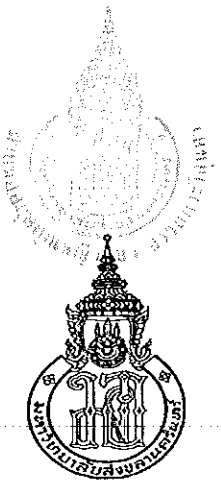

The Defense Mechanism of *Hevea brasiliensis* Against *Phytophthora* spp. and the Crosstalk between the Salicylic Acid and Jasmonic Acid Signaling Pathways Induced by Elicitors

Nion Chirapongsatonkul

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Author	Miss Nion Chirapongsatonkul
Major Program	Biochemistry

Major Advisor

.....
(Assoc. Prof. Dr. Nunta Churngchow)

Co-advisor

.....
(Assoc. Prof. Dr. Nongporn Towatana)

Examining Committee :

.....Chairperson
(Assoc. Prof. Dr. Prapaporn Utarabhand)

.....Committee
(Assoc. Prof. Dr. Nunta Churngchow)

.....Committee
(Assoc. Prof. Dr. Nongporn Towatana)

.....Committee
(Dr. Serge KAUFFMANN)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Biochemistry

.....
(Assoc. Prof. Dr. Krerkchai Thongnoo)
Dean of Graduate School

ชื่อวิทยานิพนธ์	กลไกการต้านทานโรคของยางพาราต่อเชื้อ *Phytophthora* spp. และปฏิสัมพันธ์ระหว่างวิถีกรดซาลิไซลิกและวิถีกรดจัสโมนิกเมื่อถูกกระตุ้นด้วยอิลิซิเตอร์ต่างๆ
ผู้เขียน	นางสาวนิอร จิรพงศธรกุล
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บทคัดย่อ

Phytophthora palmivora เป็นสาเหตุของโรคใบร่วงในยางพารา โดยเชื้อดังกล่าวสามารถแทรกผ่านทางรอยแผลที่กรีดเปิดหน้ายางซึ่งทำให้ผลผลิตยางพาราลดลง ได้ทำการศึกษาระบบ defense response ในใบและแคลลัสของยางพาราสองพันธุ์ คือ พันธุ์ต้านทาน (BPM-24) และพันธุ์อ่อนแอ (RRIM600) เมื่อถูกกระตุ้นด้วย zoospore และ elicitin ของเชื้อ *Phytophthora* spp. 3 สายพันธุ์ ได้แก่ *P. palmivora* ที่แยกได้จากยางพารา [P. pal (Hevea)], *P. palmivora* ที่แยกได้จากทุเรียน [P. pal (Durian)] และ *P. parasitica* ที่แยกได้จากต้นหน้าวัว [P. para] หลังจากบ่มใบยางด้วย zoospore ของเชื้อทั้งสามสายพันธุ์ที่ความเข้มข้น $5x10^6$ zoospores/ml พบว่า รอยแผลที่เกิดจากเชื้อ P. pal (Hevea) มีขนาดใหญ่ที่สุด รองลงมาคือ P. pal (Durian) และ P. para นอกจากนี้ P. pal (Hevea) ยังกระตุ้นให้มีการสร้าง scopoletin (Scp) ซึ่งเป็น phytoalexin ที่สร้างในยางพาราในอัตราและปริมาณที่สูงกว่าเมื่อเทียบกับเชื้ออีก 2 สายพันธุ์ ผลดังกล่าวแสดงให้เห็นว่าเชื้อทั้งสามมีความสามารถแตกต่างกันในการเข้าเจาะทำลายรวมถึงการเจริญเติบโตในใบยางพาราหรือมีความรุนแรงต่อยางพารต่างกัน ซึ่งสามารถเรียงลำดับจากมากไปน้อยได้ดังนี้ คือ P. pal (Hevea) > P. pal (Durian) > P. para และเมื่อบ่มใบยางด้วย elicitin (2.5 ไมโครกรัม) พบว่า elicitin จากเชื้อ *P. palmivora* ทั้งที่แยกจากยางพาราและทุเรียนกระตุ้นให้พืชสร้าง Scp ได้ใกล้เคียงกันในขณะที่ elicitin จาก P. para กระตุ้นให้มีการสร้าง Scp ได้น้อยและช้ากว่า นอกจากนี้ยังได้ทำการทดลองบ่มแคลลัสของยางพาราทั้งสองสายพันธุ์ด้วย zoospore ของเชื้อ P. pal (Hevea) และ P. para ซึ่งผลที่ได้จากแคลลัสคล้ายคลึงกับผลจากการทดลองในใบ และเมื่อเปรียบเทียบที่สภาวะการทดลองที่เหมาะสมพืชพันธุ์ต้านทานจะตอบสนองในรูปแบบของ hypersensitive response (HR) ในขณะที่พืชพันธุ์อ่อนแอจะเกิดอาการของโรคหรือที่เรียกว่า disease แต่เมื่อพืชอยู่ในสภาวะการทดลองที่รุนแรงขึ้น (zoospore หรือ elicitin ที่ความเข้มข้นสูงมาก) พืชพันธุ์ต้านทานจะเกิด disease แต่ในทางตรงข้าม เมื่อสภาวะการทดลองลดความรุนแรงลง (ใช้ zoospore หรือ elicitin ที่ความเข้มข้นต่ำมาก) พืชพันธุ์อ่อนแอเองก็สามารถแสดงออกแบบ HR ได้ โดยพบว่า

ความสามารถในการกระตุ้นระบบ defense response เช่น การสร้าง Scp และการทำงานของเอนไซม์ peroxidase (POD) สัมพันธ์กับความต้านทานของพันธุ์ยางพาราที่ทดสอบ จากข้อมูลข้างต้น ปฏิสัมพันธ์ระหว่างใบยางพาราและ zoospore ของ P. pal (Hevea) จะถูกใช้เป็น plant-pathovar system ในการศึกษาต่อไป

เชื้อ *P. palmivora* กระตุ้นให้เกิดการตายของเซลล์ (cell death) ในใบยางพันธุ์ต้านทานและพันธุ์อ่อนแอ แปรผันตรงกับความเข้มข้นของ zoospore ที่ใช้ และเวลาของการทดสอบ การย้อม cell death ด้วยสี trypan blue ทำให้แยกการตายของเซลล์ในใบยางได้สองแบบ คือ แบบ disease-like cell death ซึ่งพบในเนื้อเยื่อที่มีการลามของเชื้อ และแบบ HR-like cell death ในเนื้อเยื่อบริเวณที่เชื้อถูกกักบริเวณไว้ จากผลการทดลองพบว่าระดับความต้านทานของยางพาราพันธุ์ต้านทาน BPM-24 สูงกว่าพันธุ์อ่อนแอ RRIM600 ประมาณ 10 เท่า นอกจากนี้ยังได้ศึกษาปฏิกิริยา oxidative burst ในใบยางเมื่อถูกกระตุ้นด้วย zoospore ของ *P. palmivora* ซึ่งติดตามการสร้าง superoxide anion (O_2^-) โดยการย้อมด้วย nitroblue tetrazolium (NBT) และ hydrogen peroxide (H_2O_2) โดยการย้อมด้วยสี 3, 3'-diaminobenzidine (DAB) รวมถึงการใช้ ตัวยับยั้งการทำงานของเอนไซม์ ที่เกี่ยวข้องกับการเกิด oxidative burst ได้แก่ สาร diphenylene iodonium (DPI) ซึ่งยับยั้งการทำงานของเอนไซม์ NADPH oxidase และ diethyldithiocarbamate (DDC) ซึ่งยับยั้งการทำงานของเอนไซม์ superoxide dismutase (SOD) ทำให้สามารถสรุปได้ว่า ในระบบการทดลองนี้ O_2^- ถูกสร้างขึ้นจากเอนไซม์ NADPH oxidase ในขณะที่ H_2O_2 บางส่วนนั้นสร้างมาจากเปลี่ยน O_2^- โดยอาศัยการทำงานของเอนไซม์ SOD และคาดว่า H_2O_2 อาจจะถูกสร้างจากกระบวนการอื่น ๆ ด้วย นอกจากนี้พบว่าทั้ง O_2^- และ H_2O_2 มีบทบาทเกี่ยวข้องในการเกิด cell death ซึ่งจะมีผลยับยั้งการลุกลามของเชื้อ ในขณะที่ O_2^- เท่านั้นที่จำเป็นต่อการกระตุ้นให้มีการสร้าง Scp

จากผลการทดลองระดับ field test พบว่า TTF5P (สารผลิตภัณฑ์ของบริษัท Tribo technologies สามารถกระตุ้นให้พืชหลายชนิดต้านทานต่อเชื้อโรคต่าง ๆ อย่างกว้างขวาง) TTF5P ประกอบด้วย ปุ๋ย NPK, กรดอะมิโน (amino acids; AA) และสารสกัดจากสาหร่าย (algae extract) ผลการทดลองในห้องปฏิบัติการพบว่า TTF5P สามารถกระตุ้นให้มีการสร้าง pathogenesis-related (PR)-proteins และกระตุ้นยีนที่เกี่ยวข้องในกระบวนการ phenylpropanoid pathway ในใบยาสูบ ซึ่ง PR-proteins ดังกล่าวถูกใช้เป็นเครื่องหมาย (marker) ของ salicylic acid (SA)- และ ethylene/jasmonic acid (ET/JA)-dependent defense responses เมื่อแทนที่สารสกัดจากสาหร่ายด้วย laminarin (Lam) และ กรดอะมิโนด้วย β-aminobutyric acid (BABA) ส่งผลให้ความสามารถในการกระตุ้นระบบ defense response ของพืชสูงขึ้น สิ่งที่น่าสนใจคือ Lam ลดการสร้าง PR1 ที่ถูกกระตุ้นด้วย BABA ซึ่งสอดคล้องกับผลการทดลองของ Benoît Boachon (นักศึกษาปริญญาโท ณ IBMP) ที่ได้รายงานไว้ว่า Lam ยับยั้งการแสดงออกของยีน *PR1* เมื่อทำการทดสอบด้วยสารผสมของ Lam กับ sulfated

laminarin (PS3) ผู้วิจัยจึงทำการ infiltrate สาร Lam, PS3 และ Lam+PS3 เข้าสู่ใบของพืชสองชนิด คือ ยาสูบและ *Arabidopsis* ผลการทดลองจากยาสูบและ *Arabidopsis* สายพันธุ์ปกติ (wild type) Col-0 แสดงให้เห็นถึงความสามารถของ Lam ในการยับยั้งการแสดงออกของยีน *PR1* และการสร้าง SA ในขณะที่พืช mutant ซึ่งมีความผิดปกติใน JA signaling pathway หรือสายพันธุ์ *coi1-16* ไม่พบการยับยั้งดังกล่าว ดังนั้นผลการทดลองดังกล่าวทำให้สันนิษฐานได้ว่า Lam ยับยั้ง SA-dependent defense responses โดยผ่านทาง JA นอกจากนี้พบว่า Lam (ในชุดการทดลอง Lam+PS3) มีผลให้จำนวนแบคทีเรีย *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) สูงขึ้นเมื่อเปรียบเทียบกับจำนวนแบคทีเรียในชุดการทดลองของ PS3 อย่างเดียว ซึ่งสอดคล้องกับการเกิดโรคและรอยแผล (disease lesion) ในชุดการทดลองของ Lam+PS3 หลังจากได้รับเชื้อ *Peronospora parasitica* (*Pp*) จะมากขึ้นเมื่อเทียบกับพืชที่กระตุ้นด้วย PS3 อย่างเดียว แต่ใน *coi1-16* mutant กลับพบว่าจำนวนเชื้อแบคทีเรียมีความใกล้เคียงกันในทุก ๆ การทดลอง (เมื่อถูก infiltrate ด้วย Lam PS3 และ Lam+PS3) แต่จะน้อยกว่าพืชควบคุม ซึ่งเป็นการยืนยันว่า Lam ยับยั้ง SA-dependent defense responses รวมถึงยับยั้งการต้านทานของพืชที่ควบคุมโดย SA signaling pathway ผ่านทาง JA นอกจากนี้เมื่อใช้ *Arabidopsis* ที่มีความผิดปกติใน SA signaling pathway (*sid2/nahG*) ยังพบว่า JA ก็ถูกยับยั้งโดย SA ด้วย

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Author	Miss Nion Chirapongsatonkul
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ABSTRACT

Phytophthora spp., especially *P. palmivora*, is a causal agent of most important disease of the rubber tree, *Hevea brasiliensis*. It can attack the tapping surface resulting in poor latex production. The defense responses of leaves and calli of two cultivars (resistant; BPM-24 and susceptible; RRIM600) of the rubber tree against zoospores and elicitin purified from *Phytophthora* spp. were investigated. Zoospores of three strains of *Phytophthora* spp.; *P. palmivora Hevea* isolate [P. pal (Hevea)], *P. palmivora Durian* isolate [P. pal (Durian)] and *P. parasitica Anthurium* isolate [P. para] were inoculated on *Hevea* leaves. At the same zoospore concentration ($5x10^6$ zoospores/ml), P. pal (Hevea) caused larger expanded lesions than did P. pal (Durian) and P. para. The speed and extent of scopoletin (Scp), a phytoalexin produced in *H. brasiliensis*, detected in tissues inoculated with P. pal (Hevea) was produced much higher than those treated with P. pal (Durian) and P. para. The different abilities of penetration and growing in *Hevea* plant which could be demonstrated by using different species and/or different isolate of the pathogens. The results showed that P. pal (Hevea) is the most virulent to *Hevea* followed by P. pal (Durian) and P. para, respectively. After *Hevea* leaves of BPM-24 and RRIM600 were treated with elicitins (2.5 µg/droplet), these proteins from P. pal (Hevea) and P. pal (Durian) elicited the Scp production in similar pattern whereas its accumulation triggered by P. para elicitin was delayed and much lower. The responses to zoospores of P. pal (Hevea) and P. para in *Hevea* calli were similar to those detected in *Hevea* leaves. At the optimal treatment condition, resistant cultivar normally exhibited hypersensitive response (HR) whereas susceptible one displayed disease. The HR can

shift to disease in the resistant cultivar under severe condition (very high zoospore or elicitin concentration). Likewise, the susceptible cultivar can also display HR after treatment with mild condition (low zoospore or elicitin amount). The expressed strength of defense responses, Scp accumulation and enzyme peroxidase activity, is correlated to the resistance of tested cultivars. The interaction between *Hevea* leaves and zoospores of P. pal (Hevea) was selected to use as model for further studies.

Cell death, in leaves of a resistant BPM-24 and susceptible RRIM600 cultivars of *H. brasiliensis* was induced in a dose- and time-dependent manner by *P. palmivora* zoospores. Two types of cell death; disease- and HR-like cell death, based on the trypan blue staining was observed in our system depend on the tested conditions. First type was occurred in the tissues displayed pathogen spreading or when inoculated with high zoospore amount whereas the latter was observed in the tissues restricted or stopped the pathogen expansion. Resistant responses from BPM-24 were generally 10 fold greater than those from RRIM600. The oxidative burst was followed by superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) production detected by using nitroblue tetrazolium (NBT) and 3, 3'-diaminobenzidine (DAB) staining, respectively. Diphenylene iodonium, (DPI) an inhibitor of NADPH oxidase and diethyldithiocarbamate (DDC) an inhibitor of superoxide dismutase (SOD) showed that NADPH oxidase produced O_2^-, and H_2O_2 was dismutased from O_2^- via SOD but other pathways were also taken part. Both O_2^- and H_2O_2 were involved in the HR cell death and preventing pathogen spread whereas O_2^-, rather than H_2O_2 was essential for Scp production.

TTF5P, a product from Tribo technologies composed of three components; NPK, amino acid (AA) and algae extract. Since it showed strong resistance against various pathogens in different plant species in field tests, its elicitation activity to induce defense responses in tobacco plants was determined in this study. Pathogenesis-related (PR)-proteins, which known as markers of salicylic acid (SA)- and ethylene/jasmonic acid (ET/JA)-dependent defense responses, and defense genes involved in phenylpropanoid pathway were induced after TTF5P treatment in laboratory experiments indicating its efficiency elicitation activity in wide range of plant defense responses. Replacing algae extract and AA by laminarin

(Lam) and nonprotein β-aminobutyric acid (BABA), respectively, exhibited stronger PR proteins expression. Surprisingly, Lam showed suppression activity on BABA-induced PR1 expression. This finding was comparable to that demonstrated by Benoît Boachon, a master student worked in my collaboration group in IBMP, who found that the SA-defense responses such as *PR1* expression was reduced after triggering by Lam plus its sulfated form (PS3). The following work was pursed in tobacco and *Arabidopsis* plants according to this concept. Lam, PS3 and Lam+PS3 was infiltrated into leaves of tested plants. The results from tobacco and *Arabidopsis* wild type Col-0 showed suppression effect of Lam on *PR1* expression and SA level. In addition, the restoration of *PR1* and SA content in mutants defected in jasmonic acid (JA) pathway, *coi1-16*, suggested that Lam suppresses SA-dependent defense response via JA. The resistances to bacterial *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) and *Peronospora parasitica* (*Pp*) were also tested. In Col-0, PS3 induced strong resistance to these pathogen whereas the resistance was decreased in tissues treated with Lam+PS3 resulting in enhance pathogens growth of *Pst* DC3000 and increased disease after *Pp* challenging. Conversely, in *coi1-16* mutant, the number of *Pst* DC3000 was similar in all treatments but lower than those in control. These data therefore confirmed that Lam suppresses SA-dependent defense and resistance controlled by SA through JA. However, the level of JA is the important key to confirm that SA was suppressed via JA. Moreover, wild type Col-0 and the mutant *sid2/nahG* which is defected in SA signaling pathway and cannot accumulate elevated SA showed that JA was suppressed though SA.

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LIST OF ABBREVIATIONS

A	Absorbance
AOS	Active oxygen species
APX	Ascorbate peroxidase
Avr	Avirulence
BA	6-benzylaminopurine
BABA	β-aminobutyric acid
BSA	Bovine serum albumin
BTH	Benzothiadiazole
CAT	Catalase
CCoAOMT	Caffeoyl CoA-*O*-methyltransferase
CFU	Colony forming units
COI1	CORONATINE INSENSITIVE 1
coi1	*coronatine insensitive1*
CHIB	*BASIC CHITINASE*
DAB	3, 3′-diaminobenzidine
DCINA	2,6-dichloroisonicotinic acid
DDC	Diethydithiocarbamate
DMF	Dimethylformide
DP	Degree of polymerization
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
DPI	Diphenylene iodonium
DTT	Dithiotreitol
EDS	Enhanced disease susceptibility
EDTA	Ethylenediamine tetraacetic acid
eds	*enhanced disease susceptibility*
ET	Ethylene
EtOH	Ethanol
GPX	Glutathione peroxidase

LIST OF ABBREVIATIONS (Continued)

GST	Glutatione S-transferase
HEL	*HEVEINLIKE PROTEIN*
H_2O_2	Hydrogen peroxide
HR	Hypersensitive response
hrp	*hypersensitive response and pathogenicity*
ICS	Isochorismate synthase
IPL	Isochorismate pyrovate lyase
IR	Induced resistance
ISR	Induced systemic resistance
JA	Jasmonic acid
JAR1	*JASMONIC ACID RESISTANT1*
jin	*jasmonic acid insensitive*
kDa	Kilodalton
Lam	Laminarin
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MAPK	Mitogen-activated protein kinase
MeJA	Methyl jasmonate
MeSA	Methyl salicylate
MS	Murashige and Skoog
NBT	Nitroblue tetrazolium
NIM	*NON-INDUCIBLE IMMUNITY*
NPR1	*NONEXPRESSOR OF PR1*
NO	Nitric oxide
OMT	*O*-methyltransferase
O_2^-	Superoxide anion
$OH^·$	hydroxyl free radical
OPDA	12-oxo-phytodienoic acid

LIST OF ABBREVIATIONS (Continued)

PAD	Phytoalexin-deficient
pad	*phytoalexin-deficient*
PAGE	Polyacrylamide Gel Electrophoresis
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PDF1.2	*PLANT DEFENSIN 1.2*
PI	Proteinase inhibitor
Pp	*Peronospora parasitica*
PR	Pathogenesis-related
PS3	Sulfated laminarin
Pst DC3000	*Pseudomonas syringae* pv. tomato DC3000
POD	Peroxidase
RNA	Ribonucleic acid
rpm	Round per minute
ROS	Reactive oxygen species
RT	Reverse trascriptase
SA	Salicylic acid
SAR	Systemic acquired resistance
SID	SA induction-deficient
sid	*SA induction deficient*
SAI	*SA-INSENSITIVE*
Scp	Scopoletin
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
TEMED	N,N,N',N'-tetramethyl-ethane-1,2-diamine
THI2.1	*THIONIN 2.1*
TMV	Tobacco mosaic virus

LIST OF ABBREVIATIONS (Continued)

TIR	Toll and IL-1 receptor
TRIS	Tris (hydroxymethyl) aminomethane
Triton X-100	Polyoxyethylene octyl phenyl ether
TTSS	Type III secretion system
UV	ultraviolet
V_8	V_8 agar
VSP	*VEGETATIVE STORAGE PROTEIN*
2,4-D	2,4-dichlorophenoxy acetic acid

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEWS

Introduction

Plants provide, directly or indirectly, all the food and many other products upon which humans and animals depend. Since diseases can affect plants in many ways, such as by reducing the quality and quantity of the crops and some cases of disease has resulted in a devastating loss of plant crops, so fighting plant diseases is a major challenge for successful agriculture. In earlier times the control of plant diseases depended on using toxic chemicals some of which were harmful to farmers, consumers and also caused environmental pollution. As the human population continues to grow rapidly, there is a resulting increased demand for cultivating land and crops for food.

Plants are exposed to a variety of potential pathogens during their lifetime, including bacteria, virus, fungi, nematodes and pests, but actual infectios occur only in limited cases (Agrios, 1997; Kombrink and Somssich, 1995). Besides their preformed physical and chemical barriers that prevent infection, plants have developed a wide variety of defense responses that are induced after pathogen attack. A plant starts its defense responses upon recognition of the pathogens or pathogen-associated molecular patterns (PAMPs) (Yamaguchi *et al.*, 2000).

For the reasons presented above, there has been much researches nowadays focused on understanding plant-pathogen interactions with the aims to find both environmentally friendly and more efficient means for controlling plant diseases. The study of plant-pathogen interactions can provide tools for developing more long-lasting approaches. Plant defense responses are a result of a complex network of signaling events. A timely response to intruding pathogens also plays a critical role in acquiring resistance. Thus, the early signaling events in response to pathogens are a major factor to provide effective resistance leading to a resistant or susceptible reaction by the plants (Maleck *et al.*, 2000). One of the earliest responses activated after host plant recognition is the oxidative burst, in which levels of active or reactive oxygen species (AOS or ROS) such as superoxide anion (O_2^-) and hydrogen peroxide

(H_2O_2) rapidly increase (Bolwell *et al.*, 1995; Lamb and Dixon, 1997). ROS has been proposed to act as a signal molecule triggering either directly or indirectly the development of other defense responses, as well as being a direct response to invading pathogens by having anti-microbial functions (Wu *et al.*, 1995; Delledonne *et al.*, 2001). Moreover, after pathogen recognition, the signal transduction pathways trigger by endogenous signaling hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) will be switched on. These activate the defense and resistance mechanism. Defense pathways influence each other through a network of regulatory interactions, and thus, plant defense responses are a result of this complex interplay (Kunkel and Brooks, 2002; Bostock, 2005).

Elucidation of plant defense mechanism can provide useful tools for development strategies to protect crops. The way to speed up plant defense activation upon pathogen attack, thus improving the chances of the plant to successfully control current and future encounters with the invaders will be attractive. Improving our understanding of the complexity of signaling cascades that lead to an effective resistance by plants will be the key in engineering durable disease resistance (Stuiver and Custers, 2001). In addition, plant resistance can also be improved by using appropriate elicitors, from natural or synthesized substances, to elicit the activation of natural defense mechanisms. Various types of elicitors have been characterized, including carbohydrate polymers, lipids, (glyco)peptides, and (glyco)proteins from fungi, bacteria, or even host plants (Côté and Hahn, 1994; Ebel and Cosio, 1994) or from seaweed (Bouarab *et al.*, 1999; Klarzynski *et al.*, 2000; Potin *et al.*, 1999). Among them, β-1,3- with branched 1,6-glucans, xyloglucans, oligogalacturonides, and chitin or chitosan oligomers exhibit elicitor activity in different plant species and evoke pathogen defense responses (Côté and Hahn, 1994; Côté *et al.*, 1998; Darvill *et al.*, 1992; John *et al.*, 1997; Sharp *et al.*, 1984). Knowledge about plant-pathogen interactions will hopefully lead to achieve broad-spectrum protection, long-lasting effects, and reduced chemical input in modern-day agriculture.

This work is divided into three experimental parts;

Part 1 The study of the interaction between *Hevea brasiliensis* (Wild.) Muell.-Arg. or rubber plant and zoospores or elicitins from

Phytophthora spp., an oomycete pathogens causing important diseases in *Hevea*, an economically important crop in Thailand. From this first part, a suitable plant-pathogen system will be selected to use as a model for further studies. The details of this part are described in Chapter 2.

Part 2 The aim of the second part is to contribute the understanding of the oxidative burst, one of early signaling events leading to the onset of defense responses in *Hevea* leaves infected with zoospores of *P. palmivora*. This part is described in Chapter 3.

Part 3 For the last investigation, the host plant was switched to tobacco (*Nicotiana tabaccum*) and *Arabidopsis thaliana* because they have been known as models for studying plant defense responses. The defense responses after treatment with different elicitors proposed to be applied to crop protection including TTF5P (a fertilizer product from Tribo technologies which induced strong resistance across many plant species against pathogens), β-1,3-glucans and nonprotein β-aminobutyric acid (BABA) were therefore demonstrated in tobacco and *Arabidopsis*. Moreover, the complex involvement of signal transduction pathways after treatment with β-1,3-glucans was also established in *Arabidopsis* since various mutants defective in signaling pathways were available. The details of this part are described in Chapter 4. Even though these two model plants are not economic products in Thailand, these strategies and knowledge of their defense responses will be helpful for improving the study of plant-pathogen interactions in more important plants, for example *Hevea* plants, in Thailand.

Literature review

1 *Hevea brasiliensis*

Hevea brasiliensis (Wild.) Muell.-Arg. or Para rubber is a member of the family Euphorbiaceae. Formerly it was classified as *Siphonis brasiliensis* Wild. ex A. Juss. *H. brasiliensis* is a tropical tree and native to the Amazon Basin in Brazil and adjoining countries. *Hevea* was taken from the Amazon region to many other tropical regions of the world, such as South East Asia including Thailand, by the British Colonial office (Reed, 1976).

Kingdom : Plantae
Subkingdom : Tracheobionta
Division : Magnoliophyta
Class : Magnoliopsida
Subclass : Rosidae
Order : Malpighiales
Family : Euphorbiaceae
Subfamily : Crotonoideae
Genus : *Hevea*
Species : *Hevea brasiliensis*

From http://en.wikipedia.org/wiki/Rubber_tree

Hevea plants grow best at temperatures of 20-28 ^{0}C with a well-distributed annual rainfall of 1,800-2,000 mm. Mature *Hevea* trees on rubber plantations are 20-30 m high, with girth of 2-3 m; stems smooth and straight; black grayish; taproot well developed' leaves alternate, trifoliate, petioles 7.5-10 cm long; flowers numerous; female flowers apical; fruit a 3-lobed, 3-seeded ellipsoidal capsule, variable in size, 2.5-3 cm long, mottled brown, weighing 2-4 grams each (Figure 1.1) (Reed, 1976). Such trees are flowering once a year, and after insect cross pollination, produce large fruits containing several thimble-sized seeds with hard outer coats. Seeds are collected in July-September in India. If satisfactorily germinated and planted within 2-3 weeks, the seeds grow to produce seeding plants. Depending on conditions, the rubber trees take 5-10 years to reach "maturity", which is defined as the stage when tapping can be started. In practice, this is the time when the truck is about 500 mm in diameter at

0.75 meter above ground level. Tapping is conducted by removal of a thin cut of the bark about 1 mm deep at regular intervals, thus opening the latex vessels in the bark, which are arranged in concentric cylinders and run in counter-clockwise spirals up the trunk. Usually, the cuts run half-way around the trunk, but may encircle the tree. Trees are tapped early in the morning when the flow of latex is highest; latex flow decreases with temperature and usually ceases in about 3 h. Close to 21 million tons of rubber were produced in 2005 of which around 42% of the world consumption was natural; the rest is delivered by synthetic processes. Today South East Asia is the main source of natural rubber, accounting for around 94% of output in 2005. The three largest producing countries; Thailand, Indonesia and Malaysia, all together account for around 72% of all natural rubber production. Major consumers are United States of America, United Kingdom and France (FAO, 2006).

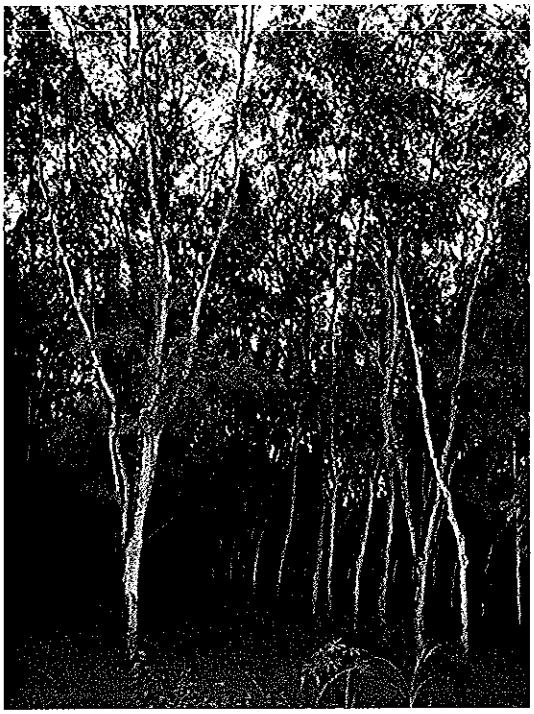

Figure 1.1 Rubber tree plantaion.
(From http://en.wikipedia.org/wiki/Image:Rubber_tree_plantation.JPG)

About 90 species of fungi are known to attack *Hevea* trees, the most prevalent ones being the following; *Botryodiplodia elactica* and *B. theobromae*, *Collectotrichum heveae* (leaf spot), *Fomes lameansis* (brown root rot), *Gloeosporium heveae* (die-back), *Oidium heveae* (powdery mildew). *Pellicularis salmonicolor* (pink disease),

Polystichus occidentalis and *P. personii* (white spongy rot), *Sphaerella heveae* (rim blight), *Sphaerostilbe repens* (red rot), *Ustulina maxima* (charcoal rot) and *Phytophthora palmivora* (causing fruit rot, leaf-fall, black stripe and die-back). It is also attacked by bacteria, nematodes, insects, white ants and snails.

The leaf-fall and black stripe in *H. brasiliensis* are frequently found in Thailand and can decrease the quality and yield of rubber latex. Several species of *Phytophthora* have been reported to be responsible for leaf-fall and black stripe. The common species are *Phytophthora palmivora* (Butl.) Butl., *P. meadii* Mc Rae and *P. botryosa* Chee. In 1984, several other species of *Phytophthora* have also been identified as causes of black stripe infection in China; for example, *P. citrophthora* (Smith & Smith) Leonion, *P. Cactorum* (Lebert & Cohn) Schrodter and *P. capsici* Leonian. The early symptoms of black stripe are not obvious. A 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 the bark is removed. As the infection progresses, the stripes form broad lesions, and finally spread to the full width of the tapping panel. Occasionally, infection occurs on untapped bark resulting in a wound, called "canker". This may arise on bark previously affected by black stripe or on wounds. The early symptoms of canker are not obvious but in the advanced stage, the bark bursts and latex oozes out. Pads of coagulated latex are formed under the bark causing it to bulge and split open. Black stripe incidence is associated with wet weather, being favored by prolonged cool and rainy periods. The fungus is commonly presented in soil and its sporangia are spread by water droplets. In areas where abnormal leaf fall occurs, the sporangia are washed down from the canopy. Canker development is common in leaf-fall areas via heavy inoculum from the tree canopy.

2 *Nicotiana tabacum*

Kingdom : Plantae

Division : Magnoliophyta

Class : Magnoliopsida

Order : Solanales

Family : Solanaceae

Genus : *Nicotiana*

Species : *Nicotiana tabacum*

From http://en.wikipedia.org/wiki/Nicotiana_tabacum

Nicotiana tabacum or cultivated Tobacco is a perennial herbaceous plant. It is found only in cultivation, where it is the most commonly grown of all plants in the *Nicotiana* genus, and its leaves are commercially grown in many countries to be processed into tobacco. It grows to heights between 1 to 2 m. Researches and experiments have been studied for examining its defenses and resistances against biotic and abiotic stresses among wild *Nicotiana* species.

3 *Arabidopsis thaliana*

Kingdom : Plantae

Division : Magnoliophyta

Class : Magnoliopsida

Order : Brassicales

Family : Brassicaceae

Genus : *Arabidopsis*

Species : *Arabidopsis thaliana*

From http://en.wikipedia.org/wiki/Arabidopsis_thaliana

A. thaliana or *Arabidopsis* is a small flowering plant native to Europe, Asia, and northwestern Africa. A spring annual with a relatively short life cycle, *Arabidopsis* is an annual (rarely biennial) plant usually growing to 20–25 cm tall. The leaves form a rosette at the base of the plant, with a few leaves also on the flowering stem. The basal leaves are green to slightly purplish in color, 1.5–5 cm long and 2–10 mm broad, with an entire to coarsely serrated margin; the stem leaves are smaller, unstalked, usually with an entire margin. Leaves are covered with small unicellular

hairs (called trichomes). The flowers are 3 mm in diameter, arranged in a corymb; their structures are that of the typical Brassicacaea. The fruit is a siliqua 5–20 mm long, containing 20–30 seeds.

Arabidopsis is widely used as one of the model organisms for studying plant sciences in biology and genetics (Rensink and Buell, 2004; Coelho *et al.*, 2007). It plays the role for agricultural sciences just as do mice and fruit flies (*Drosophila*) play in animal biology. Although *A. thaliana* has little direct significance for agriculture, it has several traits that make it a useful model for understanding the genetic, cellular, and molecular biology of flowering plants. This may be due to the small size of its genome with about 157 million base pairs and five chromosomes (Bennett *et al.*, 2003), its genome had been completely sequenced by the Arabidopsis Genome Initiative (2000), small in size, rapid life cycle and it produces many seeds.

