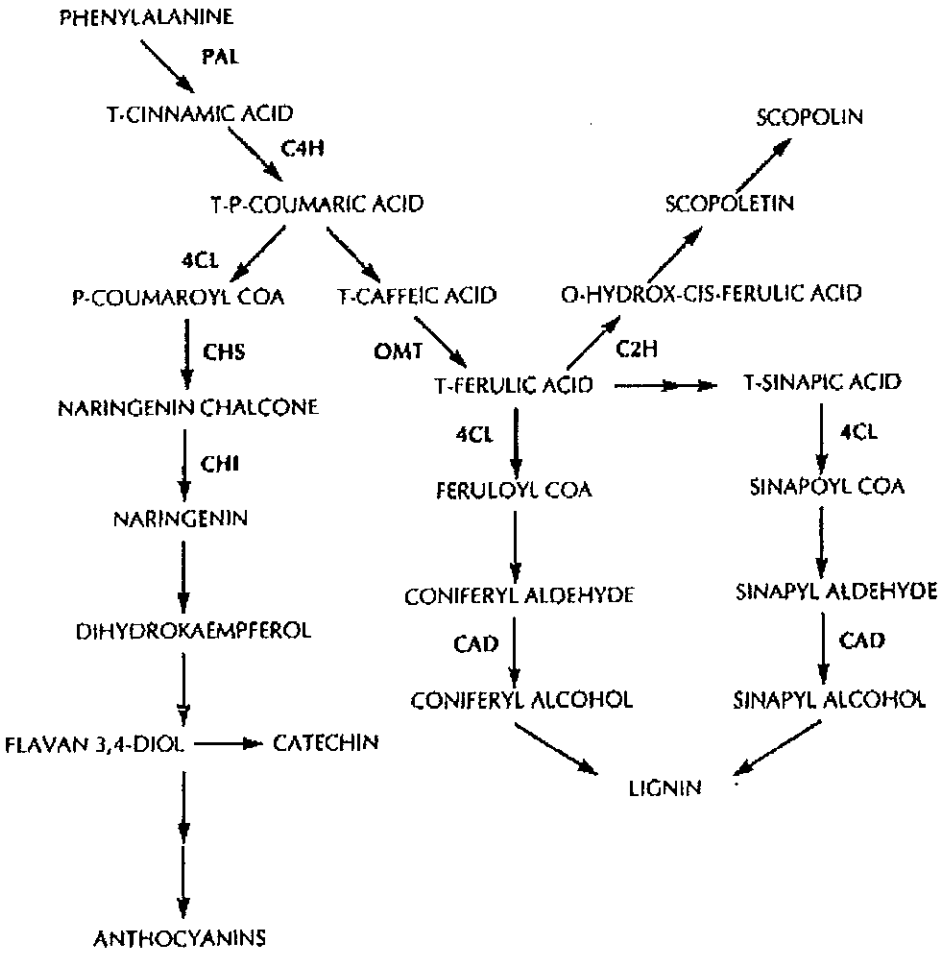


Fig.9 Phenylpropanoid pathway to produce scopoletin.
(http://www.fao.org/docrep/V4510E/V4510E01.gif)

4.1.4 Cell wall reinforcement

Most pathogens must penetrate host cell walls at some stage, either as germ tubes, hyphae or haustoria, although penetration can sometimes occur through a wound or natural opening. If the cell can respond quickly enough to repair or reinforce the cell wall, penetration efficiency may be reduced and pathogen development is retarded. Fortifying the plant cell wall can increase resistance in various ways. Such as *Pseudomonas syringae* or *Cladosporium fulvum*, sealing the wall could impede leakage of cytoplasmic contents, thereby reducing nutrient

availability for the pathogens. *Sotrytis cinerea*, that rely on hydrolysis of the plant cell wall in advance of hyphal growth, the diffusion of toxins and enzymes to the sensitive plant cells would be retarded (Kim *et al.*, 1996). A number of different types of cell wall fortifications are produced between the host cell wall and plasma membrane, directly under the penetration peg as the defense responses such as the formation of a lignin and papilla.

Lignin is a complex phenolic polymer that reinforces the walls of certain cells in higher plants. It is mainly found in the vascular tissues, where its hydrophobicity waterproofs the conducting cells of the xylem and its rigidity strengthens the supporting fiber cells of both the xylem and phloem. It may also play an important role in defense against pathogen attack (Hawkins *et al.*, 1997). Lignins are complex aromatic heteropolymers that, in Gymnosperms, derive mainly from coniferyl alcohol, and in Angiosperms, from approximately equal parts of coniferyl and sinapyl alcohols. These monolignols are products of the phenylpropanoid metabolism, which is initiated by deamination of phenylalanine by the enzyme phenylalanine ammonia-lyase (PAL). A series of hydroxylation and *O*-methylation reactions and conversion of side-chain carboxyl to an alcohol results in the building blocks of lignins (Fig.10) (Humphreys and Chapple, 2002).

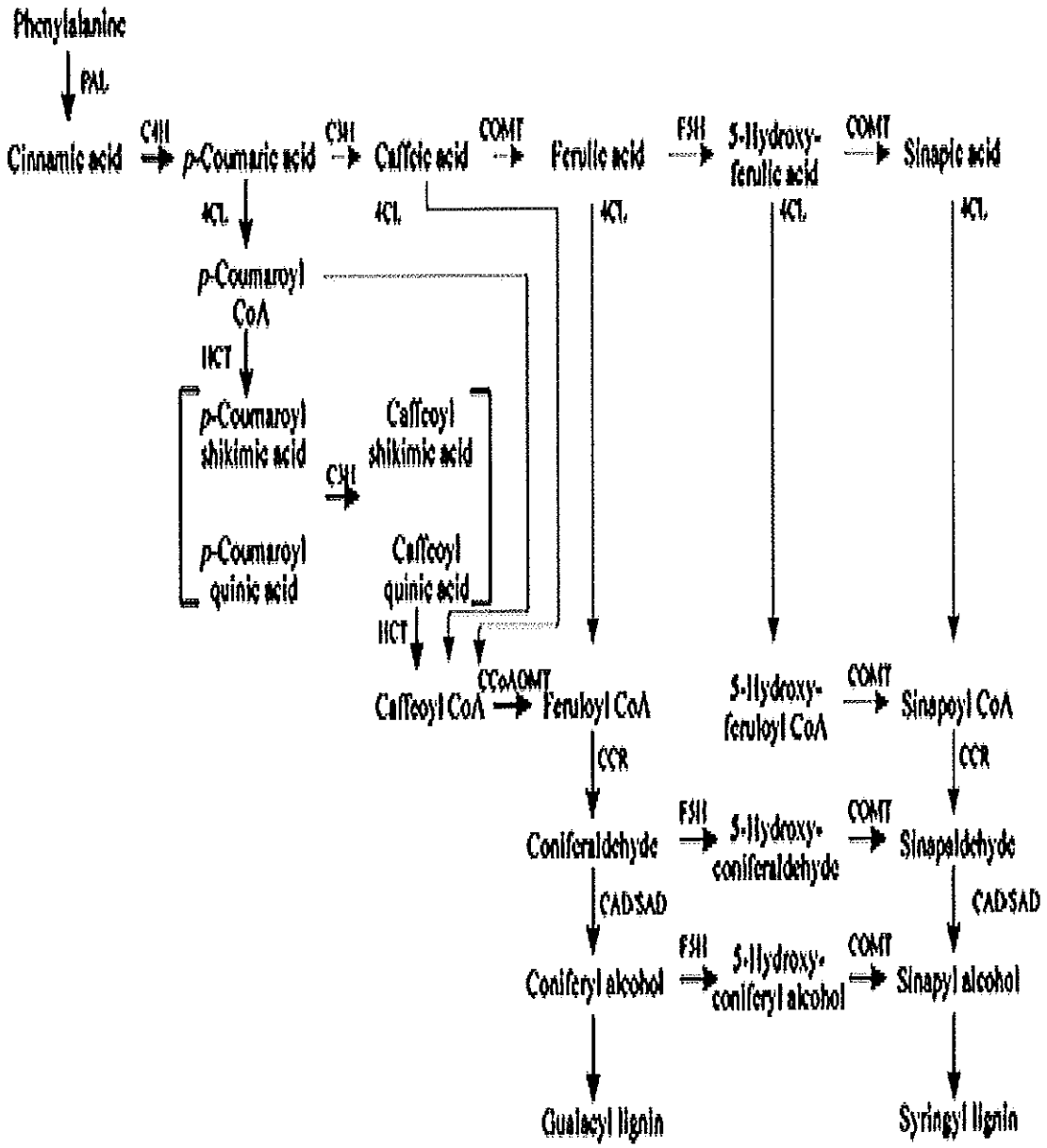


Fig.10 Phenylpropanoid pathway leading to produce monolignal, precursors of lignin. 4CL, 4-hydroxycinnamoyl CoA ligase; C3H, *p*-coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CAD, cinnamy alcohol dehydrogenase; CCoAOMT, caffeoyl CoA *O*-methyltransferase; CCR, cinnamoyl CoA reductase; COMT, caffeic acid *O*-methyltransferase; F5H, ferulate 5-hydroxylase; HCT, hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase; PAL, phenylalanine ammonia-lyase; SAD, sinapyl alcohol dehydrogenase (Humphreys and Chapple, 2002).

The rapid deposition of lignin following infection is associated with resistance to non-pathogens and avirulent pathogens in many plants. Lignin also binds to hyphal tips and bacteria cells, preventing further growth and movement and restricting the diffusion of pathogen enzymes and toxins and the uptake of water and nutrients by the pathogens. In addition, the low molecular weight phenolic precursors of lignin and the free radicals produced during polymerization reactions in the cell wall may affect pathogen membrane plasticity or inactivate pathogen enzymes, toxins, or elicitors. Hyphae themselves may also become lignified (Mauch-Mani and Slusarenko, 1996).

One type of cell wall reinforcement that occurs rapidly in response to fungal invasion is the formation of papillae (Fig.11). Papillae often form immediately beneath the penetration peg and are heterogeneous in composition (Heath, 1980). They are thought to physically block fungal penetration of host cells (Bayles *et al.*, 1990). However, it is also possible that their formation is required to provide adequate support for subsequent haustorium development, in which case they may be essential for pathogenesis (Heath, 1980).

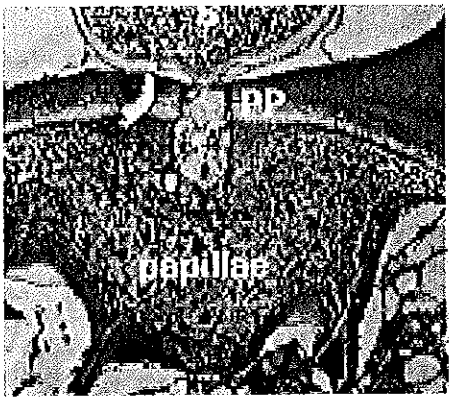


Fig.11 Papillae formation. Papillae develop beneath the penetration peg (PP) and germinating spore (S) of an avirulent isolate of fungus *Erysiphe graminis* on barley leaves (Kim *et al.*, 1996).

4.2 Systemic resistance

4.2.1 Pathogenesis-related proteins (PR-proteins)

The one important role in plant defense against pathogenic and in general adaptation to stressful environment is pathogenesis-related proteins (PR-proteins) production. The definition of PR-proteins is proteins coded by the host plant but induced particularly in pathological or related situations (Antoniw and Pierpoint, 1978; Van Loon *et al.*, 1994). Infected plants do not only accumulate PR-proteins locally but are also induced systemically to protect infection by fungi, bacteria and viruses in the future, link with production of systemic acquired resistance (SAR) (Van Loon and Van Strien, 1999). Each plant species can produce various families of PR-proteins. The function of PR-proteins in plant suggested that it may be effective in inhibiting pathogen growth, multiplication or spread, and be responsible for the state of SAR (Kombrink and Somssich, 1997; Ryals *et al.*, 1996).

At first, PR-proteins were classified into 5 groups (PR-1 to PR-5). They were characterized by biochemical and molecular-genetic techniques in tobacco (Table 1). Various members that have similar PR-proteins properties are grouped into the same class (Bol *et al.*, 1990). After that eleven families (PR-1 to PR-11) were recognized and classified for tobacco and tomato, with the families PR-8 and PR-10 being also present in cucumber and parsley, respectively (Van Loon *et al.*, 1994). Families (PR-12, PR-13 and PR-14) were recognized in radish, *Arabidopsis* and barley, respectively (Van Loon and Van Strien, 1999) (Table 1). Those of the new PR-proteins classification were proposed based on their grouping into families sharing amino acid sequences, serological relationships, and enzymatic or biological activity (Van Loon *et al.*, 1994).

Table 1 The families of pathogenesis-related proteins (Van Loon and Van Strien, 1999).

Family	Type member	Properties
PR-1	Tobacco PR-1a	Unknown
PR-2	Tobacco PR-2	β-1,3-glucanase
PR-3	Tobacco P, Q	Chitinase type I, II, IV, V, VI, VII
PR-4	Tobacco "R"	Chitinase type I, II
PR-5	Tobacco S	Thaunatin-like
PR-6	Tomato Inhibitor I	Proteinase-inhibitor
PR-7	Tomato P_{69}	Endroproteinase
PR-8	Cucumber chitinase	Chitinase type III
PR-9	Tobacco "lignin-forming peroxidase"	Peroxidase
PR-10	Parsley "PR1"	"Ribonuclease-like"
PR-11	Tobacco class V chitinase	Chitinase type I
PR-12	Radish Rs-AFP3	Defensin
PR-13	Arabidopsis THI2,1	Thionin
PR-14	Barley LTP4	Lipid-transfer protein

New families of PR-proteins were grouped by criteria that proteins must be induced by a pathogen in tissues that do not normally express the proteins and induced expression must occur in at least two different plant-pathogen combinations, or expression in a single plant-pathogen combination must be confirmed independently in different laboratories (Van Loon and Van Strien, 1999).

Peroxidase (POD) (E.C. 1.11.1.7) is grouped into PR-9 family associated with the plant defense pathway and is elicited when challenged with elicitors or infection with pathogens (Perera *et al.*, 2004; Rocío *et al.*, 2004). The peroxidase involves in phenylpropanoid pathway as an important enzyme in producing lignin. The substrate of phenylpropanoid pathway is phenylalanine. Then it is first converted to cinnamic acid by the action of the enzyme phenylalanine ammonia-lyase (PAL). In a series of hydroxylations, methylations and reductions, the monolignols are formed. Monolignols are polymerised to lignin in a peroxidase or laccase catalysed radical reaction (Fig.12).

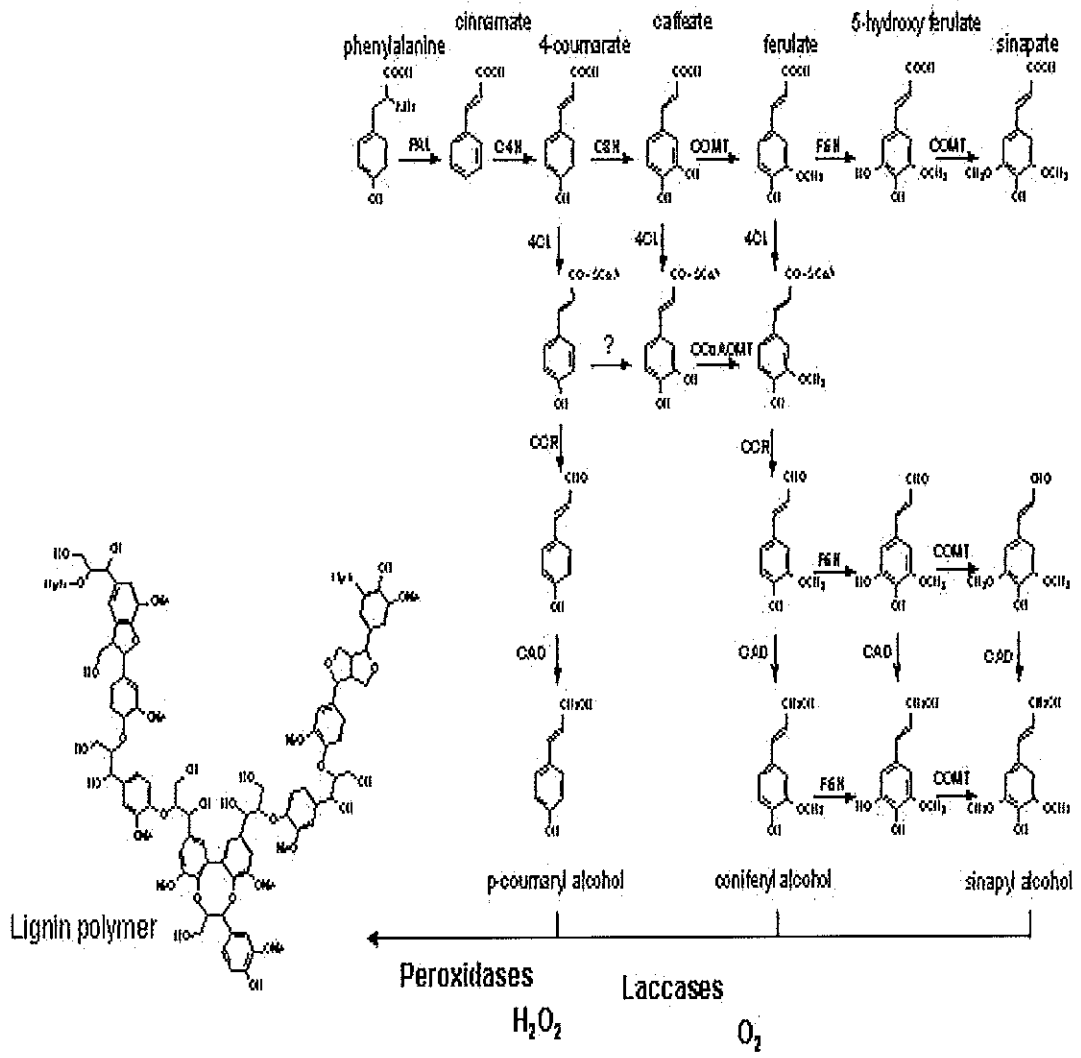


Fig.12 Phenylpropanoid pathway to produce lignin polymer.
(http://www.mm.helsinki.fi/MMSBL/english/research/Gerberalab/lignin_phenylpropanoid.html)

The role of a peroxidase enzyme in the metabolism of the phytoalexin has been investigated in leaf discs of sunflower by using scopoletin as substrate. The disappearance of scopoletin was associated with the increased activity of the peroxidase that converted the coumarin to a colored insoluble metabolite (Edwards *et al.*, 1997). The anionic peroxidase 45 kDa associated with the suberization response was obtained from potato (*Solanum tuberosum* L.) tubers during wound healing (Mark *et al.*, 1999). However, the size of anionic peroxidase found in sycamore

maple (*Acer pseudoplatanus* L.) is different from potato anionic peroxidase. That is molecular weight of sycamore maple anionic peroxidase purified from cell suspension is 42 kDa having a pl of 3.6 (Jeffrey *et al.*, 1994).

4.2.2 Systemic acquired resistance (SAR)

Systemic acquired resistance (SAR) is the form of induced resistance to pathogens in plants induced by previous infection or treatment with elicitors that result in resistance (or tolerance) against subsequence challenge by a pathogen or parasite (Glynn, 2001; Gary and Robert, 2004). Then, the first infection predisposes the plant to resist attacks in future. This latter aspect makes it comparable to immunization in animals and humans (Brigitte and Jean, 1998). SAR can enhance resistance in plants to broad range of pathogens, including fungi, bacteria, viruses, nematodes, parasitic plants, and even insect herbivores (Kessler and Baldwin, 2002; McDowell and Dangl, 2000; Sticher *et al.*, 1997; Van Loon *et al.*, 1998). This resistance is expressed locally at the site of pathogen attack and systemically, in uninfected parts of the plant.

After exposing the plant to virulent, avirulent, elicitor and nonpathogenic microbes, or artificially with chemicals such as salicylic acid (SA), 2,6-dichloro-isonicotinic acid (INA) or benzo (1,2,3) thiadiazole 7-carbothioic acid *S*-methyl ester (BTH), the SAR is triggered in the plants (Sticher *et al.*, 1997). For example, *Bacillus subtilis* produces lipopeptides to induce resistance in barley, especially against powdery mildew (Steiner *et al.*, 1988). While the culture filtrates of *Penicillum janczewskii* are infiltrated into melon and cotton, the peroxidase activity increases to protect against *Rhizoctonia solani* (a response associated with SAR) (Madi and Katan, 1998). The period of time required for the establishment of SAR is different in each plant depending on the mechanism, the ability of plant and elicitor that corresponds to the time required for the coordinated accumulation of pathogenesis-related proteins (and transcripts) and salicylic acid throughout the plant (Cameron *et al.*, 1994; Uknes *et al.*, 1992; Ward *et al.*, 1991). After induction of SAR, the symptoms remained very limited in tobacco when inoculation with *Phytophthora nicotianae* after inducing necrosis by elicitation on the stem of tobacco with elicitors whereas in the water-treatment control, fungal invasion progressed downwards at a constant rate (Ricci *et al.*, 1989).

The SAR has SA acts as an endogenous signal involved in defense signaling (Fig.13) (Brigitte and Jean, 1998). In upper, non-infected leaves, of infected tobacco plants, SA was accumulated up to 70% resulting from SA that was transported from the infected leaf (Shulaev *et al.*, 1995). The volatile methyl salicylate (MeSA) represents an inactive precursor of SA that can be translocated and converted to SA in tobacco after infection and can induce defense responses by conversion to SA (Shulaev *et al.*, 1997). The 4-hydroxy benzoic acid (4HBA) as well as SA increases in phloem sap concomitantly with an increase in activity of phenylalanine ammonia-lyase in the petiole. Then both SA and 4HBA are produced in stems and petioles in response to a mobile signal from the leaf lamina (Smith-Becker *et al.*, 1998).

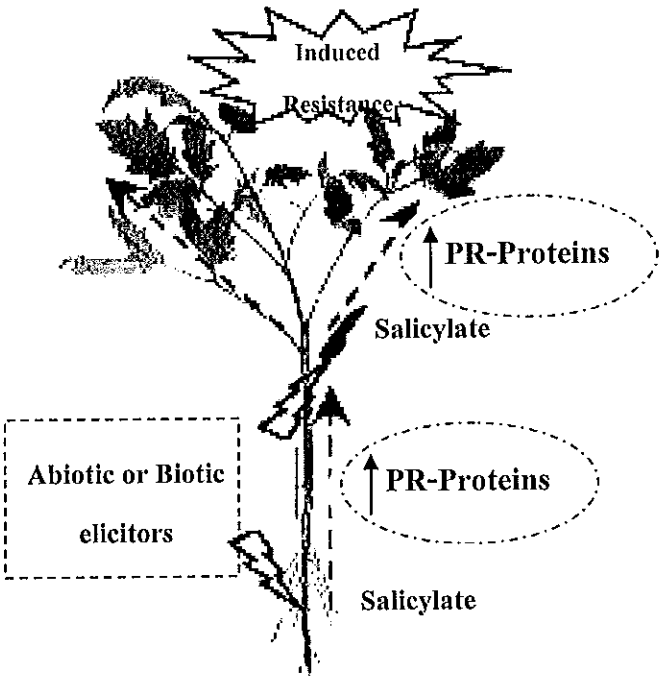


Fig.13 Systemic acquired resistance, induced by the exposure of root or foliar tissues to abiotic or biotic elicitors, is dependent of the phytohormone salicylate (salicylic acid), and associated with the accumulation of pathogenesis-related (PR) proteins (Gary and Robert, 2004).

Objectives

1. To study induction of plant defence mechanisms in *H. brasiliensis* using elicitin and new protein elicitor from *P. palmivora.* The defence mechanisms observed are induction of phenolic compounds, accumulation of scopoletin, induction of peroxidase enzymes and induction of local resistance.

2. To study inhibition of protease in *H. brasiliensis* by protease inhibitor from *P. palmivora.* The investigation includes isolation of protease inhibitor from *P. palmivora* by RT-PCR, nested RACE and cloning methods then tests the purified protease inhibitor with protease from rubber leaf.

CHAPTER 2
MATERIALS AND METHODS

1 Elicitors from *P. palmivora* inducing defense mechanisms in *H. brasiliensis*

1.1 *P. palmivora*

1.1.1 Isolation and cultured condition of *P. palmivora*

P. palmivora, isolated from *H. brasiliensis*, was obtained from the Rubber Research Institute of Thailand (RRIT). The fungus was further purified by monospore isolation prior to being used in this research. The monospore was produced by growing the fungus on V_8 agar for 1 week. Then 10 ml of sterile distilled water was added onto the growing mycelium of *P. palmivora* and incubated at 4°C for 15 minutes. There after, *P. palmivora* was further incubated at 25°C for 30 minutes to release zoospores from sporangium. The monospore was isolated by spreading the suspension of zoospores on potato dextrose agar (PDA) plate and incubated at 25°C for 3 days. A single colony of *P. palmivora* was picked and grown on the new PDA plate at 25°C for 1 week, then cells were used in this research (Fig.14). Cells were maintained on PDA plate at 25°C in the dark and subcultured under sterile condition every week.

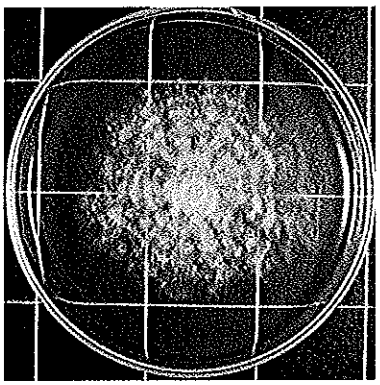


Fig.14 The mycelium of *Phytophthora palmivora* grown on potato dextrose agar.

1.1.2 Zoospores preparation of *P. palmivora*

The mycelium of *P. palmivora* grown in PDA plate for 1 week was cut with a 5 mm diameter cork borer and transferred to V_8 agar. The mycelium of *P. palmivora* on V_8 agar was grown at 25°C for 1 week (Fig.15) after that 10 ml of sterile distilled water was added and incubated at 25°C overnight after that incubated at 4°C for 15 min. The *P. palmivora* was further incubated at 25°C for 30 min to release zoospores from sporangium. The suspension of zoospores was dropped on Petroff Hauser and counted under light microscope. The concentration of zoospores was calculated using the formula below.

$$\text{Zoospores concentration in 1 ml (zoospores/ml)} = \frac{\text{(Number of zoospores in field)}}{\text{Volume on slide } (4\times10^{-6} \text{ ml})}$$

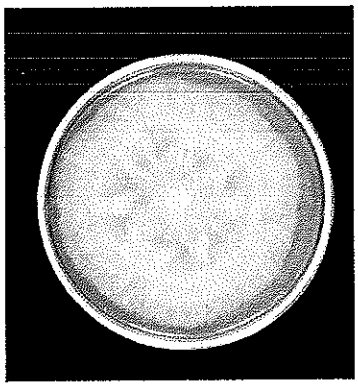


Fig.15 The mycelium of *Phytophthora palmivora* grown on V_8 agar.

1.1.3 The culture filtrate preparation from *P. palmivora* for elicitor production

The mycelium of *P. palmivora* grown for 1 week on PDA plate (Fig.13) was cut with 5 mm diameter cork borer. Then 15 pieces of *P. palmivora* mycelium were transferred to 150 ml of sterile Henninger medium (appendix) and grown on a rotary shaker at 100 rpm, 25°C in the dark for 15 days (Fig.16). The mycelium in *P. palmivora* culture filtrate was eliminated by filtration with two layers of Whatman paper No.1. Then the filtrate was kept at -20°C until purification of elicitors.

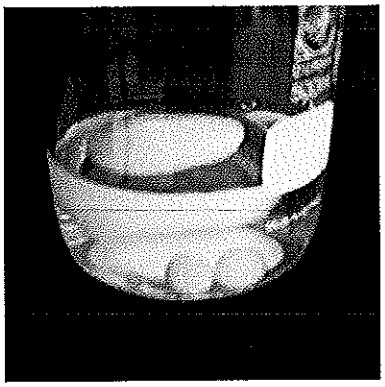


Fig.16 The culture of *Phytophthora palmivora* grown on Henninger medium for 15 days.

1.2 Plant materials

1.2.1 Preparation of tobacco plants for partial chemical characterization of elicitiors

Tobacco plants (*Nicotiana tabacum*) were grown in a field for 1 month. After that it was transferred to the growth chamber and grown with a photoperiod of 12 h of light and 12 h of dark at 25°C. The 8-week-old tobacco plants without infection were used for partial chemical characterization of elicitiors.

1.2.2 Callus induction from integument of *H. brasiliensis* immature seed

The fruit of *H. brasiliensis* (BPM-24 cultivar), 6-8 weeks old, was cleaned with distilled water. Then it was sterilised with 95% alcohol and held it over the flame 2-3 times before taking the seed out from the fruit by cutting the bark with sterilized razor blade. After that the 4 pieces of *H. brasiliensis* immature seed were transferred to grow in Murashige and Skoog's (MS) medium supplemented with 2 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D), pH 5.7 at 25°C in the dark for 1 month. When the callus growed from integument, it was transferred to the new Murashige and Skoog's (MS) medium supplemented with 2 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D), pH 5.7 and subcultured every 4 weeks (Fig.17).

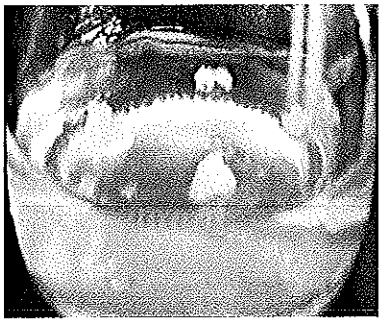


Fig. 17 The callus induced from integument of *Hevea brasiliensis* immature seed.

1.2.3 Cell suspension preparation of *H. brasiliensis* for biological assays of elicitors

Cell suspension was generated from an integument derived callus of *H. brasiliensis* (BPM-24 cultivar) (Fig.16) by transferring the 4-week-old callus to Murashige and Skoog's (MS) medium supplemented with 2 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) and 0.1 mg/l thidiazuron (TDZ), pH 5.7 and grown on a rotary shaker at 100 rpm, 25°C for 14 days (fig.18). After shaking for 14 days, cell suspension was generated from the callus. It was further subcultured every 14 days many times to produce a homogeneous cell suspension. The 14-day-old cells were equilibrated in 5% MS, 10 mM 4-Morpholineethanesulfonic acid (MES) and 3% sucrose for 3 days before treatment. The 14-day-old cells were also grown in the same medium used for equilibrating cells during the treatment.

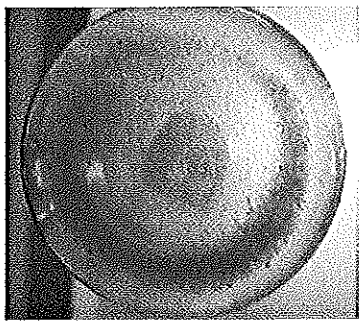


Fig.18 The cell suspension of *Hevea brasiliensis* grown in Murashige and Skoog's (MS) medium supplemented with 2 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) and 0.1 mg/l thidiazuron (TDZ), pH 5.7 for 14 days.

1.2.4 Preparation of rubber seedlings for local resistance induction and biological assays of elicitors

Rubber plants (RRIM600 cultivar) were grown in a growth chamber with a photoperiod of 12 h of light and 12 h of dark at 25°C. The 8-week-old rubber plants without infection were used for induction of local resistance and biological assays by elicitors.

1.3 Purification of the elicitors from *P. palmivora*

Proteins with elicitor activity were purified from the culture filtrate of *P. palmivora*. A 15-day-old-fungal culture was filtered through two layers of Whatman filters (No.1). Protein was precipitated with 90% $(NH_4)_2SO_4$ at 4°C overnight with gentle stirring. The pellet was collected by centrifugation at 12,000g for 20 min at 4°C and then resuspended with distilled water. The 1 ml of solution was desalted by loading onto PD-10 column and eluted with distilled water. The first 1 ml fraction was discarded and the next 6 fractions were kept by collecting 1 ml per fraction on ice immediately after elution. The fractions from the PD-10 column were monitored for protein content at an absorbance of 280 nm. The eluted fractions with high protein content were pooled and further purified by DEAE cellulose and hydrophobic columns to gain elicitin and the new protein elicitor. The amount of protein was measured at 280 nm.

1.3.1 Elicitin purification

The fractions with high protein content from the PD-10 column were further purified for elicitin using DEAE cellulose column in 20 mM Tris-HCl buffer pH 7.5 at 4°C. The DEAE cellulose 30 g was saturated with water at room temperature. Then incubated in 30 ml of 0.5 M hydrocholic acid (HCl) and mixed with stirrer. It was allowed to stand at room temperature for 1 hour or until the DEAE cellulose precipitates. After that the supernatant was discarded and the DEAE cellulose was washed with distilled water many times until the upper solution has pH about 7. Then the 30 ml of 0.5 M sodium hydroxide (NaOH) was added into the DEAE cellulose and mixed with stirrer. The incubation with 0.5 M NaOH of DEAE cellulose and washing were the same with methods of DEAE cellulose incubation with 0.5 M HCl. After that the DEAE cellulose was incubated with 20 mM Tris-HCl

buffer pH 7.5 before packing in a 10 x2.5 cm column at 4°C. The DEAE cellulose column was adjusted with 150 ml of 20 mM Tris-HCl buffer pH 7.5 at 4°C at a flow rate of 30 ml/h. Then 4 ml of the high protein fractions (0.05 mg/ml) from the PD10 column was loaded into DEAE cellulose column at 4 °C. The DEAE cellulose column was washed with the 20 mM Tris-HCl buffer pH 7.5 and kept 3 ml per fraction at a flow rate of 30 ml/h at 4°C until the washed fractions had no protein (monitored from the absorbance at 280 nm). Elution was achieved with the same buffer containing 0.1 M NaCl at the same flow rate of the column washing at 4°C. The 1 ml/fractions were collected and measured for their protein contents with absorbance at 280 nm. The fractions with high protein content were checked for the purity of elicitin by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE). The pure elicitin fractions that identified by SDS-PAGE were pooled and concentrated by ultrafiltration, molecular weight cut off 10 kDa. The sample 5 ml in ultrafiltration tube was centrifuged at 3,000 rpm, 4°C for 10 min or until sample retained 0.5 ml in the tube. Then the concentrated protein was collected and measured protein concentration by Bicinchoninic acid (BCA) method.

1.3.2 New protein elicitor purification

After elicitin was eluted with 20 mM Tris-HCl buffer pH 7.5 containing 0.1 M NaCl, the new protein elicitor was further eluted with the same buffer but containing 0.3 M NaCl. The 1 ml/fractions were collected and measured for their protein contents at an absorbance of 280 nm. The fractions with high protein content were checked for the purity of new protein elicitor by SDS-PAGE. The new protein elicitor fractions were pooled and adjusted to 1 M $(NH_4)_2SO_4$ before loading onto a HiTrap Phenyl FF (high sub) column (5 ml). The HiTrap Phenyl FF column was washed with 25 ml of sterilized distilled water and followed by 25 ml of 20 mM Tris-HCl pH 7.5 at a flow rate of 1 ml/min. After that it was equilibrated with 25 ml of 20 mM Tris-HCl pH 7.5 containing 1 M $(NH_4)_2SO_4$ at a flow rate of 5 ml/min. After loading sample, the column was washed with 15 ml of 20 mM Tris-HCl pH 7.5 containing 1 M $(NH_4)_2SO_4$ and 15 ml of the same buffer containing 0.5 M $(NH_4)_2SO_4$. The purified protein with high elicitor activity was eluted with 20 ml of 20 mM Tris-HCl pH 7.5 containing 0.3 M $(NH_4)_2SO_4$. The 1 ml/fractions were collected and measured for their protein contents by an absorbance at 280 nm and checked for

purity by SDS-PAGE. Then the pure elicitor fractions were pooled and concentrated by ultrafiltration, molecular weight cut off 10 kDa. The obtained protein was measured for protein content by the BCA method.

1.4 Characterization of elicitors by Tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis (Tricine-SDS-PAGE)

The high protein content fractions of elicitin from DEAE cellulose column and the new protein elicitor from HiTrap Phenyl FF column were checked for protein purity by using Tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis (Tricine-SDS-PAGE). Sodium dodecyl sulphate polyacrylamide gel was prepared as shown on table 2.

Table 2 The component of sodium dodecyl sulphate polyacrylamide gel for elicitor electrophoresis.

Component	Stacking gel (4%)	Separating gel (16.5%)
Acrylamide-bisacrylamide mixture (49.5%)	0.40 ml	4.33 ml
3.0 mM Tris-HCI, pH 8.45+0.3% SDS	1.24 ml	4.33 ml
10% Ammonium persulfate	50 µl	130 µl
TEMED	10 µl	13 µl
Deionized water	3.31 ml	4.20 ml
Total volume	5.0 ml	13.0 ml

The sample was mixed with sample buffer (0.5 M Tris-HCl, pH 6.8 + 2.4 ml glycerol + 1 ml 10% SDS + 0.2 ml β-mercaptoethhanol + 0.4 ml 0.5% bromophenol blue, the total volume was adjust to 10 ml with buffer) in the ratio 3:1 and boiled about 5 min before loading 20 µl sample in each lane. The electrophoresis buffers were composed of two buffers: anode buffer (0.2 M Tris-HCl, pH 8.9) and cathode buffer (0.1 M Tris-HCl + 0.1 M Tricine + 0.1% SDS, pH 8.25). The gel was

performed electrophoresis with 60 volts for 2 h and stained with silver nitrate. The silver staining kit (GE Healthcare, Bio-Sciences) was used to stain the gel following the manufacturer's instructions. A value of the relative molecular mass (*Mr*) of the protein band was estimated by comparison with a broad molecular weight marker (6.5-200 kDa).

1.5 Protein measurement by Bicinchoninic acid method (BCA method)

The Bicinchoninic acid method was modified from Smith *et al.* (1985). The 2 ml of solution C (appendix) was reacted with 100 µl of sample then mixed by shaking and incubated at 34 °C for 30 min. The mixture was measured absorbance at 562 nm. The protein was calculated by comparing with standard curve of Bovine Serum Albumin (BSA) (Fig.18).

The standard curve of protein by Bicinchoninic acid method was performed by dilution the stock solution of BSA (0.5 mg/ml) to 5, 10, 15, 20 and 25 µg in 100 µl of distilled water. Then it was reacted with 2 ml of solution C and mixed by shaking and incubated at 34°C for 30 min. After that the mixture was measured the absorbance at 562 nm (Fig.19).

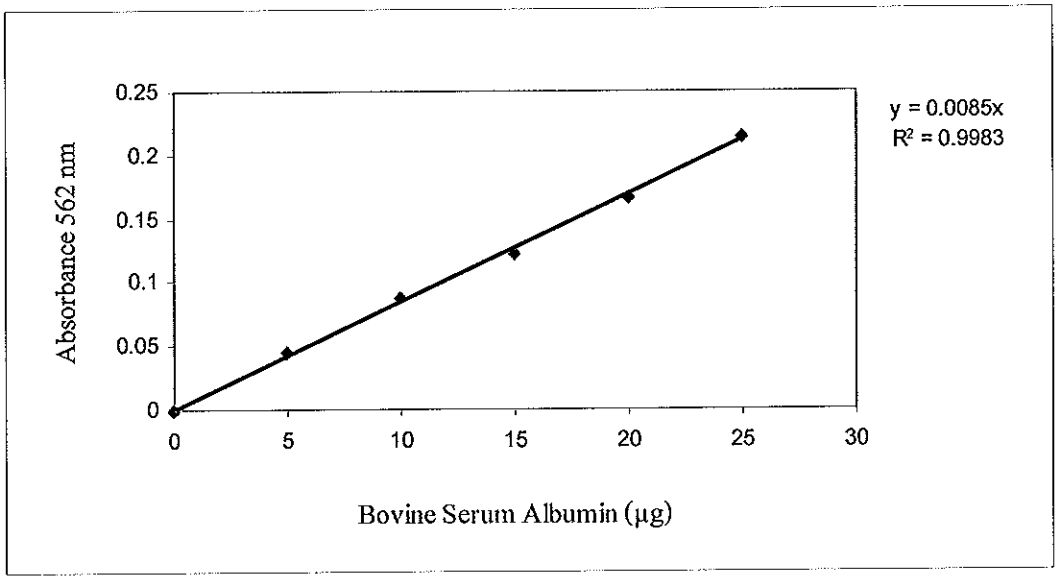


Fig.19 Standard curve of Bovine Serum Albumin at absorbance 562 nm using the Bicinchoninic acid method.

1.6 Protein measurement by Bradford method

The Bradford method was modified from Bradford (1976). The protein solution (100 µl) was reacted with 1 ml of Bradford solution (appendix) and incubated at room temperature for 10 min. Then the solution was measured protein at the absorbance 595 nm. The protein was calculated by comparing with standard curve of BSA at 595 nm (Fig.20).

The standard curve of protein by Bradford method was performed by dilution the stock solution of BSA (0.5 mg/ml) to 5, 10, 15, 20 and 25 µg in 100 µl of distilled water. Then it was reacted with 1 ml of Bradford solution and incubated at room temperature for 10 min. The absorbance at wavelength 595 nm was used to generate the standard curve (Fig.20).

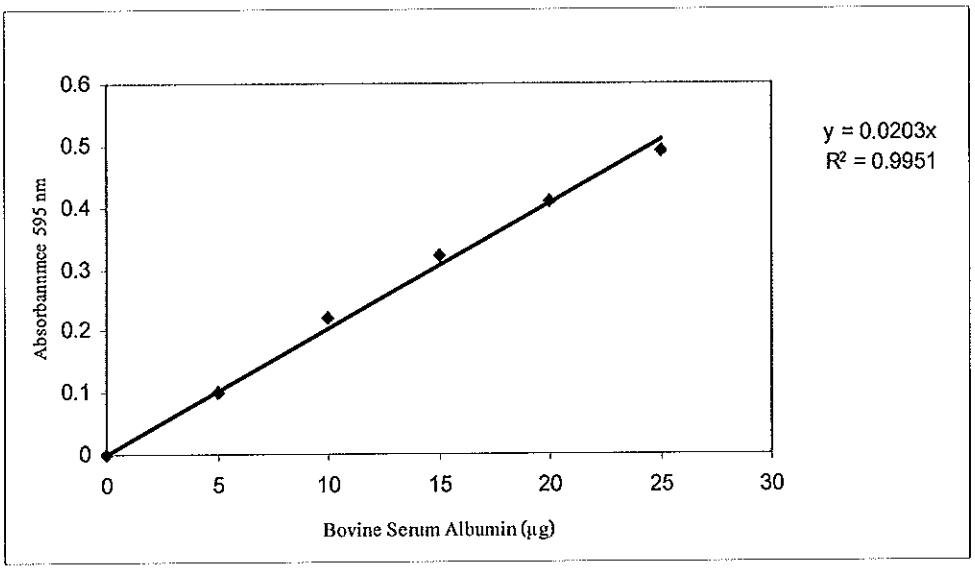


Fig.20 Standard curve of Bovine Serum Albumin at absorbance 595 nm using Bradford method.

1.7 Partial chemical characterization of elicitin and new elicitor

Characterization of elicitin and the new elicitor was assayed on 8-week-old tobacco plants. The 10 µl of elicitor solution was infiltrated through the stomatal pores into the mesophyll tissue of fully developed leaves with a 1 ml syringe without a needle. Hypersensitive cell death was detected by the appearance of a brown necrotic region in the infiltrated area after elicitor infiltration for 2 days. For all

of the bioassays, the final concentration of elicitors before infiltrating was 50 nM. Every bioassay was repeated three times.

1.7.1 Heat stability determination

100 µl of protein elicitors were incubated at 80, 90 and 100°C for 5 min then kept on ice immediately and centrifuged for 5 min at 12,000 rpm. After the temperature of the solution returned to room temperature, 10 µl of the solution was infiltrated into tobacco leaves to evaluate its ability to induce hypersensitive cell death. One leaf of tobacco plant was infiltrated with six samples following Fig. 21 and the pattern of infiltration was the same in each leaf. The positive control was 10 µl of non heat elicitor and negative control was 10 µl of distilled water.

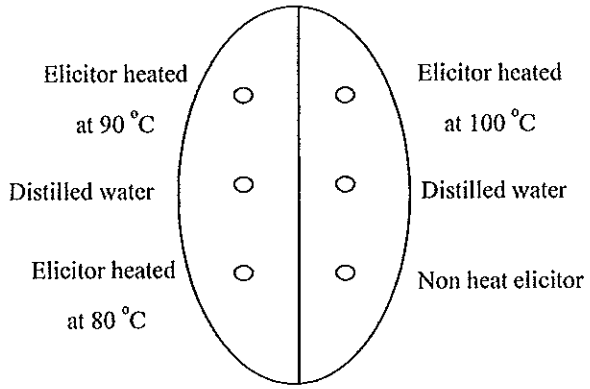


Fig.21 The model of infiltration tobacco plant for heat stability test of elicitors.

1.7.2 Acid and base stability

100 µl of protein elicitors were incubated in a series of buffer solutions with pH values of 2, 4, 5, 6, 7, 8, 9, 10, respectively. The final pH of solution was determined by pH paper. The mixtures were allowed stand at room temperature for 60 min. Then the solution was adjusted to pH 7.5 with 20 mM Tris-HCl pH 7.5 before infiltration into tobacco leaves. One tobacco plant was infiltrated four leaves and one leaf was infiltrated with four different samples following Fig. 22. Water replaced the elicitors in the experiments for controls.