Finally, plant transformation in *Arabidopsis* is routine, using *Agrobacterium tumefaciens* to transfer DNA to the plant genome. The current protocol, termed "floral-dip", involves simply dipping a flower into a solution containing *Agrobacterium* with the inserted DNA of interest, and a detergent. This method avoids the need for tissue culture or plant regeneration.

4 *Phytophthora* spp.

The name of *Phytophthora* (the plant-destroyer) is derived from Greek "phytón" and "phthorá", which literally means plant and destruction, respectively. It is the *Oomycetes* (water moulds). Heinrich Anton de Bary described it for the first time in 1875.

Domain : Eukaryota
Kingdom : Chromalveolata
Phylum : Heterokontophyta
Class : Oomycetes
Order : Peronosporales
Family : Pythiaceae
Genus : *Phytophthora*

From http://en.wikipedia.org/wiki/Phytophthora

Phytophthora is a major genus of plant pathogens within the diploid, algae-like oomycete fungi. Currently, this genus is assigned to the order Pythiales, phylum Oomycota within the group of heterokont, biflagellate organisms that comprise the Kingdom Chromista (Cavalier-Smith, 1986). *Phytophthora* is fungus-like which is supported by a number of features including biflagellate zoospores, aseptate hyphae, a diploid thallus and the cell wall is composed of cellulose and glycan rather than chitin.

4.1 Fungi resemblance

Phytophthora is sometimes referred to as a fungal-like organism but it is classified under a different kingdom altogether: Stramenopila (previously named Chromista). This is a good example of convergent evolution: *Phytophthora* is morphologically very similar to true Fungi yet its evolutionary history is quite distinct. In contrast to fungi, stramenopiles are more closely related to plants than animals. Whereas fungal cell walls are made primarily of chitin, stramenopile cell walls are constructed mostly of cellulose. Ploidy levels are different between these two kingdoms as are biochemical pathways.

4.2 Biology

Phytophthora may reproduce sexually or asexually. In many species, sexual structures have never been observed, or have only been observed in laboratory matings. In homothallic species, sexual structures occur in single culture. Heterothallic species have mating strains, designated as A1 and A2. When mated, antheridia introduce gametes into oogonia, either by the oogonium (female) passing through the antheridium (amphigyny; male) or by the antheridium attaching to the proximal (lower) half of the oogonium (paragyny), and the union producing oospores. 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 (Figure 1.2).

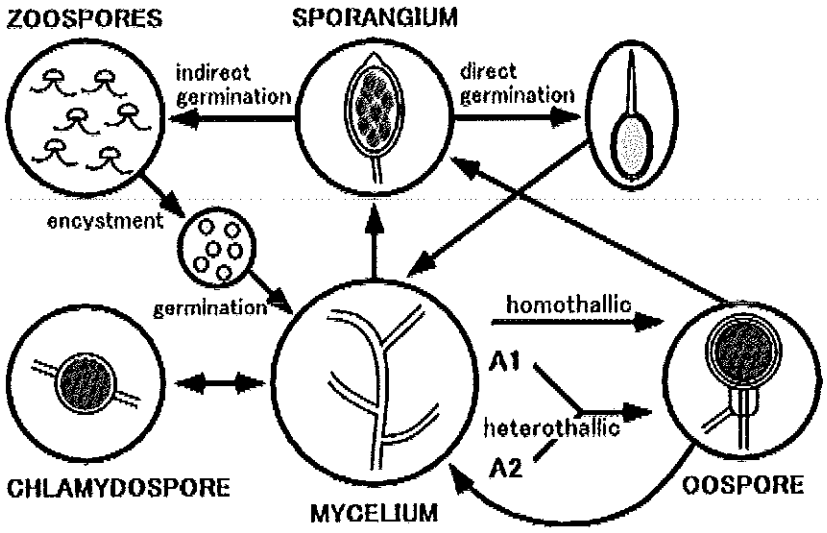

Figure 1.2 The life-cycle of *Phytophthora* spp.
(From http://en.wikipedia.org/wiki/Image:Phytophthora_life_cycle.png).

Phytophthora species are mostly pathogens of dicotyledon plants, and are relatively host specific parasites. They cause root, stem and fruit rot in more than 100 plant species. Many species of *Phytophthora* are plant pathogens of considerable economic importance including rubber tree (*H. brasiliensis*). *P. palmivora*, *P. botryosa*, *P. heveae*, *P. meadii* and *P. parasitica* have been described as pathogens of the rubber tree. *P. palmivora* and *P. meadii* are the most frequently isolated and described as the causal agents of black stripe, green pod rot and abnormal leaf-fall. In Malaysia and Thailand, these diseases are considered to be caused by *P. palmivora* and *P. botryosa* (Erwin and Ribeiro, 1996).

P. palmivora (Bulter) is a ubiquitous pathogen with a wide host range (Erwin and Ribeiro, 1996). It causes many diseases such as black pot, stem canker and wilt of cocoa, root and fruit rot of papaya, fruit rot and premature nut full in coconut, foot rot in black pepper, bud-rot of palms and black stripe, patch canker and pod rot in rubber tree. *P. palmivora* is heterothallic and distinguished from other species of *Phytophthora* mainly by the prominent papillate sporangia that are ellipsoidal to avoid in shape, dedicuous and have a short pedicel. However, the morphological characteristics of *P. palmivora* exhibit considerable variation depending on the isolate and the host. *Phytophthora* is a soil-borne pathogen; it causes root rot and can spread

easily from root to root contact, through the movement of infested soils, through irrigation systems, rain splash, insects and pruning equipments (Hoilliday, 1980).

P. parasitica (Dastur) or *P. nicotianae* (Breda de Haan) causes root rot, foot rot, leaf blight, and fruit rot in a variety of economically important crops (Erwin and Ribeiro, 1996). Although isolates that were morphologically identified as *P. parasitica* are pathogenic to a wide range of plant species, considerable evidence shows that some isolates are specifically pathogenic to a few hosts or a single host, such as tobacco (Erwin and Ribeiro, 1996).

To date, more than 30 *Phytophthora* species have actually been found to secrete highly conserved proteins collectively termed elicitin with molecular weights of about 10 kDa which can trigger plant defense responses (for review see Ponchet *et al.*, 1999).

5 *Peronospora parasitica* or *Hyaloperonospora parasitica*

Hyaloperonospora Constant. *parasitica* (Pers.:Fr) Fr. (formerly *Peronospora parasitica*; *Pp*) is a species from family Peronosporaceae. It causes downy mildew in brassica plants such as oilseed rape and cauliflower and can cause economically important damage by killing seedlings or affecting the quality of products intended for freezing.

Domain : Eukaryota
Kingdom : Chromista
Phylum : Heterokontophyta
Class : Oomycetes (Water molds)
Order : Peronosporales
Family : Peronosporaceae
Genus : *Hyaloperonospora*
Species : *Hyaloperonospora parasitica*

From http://en.wikipedia.org/wiki/Hyaloperonospora_parasitica

The taxonomy of the group of organisms causing downy mildew of brassicas, a genus of plants in the mustard family (Brassicaceae), has undergone a number of revisions since the genus *Peronospora* was coined originally by Codar in 1837. All isolates pathogenic to brassicas were described initially as *Pp* but Gäumann classified

isolates from different brassicaceous hosts distinctly in 1918 and thus defined 52 new species based on conidial dimensions and host range. After much debate it was decided to revert to the aggregate species of *Pp* for all brassica-infecting downy mildews, whilst recognizing that these show some isolated-specific differences. The latest re-examination of *Pp* has placed isolates of *Pp* and five other downy mildew species in a clear new subgroup on the basis of their hyaline conidiospores, recurved conidiophore branch tips and ITS1, ITS2 and 5.8S rDNA sequence comparisons; meriting the coining of the new genus '*Hyaloperonospora* Constant' (Slusarenko and Schlaich, 2003).

The class Oomycetes in the Kingdom Chromista (Straminipila) comprises fungus-like organisms with heterokont zoospores (i.e. possessing two types of flagellae, whiplash and tinsel). The oomycetes have non-septate hyphae with cellulose-based walls containing very little or no chitin. The latter is regarded as a major distinction separating the oomycetes from the true fungi, and reports of the presence of chitin had generally been regarded as due to small amounts of contamination. However, in view of recent studies showing a chitin synthase gene in an oomycete and demonstrating the presence of the polymer itself by an interaction with wheat germ agglutinin (WGA), it is perhaps safe to say that we have not seen the last taxonomic revision which will affect this group. The families within the oomycetes show a clear evolutionary trend to a lesser absolute dependence on an aqueous environment and some members of the Peronosporales, e.g. *H. parasitica*, have no zoosporic stage in their life cycle (Slusarenko and Schlaich, 2003).

6 *Pseudomonas syringae*

Pseudomonas syringae is a rod shaped, Gram-negative bacterium with polar flagella. It is a member of the *Pseudomonas* genus, and based on 16S rRNA analysis, *P. syringae* has been placed in the *P. syringae* group (Anzai *et al.*, 2000). It is a plant pathogen which can infect a wide range of plant species, and exists as over 50 different pathovars. Many of these pathovars were once considered to be individual species within the *Pseudomonas* genus, but molecular biology techniques such as DNA hybridization have shown these to in fact all be part of the *P. syringae* species.

It is named after the lilac tree (*Syringa vulgaris*), from which it was first isolated (Kreig and Holt, 1984).

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Gamma Proteobacteria

Order: Pseudomonadales

Family: Pseudomonadaceae

Genus: *Pseudomonas*

Species: *Pseudomonas syringae*

From http://en.wikipedia.org/wiki/Pseudomonas_syringae

6.1 Epidemiology

Disease caused by *P. syringae* tends to be favored by wet, cool conditions- optimum temperatures for disease tend to be around 12–25 ^{0}C, although this can vary according to the pathovar involved. The bacteria tend to be seed borne, and are dispersed between plants via rain splash.

Although it is a plant pathogen, it can also live as a saprophyte in the phyllosphere when conditions are not favorable for disease (Hirano and Upper, 2000). Some saprophytic strains of *P. syringae* have been used as biocontrol agents against post-harvest rots (Janisiewicz and Marchi, 1992).

6.2 Pathovars

Following ribotypical analysis several pathovars of *Pseudomonas syringae* were incorporated into other species, for example, *P. amygdali*, *P. tomat*, *P. coronafaciens*, *P. avellanae*, *P. helianthi*, *P. tremae*, *P. cannabina*, and *P. viridiflava*. The remaining pathovars are as follows:

Pseudomonas syringae pv. *aceris* attacks maple *Acer* species.

Pseudomonas syringae pv. *aptata* attacks beets *Beta vulgaris*.

Pseudomonas syringae pv. *atrofaciens* attacks wheat *Triticum aestivum*.

Pseudomonas syringae pv. *dysoxylis* attacks the kohekohe tree *Dysoxylum spectabile*.

Pseudomonas syringae pv. *japonica* attacks barley *Hordeum vulgare*.

Pseudomonas syringae pv. *lapsa* attacks wheat *Triticum aestivum.*

Pseudomonas syringae pv. *panici* attacks *Panicum* grass species.

Pseudomonas syringae pv. *papulans* attacks crabapple *Malus sylvestris* species.

Pseudomonas syringae pv. *pisi* attacks peas *Pisum sativum.*

Pseudomonas syringae pv. *syringae* attacks *Syringa* and *Phaseolus* species.

Note that *Pseudomonas savastanoi* was once considered a pathovar or sub-species of *P. syringae*, and in many places continues to be referred to as *Pseudomonas syringae* pv. *savastanoi*, although as a result of DNA-relatedness studies it has been instated as a new species. It exists as three host-specific pathovars, *fraxini* which causes ash canker, *nerii* which attacks oleander and *oleae* which causes olive knot.

7 Plant-pathogen interactions

There are many groups of pathogenic microorganisms causing plant diseases such as fungi, viruses, bacteria, protozoa, and nematodes that belong to the same groups as those causing diseases in animals. To be a potentially and successful pathogen, a microorganism needs to interfere with one or more of the essential functions of the host plant, in order to cause diseases. One prerequisite for pathogenicity of a microorganism, however, is the ability to gain access to the plant interior. Pathogens force their ways through plant surfaces by different means; some take advantage of natural openings such as stomata or lenticels, or enter the plant through wounds, while others simply penetrate the leaf surfaces. Fungi, such as powdery mildew, for instance, can grow a fine hyphal thread directly into the plant epidermal cells. In most fungal diseases, the fungus penetrates not only the cuticle but also the cell wall - the next obstacle for pathogens after reaching the intercellular spaces (apoplast). Oomycetes and nematodes usually use the penetration method, while bacteria utilize wounds and natural openings. Besides those methods, some pathogens use chemical activities to overcome these barriers, for example, certain bacteria secrete cutin-degrading enzymes, cutinases, while others produce an extracellular enzymes to

degrade the cell wall, including pectinases, cellulases, and polygalacturonases (Toth *et al.*, 2003).

The virulence mechanisms that the pathogen uses to reach its final goal – to take advantage of the plant requires a source of nutrients – including secretion of toxins, growth regulators, and other substances that disturb the metabolism of plant cells or interfere with the plant defenses (Agrios, 2005). The virulence strategy depends on how the pathogen intends to utilize the plant; biotrophs obtain nutrients from living plant tissue without killing the cells, whereas necrotrophs kill the cells and absorb the released nutrients during invasion (Glazebrook, 2005). Some pathogens, called hemi-biotrophs, fill the requirements of both biotrophs and necrotrophs, depending on the prevailing conditions they are in or the stages of their life cycles (Glazebrook, 2005). Most pathogens continue multiplying indefinitely within the infected tissue to obtain nutrients until the plant is dead.

To counter pathogen attacks, plants have evolved a wide variety of defense responses. Due to plants' structure (stronghold-like structure) as well as their innate abilities to recognize potential invading pathogens and activate effective defenses, plants are generally resistant to most pathogens (Heath, 2000; Nürnberger *et al.*, 2004). Thus, in order to be successful, a pathogen also needs to evade the plant surveillance system or suppresses plant defenses. The ability to detect potential pathogens has been essential to the development of modern plants (Chisholm *et al.*, 2006). Perception of the pathogen is achieved through receptors, a surveillance system capable of recognizing both conserved molecular patterns and specific effector proteins, and activation of the corresponding defenses (Montesano *et al.*, 2003). Plants defend themselves against invaders in two ways: with structural barriers that inhibit the pathogen from gaining entrance and spreading throughout the plant and with biochemical reactions that take place in the plant tissues. These reactions produce toxic substances resulting in the inhibition of the pathogen growth. The combinations of these two defense types vary between different plant-pathogen interactions (Glazebrook, 2005).

Plants can respond to pathogens by two types of reactions, namely compatible and incompatible reactions. The compatible reaction is the interaction between a susceptible host and a pathogen or in a case when the plant fails to recognize the

pathogen or an elicitor, appropriate defenses might not be mounted, resulting in disease. Conversely, the incompatible reaction is the interaction between a resistant host and a pathogen, the plant responds with a rapid and well-aimed activation of defenses thus the attempted infection is halted.

8 Plant defense

Plants have evolved numerous and complex defense mechanisms to survive attacks of pathogens. These defenses included both preformed and inducible defense systems such as strengthening of the cell wall, induction of an oxidative burst and a hypersensitive response (HR; rapid localized cell death at the site of infection), increased expression of defense-related genes [e.g. pathogenesis-related (*PR*) genes] and synthesis of phytoalexins (antimicrobial compounds, Hammond-Kosack and Jones, 1996; Nürnberger and Scheel, 2001).

Even if plants live a sessile life, they are dynamic organisms that fight the pressure of pathogens with advanced defense strategies, including both preformed and inducible defense systems. Resistance of an entire plant species to all strains of a pathogen is called nonhost resistance, the most common type of resistance expressed by plants (Heath 2000; da Cunha *et al.*, 2006). Simply put, this means that, for example, the pathogens of tomato (*Lycopersicon esculentum*) do not infect spruce (*Picea* sp.), and vice versa. Significant components of nonhost resistance are the preformed or constitutive defenses associated with plant structures and chemical compounds already present in the plant. These include structural barriers, such as the plant cell wall, as well as inhibitory compounds, e.g. phenolics and tannins (Heath, 2000; Nürnberger *et al.*, 2004; Agrios, 2005).

Inducible defenses are triggered by the recognition of the pathogens. Basal defense, a constituent of both nonhost and host resistance, provides basal-level resistance (also called innate immunity or local induced resistance) that prevents infection by a wide range of microbes (Heath, 2000; Thordal-Christensen, 2003; Nürnberger *et al.*, 2004). Some pathogens have acquired the ability to suppress basal defense responses and enhance their virulence by delivering specific effector proteins to the plant cells that interfere with plant defense. Gene-for-gene or race-cultivar-specific resistance occurs when specific members of a plant species, but not the species as a whole, have

acquired resistance to a particular pathogen. This type of resistance is usually restricted to a particular pathogen species, being expressed against specific genotypes of that pathogen (Dangl and Jones, 2001; Bonas and Lahaye, 2002; Hammond-Kosack and Parker, 2003; Chisholm *et al.*, 2006).

8.1 Preformed defenses or Passive defenses

Noninducible, preformed structural defenses, such as a dense epidermal layer and wax and cuticle coverings on leaves, are the first line of plant defense to invading pathogens. Structures found on surfaces, such as spiky hairs called trichomes, can prevent attack by insects or insect larvae. Hairs on leaves can also have a water repellent effect, hence making access more difficult, especially, for bacteria to establish contact with the plant. Rigid cell walls composed of fibrils of cellulose embedded in a matrix of several other kinds of polymers, such as pectin and lignin, also serve as an efficient barrier to the invasion of pathogens (Agrios, 2005).

Chemical defenses include various antimicrobial peptides, proteins, and nonproteinaceous secondary metabolites present in plant cells that can prevent ingress of the invader (Heath, 2000; Nürnberger *et al.*, 2004). Toxic secondary metabolites stored in special plant compartments can be activated or released upon tissue damage. Besides being directly harmful to the invader, they can operate by inactivating the extracellular enzymes secreted by the pathogen (Zhao *et al.*, 2005). Secondary metabolites are often restricted in their distributions to particular plant families, genera, or species. For example, avenacin A-1 is a triterpenoid saponin found in the roots of oat plants. It is highly effective against the fungus *Gaeumannomyces graminis* var *tritici*, a major pathogen of wheat and barley roots, but not of oats because of the presence of this secondary metabolite (Buchanan *et al.*, 2000).

8.2 Inducible defenses or Active defenses

Inducible plant defenses are triggered by the perception of a pathogen or pathogen-derived molecules called elicitors. The elicitors can be either general, common to a group of microbes, or specific to certain pathogenic strains. In addition, pathogens can release polysaccharide oligomers from the plant surface, which can induce defenses (Montesano *et al.*, 2003; Nürnberger *et al.*, 2004; Chisholm *et al.*, 2006). Many of the elicitors so far characterized serve as general or nonhost elicitors inducing defense responses in a wide range of plant cells. Recently, it has emerged

that many of these elicitors are derived from conserved molecular sequences presented among various microorganisms and known as pathogen-associated molecular patterns (PAMPs) (Parker, 2003; Zipfel *et al.*, 2004; Nürnberger and Brunner, 2002). These PAMPs are thought to be recognized by pattern-recognition receptors in plants and to trigger the expression of defense responses in the plant cells.

Perception of elicitors takes place in the receptors located either at the cell surface or inside the cell. According to current knowledge, recognition of general and specific elicitors triggers overlapping signaling responses in the plant (Kim *et al.*, 2005). Interestingly, by comparing changes in plant mRNA profiles in response to avirulent and virulent *P. syringae*, Tao *et al.* (2003) demonstrated that the induction of defense genes was more rapid and enhanced in response to specific elicitors (i.e. the avirulent strain). This indicates a difference in the speed rather than the quality of response triggered by the two elicitor types (Kim *et al.*, 2005).

Recognition of the elicitor induces several early responses (Figure 1.3): phosphorylation and dephosphorylation of plasma membrane proteins, increase of cytosolic Ca^{2+}, ion fluxes, and alkalization of the apoplast. Synthesis and deposition of callose in the form of papillae can be initiated rapidly at the site of pathogen invasion. Mitogen-activated protein kinases (MAPK) and NADPH oxidase are activated, and reactive oxygen species (ROS) is produced within minutes of contact with the elicitor (Zhao *et al.*, 2005). Activation of transcription factors and early expression of defense genes also occurs. The activated kinase cascades and ROS further amplify the defense signal to downstream reactions.

A series of alarm signals are triggered that are transmitted intracellularly and also to adjacent cells. These are sequentially followed by late defense gene activation and phytoalexin accumulation. Phytoalexins are toxic antimicrobial substances and can be, for example, flavonoids, alkaloids, and terpenoids produced in healthy cells in response to signals from damaged cells adjacent to them (Zhao *et al.*, 2005). Production of various defense-related proteins, such as pathogenesis-related (PR) proteins, which have antimicrobial activity and thus serve to contain the infection, is also activated (Wojstaszek, 1997; Van Loon and Van Strien, 1999). The formation of a hypersensitive response (HR), a rapid localized cell death that restricts the growth of the pathogen, is more frequently associated with the recognition of a specific than a

general elicitor (Greenberg, 1997; Greenberg and Yao, 2004) (Figure 1.3). The signals originating from the local infection site can then evolve into a systemic defense response involving distal, undamaged parts of the plant and conferring resistance to future pathogen infections.

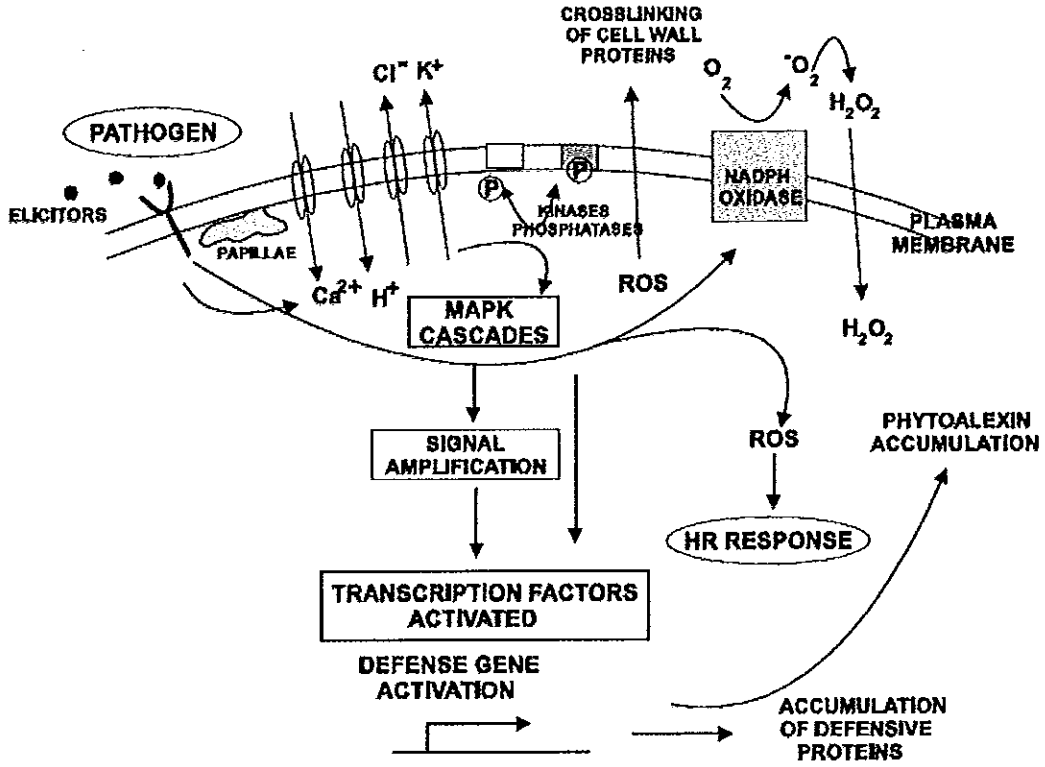

Figure 1.3 Plant responses induced by the recognition of a pathogen (adapted from Buchanan *et al.*, 2000).

9 Resistance against disease

9.1 The hypersensitive response (HR)

In plants, defense mechanisms against infectious microbes involve constitutive barriers as well as reactions induced upon contact with potential pathogens. The HR can be defined as "the rapid cell death in plants at the site of initial infection, with consequent colonisation and death of the potential pathogen" (Grant and Mansfield, 1999). This response leads to the appearance of a restricted lesion which isolates the site of attack from the surrounding healthy tissue, and although the host cells are damaged, this cell death contributes to pathogen restraint (Levine *et al.*, 1994; Lamb and Dixon, 1997; Bolwell, 1999; McDowell *et al.*, 2000).

9.2 Systemic acquired resistance (SAR)

SAR is characterized by an activation of a broad spectrum of host defense responses, locally at the site of the initial pathogen attack and systemically, in tissues untouched by the pathogen. SAR can provide resistance against widely diverse organisms such as fungi, bacteria and viruses, and is associated with induced defense reactions including biochemical and cytological changes, and depend on the production of a signal that is translocated to other parts of the plant. The first inoculation usually leads to localized necrosis, the degree of which can affect the level of protection. A major feature of SAR is that resistance is expressed against pathogens that can be widely different from the initial infecting organism. Although plants do not possess immunoglobulin, the general phenomenon can be compared to immunization in animals and humans. SAR is established in times ranging from several h to several weeks, depending on the plant and the nature of the organism employed in the first inoculation, and the duration thereof extends over long periods. SAR is associated with the accumulation of defense compounds, such as pathogenesis-related (PR) proteins, in the uninfected parts of the plant, and it is mainly effective against biotrophic pathogens (Glazebrook, 2005).

9.3 Local and systemic resistance

Wounding (mechanical injury) of plant organs leads to the rapid activation of genes that play a role in two types of responses:

a) The cells in the surrounding area of the wound undergo a local response and induce genes involved in healing.

b) Cells near the wound site trigger a systemic response in distal parts of the plant and this involves the activation of genes responsible for defense reactions (Bögre *et al.*, 1997).

9.4 Acquired resistance

As mentioned before, defense responses in plants encompass constitutive barriers that exist independently of the presence of pathogens or herbivores, as well as barriers induced upon contact with microbes and chewing insects. In this regard, acquired resistance can be explained as the systemic response of plants following pre-treatment with weakly aggressive strains, avirulent or incompatible varieties of pathogenic microbes. Here, the response results in protection against subsequent

infection with the same or related pathogens, and is defined as (i) biological control or (ii) acquired resistance of the host plant:

(i) Biological control is based on antagonistic interactions between an attenuated or non-pathogenic organism and a plant pathogen. Here the functioning mechanisms are nutritional competition and utilization of important sites for the pathogen by protecting microbe as well as production of antibiotics that act as the biocontrol agents.

(ii) Acquired resistance is based on the activation of host defense mechanism in response to pathogen attack. The resistance is expressed locally at the site of initial attack as well as systemically in tissues untouched by the initial infection.

9.5 Induced systemic resistance (ISR)

Introducing living or heat-killed pathogenic or saprophytic bacteria into tobacco leaves can induce non-specific resistance to various pathogens. Protection elicited in this manner is effective against subsequent infections by virulent pathogenic bacteria, avirulent bacteria and tobacco mosaic virus. The protection is time and light dependent and, given proper lighting conditions, becomes systemic. Although ISR is very similar to SAR in that it involves activation of the plant's defense mechanisms leading to systemic protection, it differs in one crucial aspect – accumulation of PR proteins is not associated with this resistance (Van Loon *et al.*, 1998). Non-pathogenic rhizobacteria-mediated ISR against fungi, bacteria and viruses has been demonstrated in *Arabidopsis*, bean, radish, tomato and tobacco. ISR is effective against infection by different types of pathogens such as *P. syringae* pv *tomato* and fungal pathogens *Fusarium oxysporum* and *Pp*. Bacterial strains do however vary in their abilies to induce resistance in different plants species and plants themselves exhibit diversity in the expression of ISR upon inoculation by specific bacteria. Interestingly, in a similar way to SAR, rhizobacteria-induced ISR is dependent on the regulatory protein NPR1 (NONEXPRESSOR OF PR1) (Pieterse *et al.*, 1998).

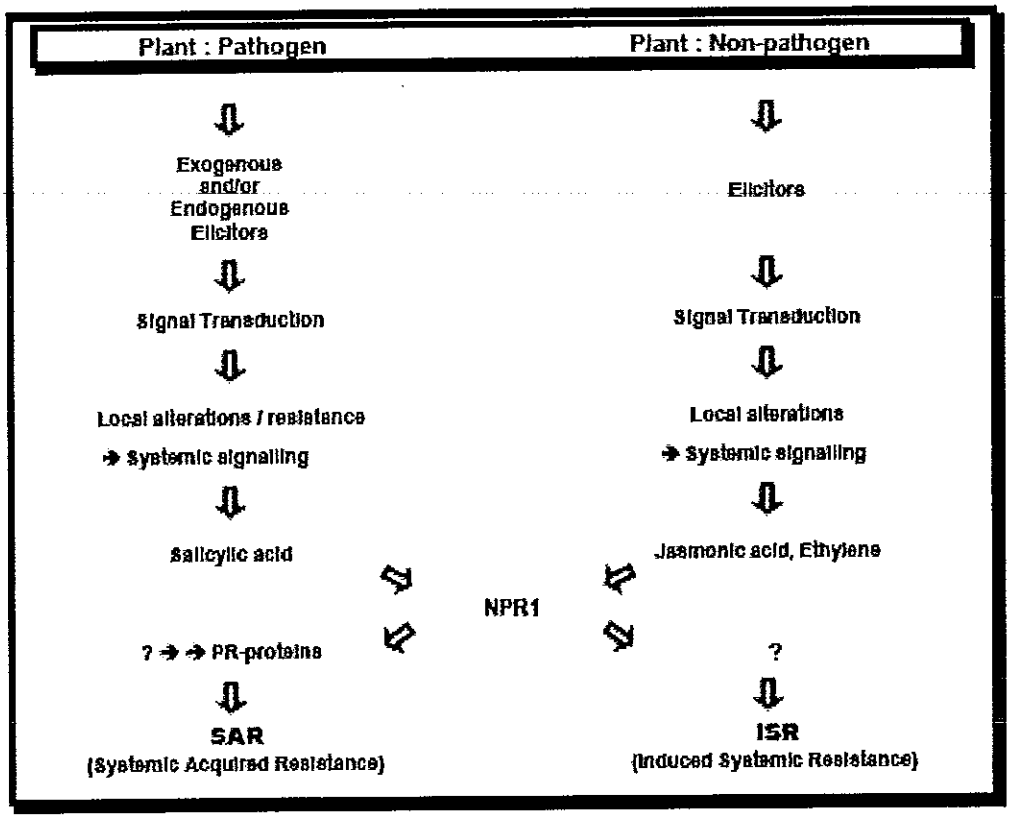

Figure 1.4 Schematic diagram showing the proposed signal transduction pathways involved in plant: pathogen interactions (SAR) and the plant: non-pathogen interaction (ISR) (Van Loon *et al.*, 1997; Van Wees *et al.*, 2000).

10 Elicitation of plant defense

10.1 General elicitors

Evolutionary ancient innate immunity, the ability to discriminate between self and nonself, is a quality of both animals and plants (Parker, 2003). It relies on the detection of pathogen-associated molecular patterns (PAMPs) characteristic of a whole class of potentially harmful microbial organisms (Heath, 2000; Nürnberger and Brunner, 2002; Nürnberger *et al.*, 2004). Pathogen-derived molecules of such patterns correspond to motifs or domains with conserved structural traits found in widely occurring compounds present in microbes but not present in their hosts and essential for microbial fitness. These molecules of diverse nature, including lipopolysaccharides (LPS), flagellins, glucans, and chitins, serve as general elicitors

that trigger basal host defense responses independently of the genotype of the individual pathogen (Figure 1.5). For example, flagellin, the protein subunit of the bacterial surface, acts as a PAMP in both animals and plants (Smith *et al.*, 2003). General elicitors are usually molecules that are indispensable in the lifestyle of microbes and thus provide a fitness penalty for the pathogen if recognized by the plant surveillance system (Nürnberger and Brunner, 2002; Nürnberger *et al.*, 2004). Endogenous plant cell wall-derived structure released by the hydrolytic enzyme activities of invading microbes can also act as general elicitors (Nürnberger *et al.*, 2004) (Figure 1.5).

10.2 Race-specific elicitors

During evolution some pathogens have developed to overcome the PAMP-triggered basal resistance by acquiring the ability to deliver effector proteins into plant cells (Chisholm *et al.*, 2006) (Figure 1.5). These effector proteins interfere with, manipulate, or suppress disease signaling, thereby enhancing pathogen growth and disease development (Chisholm *et al.*, 2006; da Cunha *et al.*, 2006; Truman *et al.*, 2006). In response, during co-evolution plants have adapted to detect these specific pathogen-derived molecules. This cultivar-specific, gene-for-gene disease resistance system is determined by pathogen-encoded effector proteins and the corresponding plant-derived R proteins (Hammond-Kosack and Jones, 1997; Bonas and Lahaye, 2002).

Many Gram-negative bacterial pathogens possess the *hypersensitive response and pathogenicity* (*hrp*) gene cluster that encodes the type III secretion system (TTSS). TTSS is utilized by the bacteria for injection of the effector proteins into plant cells (Feys and Parker, 2000; Alfano and Collmer, 2004) (Figure 1.5). Chisholm *et al.* (2006) speculated that the effectors have developed to interfere with the components of PAMP-triggered defense or to promote the pathogenicity of the microorganism by affecting a variety of host proteins (Figure 1.5). Kim *et al.* (2005) demonstrated that *P. syringae* effector proteins AvrRpt2 and AvrRpm1 suppress PAMP-triggered defense responses in *Arabidopsis* by inhibiting flagellin-induced accumulation of callose. Some avirulence factors act by suppressing the HR response (Jamir *et al.*, 2004), which is central in activating certain plant defense responses. Although many effector proteins have been cloned, the biochemical function of most

remains unknown. AvrPtoB has been shown to have ubiquitin ligase activity in vivo (Abramovitch *et al.*, 2006). Deletion of key residues from this protein eliminated ubiquitin ligase activity and the capability of AvrPtoB to inhibit cell death. Thus, this effector was suggested to act by targeting proteins responsible for regulation of programmed cell death to degradation of the mimiking ubiquitin ligase of the host (Chisholm *et al.*, 2006).