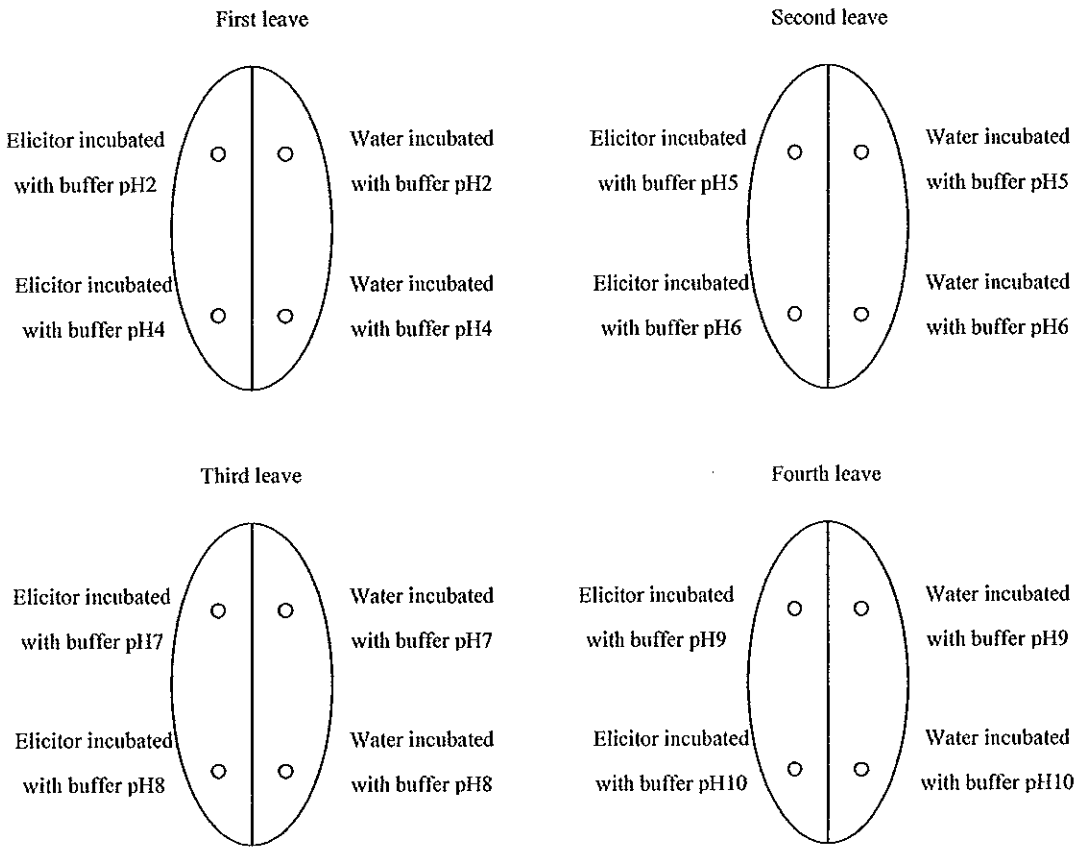


Fig.22 The model of infiltration tobacco plant for test acid and base stability of elicitors.

1.7.3 Stability to ProteaseK

100 µl of 50 nM protein elicitors were treated in 100 µl of 6 units ProteaseK solution (20µg/ml) for 30 min at room temperature, centrifuged for 5 min at 12,000 rpm and then 10 µl of solution was infiltrated into tobacco leaves. One tobacco plant was infiltrated three leaves and one leaf was infiltrated with four different samples following Fig. 23 and the pattern of infiltration was the same in each leaf. The positive control was elicitors at the concentration of 50 nM and water was used in the experiments for negative controls.

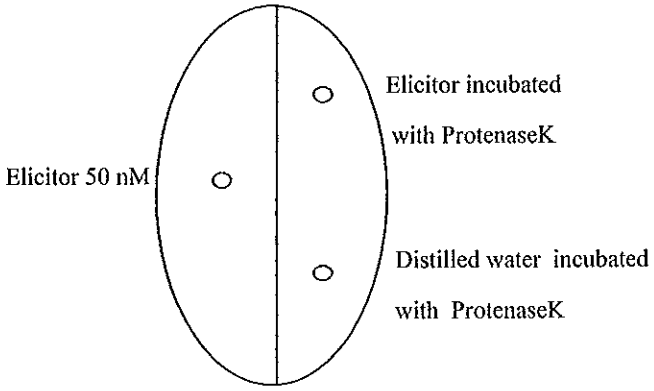


Fig.23 Model of infiltration tobacco plant for test stability to ProteaseK of elicitors.

1.8 Biological assays in cell suspension treated with elicitin and new elicitor

Cell suspension of *H. brasiliensis* (BPM-24 cultivar) (0.3 g) grown in 5% MS, 10 mM 4-Morpholineethanesulfonic acid (MES) and 3% sucrose was incubated with elicitors (150 nM for elicitin and 5 nM for the new elicitor) at room temperature with gently shaking. The cell suspension and treated medium were collected at 0, 8, 16, 24, 32, 40, 48, 56, 64, 72 h of the incubation and stored in -20°C. To assay phenolic compounds and peroxidase enzyme activity, cell suspension was extracted for protein with 1:2 w/v of extraction buffer (0.2 M sodium phosphate buffer pH 7, 0.25% TritonX-100 and 1% PVPP). After that the total protein of extraction was measured by Bradford method. The treated medium was kept for measurement of Scp content. In this experiment distilled water was used as control and every bioassay below was repeated three times.

1.8.1 Measurement of phenolic compounds

The measurement of phenolic compounds was modified from the method described by Torres *et al.* (1987). 1 ml of 1 N Folin's reagent was added into 10 µl of extracted protein from cell suspension incubated with elicitors and incubated at room temperature for 10 min. After that added 2 ml of 20% Na_2CO_3 and allowed to stand at room temperature for 15 min before measurement the absorbance at 730 nm. The phenolic compounds content was calculated by comparing with the absorbance of gallic acid at 730 nm.

Standard curve 1-10 µg of gallic acid were performed by adding 1 ml of 1 N Folin's reagent and 2 ml of 20% Na_2CO_3 into 10 µl of each concentration of

gallic acid solution (1-10 μg). The mixture was mixed to homogeneous solution before measurement the absorbance at 730 nm (Fig.24).

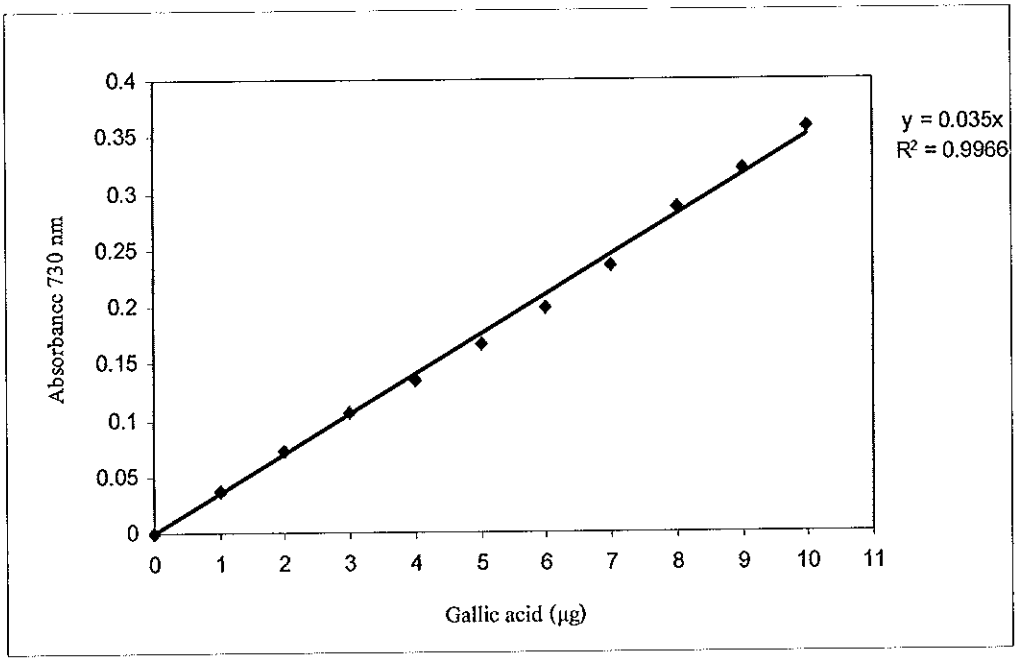


Fig.24 Standard curve of gallic acid using the absorbance at 730 nm.

1.8.2 Measurement of scopoletin content

The Scp content from the treated medium was measured with Spectrofluorometer at λ 340 nm excitation and λ 440 nm emission. Amount of Scp was calculated by comparing with standard curve of Scp and expressed in nmol/g fresh weight of cell suspension.

The stock solution of 50 mM scopoletin was diluted to 0.5, 1.0, 1.5, 2.0 and 2.5 μM and measured Scp content with Spectrofluorometer at λ 340 nm excitation and λ 440 nm emission (Fig.25).

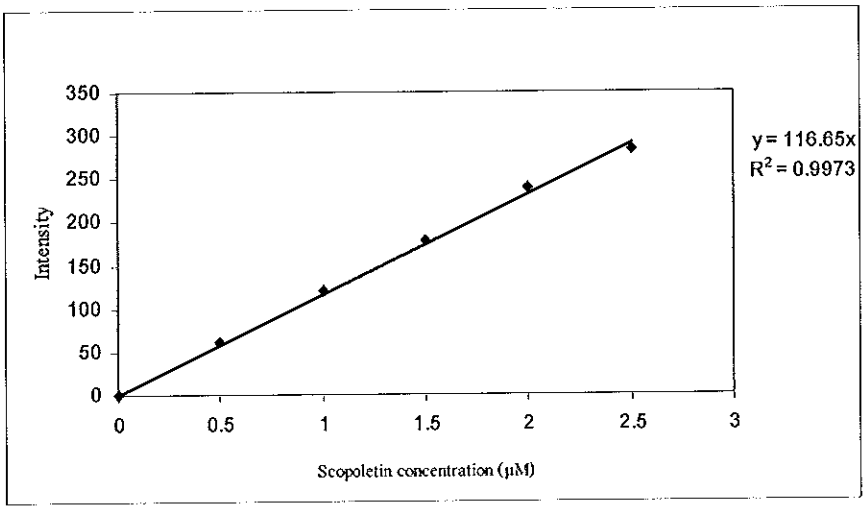


Fig.25 Standard curve of scopoletin using Spectrofluorometer at λ 340 nm excitation and λ 440 nm emission.

1.8.3 Measurement of peroxidase enzyme activity for substrate *o*-dianisidine

The peroxidase activity for substrate *o*-dianisidine was measured by the modified method of Shannon *et al.* (1966). The reaction of measurement peroxidase enzyme activity was composed of 2.775 ml of 50 mM sodium acetate, pH 5.4, 25 µl of extracted protein, 100 µl of 0.25% (w/v) *o*-dianisidine and 100 µl of 0.1 M H_2O_2 in cuvette tube. After the mixture was mixed in the cuvette tube, it was measured immediately at absorbance 460 nm. The activity was recorded every 15 second for 2 min. The peroxidase activity was calculated as specific activity.

Specific activity = International Unit per ml of extracted protein/ mg protein

The International Unit per ml of extracted protein was calculated following formula below.

Enzyme activity (unit/ml) = V_t x OD / E.D.V. (µmol/min/ml)

Where V_t = The total volume of reaction (ml)

V = The volume of extracted protein (ml)

OD = The absorbance at 1 min

E = The molar extinction coefficient: ε_M

= 11.3 $mM^{-1}cm^{-1}$ (for substrate *o*-dianisidine)

D = The distance that light passes 1 cm

1.8.4 Measurement of peroxidase enzyme activity for scopoletin using scopoletin as substrate

The peroxidase activity for Scp was measured by the modified method of Edwards *et al.* (1997). The reaction was composed of 970 µl of 0.1 M potassium phosphate pH 6.5, 10 µl of 10 mM Scp, 10 µl of 0.1 M H_2O_2 and 10 µl of extracted protein. The mixture was mixed in a cuvette and the activity was measured immediately from absorbance at 595 nm. The absorbance was recorded in every 15 second for 2 min. The peroxidase activity was calculated using a formula shown in 1.8.2.

1.9 Biological assays on young rubber tree seedlings treated with elicitin or new elicitor

1.9.1 Induction of local resistance

Induction of local resistance was assayed on rubber plant (RRIM600 cultivar) at B_1 stage. The elicitors (0.5, 1, 2, 4, 8 nM for elicitin and 0.125, 0.25, 0.5, 1, 2, 4 nM for the new elicitor) were sprayed onto the rubber leaves after treating with 1% of Triton X-100 (5 ml/plant). Two days later, the same plants were inoculated with $1x10^4$ zoospores/ml of *P. palmivora*. After infection for 2 days, the leaves of the rubber plants were checked for the disease development. In this study distilled water was used as control. All the above bioassays were repeated three times.

1.9.2 Induction of phenolic compounds and *o*-dianisidine peroxidase activity on rubber plants with elicitin and new elicitor

The rubber plants (RRIM600 cultivar) at B_1 stage were sprayed with 1% of Triton X-100 (5 ml/plant). After that the elicitors (1 nM for elicitin and 0.5 nM for the new elicitor) were sprayed onto rubber leaves. For the control experiment, elicitor was replaced by distilled water. The rubber plant leaves were collected from two plants at each time point after 0, 8, 16, 24, 32, 40, 48, 56, 64, 72, 80, 88, 96 h. Then the kept leaves were extracted with 100 mM Tris-HCl buffer pH 7.5 using buffer ratio 1:2 w/v. The phenolic compounds and *o*-dianisidine peroxidase activity were determined followed the methods described in 1.8.1 and 1.8.3.

1.9.3 Induction of *o-dianisidine* peroxidase activity during expression of local resistance

The elicitors (1 nM for elicitin and 0.5 nM for the new elicitor) were sprayed onto rubber plants (RRIM600 cultivar) after treating with 1% of Triton X-100. For the control experiment, elicitor was replaced by water. The rubber plant leaves were collected from two plants in every 8 h for 48 h. Then the plants were inoculated with $1x10^4$ zoospores/ml of *P. palmivora*. The leaves were further collected in every 8 h from 48 h to 96 h. The protein extraction and *o*-dianisidine peroxidase activity assay from rubber leaves were performed as described in 1.9.2 and 1.8.3, respectively.

2 Protease inhibitor from *P. palmivora* inhibiting protease in *H. brasiliensis*

2.1 Preparation of protease inhibitor from *P. palmivora*

2.1.1 Culture condition for *P. palmivora*

P. palmivora, isolated from infected *H. brasiliensis* plant, was maintained on PDA medium at 25°C. For RNA extraction, *P. palmivora* was grown in Henninger medium on a rotary shaker at 100 rpm and 25°C for 15 days. After harvesting, the mycelium was ground in liquid nitrogen and kept at -80 °C.

2.1.2 Primers design for *P. palmivora epi10* (*Ppepi10*)

The primers for isolation *P. palmivora epi10* (*Ppepi10*) were designed based on alignments using the program CLUSTAL-X of EPI10 from *Phytophthora infestans* (Accession number AY586282) and PramEPI10 from *Phytophthora ramorum* (Trace identified number 303447516). These sequences were obtained from the NCBI databases (www.ncbi.nlm.nih.gov). The conserved sequence of *epi10* from *P. infestans* and *P. ramorum* was used to create the primers for *Ppepi10*.

2.1.3 RNA isolation from *P. palmivora* mycelium

RNA from *P. palmivora* mycelium was extracted with RNeasy plant mini kit. 100 mg of mycelium powder was added 450 µl of buffer RLT and the mixture was centrifuged at 12,000 rpm, room temperature for 2 min. Next the supernatant was transferred to a new microcentrifuge tube then 0.5 volumes of 100% ethanol were added to the clear lysate and mixed immediately by pipetting. The solution was transferred to RNeasy mini column and centrifuged at 12,000 rpm for 15 sec. RNeasy column was added with 700 µl of buffer RW1 and transferred into a new 2 ml collection tube. 500 µl of buffer RPE was added onto the RNeasy column followed by centrifugation at 12,000 rpm, room temperature for 15 sec, to wash the column. The solution flown through the column was discarded. This step was performed 2 times and the second time the column was centrifuged for additional 2 min. To elute, the RNeasy column was transferred to a new 1.5 ml collection tube and 50 µl of RNase-free water was added directly onto the RNeasy silica-gel membrane. Finally, the column was centrifuged at 12,000 rpm, room temperature for 1 min and the RNA solution was kept at -20°C. The concentration of RNA was determined by measuring the absorbance at 260 nm. The purity of sample was calculated from the

ratio between the absorbance at 260 nm and 280 nm. The purified RNA had A_{260}/A_{280} ratio of 2±0.05.

2.1.4 The reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was treated with DNA-free (Ambion, Austin, TX) before cDNAs synthesis by dilution the sample to 10 µg nucleic acid/50 µl before adding 0.1 volumes of 10x DNase I buffer and 1 µl of rDNase I. The solution was mixed gently and incubated at 37°C for 30 min. After that it was added with 0.2 volume of resuspended DNase inactivation reagent and mixed. The solution was incubated at room temperature for 2 min and centrifuged at 10,000 g for 1.5 min. The RNA was transferred to a fresh tube before storage at -20°C. RT-PCR was performed with Superscript III reverse transcriptase RT-PCR system. The first-strand cDNAs were synthesized from 3 µg of total RNA by mixing 1 µl of 50 µM oligo $(dT)_{20}$, 11 µl of RNA and 1 µl of 10 mM dNTP together in nuclease-free microcentrifuge tube. The mixture was heated at 65°C for 5 min and incubated on ice for at least 1 min. After that the mixture was added 4 µl of 5x first-strand buffer, 1 µl of 0.1 M DTT, 1 µl of RNaseOUT Recombinant RNase Inhibitor and 1 µl of Superscript III reverse transcriptase and incubated at 50°C for 60 min. The reaction was inactivated by heating at 75°C for 15 min. The first strand cDNA was diluted to 1:10 before using as template in PCR. The forward primer (5'-TTTGGATGCCTCGACGTGTA-3') and degenerate reverse primer (5'-CGGAGCCGCACACAGGRGCATAGTTGTC-3') were used for RT-PCR. The reaction mixture for RT-PCR contained: 3 µl of forward primer, 3 µl of reverse primer, 24 µl of sterilized water, 5 µl of 10x PCR buffer, 5 µl of 10x dNTPs, 5 µl of 10x DMSO, 1 µl of Taq polymerase and 3 µl of the first strand cDNA template. PCR cycle was 95°C for 1 min, and amplified with denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 2 min for 35 cycles with final extension at 72°C for 10 min. The amplified DNA was run on 0.8% agarose gel with 100 µl of 1x TAE buffer and 5 µl of ethidium bromide at 160 volts for 30 min.

2.1.5 DNA purification from gel

The DNA was eluted from the gel by GeneElute gel extraction kit (Sigma). After electrophoresis, band of interest was cut out from the gel with a razor blade and placed in a 1.5 ml tube. 3 gel volumes of gel solubilization solution was

added into the tube and incubated at 55°C for 10 min. While gel melted, 500 µl of column preparation solution was added to column tube and centrifuged for 1 min. The solution flown through the column was discarded. When gel was melted, 1 gel volume of 100% isopropanol was added and mixed. After that, the mixture of gel-isopropanol was added to the spin column and centrifuged at 10,000 rpm for 1 min. The solution flown through the column was discarded and 700 µl of wash solution was added into the column then centrifuged at 10,000 rpm for 1 min. After discarded flow-trough, the column was centrifuged again at 10,000 rpm for 1 min to dry column. After that the column was transferred to clean 1.5 ml tube and added 100 µl of elution solution. It was allowed stand for 1 min at room temperature and centrifuged at 10,000 rpm for 1 min. DNA was concentrated to 50 µl before electrophoresis on 0.8% agarose gel. If the gel show single band of DNA, the DNA will be sequenced.

2.1.6 DNA preparation for sequencing

The 9 µl of purified DNA sample was mixed with 3 µl of 10 µM forward or reverse primer and kept at -20°C before sequencing.

2.1.7 The rapid amplification of cDNA ends (RACE)

Nested RACE-PCR reactions were employed to obtain the full-length *Ppepi10* cDNA sequence, using the Smart RACE cDNA amplification kit (Clontech) following the manufacturer's instructions. The RACE-PCR reaction was composed of 5'-RACE-PCR and 3'-RACE-PCR reaction. The setting of 5'-RACE-PCR reaction contained 3 reactions following table 3 and 3'-RACE-PCR reaction also contained 3 reactions following table 4.

Table 3 Setting up 5'-RACE-PCR reaction.

Component	5'-RACE sample	UPM only (negative control)	5'-RACE primer only (negative control)
5'-RACE-ready cDNA	5 µl	5 µl	5 µl
10x UPM	1 µl	1 µl	-
5'-RACE primer	1 µl	-	1 µl
3'-RACE primer	-	-	-
Water	1.5 µl	2.5 µl	2.5 µl
Master mix	41.5 µl	41.5 µl	41.5 µl
Total volume	50 µl	50 µl	50 µl

Table 4 Setting up 3'-RACE-PCR reaction.

Component	3'-RACE sample	UPM only (negative control)	3'-RACE primer only (negative control)
3'-RACE-ready cDNA	5 µl	5 µl	5 µl
10x UPM	1 µl	1 µl	-
5'-RACE primer	-	-	-
3'-RACE primer	1 µl	-	1 µl
Water	1.5 µl	2.5 µl	2.5 µl
Master mix	41.5 µl	41.5 µl	41.5 µl
Total volume	50 µl	50 µl	50 µl

Where UPM = Universal primer A mix

Master mix = 34.5 µl of PCR-grade water, 5 µl of 10x advantage 2 PCR buffer, 1 µl of 10 mM dNTP mix and 1 µl of 50x advantage 2 polymerase mix

The 3'-RACE primer (5'-ATGCTACTTGCGCCTTGCGTCTTGC-3') and 5'-RACE primer (5'-CACAGGGGCATAGTTGTCGGGACAC-3') were used in the first round of RACE-PCR reactions. The PCR products from the first RACE-PCR reaction were taken as the template in the nested RACE-PCR reactions, which used oligonucleotides (5'-TGCCTGGACGTGTACGACCCGGTG-3') and (5'-GACCCTCGTTCGAGTATTCCTTTCCA-3') as nested 3'-RACE primer and nested 5'-RACE primer, respectively. The primers for RACE-PCR reactions were designed based on the obtained partial *Ppepi10* sequence. The amplified DNA was run on 0.8% agarose gel with 100 µl of 1x TAE buffer and 5 µl of ethidium bromide, at 160 volts for 30 min.

2.1.8 DNA Transformation to *Escherichia coli* TOP10 cells

PCR product from RACE-PCR was transformed to *Escherichia coli* (*E. coli*) TOP10 cells by mixing 4 µl of PCR product, 1 µl of TOPO PCR 2.1 Vector and 1 µl of NaCl solution. The mixture was incubated at room temperature for 15 min then 5 µl of mixture was added to *E. coli* TOP10 cells. The mixture was placed in ice for 30 min, then at 42°C for 45 sec. The reaction was put in ice for 1 min. The mixture was removed from ice and 300 µl of Luria Bertani media broth (LB media) was added and grown at 37°C, 250 rpm for 1 h. 200 µl of the mixture was spread on Luria Bertani media agar (LA media) containing Kanamycin (25 µg/ml) and incubated at 37°C, overnight. Twenty colonies were picked and struck on new LA media containing Kanamycin (25 µg/ml) and incubated at 37°C for 3-4 h. All of colonies were preformed PCR master mix to select the cloned cell.

2.1.9 PCR master mix and miniprep

The reaction of PCR master mix contained: 2 µl of M13 forward primer, 2 µl of M13 reverse primer, 30 µl of sterilized water, 5 µl of 10x PCR buffer, 5 µl of 10x dNTPs, 5 µl of 10x DMSO, 1 µl of Taq polymerase and *E. coli* cell. The PCR cycle was the same as RT-PCR. PCR product was run on 0.8% agarose gel with 100 µl of 1x TAE buffer and 5 µl of ethidium bromide at 160 volts for 30 min. The cloned cell indicated by gel was inoculated into 3 ml of LB medium containing Kanamycin (25 µg/ml) and incubated at 37°C, 250 rpm overnight. Plasmid extraction from *E. coli* was performed with QIAprep spin miniprep kit.

2.1.10 Signal peptide, Kazal domains and sequence analyses

Ppepi10 was predicted signal peptide by SignalP 3.0 analysis. The Kazal domains of *Ppepi10* were analyzed by searching against InterPro database (http://www.ebi.ac.uk/tool/InterProScan). Multiple alignments of the Kazal domains from *P. palmivora* (PpEPI10), *P. infestans* (EPI1 and EPI10), the crayfish *Pacifastacus leniusculus* (PAPI-1), and the apicomplexan *Tosoplasma gondii* (TgPI1) were conducted using the program CLUSTAL-X. The sequences were obtained from the NCBI databases (www.ncbi.nlm.nih.gov). The *P. palmivora* sequence in this research was deposited in GenBank under accession number FJ643536.

2.1.11 Plasmid and bacterial strain for production of rKazal1, rKazal2 and rKazal3

The construction of plasmid pFLAG-Kazal1, pFLAG-Kazal2 and pFLAG-Kazal3 were performed by cloning PCR-amplified DNA fragments of Kazal1, Kazal2 and Kazal3, respectively into the *Eco*RI and *Kpn*I sites of pFLAG-ATS. *E. coli* BL21 was used for transformation.

2.1.12 Transformation for production of rKazal1, rKazal2 and rKazal3

The cloned cell contained *Ppepi10* was selected to amplify the fragments for *kazal1*, *kazal2* and *kazal3* by using PCR. The oligonucleotides Kazal1-F (5'-GCGGAATTCCATCGACGACGACAAGTGCTCATTC-3'), Kazal1-R (5'-GGGGTACCCTAGTCTGCGGGGCCGCTGG-3'), Kazal2-F (5'-GGGAATTCCATG TGCCCGGACGCTTGCCTG-3'), Kaza2-R (5'-GGGGTACCCTACGGTGGTCCCG TGTAGCC-3'), Kazal3-F (5'-GGGAATTCCATGTGCGCTGACATGTTGTGTCC -3') and Kazal3-R (5'-GCGGGTACCTTACAGATTTAAAGTTTGAGAATAGGTC-3') were used to amplify the fragments. The introduced *Eco*RI and *Kpn*I restriction sites are underlined.

The DNA of cloned cell containing *Ppepi10* for amplifying was obtained by miniprep. The PCR reaction was composed of 1 µl of 1:25 DNA sample, 2 µl of forward primer, 2 µl of reverse primer, 34 µl of sterilized water, 5 µl of 10 x Pfu buffers, 5 µl of 10x dNTPs and 1 µl of pfu Turbo. The PCR cycle was the same as RT-PCR. The amplified DNA was run on 0.8% agarose gel with 100 µl of 1x TAE buffer and 5 µl of ethidium bromide at 160 volts for 30 min to check the quality of DNA. DNA from PCR reactions was purified by using DNA purification kit. After

obtaining *kazal1, kazal2, kazal3* fragments and pFlag-ATS plasmid from miniprep by growing the cell in LB medium containing 50 µg/ml of Carbenicillin, they were digested with *Eco*RI and *Kpn*I restriction enzymes. The reaction of digestion contained: 30 µl of PCR product or pFlag-ATS plasmid, 4 µl of 10x buffer1, 4 µl of 10x BSA, 1 µl of *Eco*RI and 1 µl of *Kpn*I. The mixtures were incubated at 37 °C for 3 h and run gel immediately or kept in -20°C. The DNA band on the gel was cut out and performed gel purification. The 3 µl of DNA was run on 0.8% agarose gel with 100 µl of 1x TAE buffer and 5 µl of ethidium bromide at 160 volts for 30 min to check quality of DNA. Next all of the digested DNA samples were used to perform ligations. The control reactions contained: 1 µl of pFlag-ATS, 8 µl of sample, 1.5 µl of 10x T4 DNA ligase buffer, 1 µl of T4 DNA ligase and 3.5 µl of sterilized double distilled water. The reaction of control contained: 1 µl of pFlag-ATS, 1.5 µl of 10x T4 DNA ligase buffer, 1 µl of T4 DNA ligase and 11.5 µl of sterilized double distilled water. The mixture was incubated at room temperature for 2-3 h or incubated at 16°C overnight using PCR machine. Then, 3 µl of sample from ligation was mixed with 100 µl of *E. coli* BL21 and set electrode at 2,500 volts to transform DNA to *E. coli*. After that 200-300 µl of LB medium was added into the transformation tube and incubated at 37°C, 250 rpm for 1 h. The 20 or 200 µl of sample was spread on LA medium containing Carbenicillin (50µg/ml) then incubated at 37 °C overnight. The single colony about 20 colonies were picked and stuck on the new LA medium containing Carbenicillin (50µg/ml) following incubated at 37 °C overnight. All of colonies were preformed PCR master mix and selected only the cloned cell for sequencing.

2.1.13 Expression of rKazal1, rKazal2 and rKazal3

The cultures of *E. coli* BL21 containing pFLAG-Kazal1, pFLAG-Kazal2 and pFLAG-Kazal3 were grown overnight at 37°C, 250 rpm in 5 ml of LB medium containing Carbenicillin (50µg/ml). After that it was diluted (1:100) in LB medium containing Carbenicillin (50µg/ml) and incubated at 37°C, 250 rpm then added isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.4 mM when the absorbance 600 nm of the cultures reached 0.3. The cultures were further incubated overnight at 28°C, 250 rpm and centrifuged at 6,500 rpm and 4°C for 30 min. The culture supernatants of pFLAG-Kazal1, pFLAG-Kazal2 and pFLAG-Kazal3

were taken to purify for rKazal1, rKazal2 and rKazal3 using anti-FLAG M2 affinity gel. Before protein purification, all of the supernatants were analyzed with Western blot to check expression of those clones.

2.1.14 SDS-PAGE

SDS-PAGE was performed on 15% (w/v) polyacrylamide gel and the gel was prepared following table 5

Table 5 The component of sodium dodecyl sulphate polyacrylamide gel for Western blot analyses.

Component	Stacking gel (5%)	Separating gel (15%)
Acrylamide-bisacrylamide mixture (40%)	0.63 ml	3.75 ml
1.5 M Tris-HCI, pH 8.8	-	2.50 ml
0.5 M Tris-HCI, pH 6.8	1.25 ml	-
10% Ammonium persulfate	50 µl	50 µl
10% SDS	50 µl	100 µl
TEMED	5 µl	5 µl
Distilled water	3.0 ml	3.6 ml
Tota l volume	5.0 ml	10.0 ml

The sample was mixed with 2x loading buffer (950 µl of 2x loading buffer (appendix) + 50 µl of β-mercaptoethhanol) in the ratio 1:1 and boiled about 5 min before loading 20 µl of sample in each lane. The 1x SDS-PAGE running buffer (appendix) was used for electrophoresis. The gel was performed electrophoresis with 90 volts for stacking gel and 120 volts for separating gel. A value of the relative molecular mass (*Mr*) of the protein band was estimated by comparison with a BenchMark pre-stained protein ladder (6-180 kDa).

2.1.15 Western blot analyses

For Western blot analyses, proteins from the gel were transferred to nitrocellulose membranes using a Mini trans-blot apparatus. The 5x transfer buffer contained: 25 mM Tris-HCl, 192 mM Glycine, 20% (v/v) methanol and distilled water 1 l, pH 8.3. It was diluted to 1x transfer buffer before use by mixing 200 ml of 5x transfer buffer, 200 ml of methanol and 600 ml of distilled water together. The 1x transfer buffer was pre-cooled in -20°C before using. The protein in the gel was transferred to nitrocellulose membranes at 100 volts for 1.30 h. The membrane was washed with distilled water 1 time and incubated in 1x Phosphate buffered saline Tween (PBST buffer) (appendix) + 5% milk at room temperature and 100 rpm for 1 h to block the membrane. After that the membrane was washed with 1X PBST 1 time and incubated in 10 µl of anti-FlagHRP plus 10 ml of 1x PBST + 5% milk at room temperature and 100 rpm for 1 h. The membrane was rinsed twice with 1X PBST and incubated in 1X PBST for 10 min tree times. Detection of antigen-antibody complexes was carried out with anti-Flag M2-peroxidase and Super Signal West Pico chemiluminescent substrate.

2.1.16 Purification of rKazal1 and rKazal2

The culture supernatants of pFLAG-Kazal1 and pFLAG-Kazal2 were taken to purify rKazal1 and rKazal2, respectively using anti-FLAG M2 affinity gel. The column was washed with cool Tris buffered saline (TBS buffer) (appendix) 2 times and added anti-FLAG M2 affinity gel into the column. The 1 ml of anti-FLAG M2 affinity gel column was added 3 ml of 0.1 M glycine, pH 3.5. After that 5 ml of TBS buffer was loaded into column at 4°C before loading the sterilized sample. The sample was sterilized by filtration with membrane filter, 0.45 µm. The column was washed with 18 ml of TBS buffer and the eluted protein was kept 1 ml per fraction with 0.1 M glycine, pH 3.5, and equilibrated to neutral pH with 20µl of 1 M Tris, pH 8.0, for each 1 ml eluted fraction. The eluted fraction was kept 6 fractions per column. The pure protein fractions that identified by SDS-PAGE stained with Coomassie Brilliant Blue were pooled and concentrated by ultrafiltration, molecular weight cut off 10 kDa. Protein concentration was measured at 280 nm and calculated using an extinction coefficient of $M^{-1}cm^{-1}$ determined with the approach of Gill and von Hippel (1989). The extinction coefficient was obtained from calculation of the amount of

amino acid by using http://ca.expasy.org. The formula for calculation protein concentration is

Protein concentration (mg/ml) = OD_{280}/average of extinction coefficient of protein (ε).

Where ε of rKazal1 = 0.635

ε of rKazal2 = 0.837

2.2 Plant growth and protein extraction from *H. brasiliensis*

Rubber plants (RRIM600 cultivar) were grown in a growth chamber with a photoperiod of 12 h of light and 12 h of dark at 25°C. Total protein was extracted from the leaves of a 12-week-old rubber plant with 100 mM Tris-HCl buffer pH 7.5 followed by precipitating 61.1 g of $(NH_4)_2SO_4$ per 100 ml of protein solution at 4°C. The pellet was collected by centrifugation at 12,000g for 20 min at 4°C and then redissolved in distilled water. The solution was desalted by loading onto PD-10 column and eluted with distilled water. The fractions from the PD-10 column were monitored for protein content at an absorbance of 280 nm. The eluted fractions with high protein content were pooled.

2.3 Protease inhibition assays

2.3.1 Inhibition of commercial Ser proteases

The inhibition of commercial Ser proteases with rKazal1 and rKazal2 was tested by using the colorimetric Quanti-cleave protease assay kit. The 20 pmol of rKazal1 or rKazal2 was incubated with 20 pmol of chymotrypsin, subtilisin A, or trypsin, in a volume of 50 µl for 30 min at 25°C. 100 µl of succinylated casein (2 mg/ml in 50 mM Tris buffer, pH 8) was added into the reaction and incubated at room temperature for 20 min. After that, added 50 µl of chromogenic reagent 2,4,6-trinitrobenzenesulfonic acid and incubated for 20 min at room temperature before measuring protease activity at 450 nm by spectrophotometry.

2.3.2 Inhibition of plant protease from *H. brasiliensis*

Bio-Rad zymogram buffer system was used for inhibition test of plant protease by rKazal1 and rKazal2. The 20 pmol of rKazal1 or 80 pmol of rKazal2 was incubated with 8 µl (0.08 mg) of rubber leaf proteins for 30 min at 25°C before mixing with zymogram sample buffer in the ratio 1:1. The mixture was loaded onto

8% SDS-polyacrylamide gel without boiling or addition of reducing reagents. The gel was performed electrophoresis with 90 volts for stacking gel and 120 volts for separating gel. After running, the gel was incubated in 1x zymogram renaturation buffer for 30 min at room temperature. Then the gel was equilibrated in 1x zymogram developing buffer for another 30 min at room temperature, followed by 4 h of incubation at 37°C in fresh 1x zymogram developing buffer before staining with 0.5% Coomassie Brilliant Blue. The zymogram gel was prepared following table 6.

Table 6 The component of sodium dodecyl sulphate polyacrylamide gel for zymogram electrophoresis.

Component	Stacking gel (5%)	Separating gel (8%)
Acrylamide-bisacrylamide mixture (40%)	0.63 ml	2.0 ml
1.5 M Tris-HCl, pH 8.8	-	2.50 ml
0.5 M Tris-HCl, pH 6.8	1.25 ml	-
Gelatin (10 mg/ml)	0.5 ml	1 ml
10% Ammonium persulfate	50 µl	50 µl
10% SDS	50 µl	100 µl
TEMED	5 µl	5 µl
Distilled water	2.5 ml	4.345 ml
Total volume	5.0 ml	10.0 ml

2.4 Co-immunoprecipitation

The FLAG-tagged protein immunoprecipitation kit was used for co-immunoprecipitation of rKazal1, rKazal2 and rubber leaf proteins. A total of 800 pmol of purified rKazal1 or rKazal2 were incubated with 200 µl (2 mg) of rubber leaf proteins for 30 min at 25°C. Then 40 µl of anti-FLAG M2 resin was added and incubated at 4°C, overnight with gentle shaking. The mixture was centrifuged at 8,000 g for 30 sec. The supernatant was discarded and the resin was washed with 1 ml of 1xTBS 4 times. Then the 60 µl of FLAG peptide solution (150 ng/µl) was added into the resin and incubated for 30 min at 4°C with gentle shaking to elute bound protein complexes and followed by centrifugation at 8,000 g for 30 sec. The supernatant was kept at -20°C. The analysis was performed with 8% gel of SDS-PAGE and silver nitrate staining.

CHAPTER 3
RESULTS

1 Elicitors from *P. palmivora* inducing defense mechanisms in *H. brasiliensis*

1.1 Purification of elicitors from *P. palmivora*

Before purification of elicitors, culture filtrate of *P. palmivora* was precipitated with $(NH_4)_2SO_4$ then the precipitate was redissolved in distilled water and desalted by a PD-10 column. The fractions from the PD-10 column were monitored for protein content at an absorbance of 280 nm. The eluted fractions with high protein content were pooled and loaded onto Tricine SDS-PAGE. Two major proteins: 10 kDa and 75 kDa detected in the culture filtrate of *P. palmivora* (Fig.26) were selected for further purification.

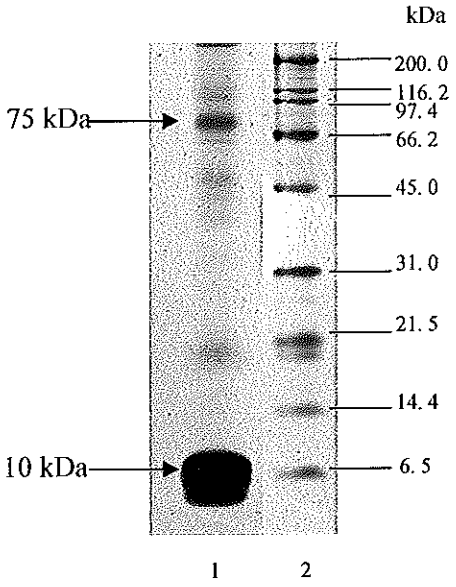


Fig.26 Silver-stained Tricine-SDS-PAGE of total proteins from culture filtrate of *P. palmivora*. Lane 1 is SDS-PAGE analysis of filtrate from *P. palmivora*. Lane 2 is standard protein markers, broad molecular weight kit.

1.1.1 Elicitin purification

The fractions with high protein content from the PD-10 column were pooled. Elicitin was purified using DEAE cellulose column and eluted with 20 mM Tris-HCl buffer pH 7.5 containing 0.1 M NaCl. The protein contents were measured at an absorbance of 280 nm. The fractions with high protein content were checked for a purity of elicitin by Tricine SDS-PAGE (Fig.27). The purified elicitin fractions were pooled and concentrated by ultrafiltration, molecular weight cut off 10 kDa. The retentate was measured for protein content by the BCA method.

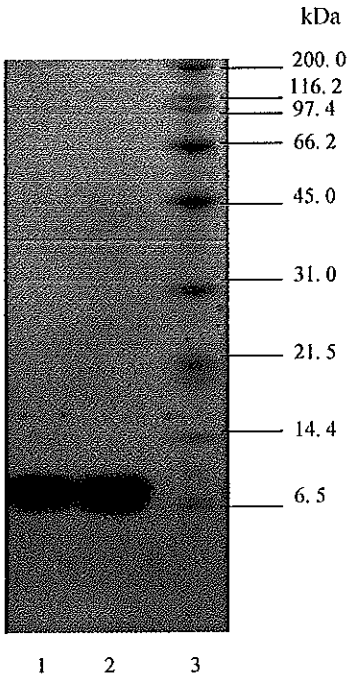


Fig.27 Tricine SDS-PAGE analysis of elicitin from DEAE cellulose column eluted with 0.1 M NaCl in 20 mM Tris-HCl buffer pH 7.5 (lanes 1,2). Lane 3 is standard protein markers, broad molecular weight kit.

1.1.2 New protein elicitor purification

New protein elicitor was purified by using DEAE cellulose column and hydrophobic column. The new protein elicitor was eluted with 20 mM Tris-HCl buffer pH 7.5 containing 0.3 M NaCl from the DEAE cellulose column. The fractions were measured for their protein contents at an absorbance of 280 nm. The fractions with high protein content were checked for a purity of the new protein elicitor by Tricine SDS-PAGE. The eluted fractions shown in the Tricine SDS-PAGE elucidate that the new protein elicitor from the DEAE cellulose column was only partially purified by elution with 20 mM Tris-HCl buffer pH 7.5 containing 0.3 M NaCl. Then the new protein elicitor fractions were pooled and further purified by a hydrophobic column, HiTrap Phenyl FF (high sub) (5 ml). The elution of the new protein elicitor with 20 mM Tris-HCl buffer pH 7.5 containing 0.3 M $(NH_4)_2SO_4$ from the hydrophobic column revealed only one protein peak which after electrophoresis in Tricine-SDS-PAGE had a molecular mass of about 75 kDa (Fig.28).

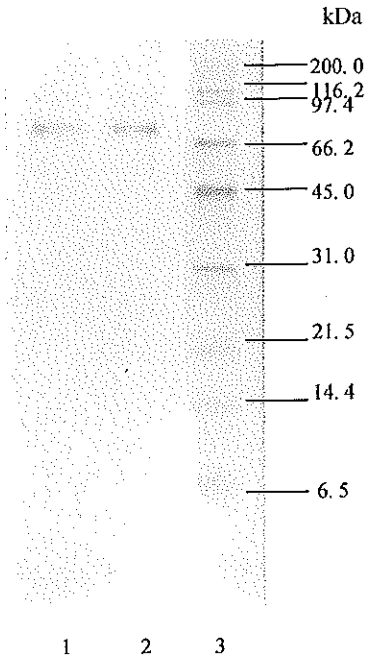


Fig.28 Tricine SDS-PAGE analysis of new protein elicitor from hydrophobic column eluted with 0.3 M $(NH_4)_2SO_4$ in the 20 mM Tris-HCl buffer pH 7.5 (lanes 1,2). Lane 3 is standard protein markers, broad molecular weight kit.

1.2 Partial chemical characterization of elicitin and new elicitor

Properties of the elicitors were assayed on tobacco plants. Each 10 µl solution of elicitor was infiltrated into mesophyll tissue of fully developed leaves using a 1 ml syringe without a needle through stomatal pores. Hypersensitive cell death was observed by the appearance of a brown necrosis in the infiltrated area 2 days after elicitor infiltration.

1.2.1 Heat stability determination

Both elicitin and the new elicitor still retained their abilities to induce hypersensitive cell death after incubation at 80, 90 and 100°C for 5 min, as indicated by the brown necrosis occurring in the infiltrated area (Fig.29).

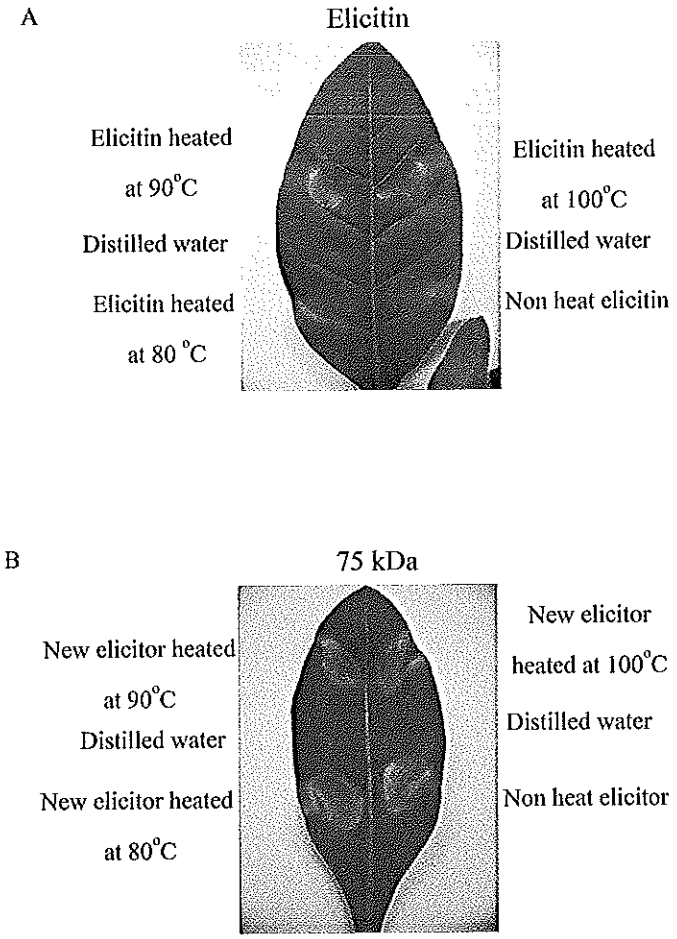
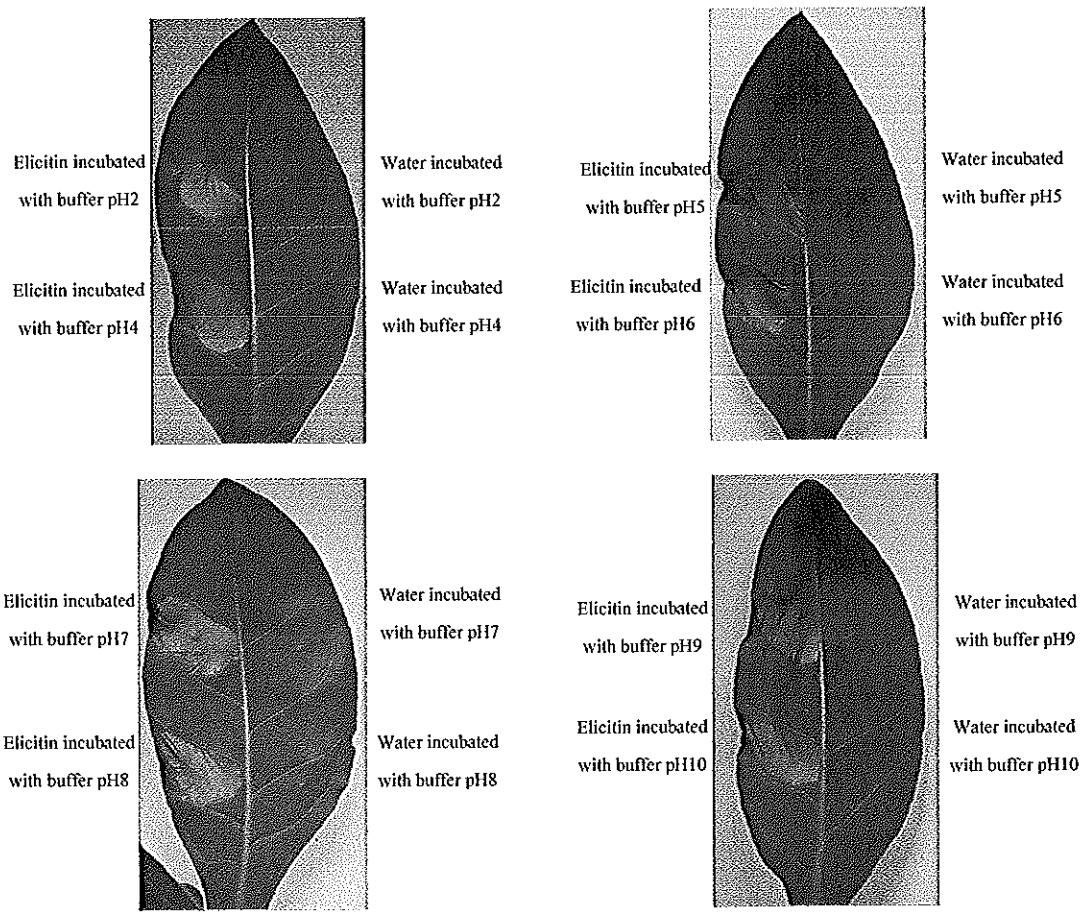


Fig.29 The hypersensitive cell death of elicitin (A) and new elicitor (B) on tobacco plants after heat treatment.