However, if the effector protein meets a matching *R* gene in the plant, it becomes a specific elicitor and the plant defense system is activated by the R protein (Figure 2). Several *R* genes confer specific resistance to fungal, viral, or bacterial pathogens carrying the matching effector gene (Staskawicz *et al.*, 2001; Bonas and Lahaye, 2002). Resistance is manifested by the HR response, one of the most prominent features of gene-for-gene resistance, and inhibition of pathogen growth (Feys and Parker, 2000; Bonas and Lahaye, 2002). The oxidative bursts in the tissues undergoing HR response also appear to be important in propagating systemic defense signals (Truman *et al.*, 2006).

11 Elicitor perception

Receptors functioning in pathogen surveillance are located either at the plant cell surface or inside, and they rapidly activate defense signaling pathways following infection. Given the vast array of different elicitors, the identification of receptors is a major challenge. Several types of putative receptors have been identified in plants, including receptor-like kinases (RLKs), which form a large family of over 400 members in *Arabidopsis* (Johnson and Ingram, 2005). RLKs are implicated in all aspects of plant biology, from early embryogenesis to disease resistance. They are composed of an extracellular domain, a single transmembrane-spanning region, and a cytoplasmic part containing a conserved kinase domain, as well as other more variable segments (Johnson and Ingram, 2005).

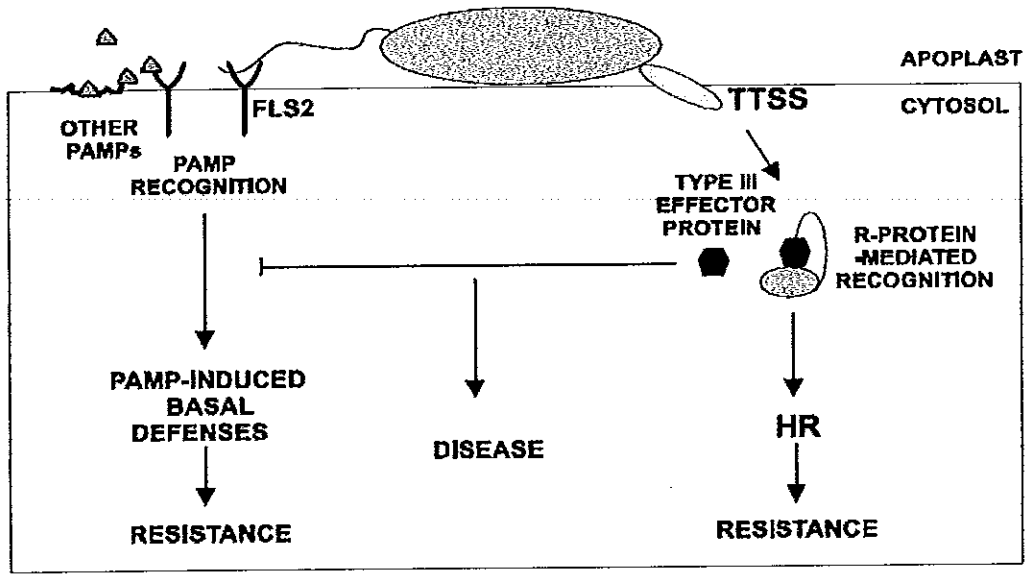

Figure 1.5 General and specific elicitors of plant defense (modified from Abramovitch *et al.* 2004 and da Cunha *et al.* 2006).

12 Defense signaling

Recognition of a pathogen triggers diverse cellular events in plants (Figure 1.3). As earlier, several immediate and local responses take place in cells, including changes in ion fluxes and alkalization of the cytoplasm (Wojstaszek, 1997). Many of these events are activated within minutes of pathogen perception. Kinase cascades involving MAPKs and CDPKs (calcium-dependent protein kinases) undergo rapid activation and amplify early responses (Ludwig *et al.*, 2005). Moreover, pathogen recognition and the early events trigger the production of the endogenous signaling hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). They operate in two major defense pathways in plants: one dependent on SA and the other dependent on JA and ET, conferring resistance to different pathogens (Thomma *et al.*, 1998). ROS and nitric oxide (NO) also contribute to the transmission of defense signals (Karpinski *et al.*, 2003). In addition, reactive electrophilic species (RES), lipid oxidation products containing α, β-unsaturated carbonyl groups, accumulate during pathogen attack. Together with ROS and NO, these have been suggested to play important roles in the activation of defense genes (Alméras *et al.*, 2003).

12.1 The oxidative burst as a secondary messenger

12.1.1 Mechanisms for the generation of ROS

One of the earliest events that occur after elicitation is the rapid production and accumulation of reactive oxygen species such as O_2^- and H_2O_2 (Doke *et al.*, 1996). This is known as the oxidative burst. Elicitation of the oxidative burst by pathogen elicitors appears to be mediated through multiple signaling cascades, which may include G-protein signaling, Ca^{2+} influx, H^+/K^+ exchange and protein phosphorylation (Low and Merida, 1996). The generation of ROS in an incompatible plant-pathogen interaction is thought to be mediated by a plasma membrane bound NADPH oxidase, a mechanism analogous to the mammalian NADPH oxidase system (Keller *et al.*, 1998).

It is proposed that this system is activated through phosphorylation. In addition to NADPH oxidase, pH-dependent cell wall peroxidases (Elstner and Heupel, 1976), germin-like oxalate oxidases (Hurkman and Tanaka, 1996) and amine oxidases (Angelini *et al.*, 1993) have been proposed as sources of H_2O_2 in the apoplast.

12.1.1.1 NADPH oxidases

The NADPH oxidase system has received the most attention of all systems involved in the oxidative burst. Chemical inhibitors of NADPH oxidases, such as diphenylene iodonium [DPI], have been shown to block or severely reduce ROS production upon biotic and abiotic stresses (Pellinen *et al.*, 1999). It was also shown that the accumulation of H_2O_2 in wounded or systemin-treated tomato leaves was inhibited by DPI (Orozco-Cardenas and Ryan, 1999). In several other model systems studied, it appears that a membrane bound NADPH complex is responsible for the oxidative burst (Keller *et al.*, 1998). Thus, these results indicated that NADPH oxidase is responsible for the synthesis of O_2^-, which is consistent with the hypothesis of Auh and Murphy (1995) that the synthesis of O_2^- is catalysed by a membrane bound enzyme.

12.1.1.2 pH dependent cell wall peroxidases

The production of ROS by pH-dependent cell wall peroxidases has been proposed as an alternative for the oxidative burst during biotic stress (Bolwell *et al.*, 1995). It seems that pH-dependent cell wall peroxidases are activated by an alkaline pH and in the presence of a reductant, H_2O_2 is formed.

Alkalinization of the apoplast upon elicitor recognition precedes the oxidative burst and the production of H_2O_2 by pH dependent cell wall peroxidases during biotic stress (Bolwell *et al.*, 1995). It was reported by Robertson *et al.* (1995) that in elicitor treated French bean cells, H_2O_2 appears to originate from a cell wall peroxidase in a three-component system requiring ion fluxes and leading to extracellular alkalinization and the release of a reductant. Evidence for a peroxidase-dependent oxidative burst in lettuce infected by *Pseudomonas syringae* pv *phaseolicola* was found by Bestwick *et al.* (1997) where the highly localized accumulation of H_2O_2 was detected cytochemically using $CeCl_3$.

12.1.2 ROS scavenging machinery in plant cells

In plant cells, enzymes and redox metabolites act in synergy to carry out ROS detoxification. Superoxide dismutase (SOD) catalyses the dismutation of O_2^- to H_2O_2, CAT dismutates H_2O_2 to oxygen and water, ascorbate peroxidases (APX) reduces H_2O_2 to water by utilizing ascorbate as a specific electron donor and glutathione peroxidase (GPX) oxidizes glutathione to form oxidized glutathione (Noctor and Foyer, 1998).

Of all these mentioned scavenging mechanisms, SOD contributes to resistance in producing H_2O_2 from O_2^-, thus playing a major role in plant defense (Gupta *et al.*, 1993).

12.1.2.1 Superoxide dismutases (SODs)

ROS are continuously produced in both stressed and unstressed plants (Apel and Hirt, 2004). Therefore plants have a well-developed defense system against ROS, involving limiting the formation of ROS as well as instituting its removal. Within a cell, the SOD's constitute the first line of defense against ROS (Ogawa *et al.*, 1997). O_2^- is produced at any location where an electron transport system is present and hence O_2^- activation may occur in different compartments of the cell. This being the case, it is not surprising that SOD's are found throughout all subcellular locations (Kliebenstein *et al.*, 1998).

Based on the metal cofactor used by the enzyme, SOD's are classified into three groups: iron SOD (Fe SOD), manganese SOD (Mn SOD) and copper-zink SOD (Cu-Zn SOD) (Alscher *et al.*, 2002). The three groups have different cellular localizations. Fe SODs are predominantly found in the chloroplasts

(Salin, 1988), Mn SOD has been located in the peroxisomes and mitochondria (Del Rio *et al.*, 1992) and Cu-Zn SOD are found throughout the plant cell (Alscher *et al.*, 2002).

There are two different groups of Cu-Zn SODs; the first group consists of cytoplasmic and periplasmic forms, which are homodimeric, the latter comprises the chloroplasmic and extracellular Cu-Zn SOD, which are homotetradimeric. Ogawa *et al.* (1997) have proposed that Cu-Zn SOD in the apoplast functions in the lignification of the cell wall and that it protects the nucleus against fatal mutations caused by O_2^- molecules. Recently Ivanov *et al.* (2005) established that after infecting susceptible and resistant (expressing HR) wheat plants with leaf rust (*P. recondita* f.sp. *tritici*), the levels of H_2O_2 and SOD activity in the resistant plants were constitutively higher than in the susceptible plants. Also in the susceptible plants, an inhibition of activities of CAT and GST was found.

12.1.2 Plant defense signaling through ROS

A well-established role for H_2O_2 is to function as a signal molecule during HR (Clarke *et al.*, 2000). When H_2O_2 is produced in response to pathogen infection, it mediates the cross-linking of cell wall proteins and plant cell wall phenolics (Grant and Loake, 2000). Although this is still somewhat controversial, H_2O_2 may also have some antimicrobial function (Wu *et al.*, 1995). However, the most important function of H_2O_2 and O_2^- is thought to act as second messengers, not only to induce plant defense related genes, but also the hypersensitive host cell death (Desikan *et al.*, 2000).

The expression of defense related genes such as GST and glutatione peroxidase has been shown to be induced by H_2O_2 in soybean (Levine *et al.*, 1994), while H_2O_2 induced the expression of GST and PAL in *Arabidopsis* suspension cultures (Desikan *et al.*, 1998). Recently, a tobacco gene encoding a proteosome subunit was identified after being induced by H_2O_2 (Etienne *et al.*, 2000). All of these genes are implicated to function during plant defense responses. GST comprises a family of enzymes involved in cellular detoxification processes following various stresses (Foyer *et al.*, 1997), GPX scavenges H_2O_2 in the ascorbate-glutatione cycle (Foyer *et al.*, 1997), PAL is an enzyme involved in the synthesis of defense-related compounds (Mauch-Mani and Slusarenko, 1996), while proteosomes are involved in

protein degradation, a common feature of the HR cell death response (Etienne *et al.*, 2000).

Microarray analysis identified a large number of up-regulated genes after H_2O_2 treatments (Desikan *et al.*, 2001). The genes found to be up regulated coded for antioxidant enzymes, defense and stress related proteins, transcription factors, protein phosphatases and protein kinases (Desikan *et al.*, 2001). This demonstrated that H_2O_2 can modulate the expression of a subset of genes within the *Arabidopsis* genome and also generates the possibility that H_2O_2 can act as a modulator of gene expression in other plants. Thus, H_2O_2 is being proposed as a major player in cell signaling.

A further role for H_2O_2 in signaling events was demonstrated by Pei *et al.* (2000). They demonstrated that calcium channels were activated by H_2O_2 in intact *Arabidopsis* guard cells. Thus, if calcium elevation is an early response to H_2O_2, it is likely that the activation of calcium dependent protein kinases may mediate downstream signaling together with protein kinases/phosphatases and other effector proteins. Researchers have suggested that reversible protein phosphorylation is a key-regulating event in the oxidative burst in response to plant-pathogen interaction (Levine *et al.*, 1994). Pharmacological data has shown that reversible protein phosphorylation is indeed involved in downstream signaling following H_2O_2 generation and/or perception.

It has been speculated that ROS would have the ability to inhibit protein phosphatases. Recent work by has confirmed these speculations since they showed that the human protein tyrosine phosphatase PTPB1 was modified by H_2O_2 at the active cysteine site. The inactivation of PTPB1 was found to be reversible and could be brought about by incubation with glutathione.

Under unstressed conditions, the formation and scavenging of ROS are in balance. However, several forms of biotic and abiotic stresses, such as pathogen invasion, excess light energy, dehydration, and low temperature, increase the generation of ROS. If the amount of ROS generated exceeds the capacity of the scavenging systems, it can result in cellular damage, manifested in inactivation of enzymes or cell death (Dat *et al.*, 2000).

Although potentially damaging, ROS has been shown to promote plant resistance to pathogens in several ways. During defense responses, ROS is produced by plasma membrane-bound NADPH oxidases, cell wall-bound peroxidases and amine oxidases in the apoplast (Mahalingam and Fedoroff, 2003; Laloi *et al.*, 2004) (Figure 1.6). One of the earliest pathogen-induced defense responses is the oxidative burst, a rapid and transient production of large amounts of ROS at the site of attempted invasion (Wojtaszek, 1997). A likely source for this apoplastic O_2^- generation is a NADPH oxidase homologous to that of activated mammalian phagocytes and neutrophils (gp91phox) (Keller *et al.*, 1998; Overmyer *et al.*, 2003; Laloi *et al.*, 2004) (Figure 1.6). *AtRBOHD* and *AtRBOHF* genes encoding NADPH oxidase in *Arabidopsis* are required for full ROS generation during bacterial and fungal challenge (Torres *et al.*, 2002). H_2O_2 is also produced in vitro by some peroxidase isoforms at an alkaline pH. Since the apoplast is alkaline following pathogen recognition, peroxidases have been suggested to contribute to the oxidative burst (Bolwell *et al.*, 1995; Wojtaszek, 1997). The accumulation of extracellular H_2O_2 induced by pathogen challenge has been proposed to crosslink the cell wall proteins, thus strengthening the wall (Neill *et al.*, 2001). The oxidative burst can be directly harmful to invading pathogens but it also contributes to cell death: ROS generated via the oxidative burst plays a central role in the development of host cell death during the HR reaction (Lamb and Dixon, 1997; Grant and Loake, 2000).

Importantly, ROS is thought to have the potential for being a signal in plant defense responses (Neill *et al.*, 2001). H_2O_2 is a relatively stable form of ROS and has the ability to diffuse across membranes and reach locations far from the site of its original generation (Wojtaszek, 1997). Increased ROS generation enhances the accumulation of SA as well as the transcripts of *PR* genes (Van Camp *et al.*, 1998; Maleck and Dietrich, 1999). Furthermore, SA has been shown to have inhibitory effects on CAT and APX activities, and this may lead to accumulation of hydrogen peroxide, free radicals, and other ROS (Chen *et al.*, 1993). SA has also been suggested to potentiate the production of NADPH oxidase-dependent O_2^- via a positive feedback loop (Van Camp *et al.*, 1998).

Plant responses to pathogens seem to share common elements with responses to excess light (Karpinski *et al.* 2003). A rapid increase in ROS

concentration, depletion of antioxidant pools, chlorosis and necrosis of leaves, local and systemic defense responses, and induction of defense gene expression are markers of both responses (Karpinski *et al.*, 2003). However, while the ROS burst during pathogen infection is considered to originate mainly from membrane bound NADPH oxidase, during excess light stress ROS is produced in the chloroplast and peroxisome (Karpinski *et al.*, 2003) (Figure 1.6). High light also induces the accumulation of SA, a central hormone in pathogen defense; Karpinski and coworkers (2003) demonstrated that high-light acclimated plants had several-fold greater foliar SA than plants cultivated in low light.

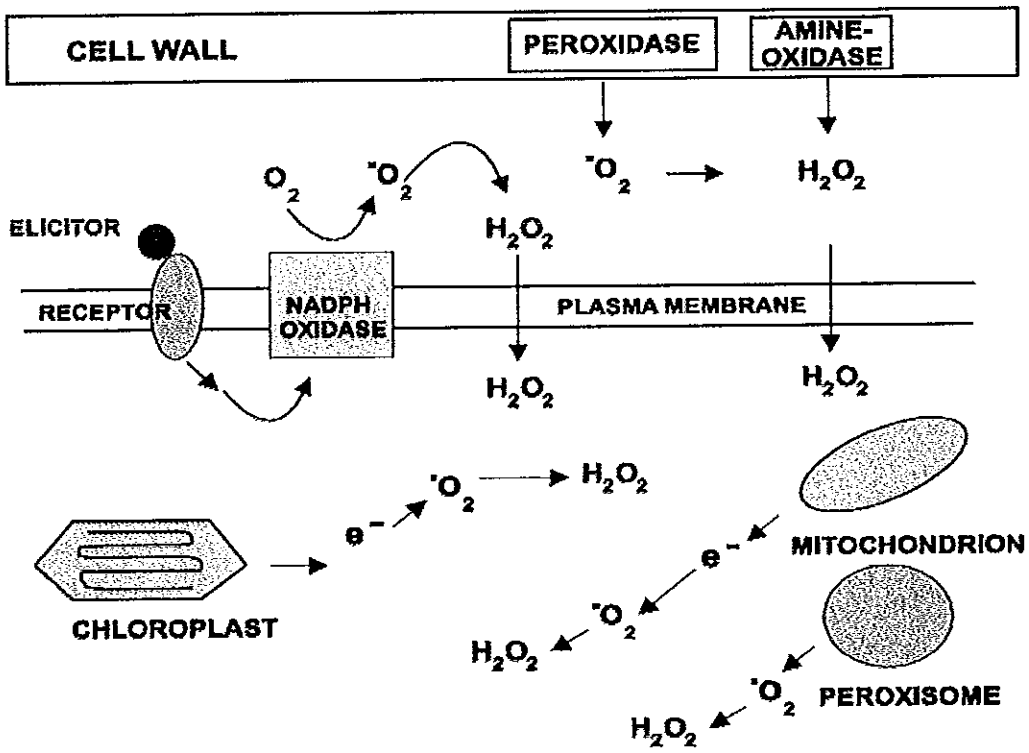

Figure 1.6 Stress-triggered formation of reactive oxygen species (ROS) in the plant cell (adapted from Mahalingam and Fedoroff 2003).

12.2 Salicylic acid-mediated defense signaling

The phytohormone salicylic acid (SA) has long been known to play a central role in plant defense signaling. SA levels increase in response to pathogen attack at the site of infection, and this is essential in resistance against various pathogens (Glazebrook, 2005). Moreover, exogenous application of SA protects plants against

pathogens and induces the expression of defense-related genes (Van Loon *et al.*, 1997). SA is required also in the establishment of systemic acquired resistance (SAR). SAR is an induced state of resistance that is manifested throughout the plant in response to pathogen-triggered localized necrosis (Malamy *et al.*, 1990; Métraux *et al.*, 1990; Durrant and Dong, 2004). It can last from weeks to even months and is effective against a wide variety of normally virulent pathogens, including viruses, bacteria, fungi, and oomycetes (Thomma *et al.*, 2001; Durrant and Dong, 2004). The induction of SA signaling and SAR is associated with accumulation of PR proteins such as beta-1,3-glucanases, thaumatin-like proteins, chitinases, and PR1, which are thought to contribute to resistance (Van Loon, 1997). Many of the PR proteins have antimicrobial activity in vitro, but their roles in the establishment of SAR are unclear. Nevertheless, they serve as molecular markers for the onset of the defense response (Van Loon, 1997; Durrant and Dong, 2004).

SA-mediated defense signaling and SAR are often induced by infection with avirulent pathogens that trigger gene-for-gene resistance and HR, but also in response to necrotizing cell death-causing pathogens (Glazebrook *et al.*, 1997; Durrant and Dong, 2004; Glazebrook, 2005). However, while virulent pathogens do not usually trigger HR, they can induce SA signaling as part of the basal defense response to contain their growth (Glazebrook *et al.*, 1997). SA-dependent defense responses are considered effective mainly against biotrophic pathogens that feed on living tissues, such as the oomycete *Peronospora parasitica* (*Pp*), the fungus *Erysiphe orontii*, and the bacterium *Pseudomonas syringae* (Glazebrook, 2005). Accordingly, impaired SA production leads to increased susceptibility to various pathogens. For example, SA production is significantly reduced in *sid2* (*SA induction deficient*) plants, resulting in increased susceptibility to both virulent and avirulent strains of *P. syringae* and *Pp* (Nawrath and Métraux, 1999). *sid2* encodes isochorismate synthase (ICS1). SA can be synthesized via two routes, firstly through the phenylalanine biosynthetic pathway by PAL from phenylalanine (Mauch-Mani and Slusarenko, 1996). Alternatively, it can be synthesized through isochorismate synthase (ICS) and isochorismate pyruvate lyase (IPL) from chorismate (Wildermuth *et al.*, 2001). The drastic reduction in the accumulation of SA in the *sid2* mutant indicates that the majority of this hormone in

Arabidopsis is produced via isochorismate (Wildermuth *et al.*, 2001) rather than via the shikimate-phenylalanine pathway, as earlier presumed (Lee *et al.*, 1995).

The first study highlighting the importance of SA in defense signaling employed transgenic *Arabidopsis* plants expressing the bacterial SA-degrading enzyme salicylate hydroxylase (*NahG*), which converts SA to catechol (Delaney *et al.*, 1994). *NahG* plants display enhanced susceptibility to several fungal, bacterial, oomycete and viral pathogens, interpreted to result from the lack of SA (Delaney *et al.*, 1994). However, recent studies comparing *NahG* plants with SA-deficient mutants indicate that the observed disease susceptibility phenotype might partly arise from the SA degradation product catechol rather than the lack of SA itself (Van Wees and Glazebrook, 2003). Treatment of *NahG* plants with catalase seems to reverse the susceptibility to *P. syringae* pv. *phaseolicola*, this suggests that the accumulation of catechol might trigger increased production of hydrogen peroxide, interfering with the true effects of the lack of SA (Van Wees and Glazebrook, 2003).

SA signaling seems to be mediated through at least two mechanisms, one dependent on *NPR1/NIM1/SAI1* (*NONEXPRESSOR OF PR1,* Cao *et al.*, 1994; *NON-INDUCIBLE IMMUNITY 1*, Delaney *et al.*, 1995; *SA-INSENSITIVE 1*, Shah *et al.*, 1997) and another independent of NPR1 (Figure 1.7) (Kachroo *et al.*, 2000). Signaling through NPR1 has been the aim of various studies. It was found that NPR1 contains at least four ankyrin repeats, which are also found on proteins that are involved in very diverse biological functions and also in protein-protein interactions. Zhang *et al.* (1999) concluded that after SA activated NPR1, it is mobilized to the nucleus where it interacts with a subclass of transcription factors in the basic leucine zipper protein family to regulate *PR-1* expression. A second mechanism where SA signaling functions without NPR1 was also described. Van Wees and Glazebrook (2003) showed that catechol, which is produced from SA by the NahG-encoded salicylate hydroxylase, is responsible for some plant defenses. Thus, other products of the phenylalanine biosynthetic pathway might be involved in defense and not only SA.

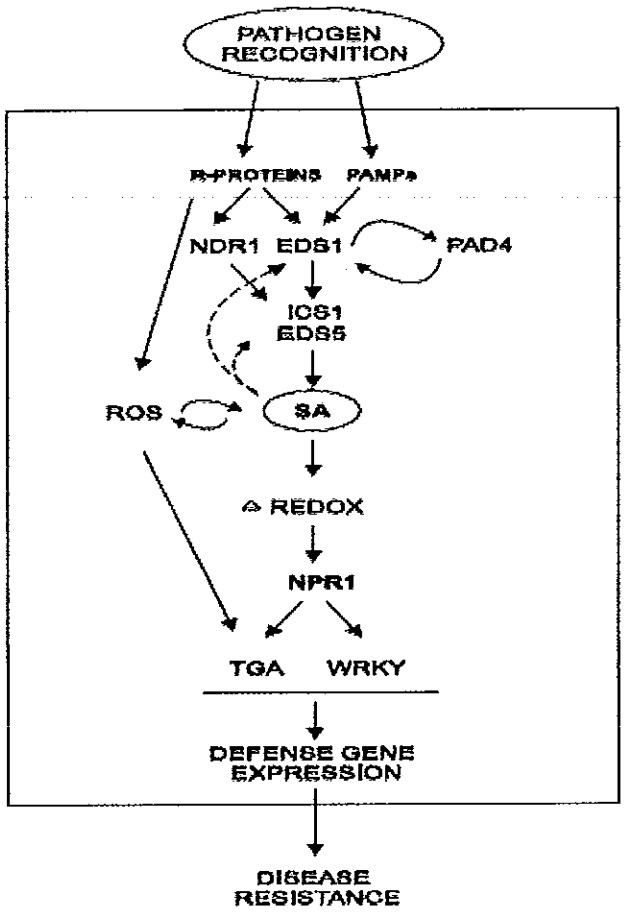

Figure 1.7 Sequence of events from pathogen recognition to gene induction in defense signaling involving salicylic acid (modified from Durrant and Dong 2004).

12.3 Jasmonic acid-mediated defense signaling

Besides SA, phytohormone jasmonic acid (JA) has also been implicated as a signal in plant defense response (Hammond-Kosack and Jones, 1996). JA is derived from linolenic acid by a lipoxygenase (LOX)–mediated oxygenated process (Hamberg and Gardner, 1992). JA and its methyl ester, methyl jasmonate (MeJA), regulate developmental processes, including embryogenesis, pollen and seed development, and root growth (Farmer *et al.*, 2003). Moreover, JAs also mediate resistance to insects, microbial pathogens, and abiotic stress responses to wounding and ozone (Reymond and Farmer, 1998; Norman-Setterblad *et al.*, 2000).

However, while JA is a terminal product of the octadecanoid pathway, it is not the only one with biological activity. Recent studies indicate that a

cyclopentenone precursor of JA, 12-oxo-phytodienoic acid (OPDA), can also induce expression of defense genes (Farmer *et al.*, 2003). JA responses are generally considered effective in defense against necrotrophic pathogens (Turner *et al.*, 2002; Farmer *et al.*, 2003). In order to better understand the working of JA in plants, mutants in the JA response have been used. Some of these mutants include *JAR1* (*JASMONATE RESISTANT1*; Staswick *et al.*, 1992), *coi1* (*coronatine insensitive 1*; Feys *et al.*, 1994) and *jin1* (*jasmonate insensitive 1*; Berger *et al.*, 1996). *Arabidopsis* mutants impaired in the synthesis (*fad3/7/8*) or perception (*coi1*) of JA exhibit enhanced susceptibility to a variety of pathogens, including the fungi *Alternaria brassicicola*, *Botrytis cinerea*, and *Pythium* sp., and the bacterium *E. carotovora* (Thomma *et al.*, 1998, 2001; Norman-Setterblad *et al.*, 2000). These pathogens have a common virulence strategy; they kill plant cells to obtain nutrients.

The perception and subsequent signal transduction of JA remain unclear. A receptor for JA has not yet been characterized. However, a central element of the JA signaling pathway seems to be the COI1 (CORONATINE INSENSITIVE 1) protein (Feys *et al.*, 1994; Xie *et al.*, 1998). *coi1* mutants of *Arabidopsis* are male-sterile, fail to express JA-regulated genes, and are susceptible to pathogens (Thomma *et al.*, 1998). COI1 is an F-box protein that forms an active SCF^{COI1} complex, which together with the COP9 signalosome (CSN) plays an essential role in JA signaling (Devoto *et al.*, 2002; Xu *et al.*, 2002) (Figure 1.8). This machinery functions in vivo as an ubiquitin ligase complex that removes repression from JA-responsive defense genes. It is thought to target regulatory proteins, including transcriptional repressors, to ubiquitin-proteasome-mediated protein-degradation (Devoto *et al.*, 2002; Xu *et al.*, 2002; Feng *et al.*, 2003). Feng *et al.* (2003) demonstrated that, like the *coi1* mutant, plants with reduced CSN function exhibit a JA-insensitive root elongation phenotype and an absence of specific JA-induced gene expression. Interestingly, the recently characterized auxin receptor TIR1 is an F-box protein that, like COI1, forms an ubiquitin protein ligase SCF^{TIR} complex (Dharmasiri *et al.*, 2005). Thus, it is tempting to speculate that, similarly to TIR1, COI1 could act as a receptor for JA.

The production of JA eventually leads to the induction of many genes, including *VEGETATIVE STORAGE PROTEIN* (*VSP*) and *THIONIN 2.1* (*THI2.1*), used as markers for JA-dependent defense responses (Berger *et al.*, 1995; Epple *et al.*,

1995; Penninckx *et al.*, 1998; Devoto and Turner, 2003). Moreover, transcription of genes that regulate JA synthesis, e.g. *DAD1, LOX2, AOS,* and *OPR3*, is induced by JA (Devoto and Turner, 2003). Some defense-related genes, such as *PLANT DEFENSIN 1.2* (*PDF1.2*), *HEVEINLIKE PROTEIN* (*HEL*), and *BASIC CHITINASE* (*CHIB*), are induced cooperatively by JA and ET in *Arabidopsis* (Penninckx *et al.*, 1998; Norman-Setterblad *et al.*, 2000) (Figure 1.8).

12.4 Ethylene-mediated defense signaling

Ethylene (ET) is a gaseous plant hormone involved in various physiological processes, including seed germination, organ senescence, leaf abscission, fruit ripening, and morphological responses of organs (Bleecker and Kende, 2000). ET also regulates plant responses to abiotic stresses, including those induced by flooding or drought, and to biotic stresses, such as pathogen attack (Penninckx *et al.*, 1998; O'Donnell *et al.*, 2003). Ethylene is formed from methionine via S-adenosyl-L-methionine and the cyclic, nonprotein amino acid 1-aminocyclopropane-1-carboxylic acid (Miyazaki and Yang, 1987).

The production of ET is one of the earliest plant responses to pathogens. Diverse viral, bacterial, and fungal microbes trigger accumulation of ET, leading to induction of defense genes, such as basic *PR1*, basic *β-1,3-GLUCANASE*, and *CHIB*, which can also be induced by ET-independent pathways (Deikman, 1997; Thomma *et al.*, 1998). ET contributes to resistance in some interactions but can promote disease development in others (Thomma *et al.*, 1998, 1999; Hoffman *et al.*, 1999; Norman-Setterblad *et al.* 2000).

Arabidopsis ethylene-insensitive 2 (*ein2*) plants display enhanced susceptibility to *B. cinerea* and *E. carotovora* (Thomma *et al.*, 1999; Norman-Setterblad *et al.*, 2000). On the other hand, infection of *ein2* with virulent *P. syringae* and *X. campestris* resulted in reduced disease symptoms (Bent *et al.*, 1992). Insensitivity to ET has also been shown to reduce foliar disease development in tomato (Lund *et al.*, 1998). ET-insensitive tobacco was susceptible to the fungus *Pythium sylvaticum*, which normally is not pathogenic to this species (Knoester *et al.*, 1998). The inability of ET response mutant *etr1* (*ethylene-resistant 1)* to develop pathogen resistance in response to nonpathogenic rhizobacteria demonstrated the requirement of ET in the establishment of ISR (Pieterse *et al.*, 1998).

Characterization of ET-response mutants in *Arabidopsis* has identified components of the ET signal transduction pathway (Figure 1.8). One class of mutations, exemplified by *etr1*, led to the identification of ET receptors (Bleecker, 1999). ET seemed to be involved in the control of several pathogenesis-related genes, including *β-1,3-GLUCANASE*, *CHIB*, and *PDF1.2* (Wang *et al.*, 2002) (Figure 1.8).

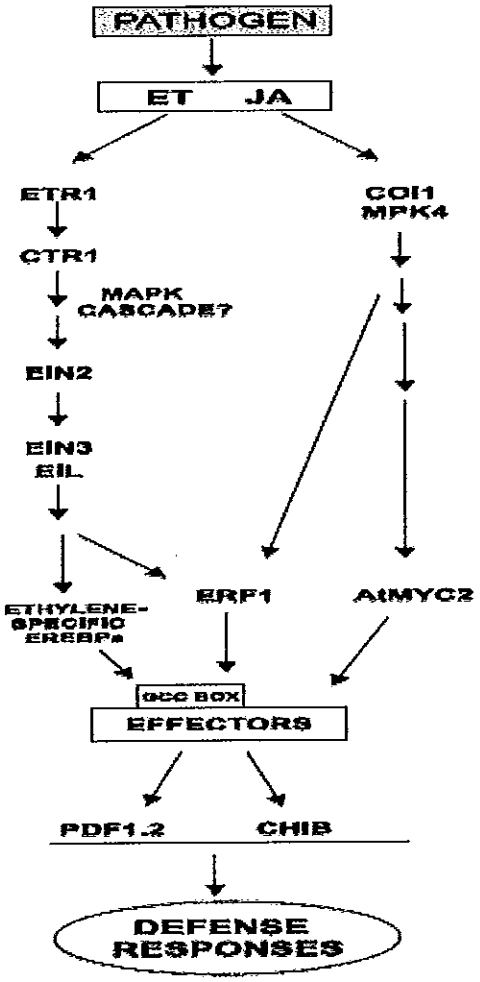

Figure 1.8 JA and ET act together to activate production of defensive proteins such as PDF1.2 and CHIB (adapted from Glazebrook, 2001 and Guo and Ecker, 2004).

13 Crosstalk between signaling pathways

Plants respond to a variety of abiotic and biotic stimuli from the environment. Following perception of stress, several signal transduction pathways are switched on, resulting in physiological and molecular changes in the plant. When pathways

operating in defense signaling are investigated, they are sometimes considered as independent units in order to simplify the interpretation. Defense pathways influence each other through a network of regulatory interactions, and thus, plant responses to various stress stimuli are a result of this complex interplay (Kunkel and Brooks, 2002; Bostock, 2005). The term crosstalk is often used when discussing interactions in defense signaling. There are however differing opinions about when it is appropriate to use this term to describe plant defense signaling (Taylor and McAinsh, 2004; Mundy *et al.*, 2006).

Uncertainty results partly because not all of the components operating in the defense pathways are known. Nevertheless, crosstalk is usually described as including a network of signal interactions in which functional outcomes can be positive, negative, or neutral (Bostock, 2005). In addition to different biotic stress signaling pathways, also biotic and abiotic pathways can "crosstalk". Several studies have described crosstalk among SA, JA, and ET signaling pathways (Kunkel and Brooks, 2003; Bostock, 2005). SA and JA signaling interact on many levels, and in most cases, this relationship seems to be mutually antagonistic (Kunkel and Brooks, 2002) (Figure 1.9). SA can inhibit the synthesis of JA and prevent the accumulation of PIs in response to JA, wounding, systemin, and oligosaccharides (Doares *et al.*, 1995). SA and its functional analogs have also been shown to prevent the expression of JA-dependent defense genes on several occasions (Gupta *et al.*, 2000; Kunkel and Brooks, 2002). Moreover, Petersen *et al.* (2000) demonstrated that MAP KINASE 4 (MPK4) regulates negative crosstalk between JA and SA in the activation of defenses. Gene induction triggered by JA is blocked in *mpk4* mutants, indicating the importance of this gene for mediation of the JA signal (Figures 1.8 and 1.9). Simultaneously, this mutant constitutively expresses SA-regulated defense genes, probably as a result of the elevated SA levels. This indicates that a MAP kinase cascade involving MPK4 represses SA biosynthesis and promotes either JA perception or response (Petersen *et al.*, 2000). A node of convergence between SA and JA signaling seems to be the plant-specific transcription factor WRKY70 (Li *et al.*, 2004) (Figure 1.9). Plants overexpressing *WRKY70* showed decreased JA- but enhanced SA-dependent defense activation, hence improving resistance to *E. carotovora* and *P. syringae* (Li *et al.*,

2004). This indicates that WRKY70 integrates defense signals, and thus, affects pathway activation (Li *et al.*, 2004).

Some evidence also supports synergism between SA and JA defenses. Simultaneous activation of both SAR and rhizobacteria-triggered ISR resulted in an additive effect on induced protection against *P. syringae* (VanWees *et al.* 2000). Moreover, ROS has been shown to stimulate accumulation of SA and induction of SAR. At the same time, SA induces the production of ROS such as hydrogen peroxide and NO (Van Camp *et al.*, 1998) (Figures 1.7 and 1.9). This synergism is thought to promote such defense responses as HR and killing of the pathogen.

Reported crosstalk between JA and ET signaling is mostly positive. Transcription factors AtMYC2/JIN1 and ERF1 are important regulators of these interactions in *Arabidopsis* (Lorenzo *et al.*, 2004) (Figure 1.8). The expression of ERF1 (and its target genes) is synergistically activated by ET and JA, and ERF1 integrates these signals into the activation of plant defenses (Lorenzo *et al.*, 2003) (Figures 1.8 and 1.9). In rare cases, JA and ET have the opposite effects; in tobacco nicotine biosynthesis, a direct defense against some herbivores is stimulated by JA and inhibited by ET (Shoji *et al.*, 2000).

14 Pathogenesis-related proteins (PR-proteins)

14.1 Terminology

Since two working groups (Van Loon and Van Kammen, 1970; Gianinazzi *et al.*, 1970), independently discovered pathogenesis-related proteins (PR, initially named "b" proteins) in tobacco leaves after induction of a hypersensitive response by infection with TMVby most research interest has focused on determining their possible involvements in plant resistance to pathogens. This assumption flowed from the initial findings that these proteins are commonly induced in resistant plants, expressing a hypersensitive necrotic response (HR) to pathogens of viral, fungal and bacterial origin. Later, however, it turned out that b-proteins are induced not only in resistant, but also in susceptible plant – pathogen interactions, as well as in plants, subjected to abiotic stress factors (Van Loon, 1985). Thus, still in 1980 Antoniw *et al.* coined the term "pathogenesis-related proteins", which have been defined as "proteins encoded by the host plant but induced only in pathological or related situations", the

latter implying situations of non-pathogenic origin. To be included among the PR-proteins, they have to be newly expressed upon infection but not necessarily in all pathological conditions. Pathological situations refer to all types of infected states, not just response to resistance but hypersensitive responses in which PR-proteins are most common; they also include parasitic attack by nematodes, insects and herbivores (Van Loon, 1999).

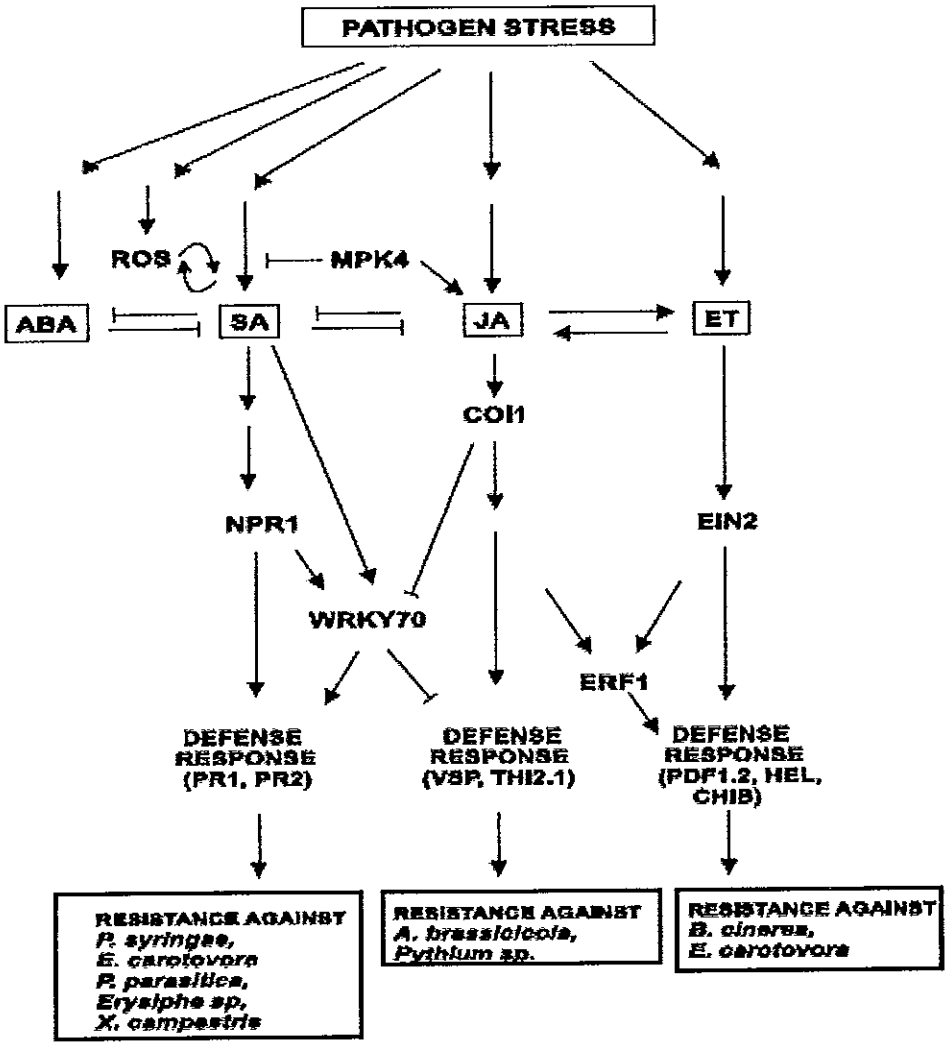

Figure 1.9 Salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) defense signaling pathways and signal crosstalk in *Arabidopsis* (modified from Kunkel and Brooks, 2002 and Durrant and Dong, 2004).

Table 1.1. PR-proteins induced in Samsun tobacco (NN genotype) by TMV infection (Bol *et al.*, 1990)

Group	Acidic PR proteins		Basic PR proteins		Function
	Name	Mol wt (kD)	Name	Mol wt (kD)	
1	1a	15.8	16 kD	16.0	Unknown
	1b	15.5			
	1c	15.6			
2a	2	39.7	Gluc.b	33.0	β-1,3-Glucanase
	N	40.0			
	O	40.6			
	Q'	36.0			
2b	O'	25.0			β-1,3-Glucanase
3	P	27.5	Ch.32	32.0	Chitinase
	Q	28.5	Ch.34	34.0	
4	s1	14.5			Unknown
	r1	14.5			
	s2	13.0			
	r2	13.0			
5a	R	24.0	Osmotin	24.0	Unknown
	S	24.0			Thaumatin-like proteins
5b			45 kD	45.0	Unknown

14.2 Classification of PR-proteins

Originally, five main groups of PR-proteins (PR-1 to PR-5) were characterized by both molecular and molecular-genetic techniques in tobacco, numbered in order of decreasing electrophoretic mobility. Each group consists of several members with similar properties (Bol *et al.*, 1990) (Table 1.1). The PR-1 group is the most abundant, constituing up to 1-2% of total leaf proteins. In 1994 a unifying nomenclature for PR-proteins was proposed based on their grouping into families sharing amino acid sequences, serological relationships, and enzymatic or biological activity. By then 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. Later three novel families (PR-12, PR-13 and PR-14) were recognized in radish, *Arabidopsis* and barley, respectively (Van

Loon and Van Strien, 1999). Table 1.2 contains all the recognized families of PR-proteins as well as their specific properties.

Table 1.2. Recognized and proposed families of pathogenesis-related proteins

Families	Type member	Properties	Reference
PR-1	Tobacco PR-1a	Antfungal	Antoniw *et al.*, 1980
PR-2	Tobacco PR-2	β-1,3-glucanase	Antoniw *et al.*, 1980
PR-3	Tobacco P, Q	Chitinase type I, II, IV, V, VI, VII	Van Loon, 1982
PR-4	Tobacco "R"	Chitinase type I, II	Van Loon, 1982
PR-5	Tobacco S	Thaumatin-like	Van Loon, 1982
PR-6	Tomato Inhibitor I	Proteinase-inhibitor	Green and Ryan, 1972
PR-7	Tomato P69	Endoproteinase	Vera and Conejero, 1988
PR-8	Cucumber chitinase	Chitinase type III	Métraux *et al.*, 1988
PR-9	Tobacco "lignin-forming peroxidase"	Peroxidase	Lagrimini *et al.*, 1987
PR-10	Parsley "PR1"	"Ribonuclease-like"	Somssich *et al.*, 1986
PR-11	Tobacco "class V" Chitinase	Chitinase type I	Melchers *et al.*, 1994
PR-12	Radish Rs-AFP3	Defensis	Terras *et al.*, 1992
PR-13	Arabidopsis THI2.1	Thionin	Epple *et al.*, 1995
PR-14	Barley LTP4	Lipid-transfer protein	García-Olmedo *et al.*, 1995
PR-15	Barley OxOa (germin)	Oxalate oxidase	Zhang *et al.*, 1995
PR-16	Barley OxOLP	"Oxalate oxidase-like"	Wei *et al.*, 1998
PR-17	Tobacco PRp27	Unknow	Okushima *et al.*, 2000

14.3 Relevance of PR-proteins to disease resistance

Presently, it is still difficult to assign a causative role of PR-proteins in plant resistance to pathogens.

14.3.1 Stronger accumulation of PR-proteins in inoculated resistant as compared to susceptible plants. Besides previous data, substantiating this statement (Van Loon, 1985), differential responses of resistant/susceptible plants were recently reported in tomato plants, inoculated with *Cladosporium fulvum* (Wubben *et al.*,

1996); *Phytophthora infestans*-infected potato (Tónon *et al.*, 2002); *Venturia inaequalis*-inoculated apple (Poupard *et al.*, 2003); *Pseudomonas syringae*-infected grapevine (Robert *et al.*, 2001), etc.

14.3.2 Important constitutive expression of PR-proteins in plants with a high level of natural disease resistance. This correlation was observed in several pathosystems, such as apple – *Venturia inaequalis* (Gau *et al.*, 2004), tomato – *Alternaria solani* (Lawrence *et al.*, 2000), and potato – *Phytophthora infestans* (Vleeshouwers *et al.*, 2000). Moreover, Vleeshouwers and coworkers (2000) proposed PR mRNAs as a molecular marker in potato breeding programs.

14.3.3 Significant constitutive expression of PR-ptoteins in transgenic plants overexpressing PR genes accompanied by increased resistance to pathogens. The increased tolerance to *Peronospora tabacina* and *P. parasitica* var. *nicotianae* was demonstrated in tobacco overexpressing PR1a gene. Transgenic rice and orange plants overexpressing thaumatin-like PR-5 possessed increased tolerance to *Rhizoctonia solani* and *P. citrophthora*, respectively (Fagoaga *et al.*, 2001), while transgenic potato overexpressing PR-2 and PR-3 had improved resistance to *P. infestans*. PR-2 and PR-3 genes, encoding for β-1,3-glucanase and chitinase, respectively, confer resistance of carrot to several fungal pathogens. The simultaneous expression of tobacco β-1,3-glucanase and chitinase genes in tomato plants results in increased (resistance to fungal pathogens). On the contrary, silencing of the PR-1b gene in barley facilitates the penetration of the fungal pathogen *Blumeria graminis* f. sp. *hordei* into the leaves (Schultheiss *et al.*, 2003).

14.3.4 Accumulation of PR-proteins in plants in which resistance is locally or systemically induced. Generalizing this broad research area it can be stated that PR-proteins are recognized as markers of the systemic acquired resistance (SAR), and PR-proteins genes are involved in the list of the so-called SAR-genes (Ward *et al.*, 1991). It has largely been demonstrated that SAR and the accompanying set of PR-proteins are induced by different pathogens, as well as by a range of chemicals predominantly in a SA-dependent pathway; SAR is active against a broad spectrum of pathogens. Some SAR-inducing chemicals, such as benzothiadiazole (BTH), β-aminobutyric acid (BABA) or 2,6-dichloroisonicotinic acid (DCINA) are harmless commercially supplied compounds and have promising practical applications as novel

tools in plant protection (Van Loon, 1997; Kuč, 2001; Edreva, 2004). It is essential to emphasize that PR-proteins members induced in resistant or SAR- expressing plants as well as those from transgenic resistant plants exhibit high antimicrobial activity (Anfoka and Buchenauer, 1997; Rauscher *et al.*, 1999; Tonón *et al.*, 2002; Anand *et al.*, 2004), this indicating their direct role in disease resistance.

CHAPTER 2

DEFENSE RESPONSE IN *HEVEA BRASILIENSIS* AGAINST *PHYTOPHTHORA* SPP.

Introduction

Hevea brasiliensis (Wild.) Muell.-Arg. or rubber plant is an economically important crop in Thailand, the latex of which is a major Thai export product. Nowadays South East Asia, especially in Thailand, Indonesia and Malaysia are the main source of natural rubber (FAO, 2006). However, one consequence of frequent tapping is that caused infection occurred at the raw surface of tapping site. This problem is common in the southern part of Thailand where the humidity is high and suitable for pathogen growth. In the southern part of Thailand, RRIM600 is the commonly used plantation cultivar because it gives a high-yield of latex but the disadvantage of this cultivar is its susceptibility to *Phythophthora* spp. while BPM-24 is considered to be a more resistant cultivar.

Plant pathogenic oomycetes have unique physiological characteristics and devastating effects on crops and natural ecosystems. *Phytophthora* leaf disease, caused by members of the oomycete *Phytophthora* spp., is the most important disease of the rubber tree. *Phytophthora* spp. described as pathogens of the rubber tree include *P. palmivora*, *P. botryosa*, *P. heveae*, *P. meadii* and *P. parasitica*. In the south of Thailand *P. palmivora* and *P. botryosa* are the most frequently isolated pathogens that cause black stripe, green pod rot and abnormal leaf fall (Butler, 1996; Erwin and Ribeiro, 1996). *P. palmivora* attacks the petioles causing mature leaves to fall prematurely and attacks the tapping surface resulting in poor latex production.

Plants are capable of responding quickly to an initial infection by oomycete pathogens. Documented responses include a rapid increase in cytoplasmic Ca^{2+} concentration, formation of wall appositions beneath the invading hypha and synthesis of reactive oxygen species (ROS), pathogenesis-related (PR)-proteins and phytoalexins. Some of these processes are also associated with hypersensitive cell death (tissue necrosis) (Keller *et al.*, 1996; Blume *et al.*, 2000; Fellbrich *et al.*, 2002).

In general, the incompatible reaction occurs in the resistant host whereas the compatible reaction causes disease in the susceptible host.

To date, more than 30 *Phytophthora* and *Pythium* species have been found to secrete highly conserved proteins, collectively termed elicitins, with molecular weights of about 10 kDa (for review see Ponchet *et al.*, 1999). Elicitins induce defense responses on a restricted number of plants, for example, *Nicotiana* species within the Solanaceae family (Kamoun *et al.*, 1993; Bonnet *et al.*, 1996), some members of Brassicaceae (Bonnet *et al.*, 1996; Keizer *et al.*, 1998) and cranberry (Ivanova *et al.*, 2002).

Since leaves are most likely to be infected during the rainy season this reduces the availability of plant materials and may cause variations in the data collected. Tissue culture of plants or callus is a tool for studying plant-cell resistance to pathogens or elicitors since it is a good system for controlling contamination from the environment and displays defense responses similar to those of the normal plant tissues (Plażek *et al.*, 2003; Maksimov *et al.*, 2004; Iyozumi *et al.*, 2005). Furthermore, cell or tissue cultures are used worldwide for their conveniences. However, some plant responses are difficult to observe in *in vitro* probably because of hormonal dependence, loss of cell differentiation, water saturation or poor gas phase exchange.

In this chapter, the induced defense responses in *H. brasiliensis* against three strains of *Phytophthora* spp.; *P. palmivora Hevea* isolate, *P. palmivora Durian* isolate and *P. parasitica Anthurium* isolate were investigated. The accumulations of Scp and the enzyme peroxidase (POD) were monitored in *Hevea* leaves and callus cultures of both resistant (BPM-24) and susceptible (RRIM600) cultivars after being treated with the zoospores or elicitins secreted by these three strains.

Objectives

1. To study the distinctive of defense responses in *H. brasiliensis* leaves against zoospores and elicitins from three strains of *Phytophthora* spp.
2. To study the defense responses; necrosis, scopoletin (Scp) biosynthesis and enzyme peroxidase activity, in the interaction between *Hevea* leaves and zoospore of *P. palmivora Hevea* isolate [P. pal (Hevea)].
3. To study the defense responses in *Hevea* calli (integument derived calli) against zoospores of *Phytophthora* spp.

Materials and Methods

1 Plant materials

1.1 *Hevea brasiliensis* leaves

1.1.1 Selection of *H. brasiliensis* cultivars

The *H. brasiliensis* cultivars were selected for their degree of resistance according to the classification by the Rubber Research Institute of Thailand (RRIT). Those are BPM-24 and RRIM600 which are considered to be the resistant and susceptible cultivar, respectively.

1.1.2 Preparation of *Hevea* leaves for treatment

The 6- to 8-day-old leaflets or B_2-C stage of rubber leaves as described by Halle' and Martin (1968) and Breton *et al.* (1997) of the selected cultivars, BPM-24 and RRIM600, were detached (Figure 2.1). The healthy leaves were rinsed with sterile distilled water to remove dirt.

1.2 *H. brasiliensis* callus

1.2.1 Callus formation from the integument of *Hevea* immature seeds

Hevea fruits of both cultivars; BPM-24 and RRIM600 were harvested at 6-to-8-weeks of age. Within one week after collection the surface was sterilized by immersion in 95% (v/v) ethanol for 30 s then burning off the ethanol with a flame, and repeated 2 or 3 times. The outer covering of *Hevea* fruits was removed by using a sterilized scalpel and forceps prior to taking out the seeds. Each seed was cut throughout the diameter and then divided into 4 or 6 small pieces (depend on its size). The small pieces were then cultured on Murashige and Skoog's (MS) medium

supplemented with 8% sucrose, 2 mg/l of 2,4-dichlorophenoxy acetic acid (2,4-D) and 2 mg/l of 6-benzylaminopurine (BA) and maintained under continuous dark conditions at 25±2 ^{0}C (Te-chato *et al.*, 2002). After 4 weeks, the calli that grew out of the integument were transferred into the new MS medium with a similar formula to that for callus induction with sucrose reduced to 3%. The calli were subcultured monthly onto fresh medium with the same composition and maintained under continuous dark conditions at 25±2 ^{0}C (Te-chato *et al.*, 2002).

1.1.2 Preparation of *Hevea* calli for treatment

The 3-week-old calli with an approximate diameter of 0.7 cm (0.3-0.4 g each) were selected and used as the experimental tissue. The calli of approximately 1.0 g were used for each treatment.

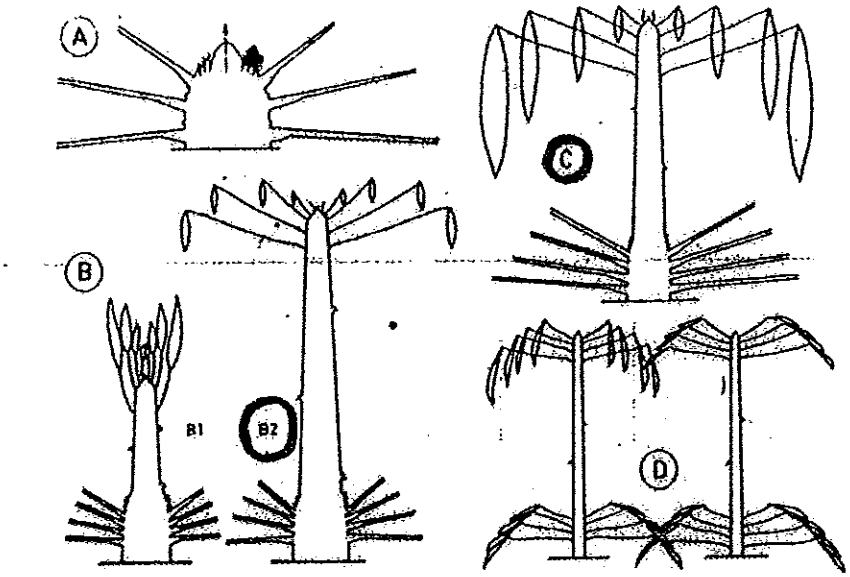

Figure 2.1 The diagram of leaf patterns and various stages of rubber leaves. A, B (B_1 and B_2), C and D represent each stage of rubber leaves.

2 *Phytophthora* spp.

2.1 Strains of *Phytophthora* spp.

2.1.1 *P. palmivora Hevea* isolate

P. palmivora isolated from a rubber tree showing disease symptoms (*P. palmivora Hevea* isolate) was kindly provided by the Songkhla Rubber Research Center, Songkhla. The fungal isolate (No. KBNM 9) was identified as *P. palmivora*

by a PCR based diagnostic assay using *Phytophthora* genus primers, generously provided by Prof. André Drenth of the University of Queensland, Australia (Drenth and Irwin, 2001). This strain would be named P. pal (Hevea). It was further purified by monospore culture in our laboratory then maintained and subcultured in sterile condition every week on potato dextrose agar (PDA) and kept at 25±2 ^{0}C (Figure 2.2).

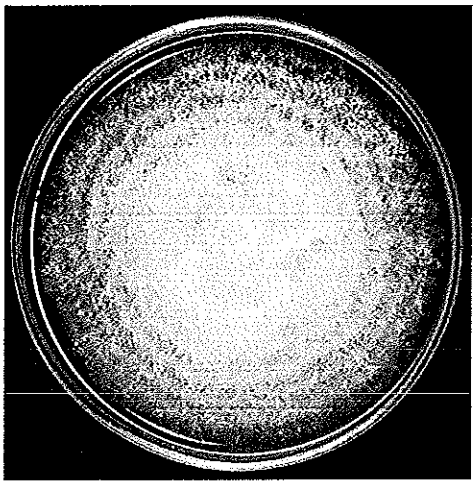

Figure 2.2 The 7-day-old mycelium of *P. palmivora Hevea* isolate on potato dextrose agar.

2.1.2 *P. palmivora Durian* isolate

P. palmivora isolated from Durian (*Durio zibethinus*) tree was kindly provided by Rungsi Charaensatapon, Field Crops Research Institute, Department of Agriculture, Thailand. In this thesis, it will be called P. pal (Durian).

2.1.3 *P. parasitica Anthurium* isolate

P. parasitica isolated from flamingo flower (*Anthurium andraeanum*) was kindly provided by Rungsi Charaensatapon, Field Crops Research Institute, Department of Agriculture, Thailand. The abbreviation P. para will represent the *P. parasitica Anthurium* isolate and used throughout this thesis. It was further purified by monospore culture in our laboratory then maintained and subcultured in sterile condition every week on potato dextrose agar (PDA) and kept at 25±2 ^{0}C. (Figure 2.3)

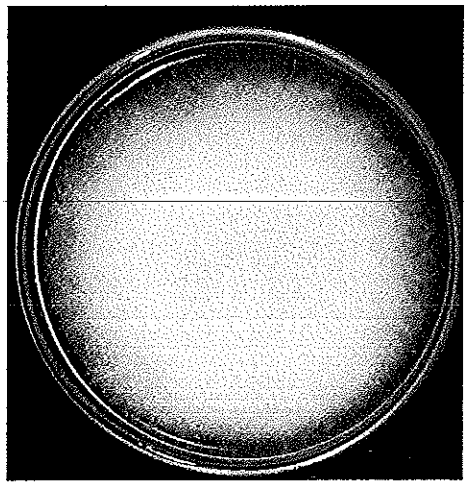

Figure 2.3 The 7-day-old mycelium of *P. parasitica Anthurium* isolate on potato dextrose agar.

2.2 Preparation of zoospores of *Phytophthora* spp.

A 0.5 cm diameter plug was cut with a cork borer from the edge of the growing mycelium, after being cultured on PDA for 7 days, and transferred to a V_8 agar. Five days later, the growing mycelium on V_8 agar was chilled for 15 min using sterile water (8-10 ^{0}C) to trigger zoospores release from the sporangium. Zoospores were collected 30 minutes later. The zoospore suspension was dropped on Petroff Hauser and counted under a microscope. The concentration of zoospores was calculated by the following formula:

$$\text{Zoospores concentration (zoospores/ml)} = \frac{\text{(No. of zoospores in field) X 1000}}{\text{Volume on slide}}$$

$$\text{(Volume on slide} = 4 \times 10^{-3}\ \mu\text{l)}$$

The desired concentrations of zoospore were subsequently adjusted by sterile distilled water. The zoospore suspensions were prepared at concentrations that ranged from $1x10^5$ to $1x10^7$ zoospores/ml (by 10-fold-dilutions). The suspensions were used within 30 minutes before the germination occurred.

3 Elicitins

3.1 The culture filtrates of *Phytophthora* spp.

For preparation of the elicitin, 15 pieces of a 0.5 cm diameter plug cut from the edge of the growing mycelium on PDA were transferred to 150 ml of sterile potato dextrose broth (PDB) in a 500 ml conical flask and shaken at 100 rpm and 25 ^{0}C in the dark for 3 weeks. The small amount of mycelium in PDB culture was transferred to PDA in order to check in the meanwhile for possible contamination from other microorganisms in the culture filtrate. Three weeks later, the mycelium was filtered through a Whatman filter paper No. 4 and the filtrate was then kept at -20 ^{0}C for elicitin purification following an adapted method described by Churngchow and Rattarasarn (2000).

3.2 Partial purification of elicitins

One liter of culture filtrate was slowly added with gentle stirring with 657 grams of $(NH_4)_2SO_4$ (ammonium sulphate) to bring the filtrate to 90% saturation at 4 ^{0}C. The solution was then centrifuged 10,000 rpm at 4 ^{0}C for 20 min. The supernatant was discarded while the pellet was dissolved with sterile distilled water. The 1 ml of solution was desalted by loading onto PD-10 column (Amersham), which containing Sephadex G-25, and eluted with distilled water. The first 1 ml of solution was discarded while the remaining 1 ml fractions were kept and their protein contents were measured at 280 nm. The fractions with a high protein content that caused necrosis in *Hevea* leaves (fraction 3 and 4) were pooled. The partially purified elicitins were measured for their concentrations based on the Bicinchoninic acid method (BCA) (Smith *et al.*, 1985) and monitored for the purity by the silver staining of a 16.5% polyacrylamide gel (Tricine-SDS-PAGE) (Schägger and Jagow, 1987).

3.3 Protein quantification BCA method

Protein contents were quantified by the method modified from Smith *et al.* (1985). A 100 µl of sample was reacted with 2 ml of solution C (appendix) and shaken for a while. The mixture was left standing for 30 minutes at 34 ^{0}C and the absorbance measured at 562 nm. The amount of protein was calculated by comparing with the standard curve of Bovine Serum Albumin (BSA).

3.4 Characterization of elicitin by Tricine-SDS-PAGE (Tricine-Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis)

3.4.1 Preparation of Sodium dodecyl sulphate (SDS) polyacrylamide gel

The slab gel was set up in size 7x8 cm^2, 0.75 mm thick. The composition of the gel was described in table 2.1.

Table 2.1. The ingredients of the gel used for Tricine-SDS-PAGE

Ingredients	Stacking gel (4%)	Separating gel (16.5%)
49.5% Acrylamide-bisacrylamide	0.40 ml	4.33 ml
3.0 M Tris-HCl, pH 8.45, 0.3% SDS	1.2 ml	4.33 ml
10% Ammonium persulfate	50 µl	200 µl
TEMED	10 µl	13 µl
Deionized water	3.34 ml	4.00 ml
Total volume	5.0 ml	13.0 ml

3.4.2 Electrophoresis

The samples were mixed with the sample buffer in the ratio of 3:1, then heated at 95 ^{0}C for 5 min. The samples and molecular marker were applied in each lane of the slab gel. The marker contains Phosphorylase b 97 kDa, Albumin 66 kDa, Ovalbumin 45 kDa, Carbonic anhydrase 30 kDa, Trypsin inhibitor 20.1 kDa, and α-Lactalbumin 14.4 kDa. The electrophoresis was run with 60 volts or 30 mA at 4 ^{0}C for 2-3 h until the bromphenol blue dye move to 0.5 cm from the edge of gel. The electrophoresed gel was then stained with silver nitrate according to the procedure of Pharmacia Biotech.

3.4.3 Silver Staining

The gel was fixed in 40% ethanol and 10% glacial acetic acid for 30 min and sensitized with 30% ethanol, 25% (w/v) glutaraldehyde, 0.2% (w/v) sodium thiosulphate and 6.8% (w/v) sodium acetate for 30 minutes. After washing with deionized water 3 times, 5 min each, the gel was then reacted with 37% (w/v) formaldehyde and 2.5% (w/v) silver nitrate solution for 20 min followed by washing 2

times, 1 min each, with deionized water. The gel bands were developed by using 2.5% (w/v) sodium carbonate and 37% (w/v) formaldehyde and gently shaking for 2-5 minutes. When the dark-brown bands appeared, the gel was immediately immersed in stopping solution containing 1.46% (w/v) Na_2-EDTA solution for 10 minutes. The gel was subsequently washed with deionized water 3 times for 5 minutes and preserved by soaking in 30% ethanol and 87% (w/v) glycerol. The photograph was taken by Sony V1 digital camera.

4 Interaction between zoospores and *Hevea* leaves

4.1 Treatment procedures

4.1.1 Zoospore droplet method

The leaves of BPM-24 (resistant cultivar) and RRIM600 (susceptible cultivar) were prepared in an 11 cm-diameter Petri dish after being rinsed with sterile water to clean the leaf surface. The leaves were then placed and turned abaxial surface upward on the moist Whatman paper. Twenty µl droplets of $5x10^4$, $5x10^5$ and $5x10^6$ zoospores/ml were applied on the abaxial surface of rubber leaves and droplets of sterile distilled water were used as control. The inoculated leaves were kept at 25 ^{0}C under 12 h of daylight (Figure 2.4).

4.1.2 Leaf disc or leaf piece method

Hevea leaves were cut into small pieces of 2x2 cm^2. Twenty pieces from 3 individual plants were placed in the 9 cm Petri dish containing 10 ml of zoospore suspensions or sterile distilled water (a control). After the abaxial surface of each piece was placed facing the zoospores for 1 h, all leaf pieces were rinsed with sterile water to remove the uninfected zoospores and replaced with 10 ml of sterile distilled water. The inoculated leaves were kept at 25 ^{0}C under 12 h of daylight. Both the inoculated leaves and the soaking solution were collected for testing at 0, 12, 24, 36, 48, 72 and 96 h.

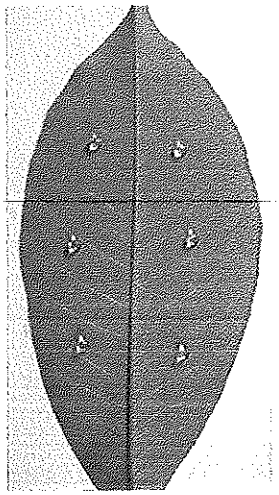

Figure 2.4 The *Hevea* leaf was treated by 20 µl droplets of zoospore suspension on the abaxial surface.

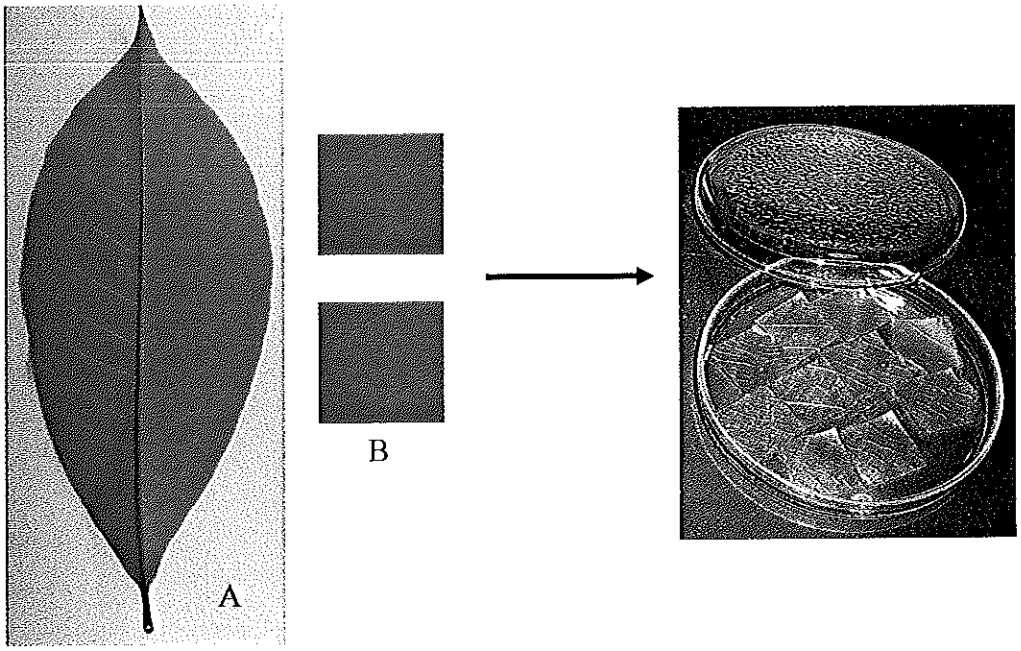

Figure 2.5 The process of leaf disc or leaf piece method.
(A) *H. brasiliensis* leaf.
(B) The *Hevea* leaf was cut into 2x2 cm^2
(C) The leaf discs or leaf pieces were dipped into the zoospore suspension or Water.