1.2.2 Acid and base stability

Both elicitin and the new elicitor retained their activities to induce hypersensitive cell death on tobacco leaves after incubation for 60 min in a series of buffer solutions with pH values of 2, 4, 5, 6, 7, 8, 9, 10, respectively (Fig.30).

A



B

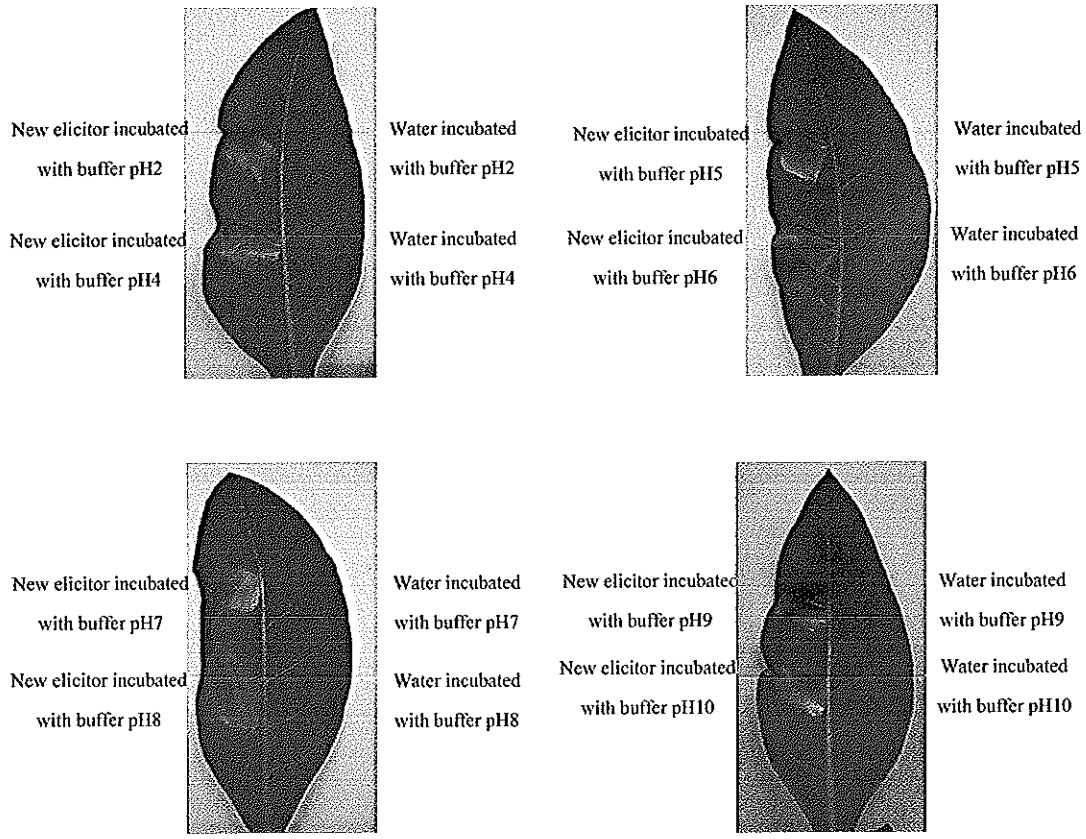


Fig.30 The hypersensitive cell death of elicitin (A) and new elicitor (B) on tobacco plants after incubation at various pH.

1.2.3 Stability to ProteaseK

For the stability to ProteaseK, 100 µl of 50 nM protein elicitors were treated in 100 µl of ProteaseK solution (20µg/ml) for 30 min at room temperature before infiltration into tobacco leaves. The positive control was each elicitor at concentration of 50 nM and water was replaced the elicitor in the experiment for negative controls. Both elicitin and new elicitor lost their activities to induce hypersensitive cell death after treatment with ProteaseK (Fig.31).

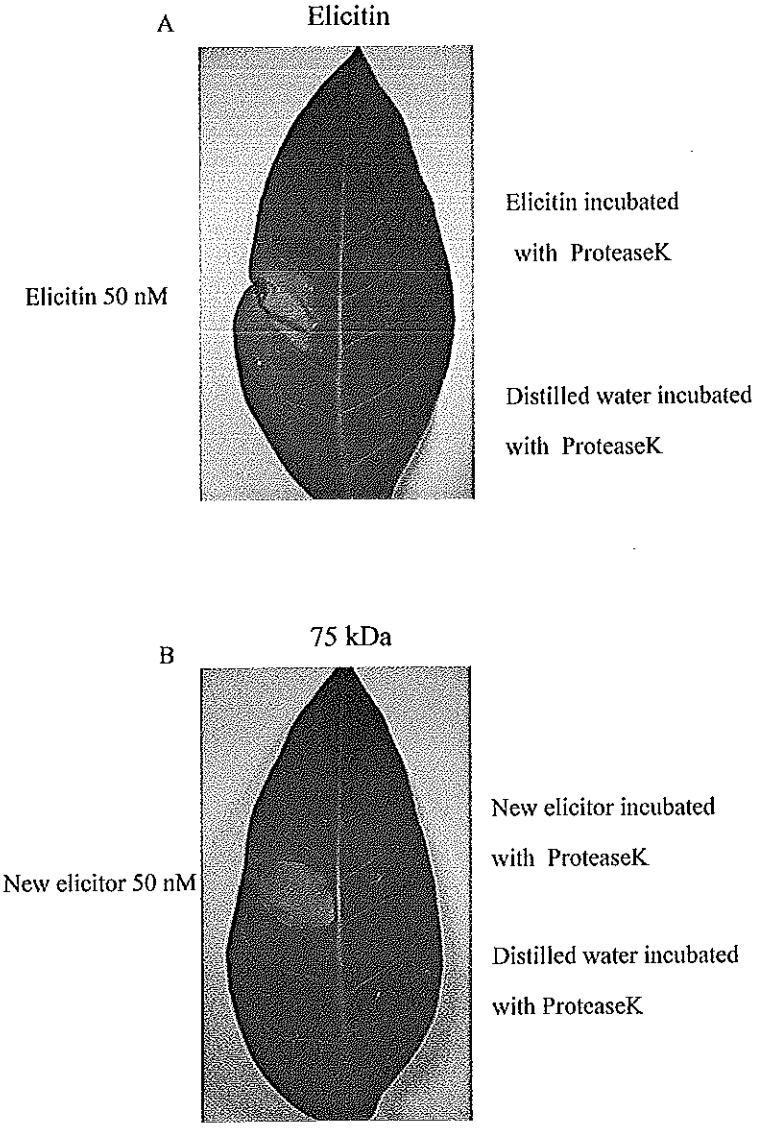


Fig.31 The hypersensitive cell death of elicitin (A) and new elicitor (B) on tobacco plants after ProteaseK treatment.

1.3 Biological assays in cell suspension of *H. brasiliensis* treated with elicitin and new elicitor

After cell suspension (BPM-24 cultivar) had been treated with elicitors (150 nM for elicitin and 5 nM for the new elicitor) for 0, 8, 16, 24, 32, 40, 48, 56, 64, 72 h respectively, the cells were collected to follow accumulation of phenolic compounds and POD activity. The treated medium was kept for measurement of Scp content. Every activity assayed was calculated from three times of experiments.

1.3.1 Synthesis of phenolic compounds

The measurement of phenolic compounds was modified from Torres *et al.* (1987). The phenolic compounds content was measured at an absorbance of 730 nm and calculated by comparing with the absorbance of gallic acid at 730 nm (Fig.24). The accumulation of phenolic compounds induced by either elicitin or new elicitor was relatively low when compared to controls. The increase in phenolic compounds reached a peak of 0.9 μg g^{-1} fresh wt by either elicitor at 64 h then declined to the basic level at 72 h (Fig.32).

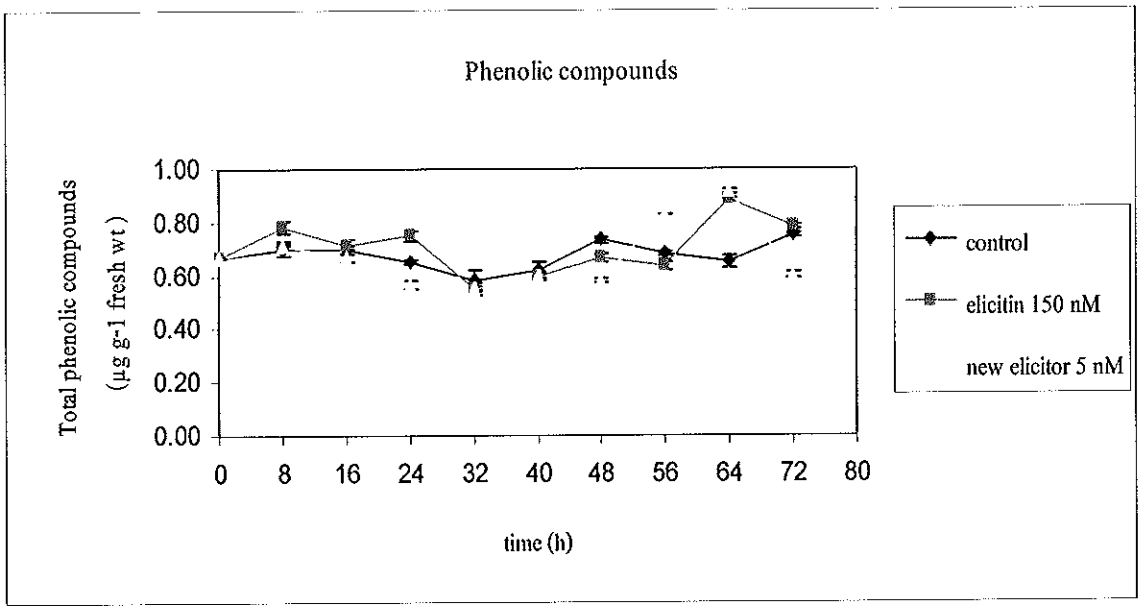


Fig.32 The accumulation of phenolic compounds in cell suspension of *H. brasiliensis* (BPM-24 cultivar) treated with either elicitin 150 nM or new elicitor 5 nM. Distilled water was used in place of elicitors in the experiment as the control. Phenolic compounds levels were calculated from three separate experiments.

1.3.2 Synthesis of scopoletin content

The Scp content from the treated medium was measured with spectrofluorometer at λ 340 nm excitation and λ 440 nm emission. The amount of Scp was calculated by comparing with standard curve of Scp (Fig.25) and expressed in nmol/g fresh weight of cell suspension. The Scp accumulation displayed similar kinetics for both elicitors. The first evidence for an increase of Scp over the control occurred at 24 h after the treatments. A peak of Scp accumulation of 8 nmole g^{-1} fresh wt was obvious at 64 h followed by a decrease at 72 h (Fig.33) when the experiment was terminated which is similar to the kinetics of phenolic compounds synthesis.

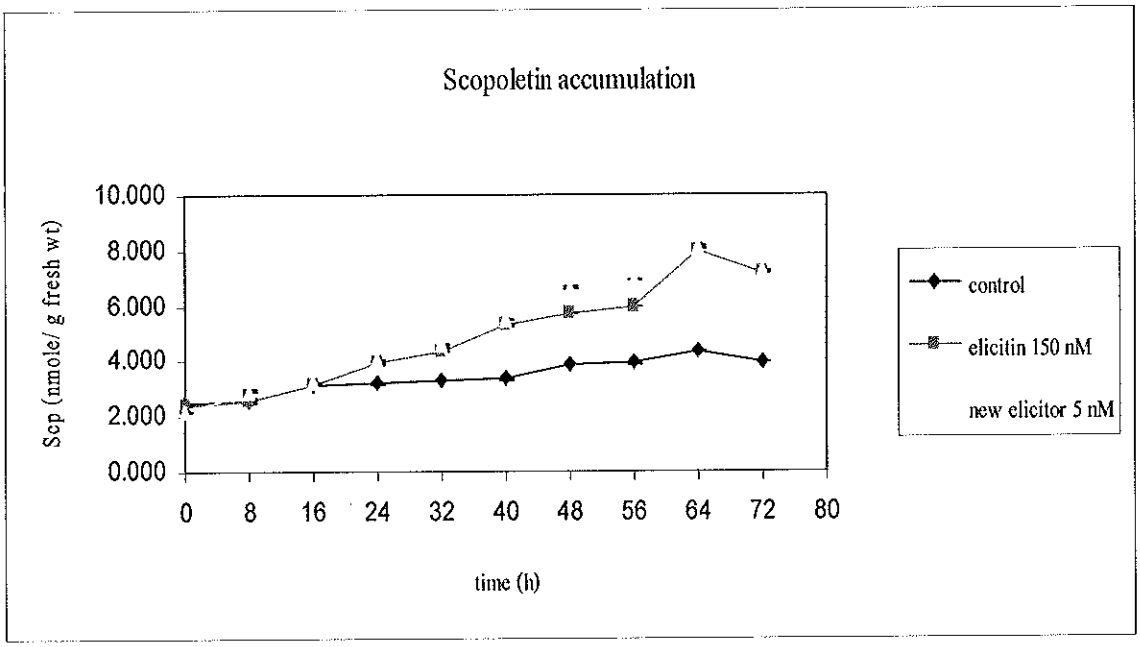


Fig.33 Time-course measurement of Scp accumulation in cell suspension of *H. brasiliensis* (BPM-24 cultivar) treated with elicitin 150 nM or new elicitor 5 nM. Distilled water was used as a control. Scp accumulation was calculated from three separate experiments.

1.3.3 Induction of peroxidase enzyme activity for substrate *o*-dianisidine

The peroxidase activity for substrate *o*-dianisidine was measured at absorbance 460 nm by the modified method of Shannon *et al.* (1966). The increase in *o*-dianisidine peroxidase activity occurred at the same time and with the same kinetics for both elicitors. The increase in *o*-dianisidine peroxidase activity started at 8 h reached a peak of 44 x 10^5 unit mg^{-1} protein for elicitin and 60 x 10^5 unit mg^{-1} protein for the new elicitor at 24 h then declined to the basic level at 72 h (Fig.34).

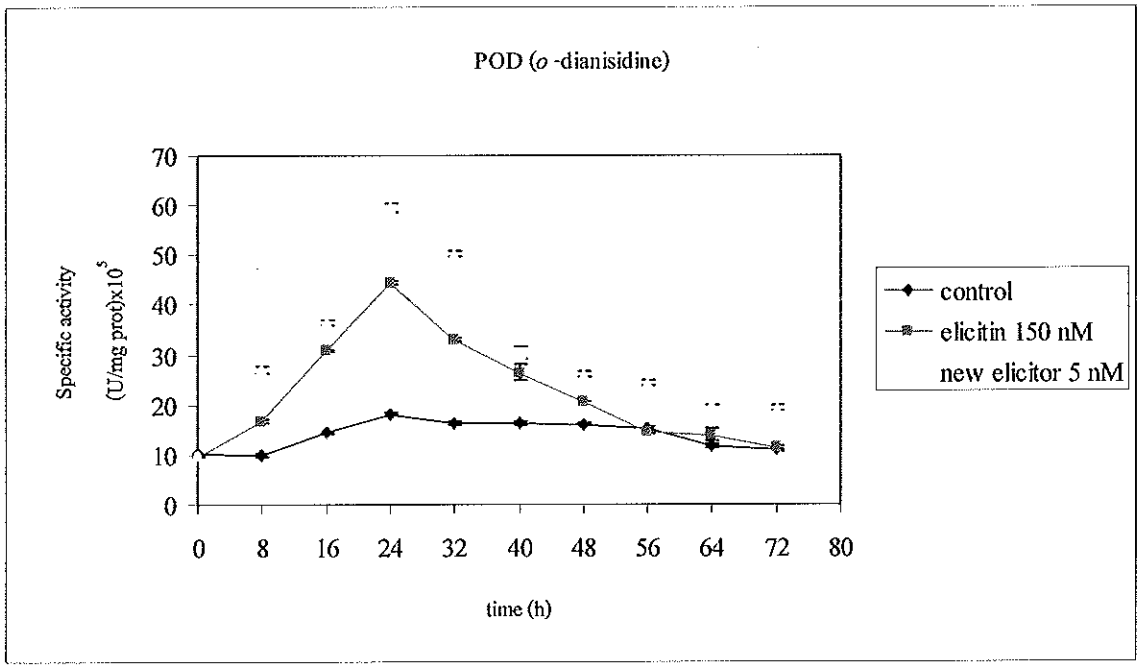


Fig.34 Time-course measurement of peroxidase activity for substrate *o*-dianisidinein from cell suspension of *H. brasiliensis* (BPM-24 cultivar) treated with elicitin 150 nM or new elicitor 5 nM. The distilled water was replaced the elicitors in the experiment for the control. POD (*o*-dianisidine) activity was calculated from three separate experiments.

1.3.4 Induction of peroxidase enzyme activity for substrate scopoletin

The peroxidase activity for substrate Scp was measured by the modified method of Edwards *et al.* (1997). The peroxidase enzyme activity was measured at an absorbance of 595 nm. The increase in scopoletin peroxidase activity occurred at the same time and with the same kinetics for both elicitors. The increase in scopoletin peroxidase activity was first observed at 8 h and the peak level of 410 unit mg^{-1} protein for both elicitors appeared at 64 h after the treatment (Fig.35) and then started to decrease at 72 h.

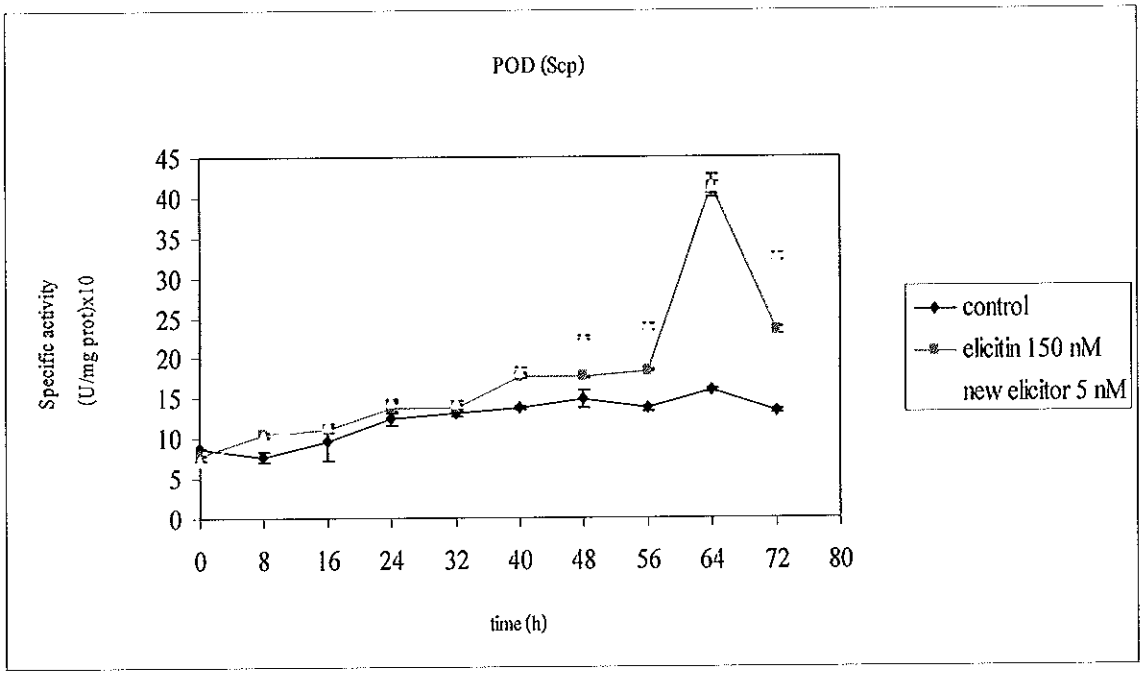


Fig.35 Time-course measurement of peroxidase activity for substrate scopoletin from cell suspension of *H. brasiliensis* (BPM-24 cultivar) treated with elicitin 150 nM or new elicitor 5 nM. Distilled water was used as a control. POD (Scp) activity was calculated from three separate experiments.

1.4 Biological assays on young rubber tree seedlings treated with elicitin or new elicitor

1.4.1 Induction of local resistance on rubber plants by elicitin or new elicitor

The ability of the elicitors to induce local resistance in leaves of rubber plants (RRIM600 cultivar) against *P. palmivora* infection was tested after the leaves were sprayed with the elicitors at dosages of 0.5, 1, 2, 4, 8 nM for the elicitin, and 0.125, 0.25, 0.5, 1, 2, 4 nM for the new elicitor and 2 days later leaves were inoculated with $1x10^4$ zoospores/ml of *P. palmivora*. Water was used as a control instead of the elicitors. The concentrations of elicitin that exhibited a strong resistance to *P. palmivora* ranged between 1-4 nM (Fig.36A) and between 0.5–2 nM for the new elicitor (Fig. 36B). After spraying rubber plant leaves with concentrations above 4 nM for elicitin and 2 nM for the new elicitor, dehydration was triggered and the leaves wilted (Fig.36). Then the minimum of each elicitor concentration that exhibited a strong resistance to *P. palmivora*, 1 nM for elicitin and 0.5 nM for new elicitor was used for biological assays on young rubber tree seedlings.