4.2 Necrosis on *H. brasiliensis*

To study the effect of zoospore concentrations on necrotic lesions on rubber leaves, the *Hevea* leaves were treated with various zoospore concentrations in both

methods; the zoospore droplet method and leaf disc or leaf piece method. The treated leaves were kept at 25 ^{0}C under 12 h of daylight for development of necrotic lesions. The necroses sizes were measured every 24 h for 72 h.

4.3 Scopoletin (Scp) analysis in *Hevea* leaves

Previous results by our group had shown that the amount of intercellular and extracellular Scp was approximately the same. Thus, the extracellular Scp or Scp in the droplets could represent the Scp produced in the cells. The droplets from each of the leaves (in the same treatment) and the soaking solution were collected and pooled for Scp assay.

4.3.1 Extracellular Scp analysis

For the droplets method, the water droplets with or without zoospores (control) were retained on the leaflets and the accumulated Scp was collected at the designated time points (every 12 h up to 72 h). The volumes of collected samples at each time point were adjusted to give the same total volume.

For the leaf disc or leaf piece method, the soaking solution with or without zoospores (control) were retained through the experiment and the accumulated Scp was collected at the designated time points every 12 h up to 72 h after treatment, then kept at -20 ^{0}C until analyzed.

The Scp was analyzed following the procedure of Churngchow and Rattarasarn (2001) using HPLC and/or spectrofluorometry (excitation and emission wavelengths of 340 nm and 440 nm, respectively). The HPLC was performed for identification of Scp produced by *Hevea* leaves and calli after treatment while the spectrofluorometry was used to measure the amount. The concentration of Scp in *Hevea* samples was given as (μM)

4.3.2 Intracellular Scp analysis

Scp production inside *Hevea* samples was assayed by grinding in 100 mM Tris-HCl, pH 7.5, the crude extract was centrifuged at 12,000 rpm for 20 minutes at 4 ^{0}C to pellet the cell debris. The supernatant was extracted with chloroform: acetic acid: H_2O (4:1:1, v/v/v) for Scp determination. The chloroform layer containing the Scp was collected, evaporated and the residue was redissolved in distilled water. The Scp content was measured as described above in 4.3.1 The concentration of intracellular Scp was given as μmole/g fresh wt using Scp (Sigma) as standard.

4.4 PR-protein (enzyme peroxidase, POD) determination

4.4.1 Crude protein extraction

One gram of control or treated leaves was ground in liquid nitrogen with a mortar and pestle then mixed with 0.5 ml of 100 mM Tris-HCl, pH 7.5 or in the ratio 2:1 of leaves to extraction buffer. The ground leaves were put into syringes plugged with a small cotton pad and the syringes were then pressed to separate the crude extract and the cell debris. The homogenate was centrifuged at 12,000 rpm for 20 min at 4 ^{0}C to pellet the cell debris. The supernatants were stored at -20 ^{0}C for further analysis of enzyme activity.

4.4.2 Peroxidase (POD) activity assay

POD activities revealed by *o*-dianisidine substrate (*o*-dianisidine POD). *o*-dianisidine POD levels were determined according to the method of Saeki *et al.* (1986) in a total volume of 3 ml. The reaction mixture consisted of enzyme extract in 50 mM acetate buffer (pH 5.4), 0.26 mM *o*-dianisidine and 3.3 mM H_2O_2. The increase in absorbance due to the oxidation of *o*-dianisidine was measured at 460 nm (ε = 11.3 mM^{-1} cm^{-1}). Enzyme activity was calculated in terms of units g^{-1} fresh weight (unit = µmole of substrate oxidized/min).

5 Interaction between elicitin and *Hevea* leaves

5.1 Scp synthesis in *Hevea* leaves

The resistant BPM-24 and the susceptible RRIM600 cultivars were used in this experiment. The B_2-C stage leaves were detached, cleaned and placed with their abaxial side up on the moist paper in a Petri dish. The leaf was scraped on both right and left sides. Ten µl of a 2.5 µg elicitin solution was dropped onto the scraped leaf surface whereas distilled water was used as a control. The Petri dish was kept at 25 ^{0}C for the development of necrosis. The droplets were collected for the Scp contents (accumulated Scp) according to 4.3.1.

6 Interaction between zoospores and *Hevea* calli

6.1 Treatment procedure

The 3-week-old calli of both cultivars; BPM-24 and RRIM600, were gently transferred from the MS medium, and as much the medium as possible were removed.

The calli (1.0 g approximately) were incubated with zoospores of *Phythophthora* spp. in a total volume of 2 ml, at $5x10^5$, $1.5x10^6$ and $4.5x10^6$ and $1.35x10^7$ zoospores/ml. Sterile distilled water was used as controls. After 1 h, the zoospore-containing solutions were removed, and replaced with 2 ml of sterile distilled water. The 2 ml of sterile distilled water was collected at 12, 18, 24, 30, 36, 42 and 48 h after treatment. The water was kept at -20 ^{0}C for the assays for extracellular Scp content assays and POD determination.

6.2 Extracellular Scp measurement in zoospores-treated calli

The Scp in the extracellular medium of zoospore-treated calli was analyzed as described in 4.3.1.

6.3 Enzyme POD assays

The enzyme activities of *o*-dianisidine POD in zoospore-treated calli were measured according to the same method as described in 4.4.2. The enzyme activities were carried out at 0, 12, 24, 48 and 72 h after elicitin treatment.

Results

1 Interaction between zoospores of *Phytophthora* spp. and *Hevea brasiliensis* leaves

1.1 Interaction between zoospores of three strains of *Phytophthora* spp. and *H. brasiliensis*

1.1.1 Necrosis (Comparison of the necrosis on *Hevea* leaves after treatment with various strains of *Phytophthora* spp.)

The leaves of two *Hevea* cultivars; BPM-24 and RRIM600, which represent resistant and susceptible to *P. palmivora*, respectively, were inoculated with zoospore droplets ($5x10^6$ zoospores/ml) of three strains of *Phytophthora* spp.; *P. palmivora Hevea* isolate, *P. palmivora Durian* isolate and *P. parasitica Anthurium* isolate. These *Phytophthora* strains are named as P. pal (Hevea), P. pal (Durian) and P. para, respectively (see method section). The progression of fungus in *Hevea* leaves was accompanied by the appearance of necrotic lesions about 24 h after inoculation. These two cultivars displayed different features of necrotic lesions during the tested period including sizes, color and border of necrosis. In the resistant BPM-24 leaves,

small black spots were observed at 24 h, additionally; the size of the necrotic lesions were similar in the treatments of P. pal (Durian) and P. para whereas it was slightly larger after inoculation with P. pal (Hevea). The necrotic sizes increased significantly in the treatment of P. pal (Hevea) at 48 and 72 h while the necrosis increased only slightly after inoculation with P. pal (Durian). Especially, P. para caused the smallest necrotic size at all indicated time points (Figure 2.6A). At the latest time points (72 h), only the leaves treated with zoospores of P. pal (Hevea) showed an expanded necrosis whereas the other two strains presented restricted necrotic lesions. The necrosis patterns, induced by zoospores of these three *Phytophthora* strains, in the susceptible RRIM600 were comparable to those in BPM-24 (Figure 2.6B). In RRIM600, the observed necrotic sizes were not much diverse at 24 h after inoculation with all strains whereas they were different at 48 and 72 h. Again, P. pal (Hevea) isolate caused the largest lesion sizes compared to those treated with the other two strains. At 48 and 72 h, a significant increase of the brown expanded lesions was detected in the RRIM600 leaves treated with both P. pal (Hevea) and P. pal (Durian) whereas P. para caused a less effect.

With all the conditions, the susceptible RRIM600 showed larger necrosis than that observed in BPM-24. Figure 2.7A and 2.7B show the necrotic symptoms of BPM-24 and RRIM600 leaves 24 h after inoculation with *Phytophthora* spp. The black spot, clear border and restricted the spreading of the pathogen is a characteristic of hypersensitive (HR) cell death. On the contrary, the large, brownish, expanded and unclear border resulting in pathogen dispersion is a disease attributes. The HR cell death was obviously observed in the interaction between the resistant BPM-24 and P. para zoospores while it was also monitored in the BPM-24-P. pal (Durian) interaction. Even though, the RRIM600 could show HR-like cell death after treatment with zoospores of P. para at 24 h, it seemed that RRIM600 could not control this pathogen spreading at 72 h. The most obvious disease phenotype was observed in the interaction between RRIM600 and P. pal (Hevea). From these results, BPM-24 showed more ability to stop the growth and dispersion of *Phytophthora* spp. than did RRIM600. This may be because of the differences of the physical barrier. The resistant BPM-24 may have more cutin or other molecules than that in the susceptible RRIM600 that can protect it from the invading pathogen. Additionally, comparing to

the invading ability of the *Phytophthora* spp., the results indicate that P. pal (Hevea), P. pal (Durian) and P. para are most, moderate and less virulent to *H. brasiliensis*, respectively. The explanation of this difference may be because of their adaptabilities consequential upon the successful penetration and ability to escape the recognition by the host.

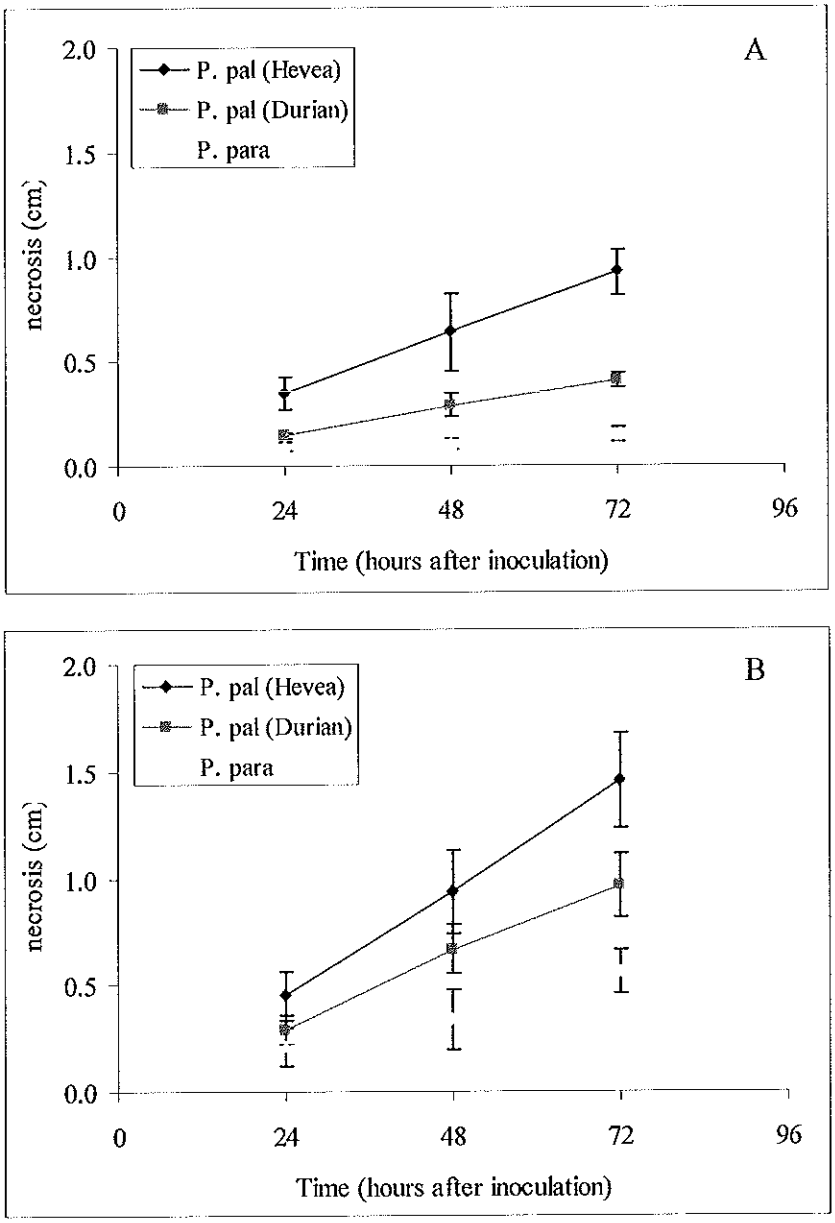

Figure 2.6 Necrotic lesion size of the resistant BPM-24 (A) and the susceptible RRIM600 (B) leaves inoculated with zoospore droplets ($5x10^6$ zoospores/ml) of three strains of *Phytophthora* spp.; P. pal (Hevea), P.

pal (Durian) and P. para. Each time point represents the mean ± SD of three independent experiments.

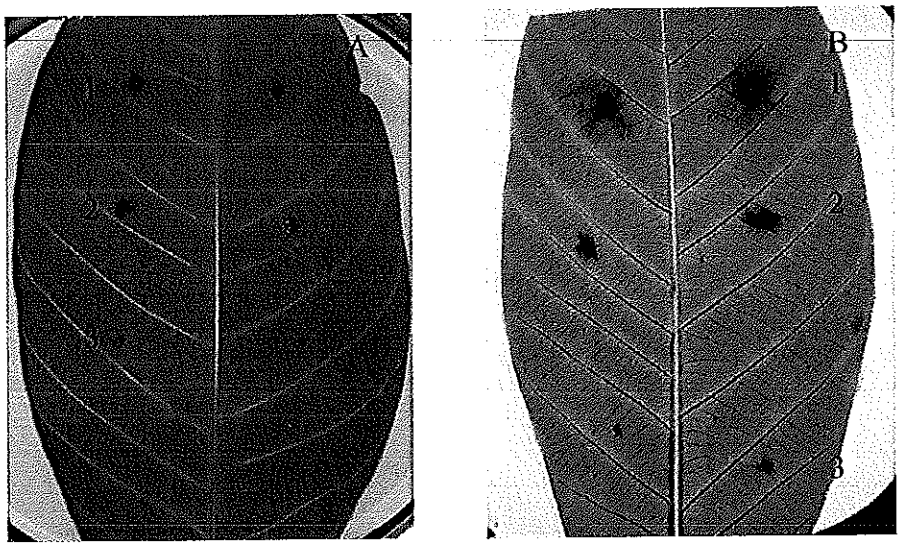

Figure 2.7 Necrotic lesions of the resistant BPM-24 (A) and the susceptible RRIM600 (B) leaves observed at 24 h after inoculation with zoospore droplets ($5x10^6$ zoospores/ml) of P. pal (Hevea). (1) P. pal (Durian) (2) and P. para (3).

1.1.2 Scopoletin (Scp) accumulation (Comparison of the Scp synthesis in *Hevea* leaves after treatments with various strains of *Phytophthora* spp.)

1.1.2.1 Scp accumulation after zoospore inoculation by the droplets method

The BPM-24 and RRIM600 leaves were inoculated with zoospore droplets ($5x10^6$ zoospores/ml) of P. pal (Hevea), P. pal (Durian) and P. para. The inoculums (droplets) were recovered 12, 24, 36, 48, 60 and 72 h after inoculation. After treatment, a bright blue fluorescence was visual in leaf tissues as well as in the inoculum under UV light. This is a characteristic of the presence of scopoletin (Scp). It was rapidly produced in both cultivars inoculated with zoospores of P. pal (Hevea). The Scp in the droplets was different in quantity after treatments with the various strains of *Phythophthora* spp. In the resistant BPM-24, the speeds of Scp synthesis were correlated with the virulence of *Phythophthora* spp. as speculated from the observed necrosis (in 1.1). The Scp amounts increased and reached a peak (12.6 μM)

at 24 h then decreased after the inoculation with P. pal (Hevea). The Scp started to increase at 12 h, peaked (6.4 μM) at 36 h and then slightly decreased afterwards in the treatment of P. pal (Durian). For the treatment of P. para, Scp rise after 12 h and achieved a maximal amount of 3 μM at 36 h, Scp was then produced constantly in range of 1.5-2 μM (Figure 2.8A). In conclusion, the Scp level in the tissue inoculated with P. pal (Hevea) was produced faster and reached a much higher level than those treated with P. pal (Durian) and P. para, respectively. The Scp accumulation in the susceptible cultivar RRIM600 was similar in pattern but differed in content and the peak time points were delayed compared to that measured for the resistant BPM-24 cultivar (Figure 2.8B). Despite the resistant and susceptible cultivars having a similar pattern of Scp accumulation in response to the *Phythophthora* spp., the maximal level of Scp in BPM-24 leaves (i.e. 12.6, 6.4 and 3 μM) was 2.5-3.5 times higher than that produced by RRIM600 (i.e. 5, 1.8 and 0.85 μM) after inoculations with P. pal (Hevea), P. pal (Durian) and P. para, respectively. The necrosis in BPM-24 was smaller than that observed in RRIM600 i.e. the converse to the Scp levels. This implies that the Scp amounts in BPM-24 were produced by less cell numbers than those from RRIM600 and therefore the ability to produce Scp in each cell was correlated to the resistance of the tested cultivars. In addition, according to the initial rate of Scp accumulation, it revealed that these three strains have a different efficiency in penetrating into *Hevea* cells which is paralleled to the virulence of the pathogens.

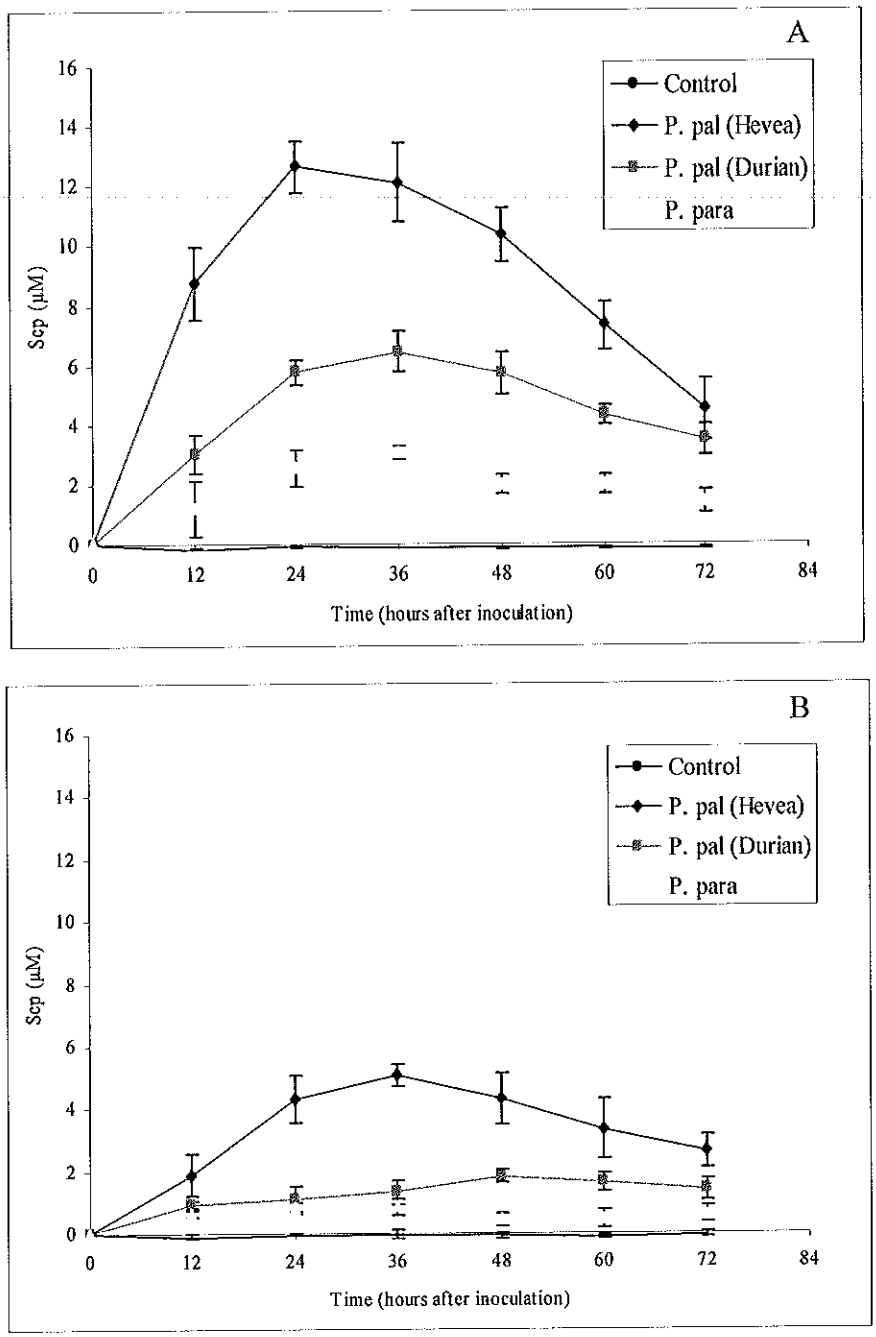

Figure 2.8 Scopoletin (Scp) accumulation in the resistant BPM-24 (A) and the susceptible RRIM600 (B) leaves inoculated with zoospore droplets ($5x10^6$ zoospores/ml) of three strains of *Phytophthora* spp.; P. pal (Hevea), P. pal (Durian) and P. para. Each time point represents the mean ± SD of three independent experiments.

1.1.2.2 Scp accumulation after zoospore inoculation by leaf piece method

The modified inoculation method was used to compare the efficiency of *Phytophthora* spp. to trigger the Scp production in *Hevea* leaves because leaf piece could present a reduced physical barrier and facilitate infection by the zoospores through the cut sites. Moreover we could compare the capability of these two cultivars in the response to the pathogens after a more successfully penetrated. The leaves were cut into a small piece 2x2 cm^2 prior to treating with the zoospore suspension ($5x10^6$ zoospores/ml). The soaking solutions were collected at 12, 24, 36, 48, 60 and 72 h after treatment which was comparable to the collected time points used for collection used in the droplets method. The rates of Scp production of both cultivars for the first 12 h were similar in all treatments. For BPM-24, Scp accumulation in P. pal (Hevea) treatment continued to increase up to 36 h and then decreased. Conversely, the Scp levels were reduced after reaching a peak at 12 h after inoculation with P. pal (Durian) and P. para (Figure 2.9A). The reduction rate of Scp accumulation was faster after P. para treatment. The detected Scp accumulation profiles were different in RRIM600. In the susceptible RRIM600 cultivar inoculated with P. pal (Hevea), Scp level was increased significantly from 12 to 24 h and then rapidly decreased whereas after reaching a peak at 24 h, the reduction after inoculation with P. pal (Durian) was much less. The Scp level retained during 12-36 h after inoculation with P. para and did not reduce significantly afterwards (Figure 2.9B). The Scp obtained from the control treatments, which was thought to result from the wounding, of BPM-24 and RRIM600 were 0.2 to 0.4 μM and 0 to 0.1 μM, respectively (Figure 2.9A and 2.9B). This may demonstrate the sensitivities of these two cultivars to wounding response. Even though the maximal level of Scp in BPM-24 and RRIM600 leaves by the leaf piece method were similar after treatment with P. pal (Hevea), the peak was delayed in BPM-24. This could be due to the rate of pathogen spreading in RRIM600 being higher. Moreover, the pathogen can spread rapidly in the susceptible cultivar and subsequently most cells were dead causing the rapid reduction in Scp accumulation. The different patterns of Scp levels, at 24 h, in the two cultivars after inoculation with P. pal (Durian) indicated that this strain can spread in the susceptible leaves from 12-24 h. The decrease of the Scp content in the

resistant cultivar after 24 h might be due to the plant resisting the spread of the pathogen. The same reason could explain the reduction of Scp production in both cultivars after inoculation with P. para 12 h which again confirms that P. para is the least virulence of to *H. brasiliensis*.

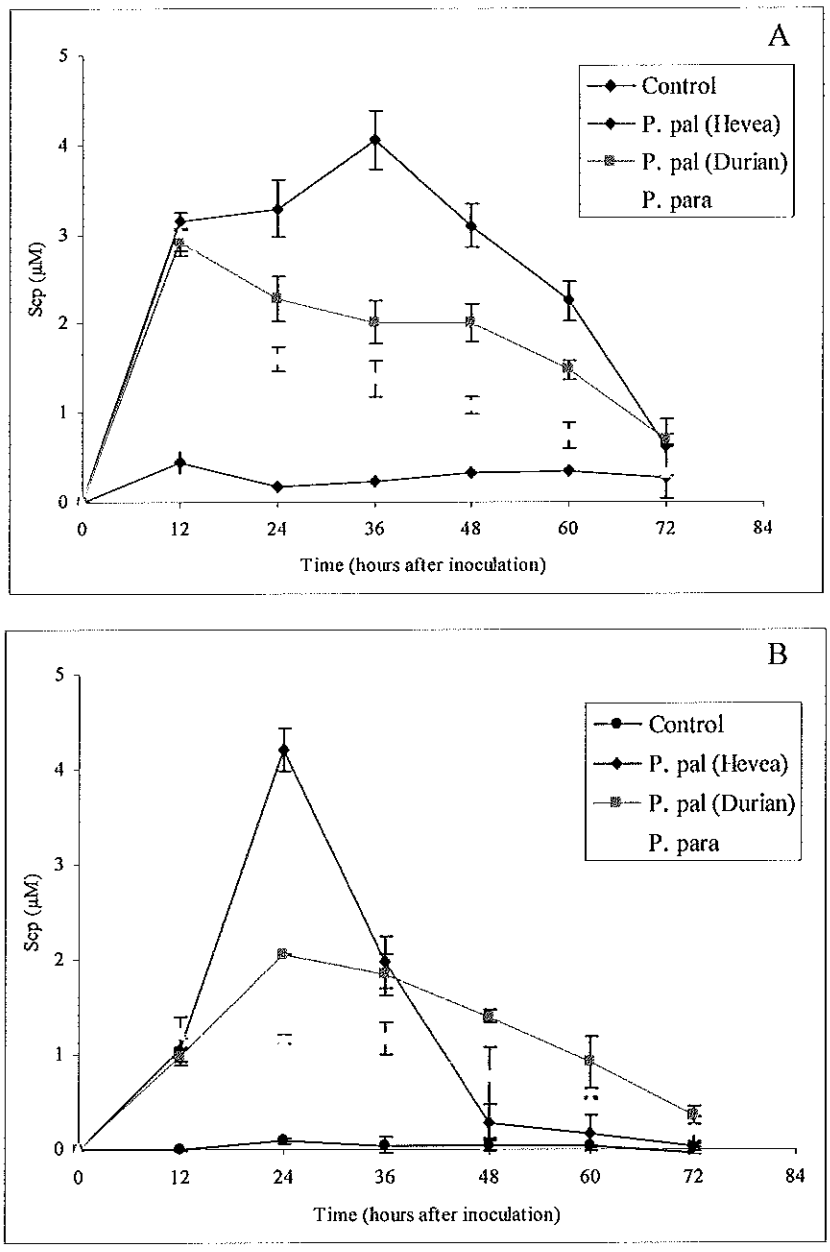

Figure 2.9 Scopoletin (Scp) accumulation in the leaf pieces (2x2 cm^2) of resistant BPM-24 (A) and the susceptible RRIM600 (B) inoculated with zoospore suspension ($5x10^6$ zoospores/ml) of three strains of *Phytophthora* spp.; P. pal (Hevea), P. pal (Durian) and P. para. Each time point represents the mean ± SD of three independent experiments.

2 Interaction between zoospores of the *P. palmivora Hevea* isolate and *Hevea* leaves by the leaf piece mathod

Since the *H. brasiliensis*-P. pal (Hevea) exhibited the strongest and clearest responses for necrosis and Scp accumulation than those from other interactions which previously shown in 1.1. This interaction was selected to establish more details of the *Hevea* resistance response to zoospore of P. pal (Hevea). The experimental procedure was followed the leaf pieces method because it provided a lot of leaf tissues which was necessary for the further studies.

2.1 Effect of zoospore concentration on necrosis size

BPM-24 and RRIM600 leaves were cut into 2x2 cm^2 pieces then inoculated with various zoospore concentrations; $5x10^3$, $5x10^4$, $5x10^5$ and $5x10^6$ zoospores/ml of P. pal (Hevea). After 12 h inoculation, no significant necrotic lesion was observed in the BPM-24 resistant cultivar inoculated with any zoospore concentrations while the RRIM600 showed some small black spots when inoculated with the high concentrations ($5x10^5$ and $5x10^6$ zoospores/ml) (data not shown). Forty-eight h after inoculation, each cultivar displayed a different set of necrotic symptoms at the same zoospore concentration. Although the necrosis of each cultivar was positively correlated to the concentration of the applied zoospores, the resistant cultivar BPM-24 exhibited smaller lesions than those in the sensitive RRIM600 cultivar (Figure 2.10). With $5x10^5$ zoospores/ml, the damage development in BPM-24 was restricted to around the cut site which is a feature of an incompatible reaction, whereas there were extended lesions in the compatible reaction of RRIM600. However with a higher zoospore concentration ($5x10^6$ zoospores/ml), the resistance of BPM-24 decreased and the necroses spread out whereas RRIM600 showed restricted lesions when inoculated with lower zoospore concentrations ($5x10^3$ and $5x10^4$ zoospores/ml). Since the leaves of RRIM600 and BPM-24 inoculated with $5x10^3$ and $5x10^4$ zoospores/ml, respectively resulted in similarly sized lesions (Figure 2.10) this indicated that the RRIM600 displayed a 10 fold lower degree of resistance than did the BPM-24.

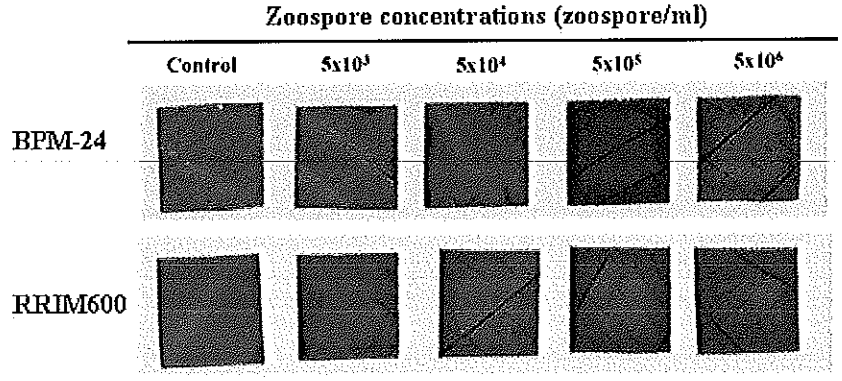

Figure 2.10 Effect of zoospore concentrations of the P. pal (Hevea) on necrosis observed in *H. brasiliensis* cut leaves. The BPM-24 (upper panel) and RRIM600 (lower panel) leaves were cut into pieces (2x2 cm^2) and inoculated with $5x10^3$, $5x10^4$, $5x10^5$and $5x10^6$ zoospores/ml. Lesion formations were investigated 48 h after inoculation. Independent experiments were repeated three times with similar results.

2.2 Effect of zoospore concentration on Scp biosynthesis

After treating the two *Hevea* cultivars with P. pal (Hevea) zoospores, the leaf piece samples were collected at 0, 12 and 48 h after inoculation. A Scp bright blue fluorescence was rapidly produced within 3-4 h in both cultivars inoculated with the high zoospore concentrations ($5x10^5$ and $5x10^6$ zoospores/ml). Scp accumulation was not detected in the tissues treated with the lowest concentration, $5x10^3$ zoospores/ml, and in the control (data not shown). For a more accurate measurement of the Scp produced in the leaf tissue, leaves treated with zoospores and untreated leaves were ground and centrifuged to pellet the cell debris and the supernatant was then further extracted with chloroform : acetic acid : H_2O (4:1:1, v/v/v) solution. The bright blue fluorescent Scp compound was measured by spectrofluorometry. At 12 h after inoculation, the Scp was present in a high amount in both cultivars that were treated with the high zoospore concentrations ($5x10^5$ and $5x10^6$ zoospores/ml), while Scp levels were low in those treated with a low zoospore concentration ($5x10^3$ zoospores/ml) or in the control. There was a close parallelism between the visualized fluorescence and the amounts of Scp (Figure 2.11). Forty-eight h after inoculation, Scp synthesis in BPM-24 kept increasing when treated with $5x10^4$ zoospores/ml

indicating an incompatible reaction. However, the increase in Scp between 12 and 48 h at the high zoospore concentration ($5x10^5$ zoospores/ml) was small whereas it was slightly reduced after inoculation with highest zoospore amount ($5x10^6$ zoospores/ml). In contrast, the Scp level in RRIM600 decreased rapidly at 48 h with zoospore concentrations of $5x10^5$ and $5x10^6$ zoospores/ml. This could be defined as a compatible reaction. However at the lower zoospore concentration ($5x10^4$ zoospores/ml), the RRIM600 leaves kept producing Scp indicating incompatibility. These results indicated that even a resistant cultivar when inoculated with a very high zoospore concentration, can act like a susceptible cultivar and the susceptible cultivar can act as resistant cultivar when treated with a very low zoospore concentration or a mild inoculation condition.

2.3 Kinetics of Scp accumulation

According to the above results (2.2), a suitable concentration of zoospores was to use to compare the kinetics of Scp accumulation in the two *Hevea* cultivars was $5x10^5$ zoospores/ml. The Scp in leaf tissues were extracted 0, 24, 48, 72 and 96 h after zoospore inoculation. The main difference between these two cultivars was that the BPM-24 displayed 2 peaks of Scp accumulation whereas only one peak appeared in the susceptible one. Scp levels increased rapidly and strongly in both cultivars after 12 h and reached their highest point at 24 h after zoospore inoculation (Figure 2.12). Even though the total amounts of Scp were similar in both cultivars, the numbers of cells penetrated by zoospores in RRIM600 were much greater than those in BPM-24 as shown by the lesion sizes. The transient accumulation of Scp in RRIM600 decreased rapidly after reaching a peak at 24 h. This profile indicated the fast spreading of the pathogen in RRIM600 leaves, therefore, most of the cells were infected in a short time. In contrast, two peaks of Scp accumulation were detected in the BPM-24 at 24 and 72 h after inoculation. The decrease of Scp observed at 48 h was correlated to the restricted HR cell death at this time point (data not shown). However at this zoospore concentration, the BPM-24 could only delay the progression of the pathogen, therefore, the Scp peaked again at 72 h. It is possible that the second phase of Scp production could also be due to the synthesis from neighboring cells induced through the signaling transduction pathway. We hypothesize that the second peak was raised according to the combination of these two possibilities i.e. a

continuing infection and signaling. In addition, a transient profile of the Scp accumulation can also be observed when the BPM-24 leaves were inoculated with a high concentration of P. pal (Hevea) $\geq 5x10^6$ zoospores/ml (Figure 2.9A).

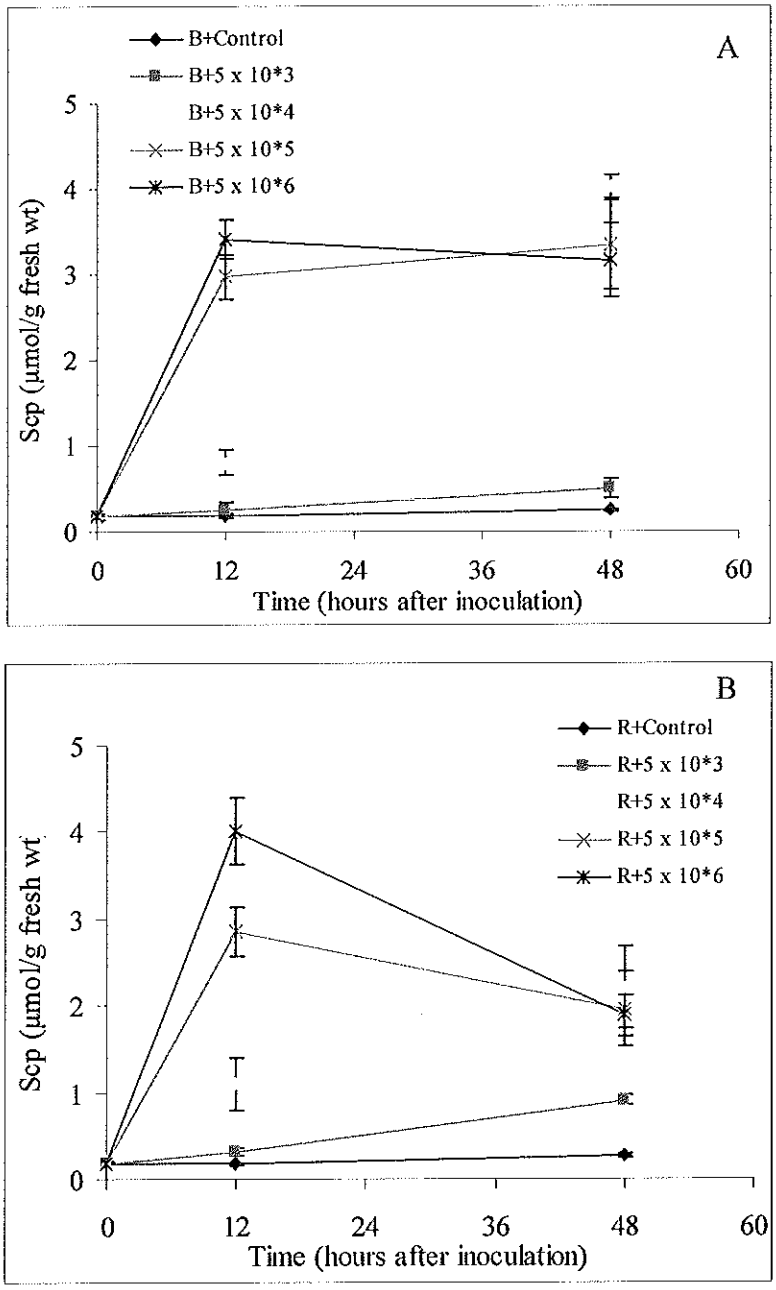

Figure 2.11 Scp accumulation in the resistant BPM-24 (A) and susceptible RRIM600 (B) cultivars after treatment with various P. pal (Hevea) zoospore concentrations. Leaf pieces (2x2 cm^2) were inoculated with $5x10^3$, $5x10^4$, $5x10^5$ and $5x10^6$ zoospores/ml of P. pal (Hevea). Control samples

were treated with sterile water. Each time point represents the mean ± SD of three independent experiments.

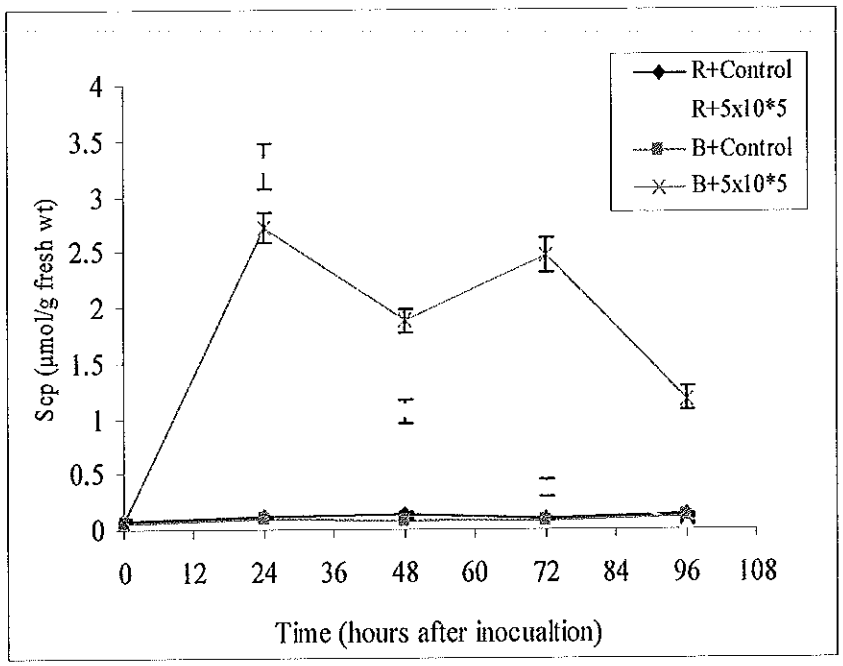

Figure 2.12 Kinetics of Scp accumulation in leaves of BPM-24 and RRIM600 treated with zoospores of P. pal (Hevea). The leaf pieces (2x2 cm^2) were treated with P. pal (Hevea) zoospores ($5x10^5$ zoospores/ml). Each time point represents the mean of three independent experiments and the standard deviation.

2.4 Induction of enzyme peroxidase (POD) activity

The intracellular POD activities revealed by *o*-dianisidine substrate (*o*-dianisidine POD) were measured in BPM-24 and RRIM600 leaves inoculated with $5x10^5$ zoospores/ml of P. Pal (Hevea). Both cultivars showed some peroxidase activity before wounding and treating with zoospores (at 0 h, Figure 2.13). Wounding slightly increased POD activity while the infection with the pathogen caused a strong induction. The patterns of POD activities were different in the two cultivars. The susceptible RRIM600 cultivar showed two peaks of activity at 24 and 72 h after inoculation while the resistant BPM-24 cultivar had only one peak at 48 h. However

peroxidase activity at 96 h in the resistant cultivar did increase from that at 72 h so a similar but delayed pattern to that of the susceptible cultivar may have occurred. To confirm this, we increased the zoospore concentration added to the BPM-24 leaves to $5x10^6$ zoospores/ml and in this case we found 2 peaks of POD activity at 24 h and 72 h, respectively (Figure 2.13).

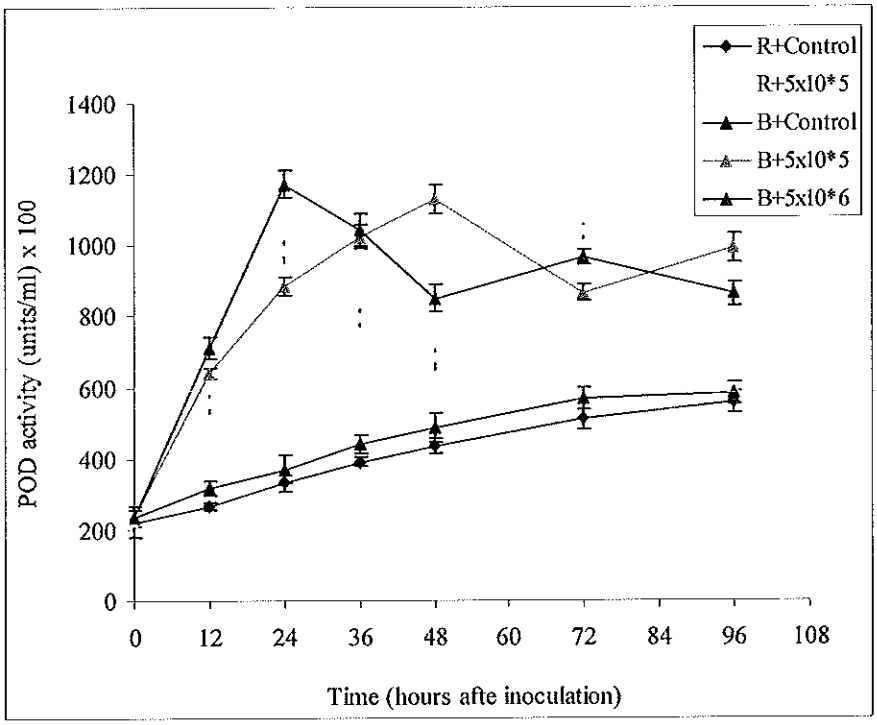

Figure 2.13 Enzyme peroxidase (POD) activity pattern in P. pal (Hevea) zoospore treated-BPM-24 and RRIM600. The leaf pieces (2x2 cm^2) of RRIM600 were treated with the zoospores of $5x10^5$ zoospores/ml whereas BPM-24 were treated with two zoospore concentrations, $5x10^5$ and $5x10^6$ zoospores/ml, to compare the POD profiles of compatible and incompatible reactions. The total POD activity was measured by using *o*-dianisidine as substrate. Each time point represents the mean $\pm$ SD of three independent experiments.

3 Interaction between elicitin purified from *Phytophthora* spp. and *H. brasiliensis* leaves

Scopoletin (Scp) was synthesized during the interaction between *Hevea* leaves and zoospores of *Phytoththora* spp. It was clearly exhibited in both resistant and susceptible cultivars after inoculation with P. pal (Hevea), P. pal (Durian) and P. para zoospores. The elicitins, a 10 kDa extercellular protein produced by three strains of *Phytoththora* spp. were tested for its ablity to trigger the induction of Scp synthesis in *Hevea* leaves. Elicitins were applied on BPM-24 and RRIM600 leaves in the concentration of 2.5 µg/droplet. The Scp appeared in the leaf tissues 2-3 h after elicitin treatment. In the resistant BPM-24, elicitins purified from P. pal (Hevea) and P. pal (Durian) elicited the Scp production in similar pattern. Two peaks of Scp were detected at 16 and 24 h after inoculation and then rapidly decreased until the amount of Scp was equal to the control (untreated). The Scp accumulation triggered by P. para elicitin was delayed and induced but about 4 fold lower than that produced in the treatments of elicitin from both of the P. pal cultures (Figure 2.14A). In the susceptible RRIM600 cultivar very low amounts of Scp, only slightly higher than its control, was produced after being treated with elicitins from all the tested *Phytoththora* strains (Figure 2.14B). These results indicated that elicitin from *P. palmivora* even P. pal (Hevea) or P. pal (Durian) had a similar activity in triggering the Scp production in *Hevea* leaves. Moreover, the response strength to elicitin was correlated with the resistance of the tested host.

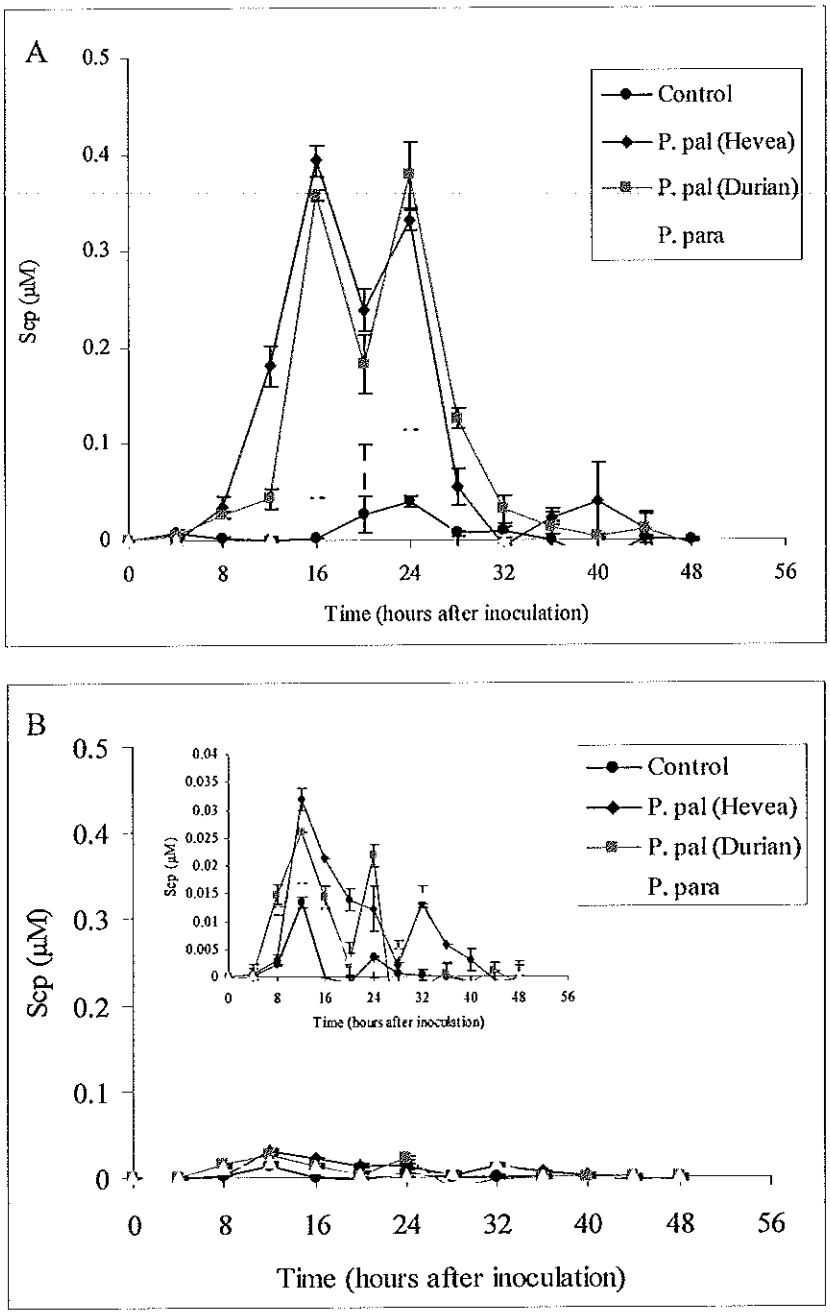

Figure 2.14 Scopoletin (Scp) accumulation in the resistant BPM-24 (A) and susceptible RRIM600 (B) leaves treated with elicitin (2.5 µg/droplet) purified from P. pal (Hevea), P. pal (Durian) and P. para. The 10-fold-expanded scale of Scp in RRIM600 was also showed in (B). Each time point represents the mean ± SD of three independent experiments.

4 Interaction between zoospores of *Phytophthora* spp. and *Hevea* calli

As the objective in these later experiments was mainly focused on whether *Hevea* cell culture (callus) exhibited similar responses to those shown by *Hevea* leaves after treatment with zoospore of *Phytophthora* spp. The defense responses (Scp accumulation and POD acvivity) in *Hevea* calli derived from integument and zoospores of P. pal (Hevea) or P. para were investigated because from the leaf experimental results, P. pal (Hevea) and P. pal (Durian) induced similar patterns of Scp accumulation while patterns for the P. para were different.

4.1 Effect of zoospore of P. pal (Hevea) concentration on Scp biosynthesis

Calli generated from two *Hevea* cultivars; BPM-24 and RRIM600 were treated with various concentrations of P. pal (Hevea) zoospores, the soaking solutions were collected every 6 h up to 48 h after inoculation. Since the Scp amounts in the extracellular medium were similar to the intracellular Scp measured in the crude calli extract (Thesis of Nak-Udom, 2005). This may be due to the textures of calli being friable and resulting in Scp release from the cell to the medium. Therefore, the Scp in the soaking medium was measured and could represent the content produced in calli. A Scp bright blue fluorescence was rapidly produced within 3-4 h in both cultivars inoculated with high zoospore concentrations. The rate of Scp production at 6 h was induced in a dose-dependent manner in both cultivars (Figure 2.15). The initial rate was correlated to the numbers of infected cell after zoospore infection. The peak of Scp appeared at 18 h in BPM-24 after being inoculated with high zoospore numbers ($1.35x10^7$ and $4.5x10^6$ zoospores/ml) whereas the observed peaks were delayed, observed at 24 and 30 h in the treatments of $1.5x10^6$ and $5x10^5$ zoospores/ml, respectively. Even though the highest zoospore concentration induced a faster Scp production rate, the maximal Scp level was observed at $1.5x10^6$ zoospores/ml which was higher than those observed in other treatments ($5x10^5$, $4.5x10^6$ and $1.35x10^7$ zoospores/ml). Diminutive and marginal amounts were observed in the control. The Scp levels in all treatments were decreased after reaching a peak. In addition, it was rapidly reduced after inoculation with the highest zoospore inoculum and this might be due to most cells being dead so they could no longer produce the Scp. In all treatments, RRIM600 showed lower Scp levels than those in BPM-24. The maximal

of Scp content detected in resistant BPM-24 was about 3-3.5 times greater than that in the susceptible RRIM600 cultivar.

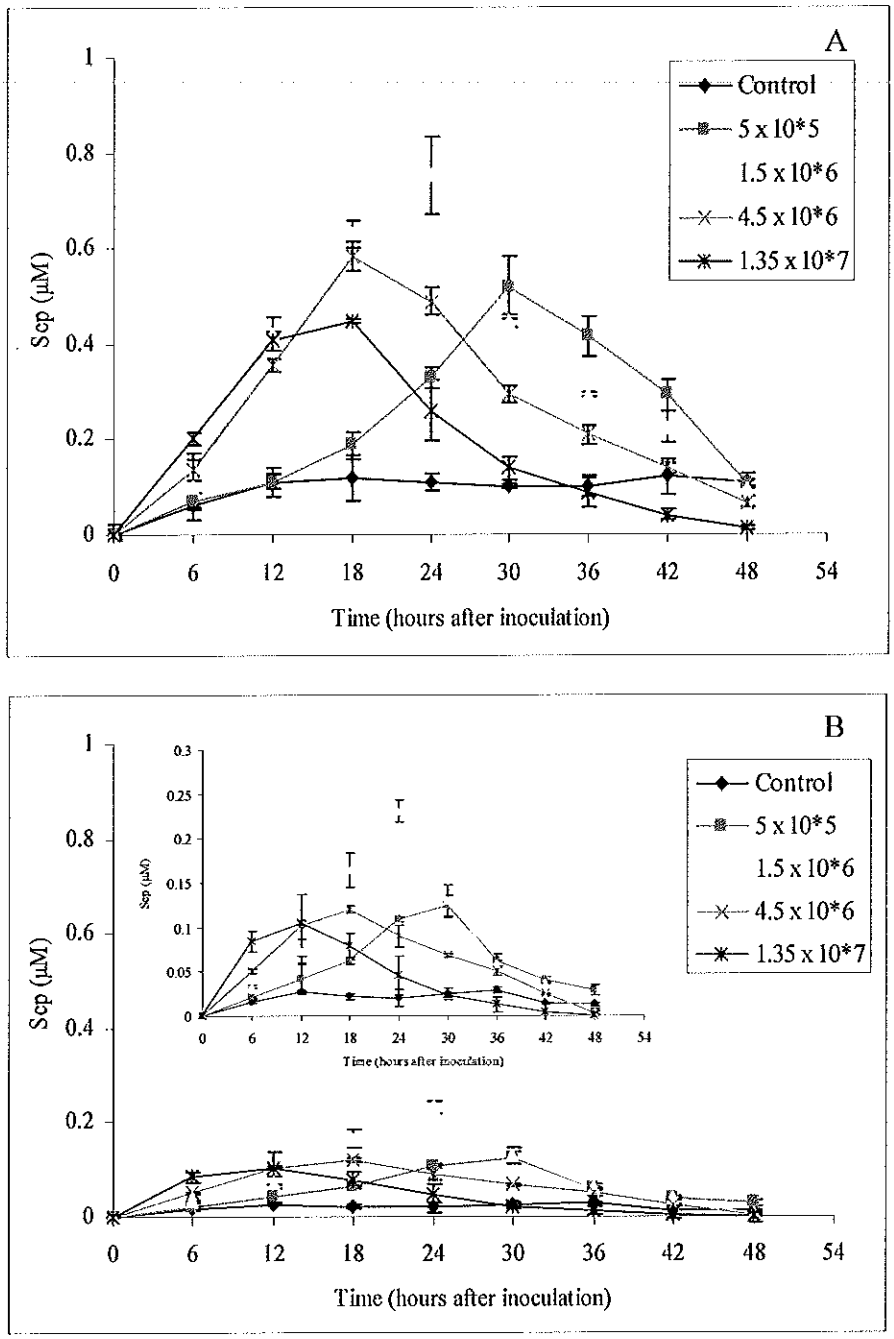

Figure 2.15 Scp accumulation in the calli of resistant BPM-24 (A) and susceptible RRIM600 (B) cultivar after inoculation with various concentrations of P. pal (Hevea) zoospore. The 3 times expanded scale of Scp in RRIM600 is also showed in (B). *Hevea* calli were inoculated with $5x10^5$, $1.5x10^6$, $4.5x10^6$ and $1.35x10^7$ zoospores/ml of P. pal (Hevea). Control samples

were treated with sterile water. Each time point represents the mean ± SD of three independent experiments.

4.2 Effect of the concentration of P. para zoospores on Scp biosynthesis

The BPM-24 and RRIM600 calli were also treated with various concentrations of P. para zoospores and the extracellular medium were collected every 6 h up to 48 h after inoculation for Scp analysis. The rate of Scp production at 6 h was induced in a dose-dependent fashion in both cultivars. The patterns of Scp accumulation were similar to those observed in calli after being treated with zoospores of P. pal (Hevea) (Figure 2.16). However, the peaks appeared at 24 and 30 h in BPM-24 after being inoculated with ($4.5x10^6$ and 1.5x10 zoospores/ml, respectively, which were delayed compared to those from the P. pal (Hevea) treatments. The optimum concentration of P. para zoospore treatment was $1.5x10^6$ zoospores/ml at which the maximal Scp level was observed. Again, the highest zoospore amounts triggered the fastest rate of Scp production but its peak is lower than those observed at the optimum zoospore amounts. A similar rate and pattern of Scp accumulation was also detected in RRIM600 as that in BPM-24. The maximal Scp content was detected in the resistant BPM-24 was about 3.5-4 times greater than those in the susceptible RRIM600 cultivar.

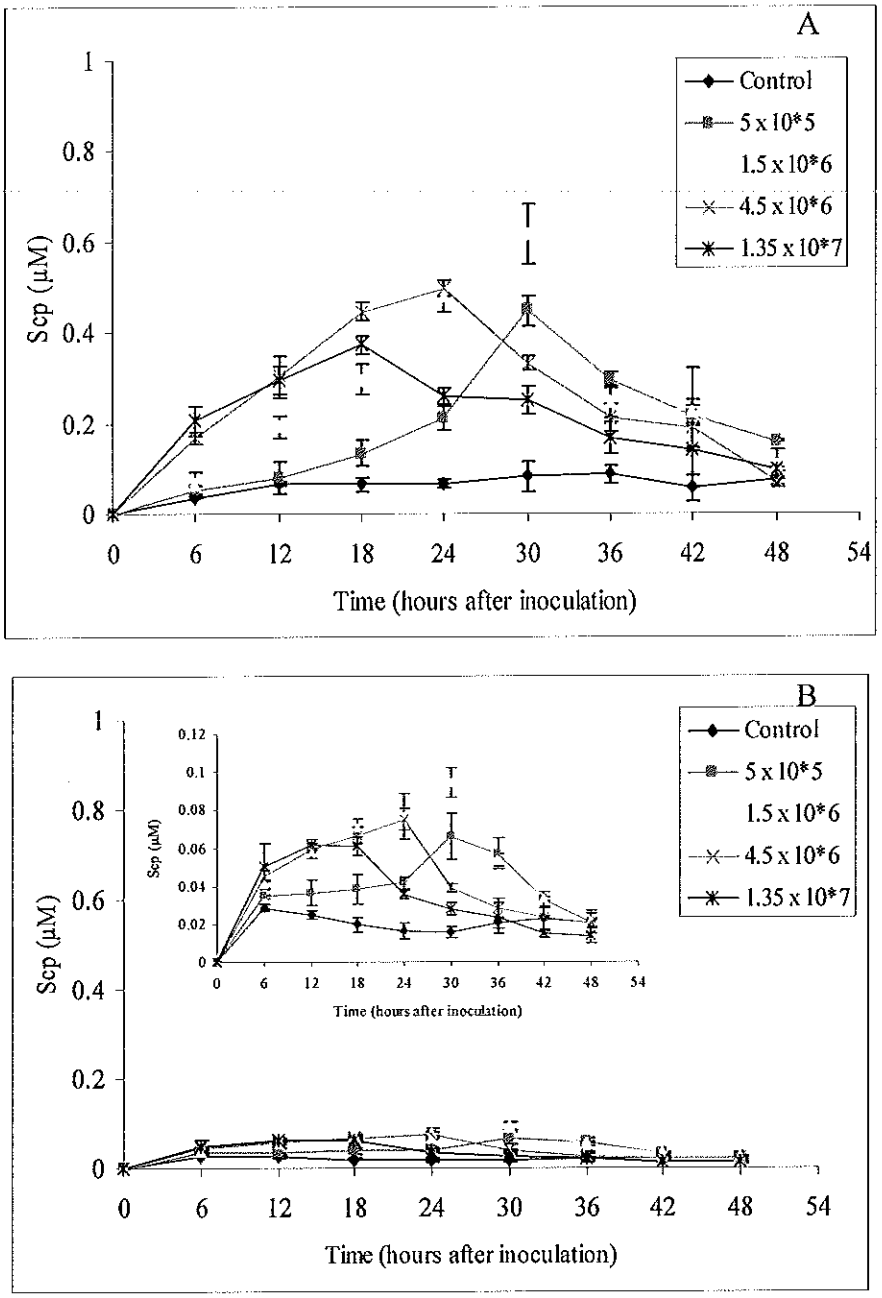

Figure 2.16 Scp accumulation in the calli of resistant BPM-24 (A) and susceptible RRIM600 (B) cultivar after inoculation with various concentrations of P. para zoospore. *Hevea* calli were inoculated with $5x10^5$, $1.5x10^6$, $4.5x10^6$ and $1.35x10^7$ zoospores/ml of P. para. The 10 times expanded scale of Scp in RRIM600 is also showed in (B). Control samples were treated with sterile water. Each time point represents the mean ± SD of three independent experiments.

5 Interaction between *H. brasiliensis* calli and P. pal (Hevea) and P. para at the optimal zoospore concentration

According to the results shown in 4.1 and 4.2, the concentration of $1.5x10^6$ zoospores/ml was selected to be the optimal numbers for comparing the response of *Hevea* calli.

5.1 Scp accumulation

At the optimal zoospore concentration of $1.5x10^6$ zoospores/ml, the rate of Scp biosynthesis was higher in the resistant BPM-24 (Figue 2.17). P. pal (Hevea) caused the faster rate of Scp production, indicating that this pathogen strain needed a shorter time to infect successfully. The peak of Scp was observed at 24 h and 30 h in both cultivars after inoculation with zoospores of P. pal (Hevea) and P. para, respectively. This evident delay could be explained by the different efficacy of the hosts' ability to recognize the different strains of pathogen. In addition, the maximal Scp content detected in the resistant BPM-24 cultivar was about 3-4 times greater than that in the susceptible RRIM600 cultivar. Hence, this revealed that the ability of *Hevea* calli to produce the Scp was correlated with the resistance of the tested cultivars.

5.2 Induction of enzyme peroxidase activity

Total POD activity was measured by using *o*-dianisidine as substrate (*o*-dianisidine POD) in crude extract of BPM-24 and RRIM600 (intracellular) calli inoculated with $1.5x10^6$ zoospores/ml of P. pal (Hevea) and P. para. Both cultivars showed constitutive levels of peroxidase activity in the control (Figure 2.18). After infection with pathogen there was a strong increasing of POD activity. In this condition, the patterns of peroxidase activities were similar for the two cultivars after inoculation either with P. pal (Hevea) or P. para. Zoospore of P. pal (Hevea) immediately induced a strong increasing of POD activity reached a maximum level at 30 h and then declined. However, delayed peak to that treatment with P. para occurred at 36 h. This behavior was comparable to the Scp accumulation obtained from the same condition, therefore the rate induced by P. pal (Hevea) is faster than that induced by P. para. The level of POD was about 1.5 fold higher when treated with P. pal (Hevea) than that treated with P. para. Moreover, the maximal POD activity exhibited in the resistant BPM-24 cultivar was 2.5 times greater than those in the susceptible

RRIM600. Again, this revealed the ability of *Hevea* calli to produce the POD was correlated with the resistance of the tested calli.

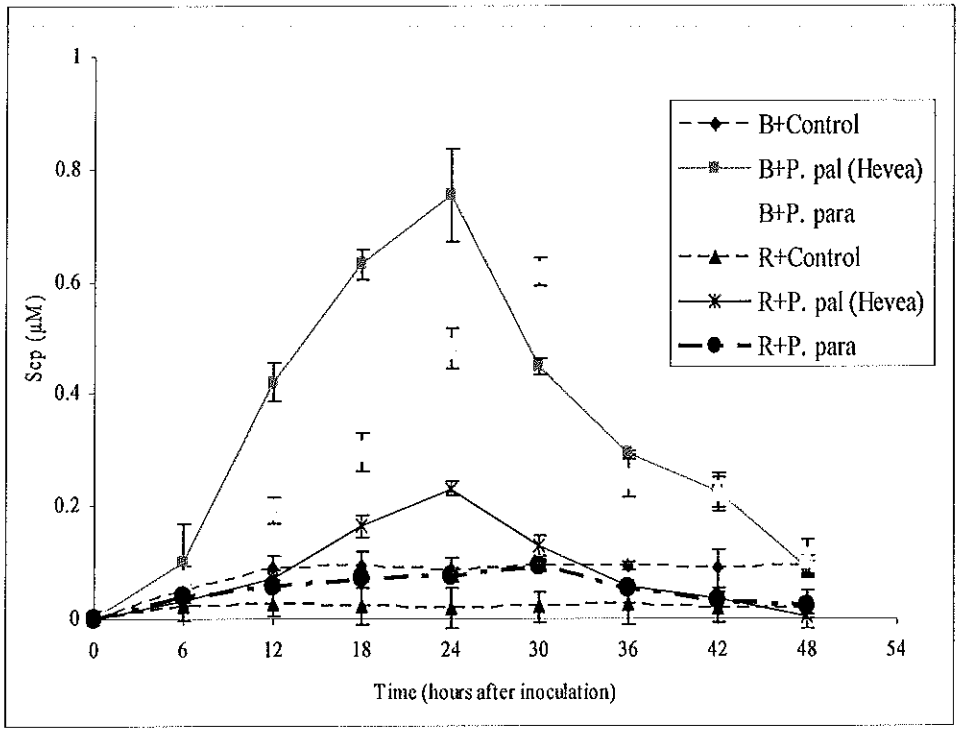

Figure 2.17 Comparison of the Scp accumulation in *Hevea* calli inoculated with optimal zoospore concentration of P. pal (Hevea) or P. para. The calli of resistant BPM-24 and RRIM600 were inoculated with $1.5x10^6$ zoospores/ml of P. pal (Hevea) or P. para. Scp was measured in the extracellular medium at 0, 6, 12, 18, 24, 30, 36 and 48 h after inoculation. Control samples were treated with sterile water. Each time point represents the mean ± SD of three independent experiments.

1.1 Necrosis

In these experiments, zoospores of three strains of *Phytophthora* spp.; *P. palmivora Hevea* isolate [P. pal (Hevea)], *P. palmivora Durian* isolate [P. pal (Durian)] and *P. parasitica Anthurium* isolate (P. para) were used to verify the different responses in *Hevea* leaves. Necrosis observed at the earliest time point could be used for comparing the invading ability of *Phytophthora* spp. The slightly difference in necrotic size at 24 h after zoospore inoculation indicated that these three *Phytophthora* strains had a similar efficiency to penetrate *Hevea* leaf tissues. *Phytophthora* zoospores exhibit chemotaxis and orient their developments with respect to the plant surface; consequently, it seems likely that molecular receptors exist on the zoospore surface are used to control these events (Bishop-Hurley *et al.*, 2002). The necrosis at later time points indicated different response to these three strains. P. pal (Hevea) caused larger expanded lesions than did P. pal (Durian) and P. para. The results suggesting that P. pal (Hevea), P. pal (Durian) and P. para are most, moderate and less virulent to *H. brasiliensis*, respectively. The explanation of this difference may be because of their adaptabilities consequential with the successful penetration and ability to escape the recognition by the host. However, Guest (1998) has suggested that a few hosts were not susceptible to some *Phytophthora* species. Necrotic lesions were initially similar, but generally with most pathogens subsequent spreading was limited. Moreover, the resistant BPM-24 exhibited smaller necrotic sizes than did the susceptible RRIM600 after treating with *Phytophthora* spp. avd this did correlated to the defined degree of resistance (this point will be addressed further in more details below in 2.1).

1.2 Scopoletin (Scp; phytoalexin produced in *H. brasiliensis*)

Plants become resistant to infection by pathogens in various ways including the production of antimicrobial compounds, named phytoalexins. *H. brasiliensis* has been demonstrated to synthesized phytoalexin after pathogen infection and it was characterized as a hydroxycoumarin called scopoletin (Scp, Giesemann *et al.*, 1986). Scp was generated in *Hevea* after treating with *Microcyclus ulei, Corynespora casiicola* and *P. palmivora* (Garcia *et al.* 1995, Breton *et al.* 1997, Churngchow and Rattarasarn 2001). The accumulation of Scp has also been reported in herbaceous plants in response to fungal (Tal and Robinson, 1986; Zeringue, 1984), bacterial

(Sequeira, 1969) and viral (Clarke and Baines, 1976) infections. Scp is exuded from living cells into the apoplast and can easily be detected under UV excitation as a blue fluorescence.