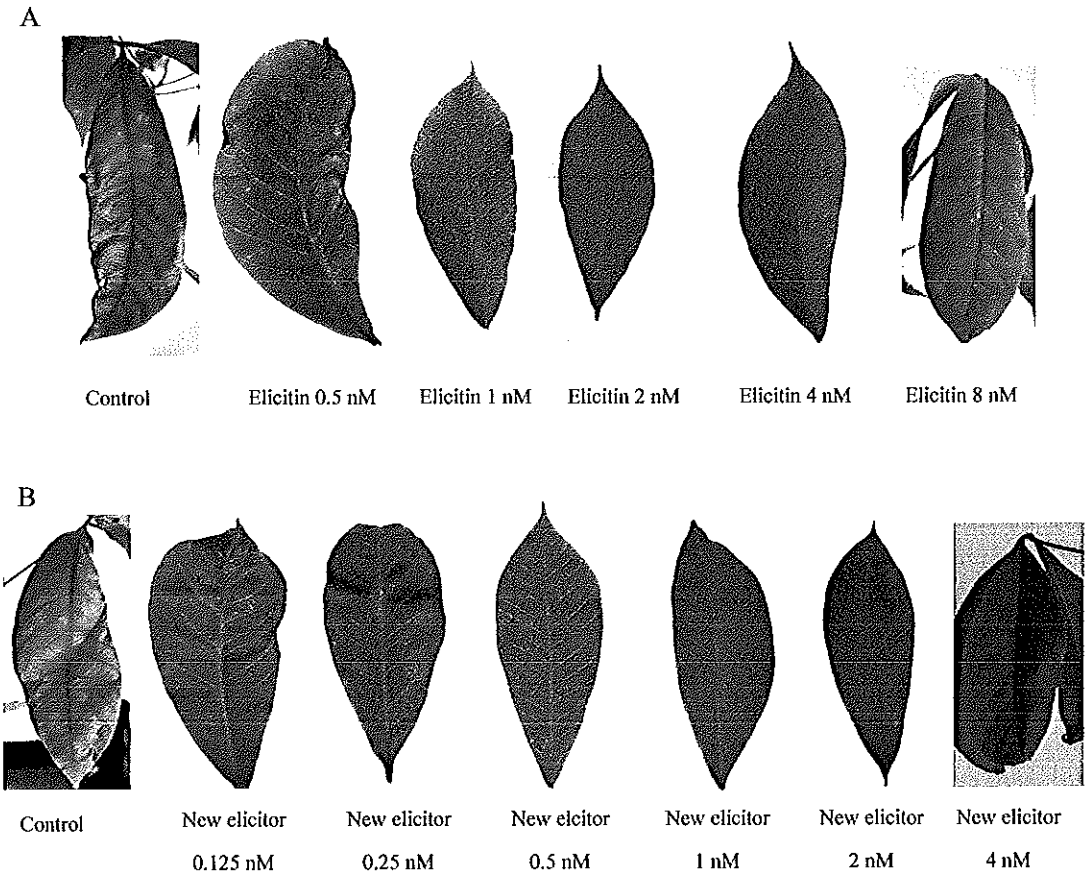


Fig.36 Induction of local resistance against *P. palmivora* was induced on rubber plants (RRIM600 cultivar) by elicitin or new elicitor. (A) Plants were pretreated with elicitin 2 days before infecting with $1x10^4$ spores of *P.palmivora*. (B) Plants were pretreated with new elicitor 2 days before infecting with $1x10^4$ spores of *P.palmivora*. Distilled water was used as a negative control.

1.4.2 Induction of phenolic compounds and *o*-dianisidine peroxidase activity on rubber plants treated with elicitin and new elicitor

After rubber plants (RRIM600 cultivar) were sprayed with elicitors (1 nM for elicitin and 0.5 nM for the new elicitor) for 0, 8, 16, 24, 32, 40, 48, 56, 64, 72, 80, 88, 96 h respectively, the leaves were collected to follow accumulation of phenolic compounds and *o*-dianisidine peroxidase activity. The induction of accumulation of phenolic compounds and peroxidase activity displayed similar kinetics for both elicitors. A peak of phenolic compounds of 6.7 µg/g freash wt for both elicitin and new elicitor was obvious at 40 h followed by a decrease at 64 h (Fig.37). The increase of *o*-dianisidine peroxidase activity started at 8 h reached a peak of 1 x 10^5 unit mg^{-1} protein for both elicitors at 48 h then declined (Fig.38).

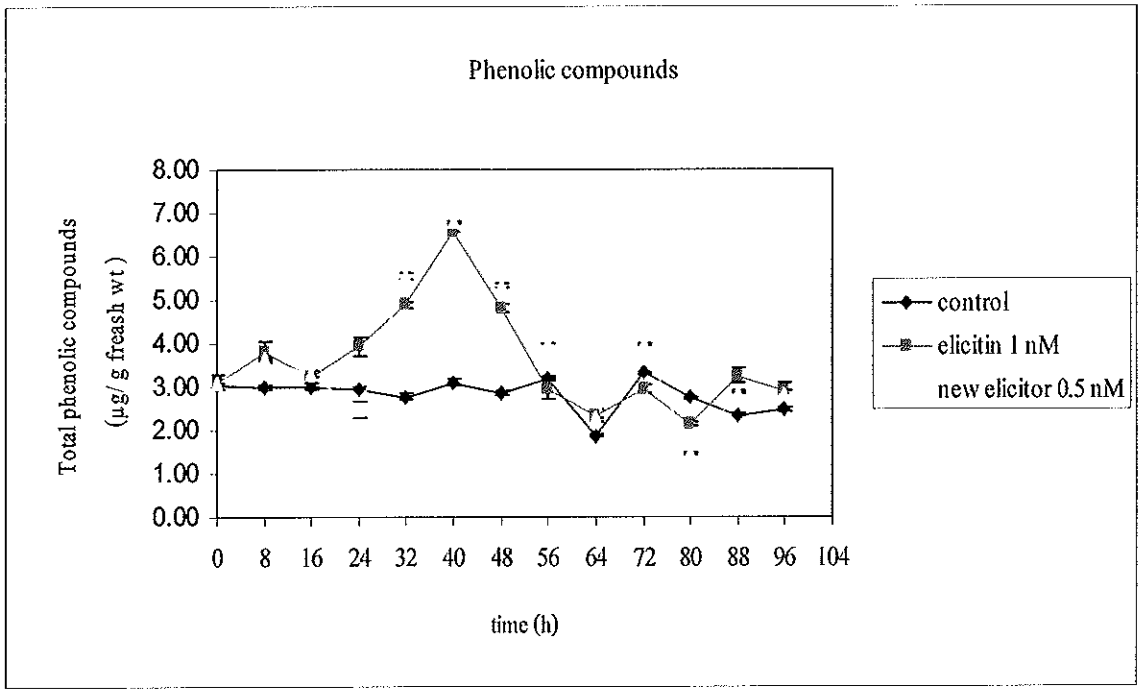


Fig.37 Accumulation of phenolic compounds in rubber leaves (RRIM600 cultivar) treated with elicitin 1 nM and new elicitor 0.5 nM. Distilled water was used as a negative control. Accumulation of phenolic compounds was calculated from three separate experiments.

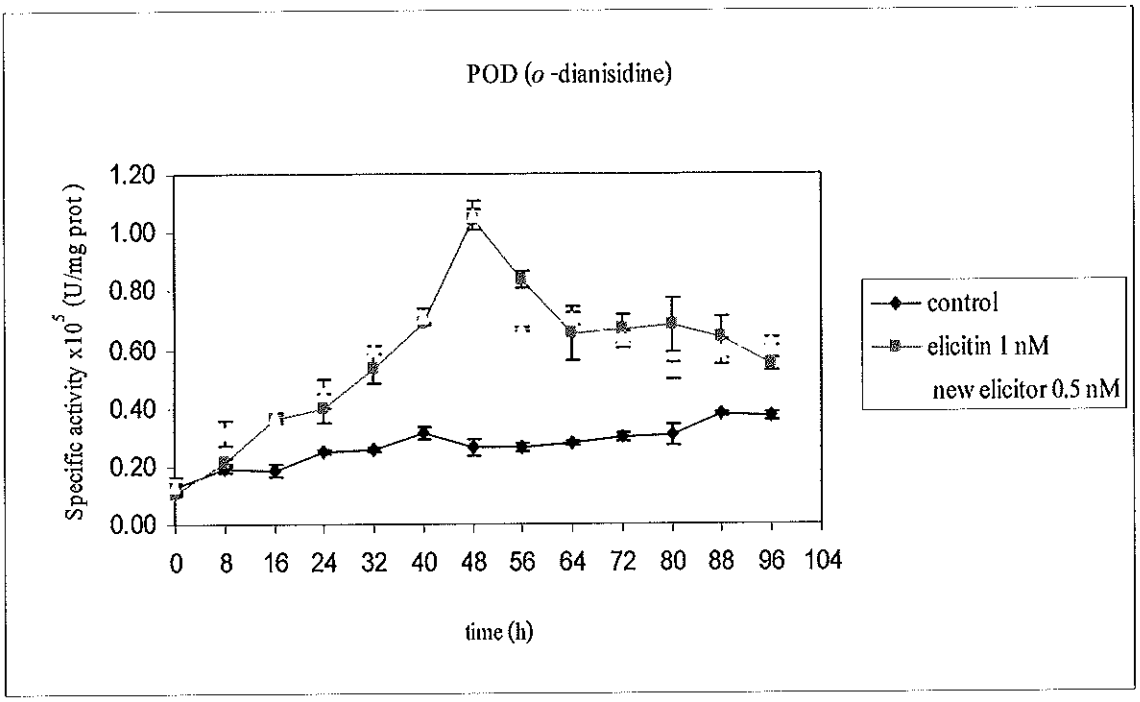


Fig.38 Induction of *o*-dianisidine peroxidase activity in rubber leaves (RRIM600 cultivar) treated with elicitin 1 nM and new elicitor 0.5 nM. Distilled water was used in the experiment as a negative control. POD (*o*-dianisidine) activity was calculated from three separate experiments.

1.4.3 Measurement of *o-dianisidine* peroxidase activity during expression of local resistance

After treating rubber plants (RRIM600 cultivar) with 1% of Triton X-100, the elicitors (1 nM for elicitin and 0.5 nM for the new elicitor) were sprayed onto rubber leaves. The leaves were collected from two plants for every 8 h until 48 h. Then the plants were inoculated with $1x10^4$ zoospores/ml of *P. palmivora*. The leaves were further collected for every 8 h from 48 h to 96 h in order to measure *o*-dianisidine peroxidase activity. The increase in *o*-dianisidine peroxidase activity started at 8 h reached a peak of 1×10^5 unit mg^{-1} protein for both elicitors at 48 h then declined at 56 h. This part of experiment was tested at the same time as the experiment in 1.4.2. Therefore the result of induction of *o*-dianisidine peroxidase activity from 8 to 48 h was the same as the experiment in 1.4.2. However, the *o*-dianisidine peroxidase activity was enhanced again at 72 h after inoculation which

reached a peak of 1.42 x 10^5 unit mg^{-1} protein for both elicitors and then decreased (Fig.39). For control the increase in *o*-dianisidine peroxidase activity started at 56 h reached a peak of 1.25 x 10^5 unit mg^{-1} protein at 80 h (Fig.39). The leaves treated with 1 nM of elicitin or 0.5 nM of new elicitor showed strong resistance to *P. palmivora* whereas the obvious disease lesions were observed in the control leaves.

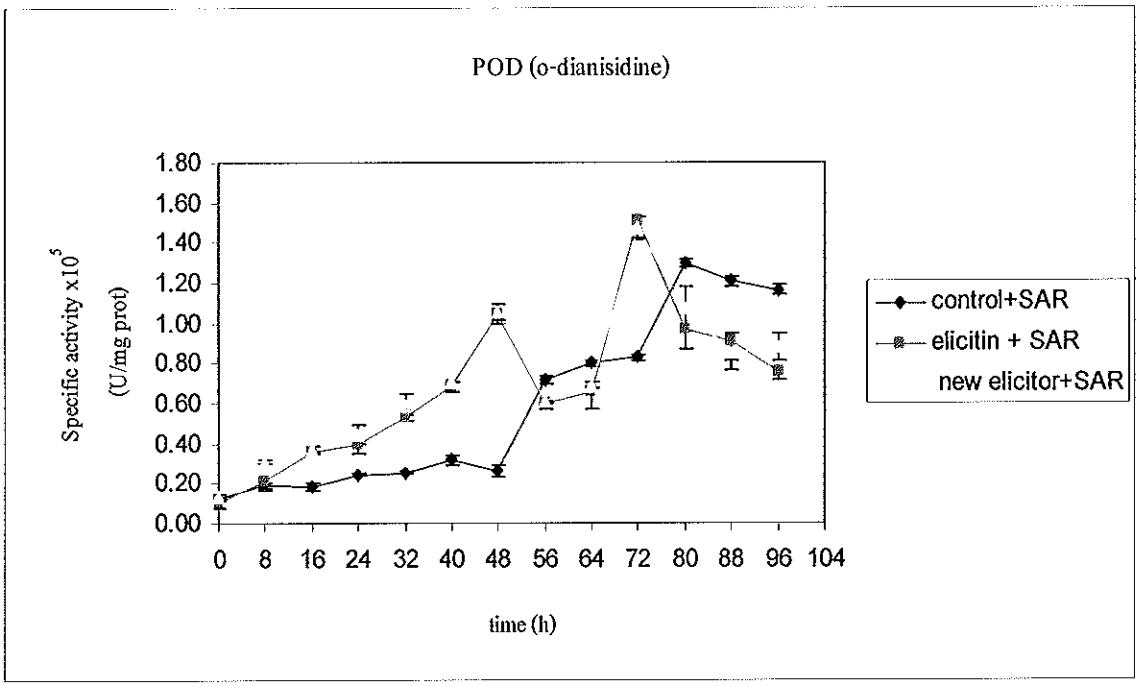


Fig.39 The *o*-dianisidine peroxidase activity during expression of local immunity against *P. palmivora* on rubber plants (RRIM600 cultivar) treated with elicitin 1 nM or new elicitor 0.5 nM. Distilled water was used as a negative control. POD (*o*-dianisidine) activity was calculated from three separate experiments.

2 Protease inhibitor from *P. palmivora* inhibiting protease in *H. brasiliensis*

2.1 Primer design for *P. palmivora epi10* (*Ppepi10*)

In order to create the *Ppepi10* primer from the unknown *epi10* sequence data of *P. palmivora*, program CLUSTAL-X was used to align sequences of EPI10 from *P. infestans* (Accession number AY586282) and PramEPI10 from *P. ramorum* (Trace identified number 303447516). The alignment of *epi10* sequences from two *Phytophthora* species revealed a highly conserved basic sequence structure. Two relatively long conserved stretches were chosen for the forward primer and the degenerate reverse primer (Fig.40).

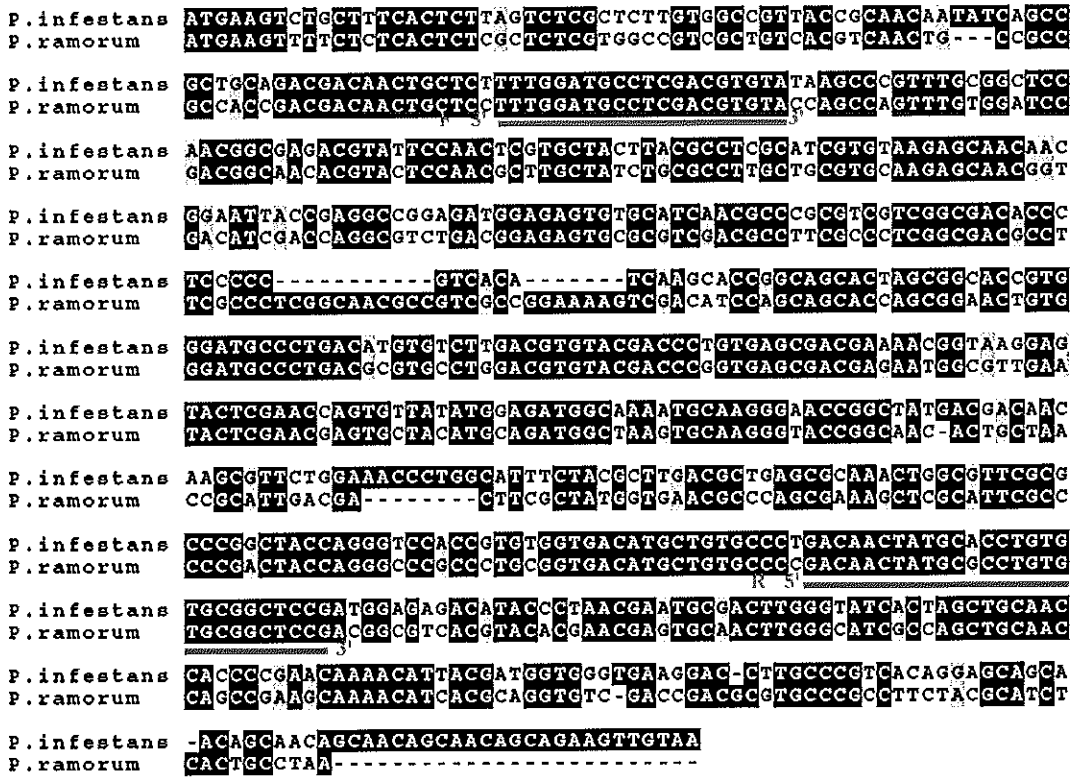


Fig.40 Alignment of *epi10* sequences from *P. infestans* and *P. ramorum* using CLUSTAL-X. The conserved sequences are highlighted in black. The underlined sequences indicated DNA primer sequences used for RT-PCR. F, forward primer; R, reverse primer.

2.2 Construction of full-length *Ppepi10*

2.2.1 RT-PCR

RT-PCR was performed with Superscript III reverse transcriptase RT-PCR system. The first-strand cDNAs were synthesized from 3 μg of total RNA. The forward primer (5'-TTTGGATGCCTCGACGTGTA-3') and reverse primer (5'-CGGAGCCGCACACAGGRGCATAGTTGTC-3') were used for RT-PCR which produced two bands of DNA (Fig.41). The two bands of DNA were cut out separately and eluted from gel by GeneElute gel extraction kit. Then each DNA sample was sequenced.

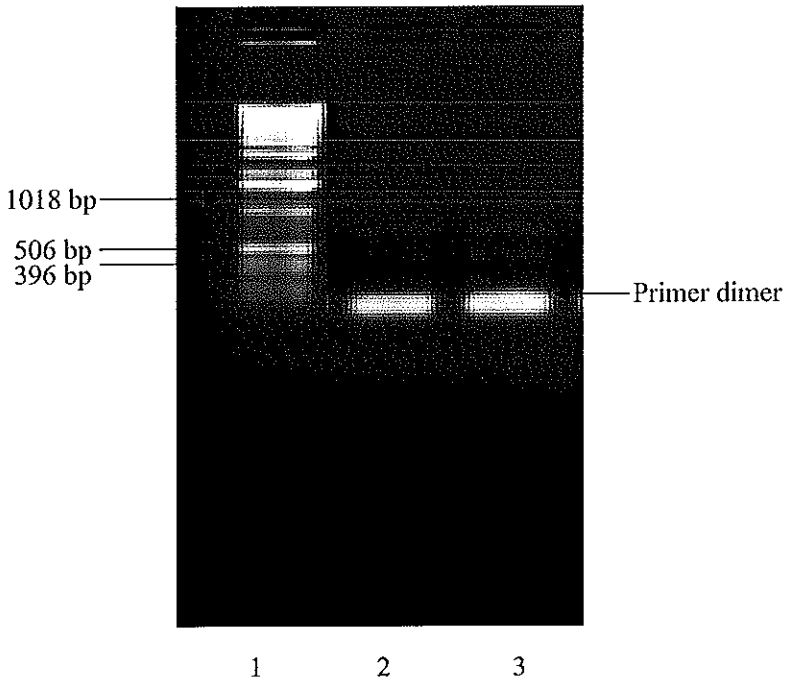


Fig.41 The DNA gel of RT-PCR product using forward primer (5'-TTTGGATGCCTCGACGTGTA-3') and reverse primer (5'-CGGAGCCGCACACAGGRGC ATAGTTGTC-3'). Lane 1 is DNA marker (1 kb DNA ladder), lane 2 and 3 are RT-PCR products.

After sequencing of *Ppepi10* cDNA amplified by RT-PCR, the partial *Ppepi10* sequence (381 bases) was obtained from the lower DNA band. From blast performing with http://blast.ncbi.nlm.nih.gov/Blast.cgi of partial *Ppepi10* sequences

shown in Fig.42, the partial *Ppepi10* sequences had high homology of Kazal-like Ser protease inhibitor EPI10 from *P. infestans*.

2.2.2 RACE-PCR

The full-length *Ppepi10* was further produced by RACE-PCR. The primers for both RACE-PCR reactions were designed from partial *Ppepi10* sequences that known from cDNA sequences amplified by RT-PCR (Fig.42).

ctc**atgctacttgcgccttgcgtcttgc**cagagcaacaacgagatcactgaggcgtccaa
3'-RACE primer
tggagagtgcgcttcgactccagcatcgtcggcaacaccttcacctaaatcgtcggcagac

aacagctccagcggccccgcagactgcccggacgct**tgcctggacgtgtacgacccg**
Nested 3'-RACE primer
gtgactgatgagaa**tggaaaggaatactcgaacgagtgc**tacatgaagatggccaagt
Nested 5'-RACE primer
gccagggcaccaacgacaagcggaatgacaaccctggcgtgtccacgctcgatgccca

gcggaagctcgcttttgcccctggctacacgggaccaccgtgcgctgacatgtt**gtgtccc**

gacaactatgcccctgtgtgcggctc
5'-RACE primer

Fig.42 Partial *Ppepi10* sequences obtained from sequencing of *Ppepi10* cDNA amplified by RT-PCR. The underlined sequences were used as RACE-PCR primers.

Two RACE-PCR reactions were used to produce full-length *Ppepi10* and found that the *Ppepi10* was not specific with both 3'-RACE and 5'-RACE primers designed from partial *Ppepi10* sequences. That is smear bands of DNA were generated using 3'-RACE and 5'-RACE primers (Fig.43). However, the full-length *Ppepi10* could be produced by using PCR product from the first RACE-PCR reaction as a template for the nested RACE-PCR (Fig.44). After DNA product from two RACE-PCR reactions was sequenced, the full-length *Ppepi10* sequence was obtained.

The full-length *Ppepi10* sequence was deposited in National Center for Biotechnology Information GenBank under accession number FJ643536 (Fig.45).

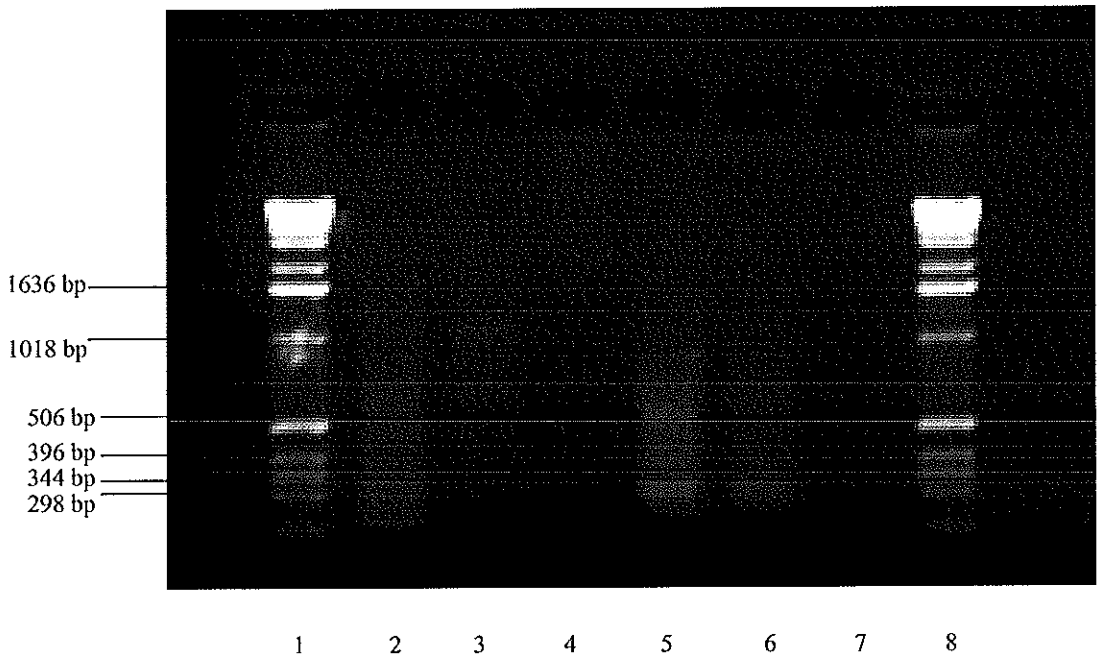


Fig.43 DNA gel of RACE-PCR using 3'-RACE and 5'-RACE primer. Lane1 and 8 are DNA marker (1 kb DNA ladder); lane 2 is 3'-RACE sample; lane 3, 6 are negative control (Universal primer A mix only); lane 4 is negative control (3'-RACE primer only); lane 5 is 5'-RACE sample; lane 7 is negative control (5'-RACE primer only).

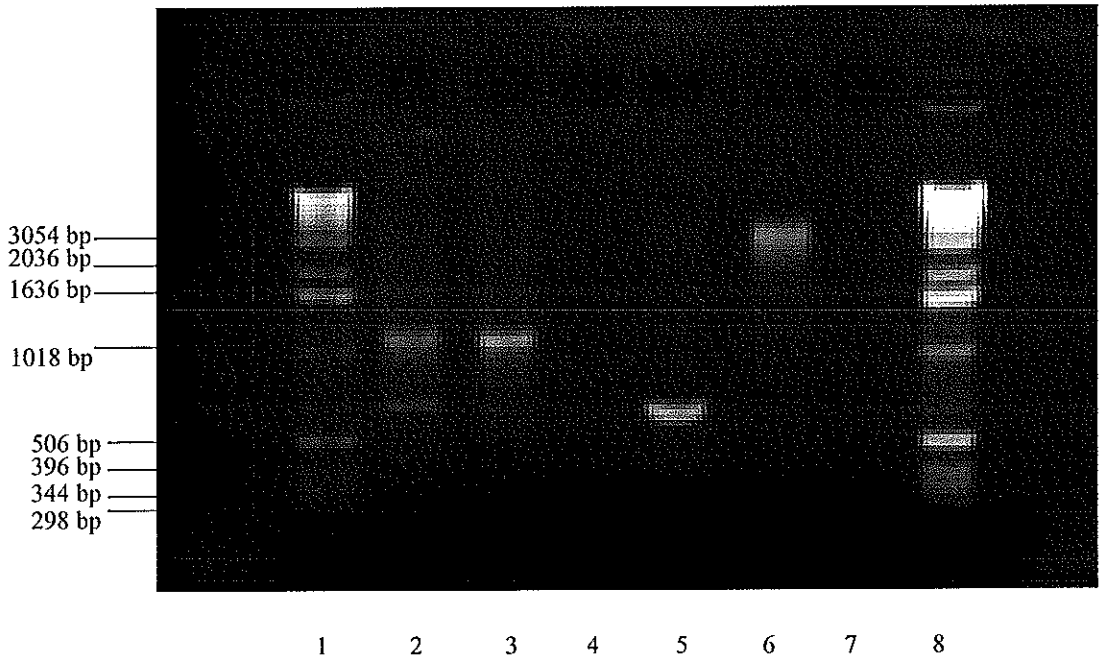


Fig.44 DNA gel from nested RACE-RCR reaction. Lane1 and 8 are DNA marker (1 kb DNA ladder); lane 2 is nested 3'-RACE sample; lane 3 and 6 are negative control (Universal primer A mix only); lane 4 is negative control (nested 3'-RACE primer only); lane 5 is nested 5'-RACE sample; lane 7 is negative control (nested 5'-RACE primer only).

ATGAAGTTTACTTTTGGTCTCGCTTTTCTGGCCATCGCTGTCGCAACGGCTACCGCCATCGACGACGACAAGTGCT
CATTCGGATGCCTCGACGTTTACAAACCGGTTTGCGGCTCTAACGGCGAGACGTACTCCAACTCATGCTACTTGCG
CCTTGCGTCTTGCCAGAGCAACAACGAGATCACTGAGGCGTCCAATGGAGAGTGCGCTTCGACTCCAGCATCGTC
GGCAACACCTTCACCTAAATCGTCGGCAGACAACAGCTCCAGCGGCCCCGCAGACTGCCCGGACGCTTGCCTGGA
CGTGTACGACCCGGTGACTGATGAGAATGGAAAGGAATACTCGAACGAGTGCTACGTGAAGATGGCCAAATGCC
AGGGCACCAACGACAAGCGGAATGACAACCCTGGCGTGTCCACGCTCGATGCCCGGCGGAAGCTCGCTTTTGCCC
CTGGCTACACGGGACCACCGTGCGCTGACATGTTGTGTCCCGACAACTACAAGCCAGTGTGCGGCTCGGATGGCGT
CACCTACACCAACGAGTGTCAGCTGGGCATCACCAGTTGCAACGAGCGCAAAAACATTACGATGGTTAATGAGGTA

Fig.45 The full-length sequence of *Ppepi10*.

2.3 Signal peptide analysis of PpEPI10

After sequencing of the full-length *Ppepi10* cDNA, it revealed that *Ppepi10* had an open reading frame of 669 bp that was corresponded to a predicted translated product of 222 amino acids using ExPASy-Tools. The amino acid sequence of PpEPI10 are shown in Fig. 46.

PpEPI10 was predicted signal peptide by SignalP 3.0 analysis. SignalP 3.0 analysis of the putative protein identified a 19-amino acid signal peptide with a significant mean S value of 0.931. The 19-amino acid signal peptide of PpEPI10 is shown in Fig.46.

M K F T F G L A F L A I A V A T A T A I D D D K C S F G C L D V Y K P V C G
S N G E T Y S N S C Y L R L A S C Q S N N E I T E A S N G E C A S T P A S S A
T P S P K S S A D N S S S G P A D C P D A C L D V Y D P V T D E N G K E Y S
N E C Y V K M A K C Q G T N D K R N D N P G V S T L D A R R K L A F A P G
Y T G P P C A D M L C P D N Y K P V C G S D G V T Y T N E C Q L G I T S C N
E R K N I T M V N E V K R M I R G F G E E T S S T Y S Q T L N L

Fig.46 The amino acid sequences of full-length *Ppepi10* and the underlined sequence is signal peptide.

2.4 Kazal domain analysis of PpEPI10

The Kazal domains of PpEPI10 were analyzed by searching against InterPro database (http://www.ebi.ac.uk/tool/InterProScan). The presence of three domains with similarity to Kazal inhibitors (InterPro IPR002350) was detected in the PpEPI10 (Fig.47).

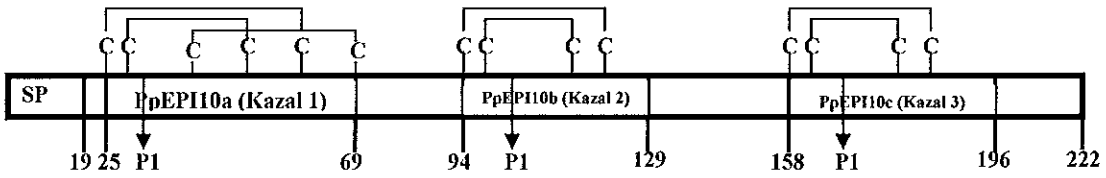


Fig.47 The structure of PpEPI10 predicted to the Kazal family of Ser protease inhibitor. SP is a signal peptide and three Kazal domains were designed as PpEPI10a, PpEPI10b and PpEPI10c, respectively. The positions of amino acid residues starting from the N terminus were indicated by numbers. The predicted P1 residues, which play central roles in determining the specificity of Kazal inhibitors, are Asp.C is the cysteine residues and the disulfide linkages predicted based on the structure of other Kazal domains are shown.

2.5 Sequence analysis of Ser protease inhibitor domains

According to a multiple alignments of the Kazal domains from *P. palmivora* (PpEPI10), *P. infestans* (EPI1 and EPI10), the crayfish *P. leniusculus* (PAPI-1), and the apicomplexan *T. gondii* (TgPI1) produced by CLUSTAL-X, the highly conserved amino acid residues of the Ser protease inhibitor were a cysteine backbone, tyrosine, and asparagine residues (Fig.48). The first domain of PpEPI10 (PpEPI10a) represented a typical Kazal domain, which contains six Cys residues (Fig.48) forming 3 disulfide bridges of Cys1-5, Cys2-4 and Cys3-6 (Fig.47). For the second (PpEPI10b) and the third domains (PpEPI10c), the Cys residues at position 6 and/or 3 were missing. For all these three domains, the predicted P1 residues, which play central roles in determining the specificity of Kazal inhibitors, are Aspartic residues, as are also found in the Kazal domains of *P. infestans* EPI1 and EPI10 (Fig.48).

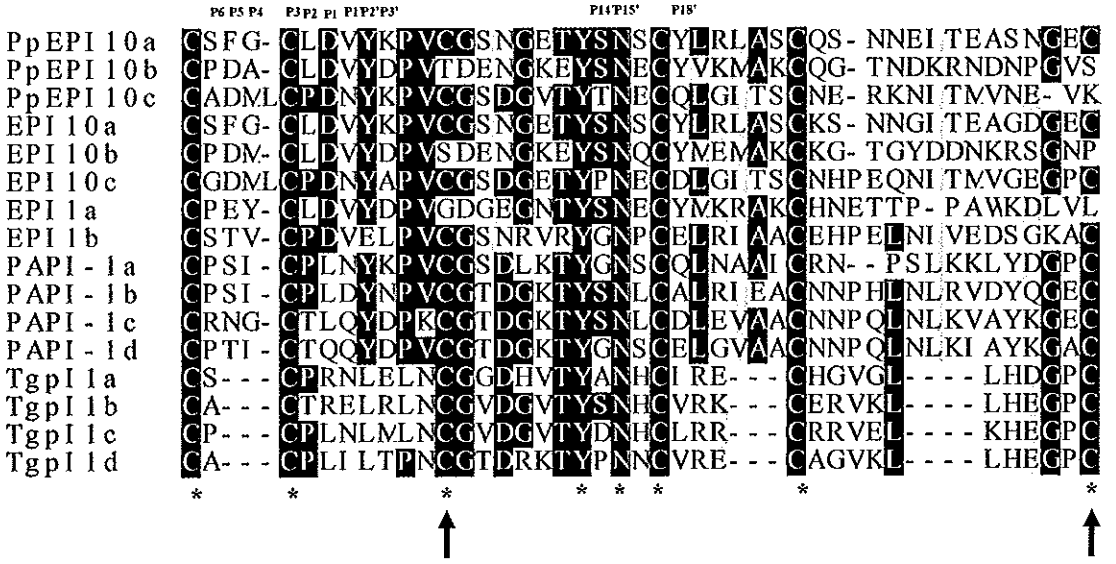


Fig.48 Sequence alignment of Ser protease inhibitor domains. Protease inhibitor PpEPI10a-c, FJ643536 from *P. palmivora*, EPI10a-c, AY586282 and EPI1a-b, AY586273 from *P. infestans*, PAPI-1a-d, CAA56043 from the crayfish *Pacifastacus leniusculus* and TgPI-1a-d, AF121778 from the apicomplexan *T. gondii*. The asterisks indicate amino acid residues that define the Kazal family protease inhibitor domains. The arrows indicate the positions of the Cys residues that was substituted in PpEPI10b or PpEPI10c. The P6, P5, P4, P3, P2, P1, P1', P2', P3', P14', P15' and P18' are contact positions of Kazal domains to interact with their Ser proteases.

2.6 Expression and purification of rKazal1, rKazal2 and rKazal3

rKazal1, rKazal2 and rKazal3 were cloned into the pFLAG-ATS protein expression vector (Sigma) in order to express and purify recombinant proteins fused with FLAG tag at the N-terminus. After performing Western blot analyses of rKazal1, rKazal2 and rKazal3 with FLAG antibody, the result shown that only rKazal1 and rKazal2 were expressed. Additional protein expression constructs (e.g., pGEX 4T-1) were made for expressing and purifying rKazal3, however, none expressed the rKazal 3 domain. Consequently only rKazal1 and rKazal2 were purified by immunoaffinity using a gravity column packed with anti-FLAG M2 affinity gel. The purified rKazal1 and rKazal2 were eluted from the column and run on SDS-PAGE, followed by staining with Coomassie Brilliant Blue. The apparent molecular weights were identified as 17 kDa for rKazal1 (Fig.49 A) and 15 kDa for rKazal2 (Fig.49 B).

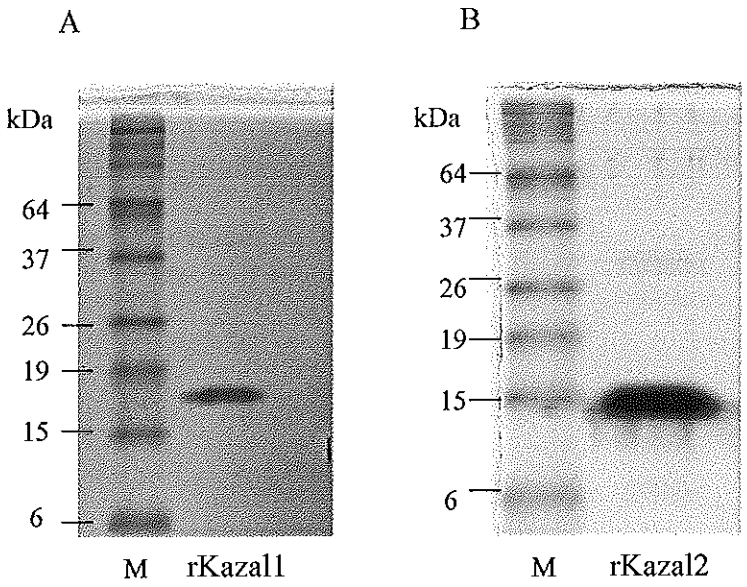


Fig.49 SDS-PAGE analysis of purified rKazal1 and rKazal2 from anti-FLAG M2 affinity column stained with Coomassie Brilliant Blue. (A) Lane M is standard protein markers, a BenchMark pre-stained protein ladder (6-180 kDa). Lane rKazal1 is purified rKazal1 from anti-FLAG M2 affinity column. (B) Lane M is standard protein markers, a BenchMark pre-stained protein ladder (6-180 kDa). Lane rKazal2 is purified rKazal2 from anti-FLAG M2 affinity column.

2.7 Inhibition of commercial Ser proteases with rKazal1 and rKazal2

The rKazal1 and rKazal2 proteins were tested for inhibition of Ser proteases using the colorimetric Quanti-cleave protease assay kit. Three major Ser proteases, chymotrypsin, subtilisin A and trypsin, were used for the inhibition assays with the purified rKazal1 and rKazal2. From experiment inhibition assays three times, the rKazal1 and rKazal2 did not inhibit chymotrypsin and trypsin. However, both of them could inhibit the activity of subtilisin A about 48.06% for rKazal1 and 26.17% for rKazal2 (Fig.50).

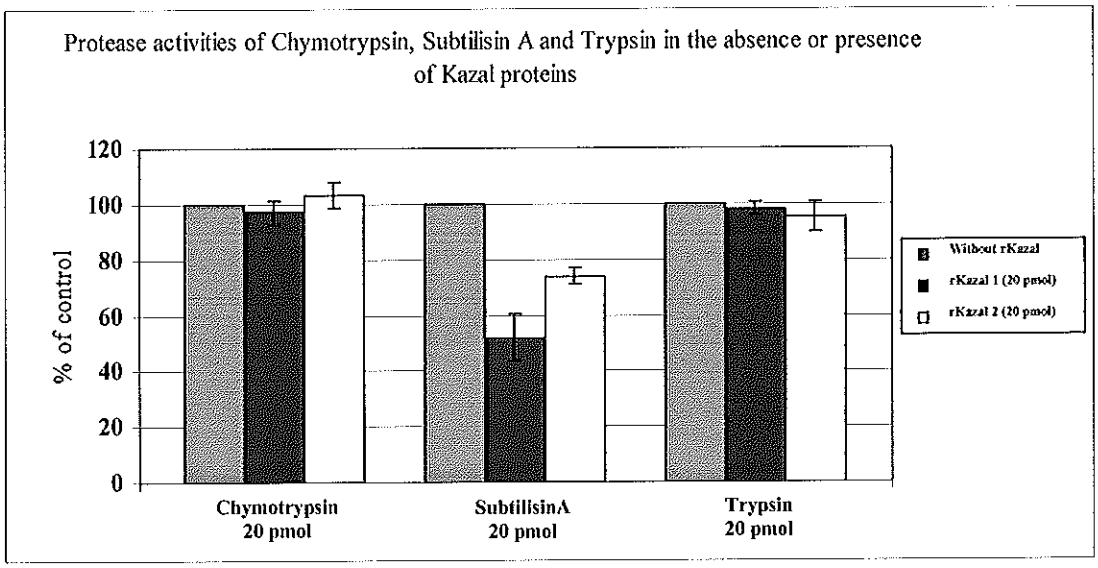


Fig.50 Protease inhibition assay of rKazal1 and rKazal2 using the colorimetric Quanti-Cleave Protease Assay Kit. Standard error of deviation was calculated from the three independent replications. Each replication was performed out of three times.

2.8 Inhibition of plant protease from *H. brasiliensis* with rKazal1 and rKazal2

The rKazal1 and rKazal2 were tested for inhibition of proteases present in rubber leaf by using the zymogram buffer system. Proteins in rubber leaf were incubated with or without rKazal1 and rKazal2 and the protease activity was detected by the gel protease assay. The zymogram gel revealed that the 20 pmol of rKazal1 did completely inhibit the protease present in rubber leaf (Fig.51 A). However, rKazal2 required a higher amount (80 pmol) to inhibit the protease completely (Fig.51 B). The zymogram gels shown that the rubber leaf had at least two proteases but only one of them (protease 95 kDa) was inhibited with rKazal1 and rKazal2 (Fig.51).

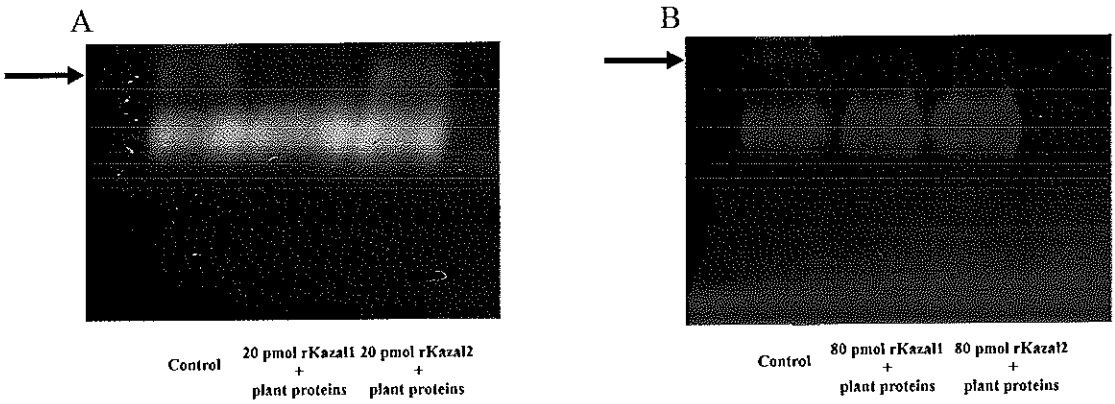


Fig.51 Protease inhibition assay using zymogram buffer system. Control is the rubber leaf proteins without incubation with rKazal1 or rKazal2. The rKazal1+plant proteins are rubber leaf proteins incubated with rKazal1. The rKazal2+plant proteins are rubber tree proteins incubated with rKazal2. (A) The zymogram buffer gel using 20 pmol of rKazal1 or rKazal2 incubated with 8 µl (0.08 mg) of rubber leaf proteins. The arrow indicates the band inhibited by rKazal1. (B) The zymogram buffer gel using 80 pmol of rKazal1or rKazal2 incubated with 8 µl (0.08 mg) of rubber leaf proteins. The arrow indicates the band inhibited by rKazal1 or rKazal2.

2.9 Co-immunoprecipitation

The rKazal1 and rKazal2 were tested for co-immunoprecipitation with rubber tree protease by using FLAG antibody covalently linked to agarose beads. The eluted samples were run on SDS-PAGE and stained with silver nitrate. The gels revealed that both rKazal1 and rKazal2 co-precipitated a 95kDa rubber tree protein, consistent with zymogram buffer system results of protease inhibition (Fig.52).

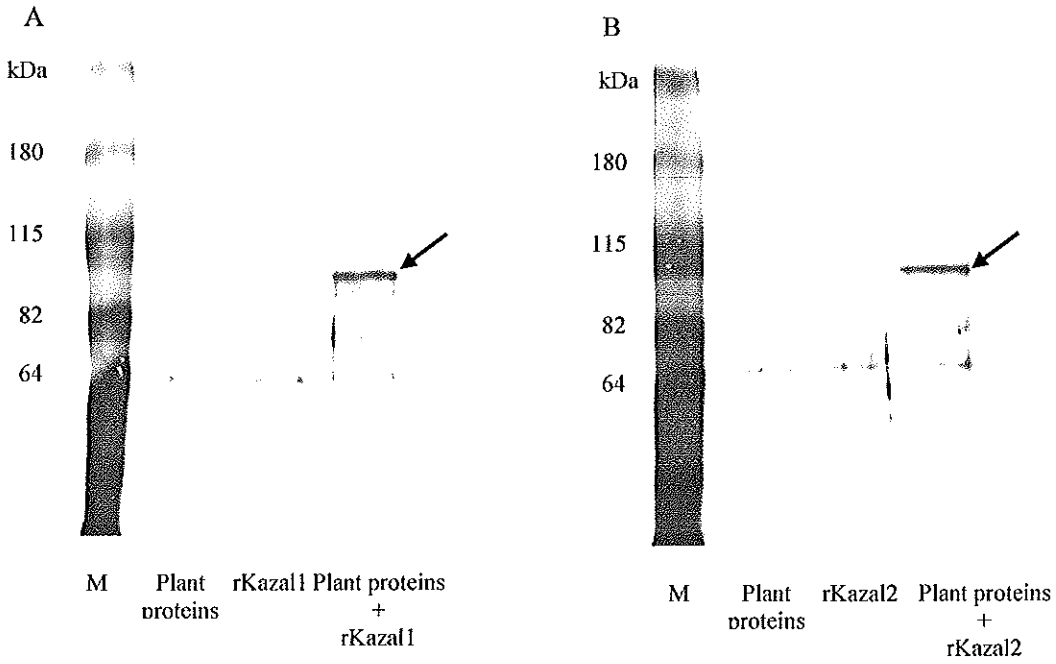


Fig.52 Co-immunoprecipitation of rKazal1, rKazal2 and rubber leaf proteins from FLAG-tagged protein immunoprecipitation kit. The samples from co-immunoprecipitation were run on SDS-PAGE gel followed by staining with silver nitrate. (A) Lane M is standard protein markers, a BenchMark pre-stained protein ladder (6-180 kDa). Lane plant protein is the rubber leaf proteins without incubation with rKazal1. Lane rKazal1 is purified rKazal1 from anti-FLAG M2 affinity column. Lane plant protein+rKazal1 is rubber leaf proteins incubated with rKazal1. The protein band pulled down with the FLAG antibody in the presence of rKazal1 was indicated by arrow. (B) Lane M is standard protein markers, a BenchMark pre-stained protein ladder (6-180 kDa). Lane plant protein is the rubber leaf proteins without incubation with rKazal2. Lane rKazal2 is purified rKazal2 from anti-FLAG M2 affinity column. Lane plant protein+rKazal2 is rubber leaf proteins incubated with rKazal2. The protein band pulled down with the FLAG antibody in the presence of rKazal2 was indicated by arrow.