In this study, Scp levels in the tissues inoculated (by droplet method) with P. pal (Hevea) produced much higher amounts than those treated with P. pal (Durian) and P. para, respectively, which could be observed for both resistant and susceptible cultivars. This also corresponded to the necrosis discussed in 1.1. Therefore, it confirms that these three strains have a different efficiency in penetrating into *Hevea* cells which parallels to the virulence of the pathogens. Despite this the pattern of Scp accumulation in response to *Phythophthora* spp. by both resistant and susceptible cultivars had a similar pattern but the peak time point in BPM-24 was faster and the maximal level of Scp in BPM-24 leaves was 2.5-3.5 times higher than that produced by RRIM600. The necrosis in BPM-24 was smaller than that observed in RRIM600 i.e. the converse of the Scp levels. This implied that Scp was produced in BPM-24 by less cell numbers than those from RRIM600, again showing a correlation between an ability of Scp production in each cell and the resistance of tested cultivars.

Moreover, when the Scp production was studied in *Hevea* leaf piece by improving access to the leaf tissue reduce the effect of any different in the physical barrier of different cultivars. This allowed a test of the spreading efficiency of different strains of *Phytophthora* spp. after the pathogen succeeded in penetration. Additionally, the pathogen spreading will be depended on the thickness of plant cell wall and the chemical barriers of each cultivar. The time of the Scp peak after infection with P. pal (Hevea) was slower in BPM-24, so this infers that the rate of pathogen spreading in RRIM600 was higher. These patterns of Scp accumulation indicated that the degree of virulence of *Phytophthora* spp. on *Hevea* leaves are as follow; P. pal (Hevea) > P. pal (Durian) > P. para. The decrease of Scp in the two cultivars might result from a different explanation in that the susceptible cultivar might have more cell dead resulting in the more rapidly reduction of Scp, conversely, the resistant cultivar after 24 h might stop the pathogen spreading. (The correlation of the degree of resistance to Scp production will be continued in more details in 2.2).

The defense response of a resistant plant can be directly linked to signaling events that lead to specific defense responses. The timely response to intruding

pathogens also plays a critical role in acquiring resistance (Maleck *et al.*, 2000). Susceptible plants often take longer to activate their defense response after infection by a pathogen. In some instances they do not respond at all (Moerschbacher *et al.*, 1999). The latter might be due to the fact that the signal transduction leading to the response is in some way blocked by the attacking pathogen (Moerschbacher *et al.*, 1999). To obtain contact with the host cell plasma membrane for establishing the initial phase of interaction, *Phytophthora* spp. has to overcome the plant cell wall by synthesizing enzymes that degrade the cell wall (Panabieres *et al.*, 2005; Le Berre *et al.*, 2007). However, plants have evolved mechanisms to recognize invading pathogens through the perception of conserved motifs in pathogen-derived molecules that are not subject to evolutionary diversification. Receptor-mediated recognition of such pathogen-associated molecular patterns (PAMPs) triggers signaling cascades which eventually activate an array of plant defense responses. The induced resistances include the production of toxic compounds and reactive oxygen species (ROS) and in some cases cellular suicide in some cases. The pathogen-induced events that lead to programmed cell death are called the hypersensitive response (HR) (Nimchuk *et al.*, 2003). Therefore, in our case, *Phytophthora* spp. may have different abilities to interact with receptor on *Hevea* resulting in different transduction pathways that lead to HR or disease. Thus, the abilities of pathogen to elicit plant defense responses and its co-evolution (host plant and pathogen) in our plant-pathogen system will be interesting to study in the future.

2 *Hevea* leaf defense responses against zoospores of P. pal (Hevea)

2.1 Necrotic lesions on *Hevea* leaves and degree of resistance

The pathogen induced necrosis of plant can be classified into hypersensitive cell death (HR-cell death) and disease lesions in the incompatible and compatible interaction, respectively. The rapid plant cell death at the infection site or HR-cell death is the most common feature of the incompatible interaction (Heath, 1997). Our results showed that the resistant BPM-24 cultivar exhibited smaller sized necrotic lesions than did susceptible RRIM600 cultivar after treating with *Phytophthora* spp. (in 1.1). This is similar to the soybean-*P. megasperma* interaction where the resistant cultivars exhibited less necrosis (Ward *et al.*, 1989). The resistant BPM-24 showed

more ability to stop the growth and dispersion of *Phytophthora* spp. than did RRIM600. This may be because of the differences in the physical barriers. The resistant BPM-24 could have more cutin or other molecules than that in susceptible RRIM600 that can protect it from the invading pathogen. However, cutting the leaves caused a reduction of resistance as the results from the leaf piece method showed that the resistance capacity in BPM-24 was decreased resulting in an increased spread of the pathogen. In addition, Hu *et al.* (2003) have reported that the HR-cell death in the resistant cultivar was effective in restricting the spread of the pathogen by cytoplasmic changes such as numerous electron-transparent vesicles, cell wall apposition and hypha were not clearly observed.

The interaction between *H. brasiliensis* and P. pal (Hevea) was selected to be a model for studying in more details because it showed very strong and clear responses. The BPM-24 and RRIM600 leaves were inoculated with various zoospore concentrations; $5x10^3$, $5x10^4$, $5x10^5$ and $5x10^6$ zoospores/ml of P. pal (Hevea). The increase of zoospore concentrations was correlated to the sizes of the necrosis. However, at $5x10^4$ and $5x10^5$ zoospores/ml, the necrotic lesions in the resistant cultivar developed at a slower rate than those detected on the susceptible one. By decreasing the zoospore concentration, the susceptible RRIM600 showed a restricted lesion or HR-cell death whereas in the resistant BPM-24 cultivar the development of necrosis had the potential to shift from HR-cell death to disease, after high or very high zoospore numbers were applied. With high concentration i.e. $5x10^5$ zoospore/ml for RRIM600 or $5x10^6$ zoospores/ml for BPM-24, the spread of the pathogen was not restricted because at this level necrosis observed in both cultivars. These results indicated that the resistant cultivar can act susceptiblity when inoculated with a very high zoospore concentration while the susceptible cultivar can act as resistance when treated with a very low zoospore amount or with a mild inoculation condition. Based on these data, we can conclude that plants have a limited tolerance to pathogens dependent on the degree of resistance. Furthermore, these results reveal the different resistance capacities of these two cultivars; the susceptible RRIM600 cultivar displayed a 10 fold lower degree of resistance than did the resistant BPM-24 cultivar.

2.2 Scp synthesized in *Hevea* leaves and the degree of resistance

The rate of Scp production in *Hevea* leaves, in both cultivars, increased in a dose-dependent manner after treatment with zoospores of P. pal (Hevea). Inoculation with high zoospore amounts ($5x10^5$ and $5x10^6$ zoospores/ml) produced more penetration sites and resultied in higher Scp level. The rapid decrease of Scp level observed in tissues treated with high zoospore concentrations indicated that many cells were destroyed and dead, so Scp could not be able produced. At lower concentration ($5x10^4$ zoospores/ml), the leaf tissues kept producing Scp whereas too low amount of zoospores could not induce Scp synthesis because plants expressed the HR responses very quickly and this restricted pathogen growth.

The kinetics of Scp accumulation was determined in a model of *Hevea* leaves treated with $5x10^5$ zoospores/ml by leaf piece method. The main difference between these two cultivars was that the BPM-24 displayed 2 peaks of Scp accumulation whereas only one peak appeared in the susceptible one. The transient accumulation of Scp in RRIM600 decreased rapidly after reaching a peak and this was caused by the fast spreading of the pathogen in RRIM600 leaves indicating that most of the cells were infected in a short time. Two peaks of Scp accumulation were detected in the BPM-24. The decrease of Scp after the first Scp peak appeared was correlated to the restricted HR cell death at that time (data not shown). Since the BPM-24 could only delay but not stop the progression of the pathogen at this zoospore concentration; therefore, a second peak of Scp was observed. The second phase of Scp production could probably be due to the continuing infection or the synthesis from neighboring cells induced through the signaling transduction pathway. The latter possible reason would agree with the data reported by Dorey *et al.* (1997) who proposed that the induction of Scp was due to the signals produced by the dead cells transferring to the neighboring cells. Even though the total amounts of Scp were similar in both cultivars, the numbers of cells penetrated by zoospores in the RRIM600 cultivar were much greater than those in the BPM-24 cultivar as shown by the lesion sizes. Thus, in our model the capability of Scp biosynthesis in response to the pathogen is correlated to the degree of resistance. There are evidences that phytoalexins accumulate in faster rate and in higher levels in resistant cultivars whereas these compounds occur more slowly and more diffuse in susceptible cultivar (Mayama *et al.*, 1981; Doke 1982;

Hahn *et al.*, 1985) and again this is comparable to our finding. Moreover, the speed and extent of Scp were correlated to the degree of resistance of *Hevea* to *M. ulei* and *Colletotrichum gloeosporioides* (Garcia *et al.*, 1995; Breton *et al.*, 1994). For further study, some other technique that could detect the Scp localization in plant tissue such as in situ hybridization might be considered.

2.3 Enzyme peroxidase (POD) activity in *Hevea* leaves and degree of resistance

Peroxidase enzymes (POD; EC 1.11.1.7) are mainly located in the cell wall, where they use hydrogen peroxide as the electron acceptor to oxidize numerous organic compounds (Chen, 2002). PODs are among the much studied enzymes in plant physiology and for technical advances. PODs are involved in many metabolic pathways, such as pathogen defenses (Wu *et al.*, 1997), cross-linking of cell wall proteins (Schnabelrauch *et al.*, 1996), oxidizing hydroxycinnamic derivates and other phenolic compounds (Bernards *et al.*, 1999) and formation of lignin and suberin (Quiroga *et al.*, 2000) . Induction of these peroxidases due to pathogen interactions has been studied in cucumber, potato, barley and rice (Rasmussen *et al.*, 1995; Ray and Hammerschmidt, 1998; Kristensen *et al.*, 1999; Manandhar *et al.*, 1999).

In this present study the untreated leaves also had basal constitutive levels of POD activity. The first peak of POD activity was closely correlated to Scp accumulation, we therefore thought that the POD in the first peak might be involved in Scp degradation during the detoxification of induced H_2O_2. This would agree with the report of Breton *et al.* (1997) that Scp peroxidase was induced in *Hevea* leaves after inoculation with the *C. cassiicola*. Scp is an antioxidant that can be readily oxidized by POD and H_2O_2 and used to limit the amount of H_2O_2 (Levine *et al.*, 1994; Chong *et al.*, 1999; Dorey *et al.*, 1999). In addition, Edwards *et al.* (1997) described Scp-degrading peroxidase from sunflower leaves. They suggested that the induced peroxidative metabolism of Scp either has a direct defensive function and/or simply protects plants from the potential phytotoxic effects of Scp. Prats *et al.* (2006) reported that an increased Scp-dependent POD activity was correlated with high levels of Scp in sunflower. They also showed that after infection Scp peroxidase activity increased during the course of the experiment in the line that was resistant to *Sclerotinia sclerotiorum* whereas it was constitutively low in a susceptible line. However, Saftić-Panković and coworkers (2006) found Scp POD activity only in a

resistant sunflower line, but not in a susceptible one, after downy mildew *Plasmopara halstedii* infection. In our work, the POD activity synthesized in each cell of the resistant leaves was much greater, as implied by the lower necrosis, than that in the susceptible RRIM600cultivar. The second peak of peroxidase might result from the living cells that may be involved in lignification. This was supported by the incident when we used guaiacol, an analogue of a lignin precursor, as substrate and found only one peak of guaiacol peroxidase activity which corresponded to the second peak we observed with *o*-dianisidine (data not shown). Guaiacol POD has been shown to be induced in plants in response to pathogens and is engaged in lignin polymerization (Gross *et al.*, 1977; Geiger *et al.*, 1989). In conclusion, the first peak represents the peroxidase isozyme being involving in Scp degradation and detoxification of H_2O_2, and the second peak represents the isozyme being used in lignin biosynthesis. It would be interest to investigate the peroxidase activities using other substrates involved in plant defense responses at both the molecular and protein levels. This would help to decide if each POD isozyme had a specific defense functions.

3 Scp synthesis in *Hevea* leaves against elicitins from different strains of *Phytophthora* spp.

More than 30 *Phytophthora* and *Pythium* species have actually been found to secrete highly conserved proteins collectively termed elicitin with molecular weights of about 10 kDa (for review see Ponchet *et al.*, 1999). Elicitins induce defense responses on a restricted number of plants, for example, *Nicotiana* species within the Solanaceae family (Kamoun *et al.*, 1993; Bonnet *et al.*, 1996), some members of Brassicaceae (Bonnet *et al.*, 1996; Keizer *et al.*, 1998) and cranberry (Ivanova *et al.*, 2002). Elicitins from *P. infestans* and other *Phytophthora* spp. induce the HR and associated biochemical changes related to defense responses in *Nicotiana* spp. but not in its hosts such as potato and tomato (Ricci *et al.*, 1989; Kamoun *et al.*, 1993, 1997).

The elicitin from *P. palmivora*, isolated from rubber tree, was purified and named palmivorien by Churngchow and Rattarsarn (2000). This elicitin was purified from PDB (potato dextrose broth) culture, vacuum filtrated, ammonium sulphate precipitation, DEAE column and Sephadex G-50 column, respectively. According to the Ph.D. thesis of Rattarasarn (2003), elicitin (10 kDa) was a major protein in a crude

culture filtrate and after ammonium sulfate precipitation, elicitin was the major protein and similar to the purified elicitin from later steps. In addition, the similar elicitation activity of a partially purified elicitin (from culture filtrate and then ammonium sulfate precipitation followed by desalted though PD-10 column) and purified elicitin had been previously demonstrated by Narthida (as in her master thesis, 2003). Therefore, in my work, elicitins were partially purified from culture filtrate of P. pal (Hevea), P. Pal (Durian) and P. para according to the method described above. After the partial purification process, these obtained elicitins were examined by Tricine SDS-PAGE and stained with the silver staining method to verify their purities. As expected, elicitins were the major protein. The elicitins from P. pal (Hevea) and P. pal (Durian) showed only one band whereas elicitin from P. para displayed a major band as protein 10 kDa (or elicitin) and a minor 40 kDa sized protein (see figure of Tricine SDS-PAGE of these elicitins in the appendix).

Elicitins were applied on BPM-24 and RRIM600 leaves at the concentration of 2.5 µg/droplet. In the resistant BPM-24 cultivar, elicitins purified from P. pal (Hevea) and P. pal (Durian) elicited the Scp production in a similar pattern whereas the Scp accumulation triggered by P. para elicitin was delayed and induced about 4 fold lower than that produced in the treatments of elicitin from both of the P. pal strains. However, the level of elicitin-induced Scp accumulation was not significantly different from the control in RRIM600. Therefore, the strength of response to elicitn was correlated to the degree of resistance of the host cell.

Based on these results together with those from the zoospore treatments, that had indicated that there might be something different in attacking the host plant of P. pal (Hevea) and P. pal (Durian), even though they are both *P. palmivora*, because pathogen spreading was obviously different. The explanation could be that the receptor on the plant cells which can recognize different elicitins and result in penetration or successful invasion. Conversely, elicitins from these two strains induced Scp accumulation with a similar pattern and amount. This suggested that the elicitation activity of elicitins was not significantly different to trigger *Hevea* defense response at least for Scp biosynthesis. Elicitin from P. para was less efficient in

triggering Scp production. This may be due to the specificity of epitopes on the elicitins and their receptors are different.

4 *Hevea* calli defense responses against zoospores of *Phytophthora* spp.

The defense responses in *Hevea* cell culture (callus) were tested in order to study whether callus exhibited similar responses to those showed in *Hevea* leaves after treatment with zoospores of *Phytophthora* spp. The Scp accumulation and POD acvivities were investigated in the *Hevea* calli derived from integument inoculation with zoospores of P. pal (Hevea) or P. para.

4.1 Scp production in *Hevea* calli induced by zoospores of *Phytophthora* spp.

In this work, the rate of Scp production induced by zoospores increased in a dose-dependent manner in both cultivars whereas the peak amount was less after treated with too high levels of zoospores ($4.5x10^6$ and $1.35x10^7$ zoospores/ml) of P. pal (Hevea) or P. para. The different levels of Scp production were consistent with the blue fluorescence observed in the callus tissues and in the media when viewed under UV light. The maximum level of Scp in *Hevea* calli was obtained when treated with $1.5x10^6$ zoospore/ml. Higher zoospore numbers than the optimal amounts induced a more rapid initial rate of synthesis but the peak levels were considerably less. Conversely, at extremely low concentrations of inducers ($5x10^5$ zoospore/ml) Scp levels were only slightly different from the controls. These phenomena were comparable to those detected in *Hevea* leaves after treatment with various zoospore concentrations of P. pal (Hevea) (as described in 2.2).

Hevea callus is a more loosely compacted structure compared with *Hevea* leaf, so the calli could have a lesser physical barrier. Thus, this allowed the zoospores easier to infect the callus tissue. The kinetics of Scp accumulation were investigated in calli, BPM-24 and RRIM600 inoculated with $1.5x10^6$ zoospore/ml of P. pal (Hevea) or P. para. In this condition, the interesting point is that zoospores of P. para needed more time than did P. pal (Hevea) to induce the Scp peak even at the initial time (only 6 h after inoculation) Scp levels were similar indicating that P. pal (Hevea) could grow and spread in calli with faster rate. In addition, the reduction rate of Scp was faster in the calli treated with P. para which may be due to it being less virulent. This is corresponds to the Scp analysis in *Hevea* leaves as discussed in discussion 1.2.

Therefore, this revealed the different efficacy of the host to recognize the strains of pathogen and confirmed that these *Phytophthora* spp. strains have different virulence properties. In addition, the maximal Scp content detected in the resistant BPM-24 cultivar was about 3-4 times greater than those in the susceptible RRIM600 cultivar. Hence, this revealed the ability to produce the Scp of *Hevea* calli was correlated with the degree of resistance of the tested cultivars.

4.2 POD activity in *Hevea* calli induced by zoospores of *Phytophthora* spp.

Zoospore of P. pal (Hevea) induced a greater POD activity stronger and reached a maximum level faster than did P. para. After the peak, POD activity was then declined. The level of POD was about 1.5 fold higher when treated with P. pal (Hevea) than that treated with P. para. Moreover, the maximal POD activity exhibited in resistant BPM-24 was 2.5 times greater than those in the susceptible RRIM600. Again, this revealed the ability to produce the POD of *Hevea* calli was correlated with the resistance of the tested calli.

Conclusion

There is a cross-reaction in triggering defense responses by zoospore and elicitin of *Phytophthora* spp. on *Hevea brasiliensis* leaves thus indicating that these species can infect *Hevea* plants. However, their abilities to penetrate and grow in *Hevea* plants were different. These differenced were observed by the spread of the pathogen which was positively correlated to the virulence. From this data, P. pal (Hevea) is the most virulent strain to *Hevea* followed by P. pal (Durian) and P. para, respectively. Even though using optimal conditions, the resistant cultivar normally exhibited an HR response whereas the susceptible one displayed disease, and the HR could be changed to disease in the resistant cultivar under severe condition (very high zoospore or elicitin concentrations). The susceptible cultivar can also display the HR response after treatment with mild condition (low zoospore or elicitin amount). Moreover, the responses of the *Hevea* calli were similar to those detected in *Hevea* leaves. The expressed strength of defense responses, Scp accumulation and enzyme peroxidase activity, is correlated to the resistance of the tested tissues.

CHAPTER 3
OXIDATIVE BURST, CELL DEATH AND SCOPOLETIN PRODUCTION IN HEVEA BRASILIENSIS LEAVES INFECTED BY PHYTOPHTHORA PALMIVORA ZOOSPORES

Introduction

Phytophthora palmivora is an oomycete plant pathogen causing root, stem and fruit rot on more than 100 plant species, including the rubber tree (*Hevea brasiliensis* (Wild.) Muell.-Arg.), the latex of which is a major Thai export product. *Phytophthora* spp. identified as pathogens of the rubber tree are *P. palmivora*, *P. botryosa*, *P. heveae*, *P. meadii* and *P. parasitica*. In the South of Thailand *P. palmivora* and *P. botryosa* are the most frequently isolated pathogens that cause black stripe, green pod rot and abnormal leaf fall (Butler, 1996; Erwin and Ribeiro, 1996).

Recognition of the pathogen will ultimately activate certain plant defense responses and lead to altered gene expression of defense related genes. The recognition elements, generally elicitors, are mainly pathogen related, either being produced by the pathogen itself or through the action of pathogenesis (Yamaguchi *et al.*, 2000). The timely response to an intruding pathogens also plays a critical role in acquiring resistance (Maleck *et al.*, 2000). Thus, the early signaling events in response to pathogens are a major contributing factor to an effective resistance in plants.

The oxidative burst, involving the striking generation and release of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), hydroxyl free radical ($OH^·$), and hydroperoxyl radical (HO_2) is one of the early events associated with a hypersensitive response (HR) in many plant-pathogen interactions (Hammond-Kosack and Jones, 1996; Lamb and Dixon, 1997). Among the effects of ROS are induction of cell death (Delledonne *et al.* 2001; Levine *et al.*, 1994), membrane deterioration (Ádám *et al.*, 1989; Benov, 2001), protein cross-linking (Bradley *et al.*, 1992; Brisson *et al.*, 1994), elicitor signal transduction (Jabs *et al.*, 1996), and systemic acquired resistance (Alvarez *et al.*, 1998; Hückelhoven and Kogel, 2003; Lamb and Dixon, 1997). Although it is well accepted that ROS is

implicated in plant cell death, it is not well established to what extent ROS is directly involved in inducing cell death or in restricting lesions (Hückelhoven and Kogel, 2003; Levine *et al.*, 1994). Since Doke (1983) discovered that superoxide generation preceded the HR in potato cells, during penetration by an avirulent isolate of *Phytophthora infestans,* superoxide has been considered one key component of the plant defense mechanisms. It has been reported that O_2^- is necessary and sufficient to trigger cell death. However, O_2^- also induced leaf lesions in the *Arabidopsis lsd1* (lesion-stimulating disease1) mutant, but not in wild type. The *lsd1* mutant forms necrotic lesions spontaneously on leaves and does not stop the spreading of cell death. In this mutant, O_2^- drastically accumulates in front of the spreading zone of cell death. This indicates that O_2^- is most likely to be a critical signal in the cell death process (Jabs *et al.*, 1996). Prior investigations have shown that O_2^- is not the only key determinant of HR in the interaction of barley with an appropriate powdery mildew fungus (Hückelhoven and Kogel, 1998).

In contrast, hydrogen peroxide (H_2O_2) has also been implicated in the induction of many plant defense responses (Buckner *et al.*, 2000; Dat *et al.*, 2000; Pennell and Lamb, 1997). H_2O_2 in plant tissues may reach levels that are directly toxic to microbes (Peng and Kuc, 1992). H_2O_2 may contribute to the structural reinforcement of the plant cell walls (Bolwell *et al.*, 1995) and trigger lipid peroxide and salicylic acid (SA) synthesis (Leon *et al.*, 1995). Moreover, H_2O_2 appears to have roles in signal transduction cascades that coordinate various defense responses, such as induction of HR and synthesis of pathogenesis-related (PR) proteins and phytoalexins (Greenberg *et al.*, 1994; Hammond-Kosack and Jones, 1996).

The catalyst for the oxidative burst(s) during host disease resistance responses in a number of plant-pathogen systems has been proposed to be an NADPH oxidase (for review, see Low and Dwyer, 1994; Higgins *et al.*, 1998; Bolwell, 1999). However, other research has implicated the possible involvement of xanthine oxidases (Montalbini and Della Torre, 1996) and peroxidases (Bolwell *et al.*, 1998).

A consequence of the debate concerning the catalyst for ROS is the question of whether H_2O_2 is generated via an $HO_2^{\cdot}/O_2^-$-dependent or -independent pathway. The mechanism of H_2O_2 generation is not yet clear. There is much evidence to implicate the involvement of a membrane-bound NADPH oxidase producing

superoxide (O_2^-) that spontaneously forms H_2O_2 (Doke, 1985; Auh and Murphy, 1995; Ros-Barceló *et al.*, 2002). Other H_2O_2-generating systems have also been suggested, e.g. cell wall cationic peroxidases (Bolwell *et al.*, 1995) and amine-oxidases (Goldberg *et al.*, 1985). It has also been suggested that the H_2O_2 generated during the auto-oxidation of coniferyl alcohol drives the oxidase activity of a basic peroxidase in Zinnia (Pomar *et al.*, 2002; López-Serrano *et al.*, 2004). However, this conversion may also be mediated in the cell wall by superoxide dismutase (SOD; EC 1.15.1.1), which catalyses the dismutation of O_2^- into molecular oxygen and H_2O_2 (Ogawa *et al.*, 1997).

In this chapter, the oxidative burst and other defense responses including HR cell death and scopoletin (Scp) production in *Hevea brasiliensis* leaves treated with the zoospores of its pathogen *Phytophthora palmivora* was studied.

Objectives

1. To identify the source of the oxidative burst (O_2^- and H_2O_2) in *Hevea brasiliensis-Phytophthora palmivora* interaction.
2. To study the relationship between the oxidative burst and other defense responses (HR cell death and scopoletin synthesis) in the *H. brasiliensis-P. palmivora* interaction.
3. To study the effect of the inhibitors of the oxidative burst generator on cell death and Scp synthesis.

Materials and Methods

1 *Hevea brasiliensis* leaves

The two cultivars of *H. brasiliensis*; BPM-24 and RRIM600, with different degrees of resistance according to the classification by the Rubber Research Institute of Thailand (RRIT), were used in these experiments. The first one, BPM-24, is considered to be the resistant cultivar while the latter represented a susceptible one.

The 6- to 8-day-old leaflets or leaflets at the B_2-C stage of BPM-24 and RRIM600 leaves were detached. Only healthy leaves were selected and rinsed with sterile distilled water to remove dirt prior to using in further studies.

2 Fungal culture and zoospore preapration

2.1 *Phytophthora palmivora*

P. palmivora isolated from a rubber tree showing disease symptoms (*P. palmivora Hevea* isolate) was kindly provided by the Songkhla Rubber Research Center, Songkhla. The fungal isolate (No. KBNM 9) was identified as *P. palmivora* by a PCR based diagnostic assay using *Phytophthora* genus primers, generously provided by Prof. André Drenth of the University of Queensland, Australia (Drenth and Irwin, 2001). It was further purified by monospore culture in our laboratory then maintained and subcultured in sterile condition every week on potato dextrose agar (PDA) and then at 25±2 ^{0}C. (Figure 2.2).

2.2 Preparation of zoospores of *P. palmivora*

A 0.5 cm diameter plug was cut with a cork borer from the edge of the growing mycelium, after being cultured on PDA for 7 days, and transferred to a V_8 agar. Five days later, the growing mycelium on V_8 agar was chilled using sterile water (8-10 ^{0}C) for 15 min to trigger zoospores release from the sporangium. Zoospores were collected 30 min later. The zoospore suspension was dropped into a Petroff Hauser counting chamber and zoospore concentrations were determined a microscopically.

The desired concentrations of zoospore were subsequently adjusted with sterile distilled water. The zoospore suspensions were prepared to the concentrations $5x10^3$, $5x10^4$, $5x10^5$ and $5x10^6$ zoospores/ml. The suspensions were used within 30 min before the germination could take place.

3 Treatment procedures

Detached *Hevea* leaves were rinsed with sterile distilled water to remove dirt, then placed and laid with abaxial surface upward in a closed plastic box containing the moist Whatman paper. Forty µl droplets of zoospore suspensions with $5x10^4$, $5x10^5$, $5x10^6$ zoospores/ml for the BPM cultivar and $5x10^3$, $5x10^4$, $5x10^5$ zoospores/ml for the RRIM600 cultivar were applied on the abaxal surface of *Hevea* leaves. Droplets

of sterile distilled water were used as a control. The inoculated leaves were kept at 25 ^{0}C in 12 h of daylight. Both leaf samples and the droplets were collected every 24 h for 72 h.

4 Localization of O_2^- and H_2O_2

The superoxide anion (O_2^-)and hydrogen peroxide (H_2O_2) localized in the zoospore-treated and control leaves, were detected by using an NBT and DAB staining method according to procedures adapted from Jabs *et al.* (1996) and Thordal-Christensen *et al.* (1997), respectively. Briefly, the leaves were immersed in 0.1% (v/v) Triton X-100 for 20 seconds and then placed in 1 mg/ml (w/v) NBT (for O_2^- detection) or 1 mg/ml (w/v) DAB (for H_2O_2 detection), then incubated at room temperature. After 8 h, the leaves were cleared by boiling for 30 min in 95% ethanol to stop the reaction and to remove chlorophyll from the leaf tissues. The intensity and pattern of NBT and DAB staining were observed visually, photographed by Sony V1 digital camera. In addition, the appearances of O_2^- and H_2O_2 were also assessed using a microscope and photographed by an Olympus E330 digital camera.

5 Scopoletin (Scp) analysis in *Hevea* leaves

For the droplets method, the droplets from the same treatment were collected and pooled for the Scp accumulation analysis. The accumulated Scp was collected at 6, 12, 24 and 48 h after inoculation. The volumes of collected samples at every time point were adjusted to the same total volume and then kept at -20 ^{0}C until analysis. The Scp was analyzed following the procedure of Churngchow and Rattarasarn (2001) using HPLC and/or spectrofluorometry (excitation and emission wavelengths of 340 nm and 440 nm, respectively). The HPLC was performed for identification of Scp produced by *Hevea* leaves after treatment while the spectrofluorometry was used for determination of the amount. The concentration of Scp was given as μM using Scp (Sigma) as the standard.

6 Necrosis on *Hevea* leaves

The treated leaves were kept at 25 ^{0}C under 12 h of daylight for development of necrotic lesions. The necroses sizes were observed visually and photographed by

Sony V1 digital camera or by using a microscope and photographed by an Olympus E330 digital camera every 24 h for 72 h.

7 Cell death assay

Cell death was monitored in untreated and zoospore-treated leaves by trypan blue staining using a method adapted and performed according to Koch and Slusarenko (1990). At the designated time points, the leaves were placed in 0.1% Triton X-100 for 10 minutes and then transferred to the 0.5% trypan blue dye for 30 min. The unbound dye was rinsed out with sterile water. The dead cells were monitored as a blue color. Cell death was observed first visually and then photographed by using a Sony V1 digital camera. In addition, it was also examined under microscope and photographed by an Olympus E330 digital camera.

8 Effect of zoospore concentrations on the oxidative burst, Scp biosysthesis and cell death

Forty µl droplets of zoospore suspension concentrations of $5x10^5$, $5x10^6$ zoospores/ml for the BPM cultivar and $5x10^4$, $5x10^5$ zoospores/ml for the RRIM600 cultivar prepared as in 2.2 were applied on the abaxial surface of the *Hevea* leaves as described in 3. Droplets of sterile distilled water were used as a control. The inoculated leaves were kept at 25 ^{0}C in a 12/12 h day/night cycle. The leaf samples were collected at 24 and 48 h after inoculation for detection of O_2^-, H_2O_2, necrotic lesions and cell death following the method described in 4, 6 and 7, respectively. The droplets were also collected for Scp as in 5.

9 Relationship between the oxidative burst, Scp accumulation and cell death

The relationships between oxidative burst and other defense responses including Scp accumulation and cell death were examined in the leaves from the resistant BPM-24 cultivar, because the oxidative burst, Scp production and cell death were displayed more clearly in the resistant cultivar. The rubber leaves were inoculated (dropped method as described in 3) with zoospore of *P. palmivora* at the concentrations of $5x10^6$ zoospores/ml. Droplets of sterile distilled water were used as the control. The inoculated leaves were kept at 25 ^{0}C in 12 h of daylight. To verify the connections of

the defense responses, the leaf samples were collected 6, 12, 24 and 48 h after inoculation for detection of O_2^-, H_2O_2, necrotic lesions and cell death following the procedure described in 4, 6 and 7, respectively. The droplets were also collected for Scp as in 5.

10 Toxicity test for oxidative burst inhibitors

10.1 Effect of oxidative burst inhibitors on the inhibition of *P. palmivora* mycelium growth

Mycelium growth inhibition was tested on the mycelium culture of *P. palmivora*. Four concentrations of DPI; 5, 25, 50 and 100 µM were serial diluted from a 1 mM stock solution of diphenylene iodonium (DPI). Three concentrations of diethydithiocarbamate (DDC); 1, 5 and 20 mM were prepared from a 100 mM stock solution of DDC. Growth inhibitions were examined in 5 cm Petri dishes by subculturing a mycelium plug (diameter of 5 mm) onto PDA containing different concentration of DPI or DDC. Fungal cultures were incubated for 4 days at 25 ^{0}C. Four days later, the fungal growth on the PDA containing inhibitors were compared with the fungus on the PDA (control) and photographed.

10.2 Toxicity of oxidative burst inhibitors on *Hevea* leaves.

For the inhibition test, the BPM-24 leaves were prepared and treated as described above in 3. The leaves were marked with marker pen in order to identify the original site for later treatments. To remove the cutin and allow the inhibitors to access the leaf tissues, Triton X-100 (0.5% (v/v)) was dropped onto the abaxial side. After 15 min, the leaves were rinsed twice with sterile distilled water to remove Triton X-100 and then replaced (at the same sites as Titron X-100) by sterile water (control) or DPI (50, 100 µM) or DDC (5 and 20 mM). The inhibitors were removed 24 h after pretreatment and then the sterile distilled water or zoospore suspension ($5x10^6$ zoospores/ml) was applied. The treated leaves were kept at 25 ^{0}C in a 12/12 h day/night cycle. The necrotic symptoms were detected 24 or 48 h after zoospore inoculation.

11 Study of the inhibition of the oxidative burst

For the inhibition test, the BPM-24 leaves were pretreated with 50 µM of DPI and 20 mM of DDC following 10.2. Twenty-four h after inhibitor pretreatment, the inhibitors were removed and further inoculated with the zoospore suspension ($5x10^6$ zoospores/ml). Droplets of sterile distilled water were used as the control. The inoculated leaves were kept at 25 ^{0}C in a 12/12 h day/night cycle. The samples were collected for the detection of O_2^- and H_2O_2 following the procedures in 4.