CHAPTER 4
DISCUSSION

1 Elicitors from *P. palmivora* inducing defense mechanisms in *H. brasiliensis*

1.1 Purification of elicitors from *P. palmivora*

In previous study, elicitin from a culture filtrate of *P. palmivora* was obtained from *P. palmivora* growing in Potato Dextrose Broth (PDB) then DEAE cellulose column and gel filtration were used for elicitin purification. In this study, two major proteins containing elicitor activity, elicitin and a new elicitor from a culture filtrate of *P. palmivora* were obtained by growing *P. palmivora* in Henninger media. The elicitin and the new elicitor from *P. palmivora* grown in Henninger media were produced at higher level than that grown in PDB. The two proteins were purified by precipitation with ammonium sulphate then loading onto a DEAE cellulose column. The elicitin was purified differently from previous study using only one step through DEAE column. By step elution with 0.1-0.5 M NaCl, two peaks of protein which had necrotic activity on tobacco leaves were detected. The purified elicitin was eluted earlier with 0.1 M NaCl and the new elicitor (a 75 kDa) was eluted later with 0.3 M NaCl. However, the fractions of new elicitor eluted with 0.3 M NaCl from DEAE cellulose column were not purified. Then the new protein elicitor fractions were furthered purified by HiTrap Phenyl FF column.

1.2 Elicitin and new elicitor classification

Elicitors may be classified into two groups "general elicitors" and "race specific elicitors". While general elicitors are able to trigger defense both in host and non-host plants, race specific elicitors induce defense responses only in specific hosts. Elicitins induce defense responses on a restricted number of plants, for example, *Nicotiana* species within the Solanaceae family (Bonnet *et al.*, 1996; Kamoun *et al.*, 1993), some members of Brassicaceae (Bonnet *et al.*, 1996) and cranberry (Ivanova *et al.*, 2002).

Elicitins from *P. infestans* and other *Phytophthora* spp. induce the HR and associated biochemical changes related with defense responses in *Nicotiana* spp. but not in its hosts such as potato and tomato (Kamoun *et al.,* 1993, 1997; Ricci *et al.,* 1989). Thus, many laboratories have used tobacco leaves and cell suspensions derived from them for studying the elicitor activity of elicitins. George (2005) has classified the elicitins as race specific elicitors which may be base on the above evidences. In this study, even though both elicitin and the new elicitor induced defense responses with different degree of intensity between two *Hevea* cultivars, they could induce HR cell death in both non-host (tobacco) and host (*Hevea*). As a consequence, they should be grouped into general elicitors.

Virulence factors are molecules produced by a pathogen that specifically cause disease or that influence their host's function to allow the pathogen to thrive. When the resistance (R) gene product appears in the host and enables to recognize the virulence factor, it is then thought of as the avirulence one. Elicitin and the new elicitor could not avoid the detection by the host which resulting in defense responses. Therefore, I proposed that both elicitors are avirulence factors. Nonetheless, with high dose of elicitors they were harmful to host cells especially elicitin which has been proposed to function as sterol carrier protein (Mikes *et al,*. 1997). Elicitin could uptake sterol from host cells which may cause cytoplasm leakage and lead to the collapse of cells. Therefore, they may represent pathogenicity factors because at certain concentrations they can cause dehydration and wilting which are similar to the disease symptoms caused by zoospore inoculation.

1.3 Biological assays in cell suspension of *H. brasiliensis* treated with elicitin and new elicitor

From field tests at several Rubber Research Centers in Thailand, RRIM600 is the most susceptible cultivar, where as BPM-24 is the most resistant cultivar to *P. palmivora*. However, the RRIM600 leaves were able to express HR cell death and synthesize Scp especially when the applied zoospores of *P. palmivora* were not overloaded (Chirapongsatonkul *et al.*, 2008). In addition, after treating seeds of both cultivars with elicitin, the induction of Scp and peroxidase isozymes was 2-3 fold higher in the BPM-24 cultivar than in the RRIM600. The responses were also 2- 3

fold stronger in the calli derived from integument of the BPM-24 cultivar than that from the RRIM600 (Chirapongsatonkul *et al.*, 2008). Therefore, in this study, BPM-24 cell suspension of *H. brasiliensis* was used to demonstrate the synthesises of phenolic compounds, Scp and peroxidase and the RRIM600 plant was selected to test for local resistance and biological assays on young rubber tree seedlings treated with elicitin and new elicitor.

Plants resist the pathogens infection in various ways. Such as accumulation of phenolic compounds and production of antimicrobial compounds, called phytoalexins, those kill the pathogen or restrict its intracellular development (Darvill and Albersheim, 1984). *H. brasiliensis* synthesizes a hydroxycoumarin phytoalexin called scopoletin (Scp) after fungal infection (Giesemann *et al.*, 1986). Peroxidase (POD) is an enzyme associated with the plant defense pathway and is elicited when challenged with elicitors. Peroxidase can oxidize various substrates due to its ability to perform a number of different types of reactions, such as peroxidation, oxidation or oxidative and catalytic hydroxylation (Hiroshi and Shiro, 2003).

Accumulation of phenolic compounds and Scp after treatment with either elicitor had similar kinetics with a peak concentration at 64 h after treatment (Fig.32, 33), suggestion that phenolic compounds were intermediate substrate for Scp accumulation. Other pathway of plant defenses in cell suspension used very low of phenolic compounds as intermediate substrate then resulting did not detect the peak of phenolic compounds at any time point except time point of the peak of Scp accumulation. For peroxidase activity, both elicitors produced similar patterns of accumulation; however, the activity of *o*-dianisidine peroxidase peaked at 24 h (Fig.34) whereas the scopoletin peroxidase peaked at 64 h (Fig.33). Therefore, the two peroxidase activities are associated with different enzymes. Suggest that the scopoletin peroxidase was produced for eliminating the Scp synthesized in cell suspension after being treated with elicitors. On the other hand, the *o*-dianisidine peroxidase may be produced for other reasons such as detoxifying of H_2O_2 from the rapid response oxidative burst. In a previous study, the role of the peroxidase enzyme in the metabolism of Scp has been investigated in sunflower (Edwards *et al.*, 1997). 10 μM $CuCl_2$ and 100 μM salicylic acid produced a rapid increase of scopoletin peroxidase activity in sunflower leaves than that with wounding. In that case, the

highest peak of scopoletin peroxidase activity appeared at 72 h after treatment with elicitors, and the disappearance of Scp was associated with the increase activity of a peroxidase that metabolized the coumarin to a coloured insoluble metabolite (Edwards *et al.*, 1997). When sunflower leaves were challenged with a fungal pathogen (Tal and Robeson, 1986) or $CuCl_2$ (Gutiérrez *et al.*, 1995), Scp accumulated and then declined after the appearance of scopoletin peroxidase. Therefore, the function of the peroxidase for substrate scopoletin may be to protect plants from the potential phytotoxic effects of Scp.

In order to produce the phenolic compounds, Scp and peroxidase production in *H. brasiliensis* cell suspension, the new elicitor required 30 fold lower concentration in comparing to elicitin. This study demonstrates that the new elicitor could activate defense responses at much less concentration than the elicitin. However, the activity of the 75 kDa elicitor was maximized at concentration about 2 fold lower than that of elicitin for induction of local resistance against *P. palmivora* on rubber plant young seedlings. When the *H. brasiliensis* leaves were treated with both elicitors, the new elicitor also required about 2 fold lower concentration when compared with elicitin for inducing phenolic compounds and *o*-dianisidine peroxidase production (Fig.37, 38). The different strength of these two elicitors in two systems may be due to the different size of them. The larger size of the new elicitor may penetrate through cell wall of leaves with more difficulty than the elicitin. Whereas, the cell suspension does not contain cell wall; therefore, the accessions by both elicitors are relatively equal. In addition, because the cell suspension was derived from an integument of *H. brasiliensis* seed, this may perceive a different recognition.

1.4 Biological assays on young rubber tree seedlings treated with elicitin and new elicitor

1.4.1 Induction of locl resistance, phenolic compounds and *o*-dianisidine peroxidase on rubber plants treated with elicitin and new elicitor

The 1 nM of elicitin and 0.5 nM of new elicitor were used for assays of phenolic compounds accumulation and inducing of *o*-dianisidine peroxidase activity on rubber plants because they are the minimum concentrations of each elicitor that exhibited a strong resistance to *P. palmivora*. This study indicated that cell suspension

of *H. brasiliensis* and rubber plants had difference in defense responses and accumulation of phenolic compounds and *o*-dianisidine peroxidase activity after inducing with each elicitor. That is *o*-dianisidine peroxidase activity in cell suspension was induced much higher and faster than that in rubber plant leaves. In contrast, accumulation of phenolic compounds in cell suspension was detected at lower level than that observed in rubber plant leaves.

1.4.2 Measurement of *o*-dianisidine peroxidase activity during expression of local resistance

The *o*-dianisidine peroxidase activity was selected to show expression of defense response after inoculation with zoospores of *P. palmivora* because it obviously expressed activity in both cell suspension and rubber plant seedlings. The obtained result indicated that both elicitors could trigger *o*-dianisidine peroxidase activity and then was enhanced again after infection with *P. palmivora.* The plants triggered earlier with both elicitors, produced second peak of *o*-dianisidine peroxidase activity stronger and more rapidly than control plants. That is plants treated with both elicitors produced second peak of 1.42×10^5 unit mg^{-1} protein for both elicitors at 72 h but for control the increase in *o*-dianisidine peroxidase activity reached a peak of 1.25×10^5 unit mg^{-1} protein at 80 h (Fig.39). Therefore, the strong resistance occurred in treated plants should be due to a higher and faster defense response at least an *o*-dianisidine peroxidase.

2 Protease inhibitor from *P. palmivora* inhibiting protease in *H. brasiliensis*

2.1 Kazal domains and sequence analyses

The *Ppepi10* gene shares approximately 72% sequence similarity to *P. infestans epi10* by analyses with the NCBI BLAST and also encodes for a protein with three Kazal domains of a Ser protease inhibitor (Fig.47). The first domain (PpEPI10a) was similar to the Kazal domains from previously identified oomycete inhibitors that contained all six highly conserved cysteine residues, suggesting both an evolutionary and functional conservation among this class of inhibitors. The signature domains for the Kazal family of inhibitor include the cysteine backbone, tyrosine, and asparagine residues (Fig.48). The second domain (PpEPI10b) and the third domain

(PpEPI10c) were atypical, lacking the third and sixth Cys for PpEPI10b and the sixth Cys for PpEPI10c; nonetheless, PpEPI10b had the same pattern of abnormal sequences to that of the EPI10b domain of *P. infestans*. That is EPI10b from *P. infestans* also lacked both the third and sixth Cys but retained the other four Cys (Fig. 48). Maybe the lack of the third and sixth Cys is a particular sequence pattern of second domain of EPI10 from *Phytophthora* species. However, this is only speculation, and this conclusion can only be made once additional Ser protease inhibitor sequences are available.

2.2 Expression and purification of rKazal1, rKazal2 and rKazal3

The rKazal1, rKazal2 and rKazal3 were expressed in *E. coli* BL21. After Western blot analyses of rKazal1, rKazal2 and rKazal3, it was found that only rKazal1 and rKazal2 expressed in *E. coli* BL21 using pFLAG-ATS vector. Additional protein expression constructs (e.g., pGEX 4T-1) were made for expressing and purifying Kazal3, however, none expressed the Kazal 3 domain. These data seem to suggest that only Kazal1 and Kazal2 domains of PpEPI10 encode a functional protease inhibitor. For the Kazal3 domain, it is possible that this protein is able to bind, but not able to inhibit the target protease. This may lead to the access of the Kazal3 protein to the protease when it stands alone without protection from Kazal1 and Kazal2 which have protease inhibitor activity. Since the inhibitors have at least one peptide bond called the reactive site that interacts with the enzyme's catalytic site and is the one that is cleaved when hydrolysis occurs (Read *et al.*, 1983). If this is true, it is likely that the Kazal3 peptide is not detected following expression in *E. coli* because it was degraded very quickly.

2.3 Inhibition of commercial Ser proteases with rKazal1 and rKazal2

Protein inhibitors of Ser proteases are well studied and much is known about the interactions between an inhibitor and its cognate enzyme (Laskowski and Kato, 1980). Most of these inhibitors act by a common mechanism. That is they bind very tightly to the enzyme (low K_m) but are hydrolyzed very slowly (Read *et al.*, 1983). The inhibitors have at least one peptide bond called the reactive site that interacts with the enzyme's catalytic site and is the one that is cleaved when

hydrolysis occurs (Read *et al.*, 1983). The concentration of protein inhibitors that reduces enzyme velocity by half is called the EC50 or IC50. If the dissociation constant for binding of inhibitor to enzyme (Ki) is low (the affinity is high), the EC50 will be low. The higher activity of rKazal1 on enzyme subtilisin A in Fig.51 inferred that Ki of rKazal1 is lower than rKazal2.

However, the lack of the third and sixth Cys for rKazal2 should not affect the Ki of rKazal2 because the contact residues of Kazal domains to interact with their Ser proteases are not the third and sixth Cys position. The contact positions of Kazal domains presented by Stephen ML. *et al.* (2001), P6, P5, P4, P3, P2, P1, P1', P2', P3', P14', P15' and P18' were shown in Fig.48. Nevertheless, the P3 (the second conserved cysteine residue) and P15' (a conserved asparagine residue) of contact positions show a low variation in each Kazal domain then the other 10 contact positions were usually used for calculation the Ki of Kazal domains (Stephen *et al.*, 2001)

2.4 Interaction and inhibition of plant protease from *H. brasiliensis* with rKazal1 and rKazal2

The amount of rKazal2 (80 pmol) to inhibit protease from rubber leaf was 4 times higher than that of rKazal1 (20 pmol) and yet only one band was removed by these two inhibitors (Fig.51). This result indicated that the other protease isozymes in rubber leaf were not subtilase type. Co-immunoprecipitation was also performed on rubber tree proteins incubated with rKazal1 and rKazal2 to identify the cognate protease from rubber tree. Both rKazal1 and rKazal2 could interact with one of the proteins from rubber tree which should be rubber tree protease (Fig.52). As a consequence, rKazal1 and rKazal2 should interact with the same rubber leaf protease because the proteins from our co-immunoprecipitation of rKazal1 and rKazal2 with rubber leaf protein each had a molecular weight of about 95 kDa (Fig.52) which concurs with the result obtained in Fig. 51. This 95 kDa protein was the only protease isozyme in rubber leaf that was targeted by the inhibitor from *P. palmivora*. I hypothesize that these 95 kDa proteins are PR-proteins found in *H. brasiliensis*, and as such, serve a primary role in plant defense.

CHAPTER 5
CONCLUSION

1 Elicitors from *P. palmivora* inducing defense mechanisms in *H. brasiliensis*

H. brasiliensis is more responsive to the new elicitor (75 kDa) than to the elicitin (10 kDa) in triggering defense responses. That is the new elicitor can trigger defense mechanism, at least accumulations of phenolic compounds, Scp, peroxidase and inducing local resistance, in *H. brasiliensis* higher than elicitin does. As a consequence, the role of 75 kDa elicitor in pathogenesis of *P. palmivora* remains to be studied.

2 Protease inhibitor from *P. palmivora* inhibiting protease in *H. brasiliensis*

PpEPI10 from *P. palmivora* was a Kazal-like extracellular Ser protease inhibitor containing tree Kazal domains. Kazal1 and Kazal 2 were the important functional domains to inhibit protease from rubber leaf and Kazal1 has a higher activity than rKazal2 to inhibit Ser protease. However, the mutation of Kazal1 and Kazal 2 should be further studied to identify the sequences of Kazal1 and Kazal 2 that have protease inhibitor function.

Applications

1. The new protein elicitor (75 kDa) could induce defense machanisms on rubber tree at lower concentration than elicitin. Therefore, it can be replaced elicitin to use as plant elicitor.
2. Kazal1 and Kazal 2 are pretease inhibitors from *P. palmivora*. They can be used to study reactions of plant defense inhibition between *P. palmivora* and rubber tree in molecular level.

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APPENDIX

1. V_8 agar

$CaCO_3$	3	g
V_8 juice	200	ml
Agar	20	g
Distilled water	800	g

V_8 juice was mixed with distilled water. Then the $CaCO_3$ and agar were added into the mixture and gently stirred. The media was sterilized prior to pouring into sterilized plates.

2. Henninger medium

KH_2PO_4	0.5	g
$MgSO_4.7H_2O$	0.25	g
Asparagine	1	g
Thiamine	1	mg
Yeast extract	0.5	g
Glucose	25	g

All of chemical above was added into 1 l of distilled water and gently stirred. The media was sterilized prior to using.

3. Ingredients of Murashige and Skoog's (MS) medium for callus induction

Table 7 Ingredients of Murashige and Skoog's (MS) medium for callus induction

Ingredients	(mg/l)
KNO_3	1900
NH_4NO_3	1650
$CaCl_2.2H_2O$	322.2
$MgSO_4.7H_2O$	180.7
KH_2PO_4	170
$MnSO_4.H_2O$	16.9
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Na_2EDTA	37.25
$FeSO_4.7H_2O$	27.85
Thiamine.HCl	0.02
Pyridoxine.HCl	0.10
Nicotinic acid	0.10
Glycine	0.4
Myo-inositol	100
BA	1.0
2,4-D	1.0
Sucrose (%)	5

Ingredients	(mg/l)
Phytagel agar (%)	0.23
pH	5.7

4. Ingredients of MS medium for cell suspension

Table 8 Ingredients of Murashige and Skoog's (MS) medium for cell suspension

Ingredients	(mg/l)
KNO_3	1900
NH_4NO_3	1650
$CaCl_2.2H_2O$	322.2
$MgSO_4.7H_2O$	180.7
KH_2PO_4	170
$MnSO_4.H_2O$	16.9
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Na_2EDTA	37.25
$FeSO_4.7H_2O$	27.85
Thiamine.HCl	0.02
Pyridoxine.HCl	0.10
Nicotinic acid	0.10
Glycine	0.4

Ingredients	(mg/l)
Myo-inositol	100
2,4-D	1.0
TDZ	0.1
Sucrose (%)	3
pH	5.7

5. The Bicinchoninic solution for protein measurement by Bicinchoninic method (BCA method)

Solution A:

1. BCA-Na_2 1% (w/v)
2. Sodium carbonate 2% (w/v)
3. Sodium tartrate 0.16% (w/v)
4. Sodium hydroxide 0.4% (w/v)
5. Sodium bicarbonate 0.95% (w/v)

The mixture is adjusted to pH 11.25.

Solution B:

Copper sulphate ($CuSO_4.5H_2O$) 4% (w/v)

Solution C:

Solution A 25 ml

Solution B 0.5 ml

The mixture is stable for 1 week at room temperature.

6. The Bradford solution for protein measurement by Bradford method

Bradford reagent: 100 mg of Coomassie brilliant blue G-250 was dissolved in 50 ml of 95% ethanol. After that 100 ml of 85% phosphoric acid was added into the mixture and mixed. Then 850 ml of distilled water was added and mixed again. The solution was filtered prior to using.

7. The 10x Phosphate buffered saline (PBS buffer)

NaCl	80 g
KCl	2 g
Na_2HPO_4	6.1 g
KH_2PO_4	2 g

All of chemical above was added into 1 l of distilled water and gently stirred. The solution did not adjust pH or autoclave prior to using. It was kept at room temperature.

8. The 1x Phosphate buffered saline Tween (PBST buffer)

10x PBS	100 ml
H_2O	900 ml
Tween-20	1 ml

The solution was kept at room temperature.

9. Tris buffered saline (TBS buffer)

The TBS buffer was composed of 50 mM Tris-HCl, 150 mM NaCl and distilled water. The buffer was adjusted pH to 7.4 and autoclaved prior to using. It was kept at 4 °C.

10. The 4x loading buffer

The 4x loading buffer contained: 125 mM Tris-HCl, pH 6.8, 50% glycerol, 4% SDS, 0.02% bromophenol blue and distilled water. The 4x loading buffer was diluted to 2x loading buffer before using.

11. The 10x SDS-PAGE running buffer

Tris-HCl	30.3 g
Glycine	144 g
SDS	10 g
Distilled water	1 l

The solution was mixed with stirrer before using and kept at room temperature.

12. The 0.1% Coomassie blue for protein gel staining

The 0.5 g of Coomassie blue was added into 200 ml of methanol. The mixture was stirred for 20 min. Then 250 ml of distilled water and 50 ml of acetic acid were added into the mixture and mixed.

The solution for destain was composed of 200 ml of methanol, 250 ml of distilled water and 50 ml of acetic acid.

13. Silver staining

The protein gel was incubated in the mixture of 53 ml of 95% ethanol, 10 ml of acetic acid and 37 ml of distilled water for 10 min. Then the gel was washed with 50% ethanol for 15 min followed by washing with distilled water 3 times for 5 min in each time. After that the gel was incubated in 0.02% sodium thiosulfate ($Na_2S_2O_3.5H_2O$) for 1 min and washed with distilled water 3 times for 30 seconds in each time. The gel was stained with silver nitrate solution (1 g of $AgNO_3$, 375 µl of 37% HCOH and 500 ml of distilled water) for 20 min followed by washing with distilled water 2 times for 20 seconds in each time. After that the develop solution (6 g of Na_2CO_3, 50 µl of 37% HCOH, 2 ml of $Na_2S_2O_3.5H_2O$ and 98 ml of distilled water) was added to the gel and incubated until the band appeared on the gel. The reaction was stopped with stop solution (53 ml of 95% ethanol, 10 ml of acetic acid and 37 ml of distilled water) and the gel was stored in 50% ethanol.

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List of Publications and Proceedings

Publications

Chinnapun, D. and Churngchow, N. 2006. Defense responses in *Hevea brasiliensis* induced by polypeptide elicitors secreted by *Phytophthora palmivora.* Agricultural Sci. J. 37 (6) (Suppl), 1035-1038.

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