12 Effect of the oxidative burst inhibitors on Scp accumulation, necrosis and cell death

The method for studying the oxidative burst inhibitors was performed following the procedure described in 10.2. The concentration of 50 µM DPI and 20 mM DDC were selected to demonstrate the effect of these inhibitors on Scp accumulation, necrosis and cell death. Twenty-four h after inhibitor pretreatment, the inhibitors were removed and further inoculated with the zoospore suspension ($5x10^6$ zoospores/ml). The inoculated leaves and the droplets were collected at 24 and 48 h for detection of O_2^-, H_2O_2, necrotic lesions, cell death and Scp following the procedure described in 4, 6, 7 and 5, respectively.

Results

1 Necrosis and cell death detected in the *H. brasiliensis-P. palmivora* interaction

1.1 Necrosis

The detached leaves of the resistant (BPM-24) and the susceptible (RRIM600) cultivars were inoculated on the abaxial surface with droplets of zoospores of *P. palmivora Hevea* isolate (*P. palmivora*) in concentrations ranging from $5x10^3$ to $5x10^6$ zoospores/ml. The necrotic sizes were observed at 24, 48 and 72 h after zoospore application. Leaves from both resistant (Figure 3.1A) and susceptible (Figure 3.1B) cultivars displayed similar patterns of necrosis after zoospores application, and both increased in a dose-dependent manner during the tested period. However, at the same zoospore concentration the resistant BPM-24 cultivar exhibited smaller lesions than those in the susceptible RRIM600 cultivar. There was no

significant increase of necrosis in the BPM-24 cultivar after treating with very low zoospore amounts ($5x10^3$ zoospores/ml) while the diameter of the lesion rapidly expanded in the susceptible cultivar after treatment with very high zoospores level ($5x10^6$ zoospores/ml). Therefore, for a more detailed comparison of the necrosis induced by the two cultivars, they were inoculated with $5x10^4$, $5x10^5$ and $5x10^6$ zoospores/ml for BPM-24 and $5x10^3$, $5x10^4$ and $5x10^5$ zoospores/ml for RRIM600. The first symptoms on *Hevea* leaf tissue infected with the *P. palmivora* zoospores appeared as necrotic spots beneath the inoculation droplets 24 h after inoculation with 40 µl of $5x10^4$, $5x10^5$ and $5x10^6$ zoospores/ml for BPM-24 and $5x10^3$, $5x10^4$ and $5x10^5$ zoospores/ml for RRIM600. Forty-eight h after inoculation, necrotic symptoms at the same zoospore concentrations were very different for these two cultivars. In the resistant BPM-24, there was no observed necrosis when treated with the low concentration ($5x10^4$ zoospores/ml), and with $5x10^5$ zoospores/ml, a resistant reaction was observed where fungal development was restricted to a few dark-brown or black spots and the progress of the damage was restricted to areas close to the infection site. A decreased resistance of BPM-24 and the spreading necrosis were observed with an application of $5x10^6$ zoospores/ml (Figure 3.2A). The susceptible RRIM600 cultivar also showed restricted lesions when being inoculated with $5x10^3$ and $5x10^4$ zoospores/ml but the lesions were extended after treatment with $5x10^5$ zoospores/ml (Figure 3.2B). At 72 h after inoculation of the BPM-24 cultivar, the spreading infection had developed into water-soaked, macerated lesions when treated with $5x10^6$ zoospores/ml while the lesions in RRIM600 spread in the same way at $5x10^5$ zoospores/ml (Figure 3.2A and 3.2B).

1.2 Cell death detected by trypan blue staining in the *H. brasiliensis-P. palmivora* interaction

To gain a more detailed comparison of the necrosis and cell death in BPM-24 and RRIM600 cultivars, zoospore concentrations of $5x10^4$, $5x10^5$ and $5x10^6$ zoospores/ml and lower concentrations of $5x10^3$, $5x10^4$, $5x10^5$ zoospores/ml were selected for BPM-24 and RRIM600 leaves, respectively because these ranges in each case covered the expression of both restricted and expanded lesions. The visible necrosis observed in both cultivars increased in a dose- and time-dependent manner during the tested period, 24, 48 and 72 h after inoculation (Figure 3.3A and 3.3B). In

addition, cell death was paralleled with the necrotic lesion (in 1.1) but the areas of cell death were larger (Figure 3.2 and 3.3). The trypan blue-stained dead cells of both cultivars was observed microscopically as shown in Figure 3.4. The results taken together from Figure 3.3 and 3.4 showed that there were two different reactions to the trypan blue staining method were observed. First, cells staining dark blue, in a HR-like cell death, appeared at the infection spot at an early time (24 h) and could be detected next to areas where the spread of the pathogen had been restricted at 48 h. The second type was light blue cells, and was termed disease-lilke cell death, with cell death being associated with areas where the pathogen had spread from the initial inoculation site with the highest zoospore inoculum. The HR-like cell death was monitored at 24 h until 48 h when the resistant BPM-24 was treated with $5x10^5$ zoospores/ml (Figure 3.4A and 3.4B) whereas the susceptible one needed lower amount, $5x10^4$ zoospores/ml (Figure 3.4E and 3.4F), to behave in the same way. For both these phenomena, the HR cell death was visible at 24 h but then converted to the disease-like cell death at 48 h were observed in both BPM-24 and RRIM600 inoculated with $5x10^6$ (Figure 4C and 4D) and $5x10^5$ zoospores/ml (Figure 4G and 4H), respectively. However, the concentration of $5x10^5$ and $5x10^4$ zoospores/ml also caused the expanded lesion at 72 h (Figure 3.3). These results reveal the different resistance properties of these two cultivars; the susceptible RRIM600 displayed a 10 fold lower degree of resistance than did the resistant BPM-24.

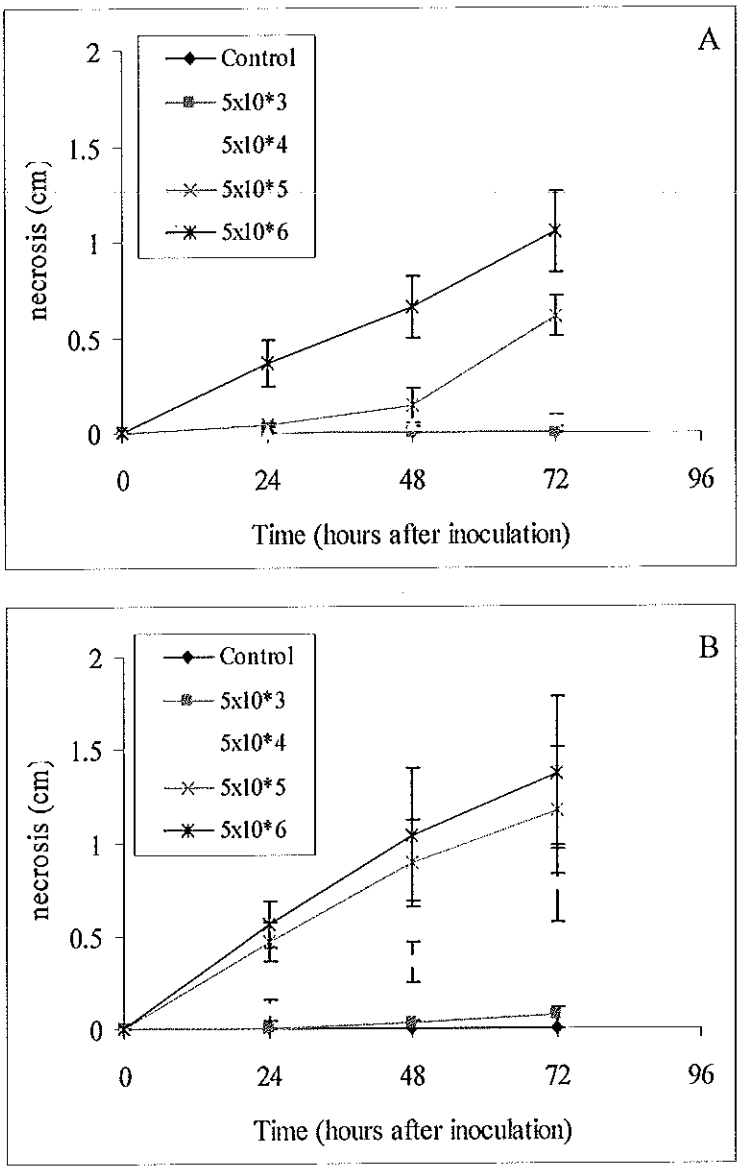

Figure 3.1 Necrotic lesion size of *Hevea* leaves inoculated with various concentrations (ranging from $5x10^3$ to $5x10^6$ zoospores/ml) of *P. palmivora* zoospores. The size of the necrosis of the resistant BPM-24 (A) and the susceptible RRIM600 (B) were examined at 24, 48 and 72 h after inoculation. The control is treated with sterile distilled water. Each time point represents the mean ± SD of three independent experiments.

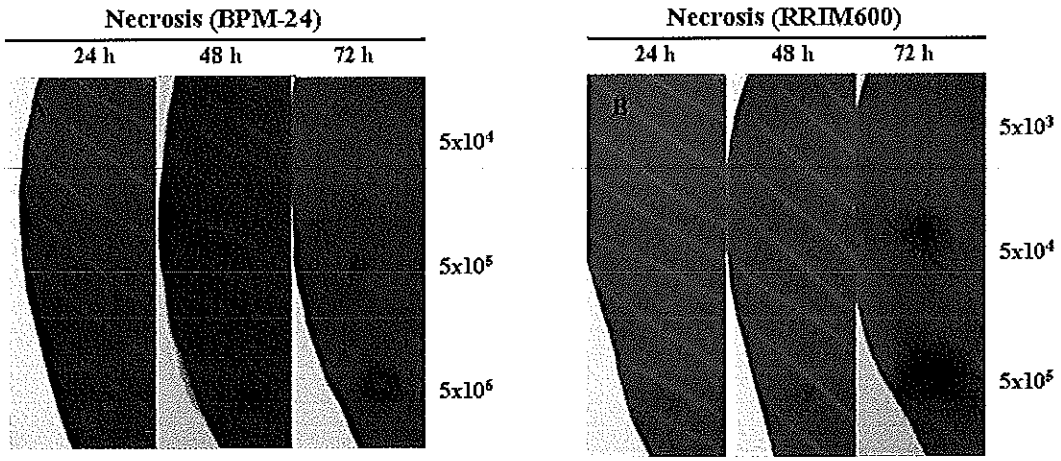

Figure 3.2 Necrosis observed in *H. brasiliensis* leaves after inoculation with various zoospore concentrations of *P. palmivora*. The droplets of zoospore were placed on the abaxial surface of *Hevea* leaves. Leaf necrosis of BPM-24 (A) and RRIM600 (B) were observed at 24, 48 and 72 h after inoculation.

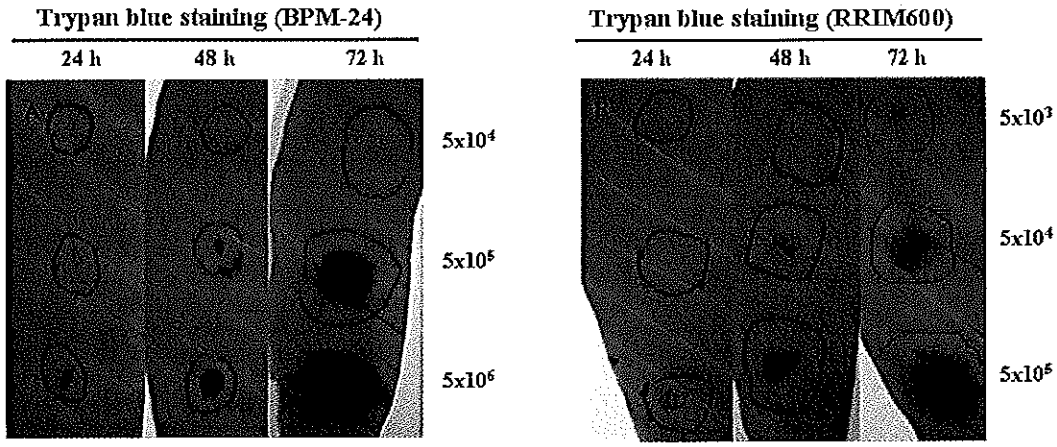

Figure 3.3 Cell death in *H. brasiliensis* leaves after *P. palmivora* zoospore treatments. Cell death was detected by trypan blue staining of BPM-24 (A) and RRIM600 (B) and observed at 24, 48 and 72 h after inoculation. The circles around the cell death areas were marked by marker pen to locate the position of the original zoospore droplets.

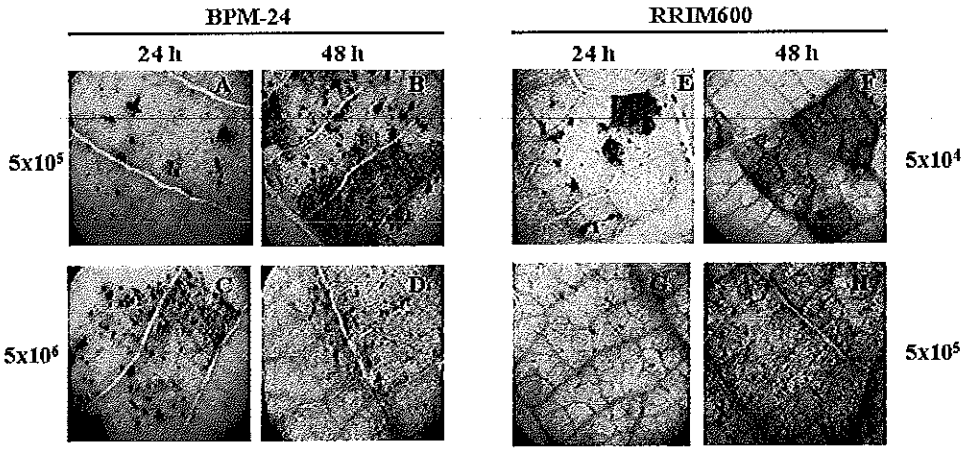

Figure 3.4 Trypan blue stained cell death in the *H. brasiliensis-P. palmivora* interaction (observed under microscope). The BPM-24 leaves were inoculated with $5x10^5$ zoospores/ml (A and B) and $5x10^6$ zoospores/ml (C and D). The RRIM600 leaves were inoculated with $5x10^4$ zoospores/ml (E and F) and $5x10^5$ zoospores/ml (G and H). In each set, the left columns are trypan blue-staining observed in the leaves at 24 and the right columns are detected at 48 h after inoculation.

2 Oxidative burst or the generation of reactive oxygen species (ROS)

2.1 Detection of superoxide anion (O_2^-) generation

After inoculation leaves of the BPM-24 and RRIM600 with $5x10^5$, $5x10^6$ zoospores/ml and $5x10^4$, $5x10^5$ zoospores/ml, respectively, O_2^- accumulation was monitored by its reaction with nitroblue tetrazolium (NBT) to produce the blue formazan, product of NBT. (Thordal-Christensen *et al.*, 1997). Twenty-four h after inoculation of the resistant BPM-24 with $5x10^6$ zoospores, abundant O_2^- was detected microscopically (Figure 3.5C) whereas lower amounts appeared in the treatment of $5x10^5$ zoospores/ml (Figure 3.5A). In both cases the O_2^- decreased at 48 h (Figure 3.5D and 3.5B). The blue formation was restricted to the area covered by the infection droplet. No significant O_2^- accumulation was detected in any untreated area. In contrast, the blue color appeared around the cut borders of both the control and treated leaves immediately after the leaf segments were immersed into NBT solution. This may result from the wounding process. Similar results on O_2^- appearance were made

with leaves from the susceptible RRIM600 cultivars but at the lower zoospore concentrations of $5x10^4$ and $5x10^5$ zoospores/ml observed at 24 h (Figure 3.5E and 3.5G) and 48 h (Figure 3.5E and 3.5G), respectively. Treatment of the leaves with concentrations of zoospores less than those used above produced no sign of invasion by the pathogen and no formazan precipitation was observed. When leaves were treated with higher concentrations of zoospores than used above some few cells showed NBT staining for O_2^- but at the highest concentrations no NBT precipitation was observed (data not shown). However, the intensity of blue precipitates was darker in the resistant BPM-24 than those in the susceptible RRIM600 when compared at the same response magnitude.

2.2 Detection of hydrogen peroxide (H_2O_2) generation

The leaves of BPM-24 and RRIM600 from the same treatment condition as in 2.1 were also assayed for H_2O_2 accumulation. The BPM-24 and RRIM600 leaves were treated with $5x10^5$, $5x10^6$ zoospores/ml and $5x10^4$, $5x10^5$ zoospores/ml, respectively. Staining with 3,3'-diaminobenzidine (DAB) was performed to detect H_2O_2 which reacted with the dye to produce brown precipitates at the sites of H_2O_2 accumulation. At 24 h, the appearance of the DAB precipitates was detected in both cultivars at the inoculation sites in a dose-dependent manner but its level was low in all zoospore treatments (Figure 3.6a and 3.6b for BPM-24 and Figure 3.6e and 3.6g for RRIM600). The intensity of DAB was intensified at 48 h (Figure 3.6b, 3.6d, 3.6f and 3.6h) in contrast to the O_2^- accumulation observed from the NBT staining. In addition, a further expansion of DAB deposits was observed at 72 h while the NBT precipitate was not observed (data not shown). Similarly, treatment of the leaves with concentration of zoospores less than those used above produced no significantly DAB staining. Again, the intensity of DAB precipitates was darker in the resistant BPM-24 than those in the susceptible RRIM600 when compared at the same response magnitude, additionally; no DAB precipitate was detected in any other area except for a small amount at the cut borders. These dramatic decreases observed for NBT staining while DAB staining was being intensified demonstrated that the detectable O_2^- in the leaf tissues preceded the production of H_2O_2.

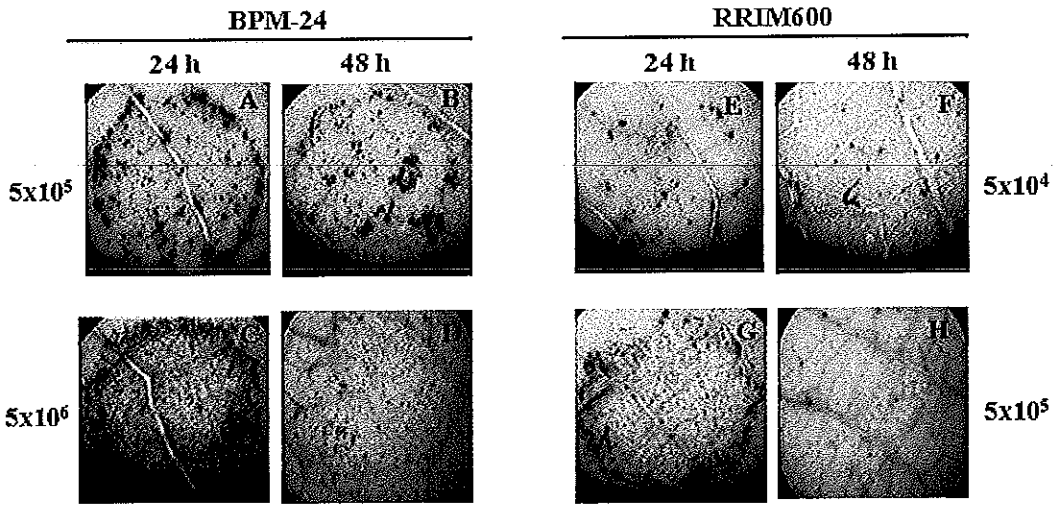

Figure 3.5 The detection of superoxide anion (O_2^-) by NBT staining in the *H. brasiliensis-P. palmivora* interaction. The BPM-24 leaves were inoculated with $5x10^5$ zoospores/ml (A and B) and $5x10^6$ zoospores/ml (C and D). The RRIM600 leaves were inoculated with $5x10^4$ zoospores/ml (E and F) and $5x10^5$ zoospores/ml (G and H). In each set, the left and right columns are NBT-staining observed in the leaves at 24 and 48 h after inoculation, respectively.

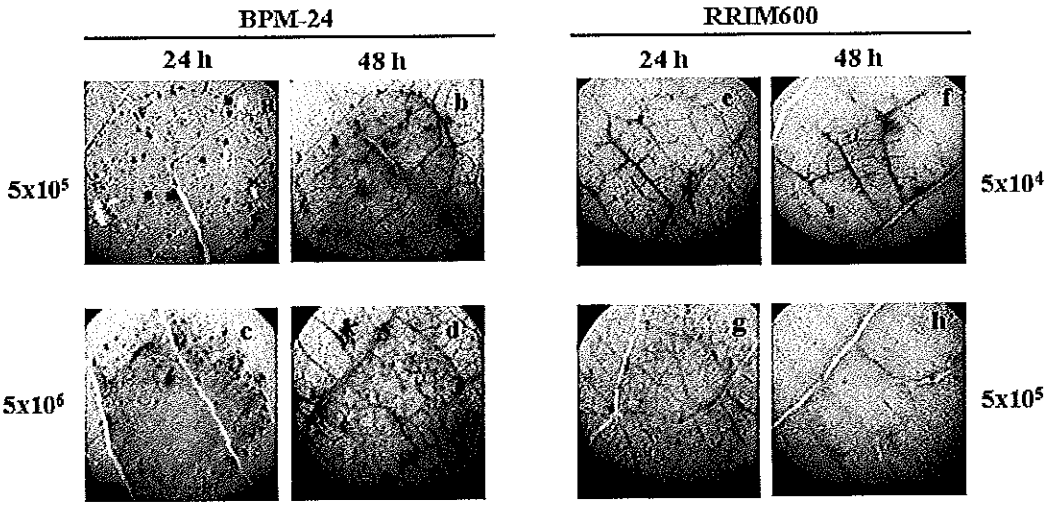

Figure 3.6 The detection of hydrogen peroxide (H_2O_2) by DAB staining in the *H. brasiliensis-P. palmivora* interaction. The BPM-24 leaves were inoculated with $5x10^5$ zoospores/ml (a and b) and $5x10^6$ zoospores/ml (c and d). The RRIM600 leaves were inoculated with $5x10^4$ zoospores/ml

(e and f) and $5x10^5$ zoospores/ml (g and h). In each set, the left and right columns are NBT staining observed in the leaves at 24 and 48 h after inoculation, respectively.

3 Establishing relationships between the oxidative burst, cell death and scopoletin (Scp) accumulation

To establish the sequence of the appearances of oxidative burst, cell death and Scp production, the experiments were performed with BPM-24 leaves because they displayed more definite defense reactions than did leaves from the susceptible RRIM600 cultivar at the same zoospore concentrations. BPM-24 leaves were therefore inoculated with *P. palmivora* zoospore suspensions of $5x10^6$ zoospores/ml. The samples were taken after 6, 12, 24 and 48 h for determination of HR cell death using trypan blue staining, Scp accumulation in the droplets, and staining for NBT and DAB for the presence of O_2^- and H_2O_2 respectively.

3.1 Oxidative burst

Around the inoculation site, the highest amounts of O_2^- were observed at 6 h, then decreased and could not be detected at 48 h after inoculation (Figure 3.7A-3.7D). H_2O_2 was first observed at 12 h and continued to accumulate during the experiment (Figure 3.7E-3.7H). Although, there were some difficulties to identify the location of H_2O_2 from the brown precipitates, especially when the necrotic lesions occurred because the necrotic tissues were also brown, however, the background from NBT staining could be used to compare and confirm that the brownish color resulted from DAB staining. The results of DAB staining at 12, 24 and 48 h showed significant accumulation of H_2O_2 after subtracting with the NBT background.

3.2 Cell death

Cell death was monitored by staining with trypan blue. The HR-like cell death was first observed, as small dark blue spots at 12 h and the dark blue area was increased at 24 h after inoculation whereas the expanded disease-like lesions started to appear at 48 h (Figure 3.7I-3.7L).

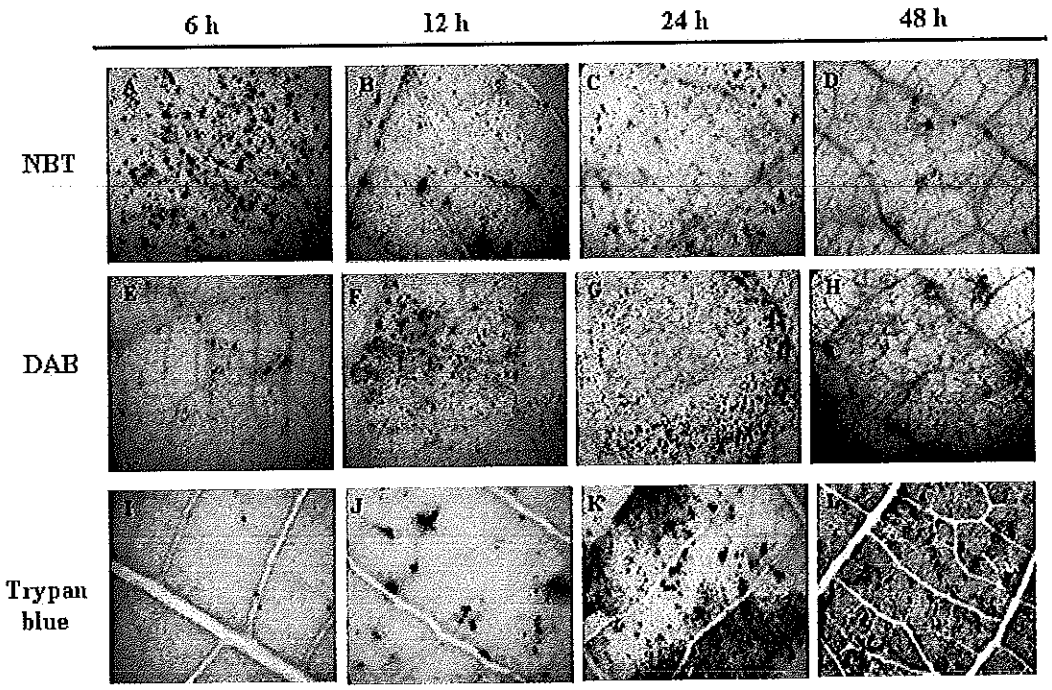

Figure 3.7 The ROS generation, cell death and scopoletin (Scp) accumulation during the *H. brasiliensis-P. palmivora* interaction. The resistant BPM-24 leaves were treated with *P. palmivora* $5x10^6$ zoospores/ml. O_2^- accumulation was detected by NBT staining at 6, 12, 24 and 48 h after inoculation (A, B, C and D). At the same time points; H_2O_2 accumulation was detected by DAB staining (E, F, G and H), cell death was monitored by trypan blue staining (I, J, K and L).

3.3 Scopoletin accumulation

The BPM-24 leaves were inoculated with *P. palmivora* zoospore suspensions of $5x10^6$ zoospores/ml and the Scp content in the droplets were measured after 6, 12, 24 and 48 h. A bright blue fluorescence noticed under UV-light or scopoletin (Scp) was visible in the tissues as well as in the droplets 3-4 h after zoospore application. The level of Scp increased rapidly and strongly after 12 h, reached its highest point at 24 h after zoospore inoculation and then declined at 48 h (Figure 3.8).

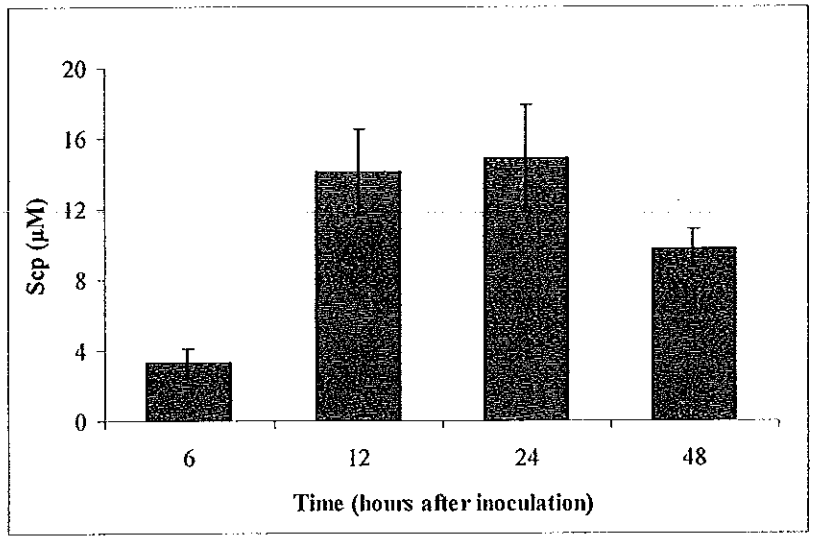

Figure 3.8 Scopoletin (Scp) accumulation. The resistant BPM-24 leaves were treated with zoospore of *P. palmivora* $5x10^6$ zoospores/ml and Scp level in the droplets were measured. Zoospores were replaced by sterile distilled water in the controls. Each time point represents the mean ± SD of three independent experiments.

Since the Scp production and HR cell death were examined in the same tissue we concluded that the first detectable reaction was the production of the formazan precipitate at 6 h (Figure 6A) followed by the appearance of Scp and the HR cell death were clearly visible at 12 and 24 h, respectively, (Figure 7 and 6K). These data indicated that O_2^- might be involved in triggering the Scp production and the HR cell death, whereas H_2O_2 is more likely to be involved in cell death than in Scp synthesis.

4 Effect of the inhibitors ROS generator on ROS accumulation, cell death and Scp production

4.1 Effect of the NADPH oxidase inhibitor (diphynylene iodonium; DPI)

4.1.1 Fungitoxicity of DPI on the growth of *P. palmivora*

Diphenylene iodonium (DPI) is an inhibitor of mammalian NADPH oxidase, and has also been shown to block the oxidative burst in plant cells (Levine *et al.*, 1994). The toxicity of DPI on mycelium growth was measured prior to using it to test its action on ROS generation and other plant defense responses in our system. The final concentrations of tested DPI in the fungus growth medium were 5, 25, 50 and

100 μM. It was shown that only the maximum concentration of 100 μM DPI showed a significant inhibit the mycelium growth and 50 μM DPI caused a slight reduction in the growth of *P. palmivora* whereas any concentrations lower than 50 μM (5 and 25 μM) had no effect (Figure 3.9).

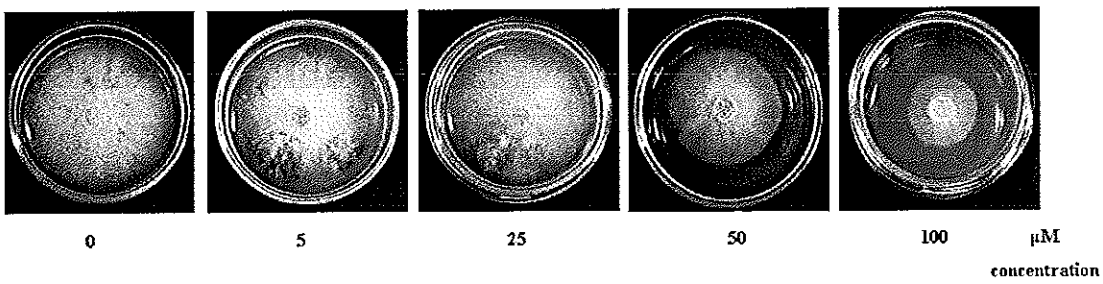

Figure 3.9 Effect of various DPI concentrations on the growth inhibition of *P. palmivora* at 4 days.

4.1.2 Effect of DPI on *Hevea* leaf tissue damage

Leaf tissue damage caused by DPI was measured. Only 100 μM of DPI was toxic to the *Hevea* tissue and caused the observed tissue damage whereas there was no observed injured tissue when treating with 50 μM DPI (Figure 3.10). In addition, the effect of the DPI concentrations on necrosis after challenging the leaves with *P. palmivora* zoospores was also observed. Pretreatment with 50 and 100 μM of DPI had similar dose-dependent effect on the necrotic lesion (Figure 3.10). The control represents the area treated with 0.5% Titron X-100 for 15 min to remove the cutin before application with other chemical or zoospores. The result showed that Titron X-100 had no toxic effect on the leaf tissue but had a slight effect on enhancing the pathogen penetration. Since 100 μM DPI caused tissue damage and also inhibited pathogen growth, therefore, 50 μM DPI was chosen for further studies.

4.1.3 Effect of DPI on ROS generation in the *H. brasiliensis-P. palmivora* interaction

The BPM-24 leaves were pretreated with the DPI 24 h prior to being inoculated with $5x10^6$ zoospores/ml. Fifty μM of DPI was selected for further studies because it gave more obvious results and was less toxic to *P. palmivora*. Under these conditions, BPM-24 pretreated with 50 μM DPI, the formation of O_2^- was abolished at 6 h (Figure 3.11A and 3,11B) while the H_2O_2 production was significantly reduced

after zoospore inoculation at 12 h (Figure 3.11C and 3.11D). Thus, this inhibitor had the efficiency in diminishing the O_2^- production in the *H. brasiliensis-P. palmivora* interaction.

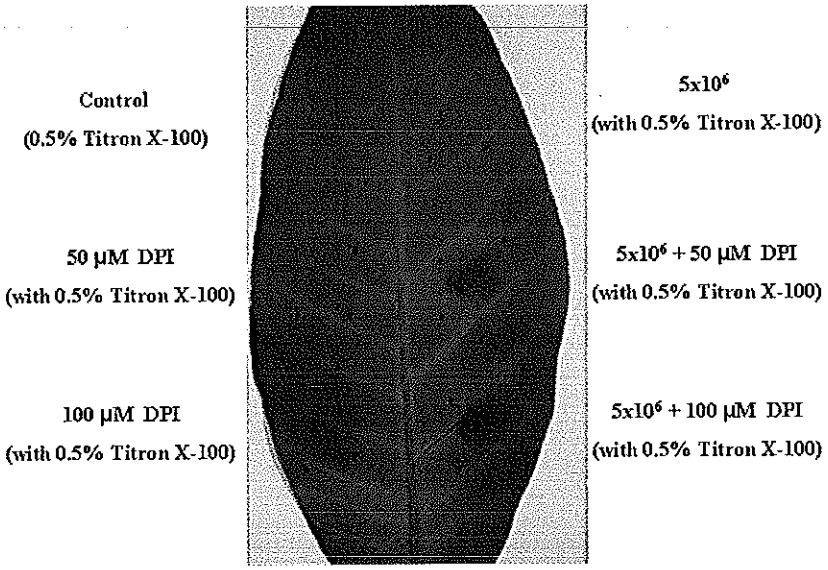

Figure 3.10 The effect of DPI on *Hevea* tissue damage and necrotic symptoms after challenging with *P. palmivora* zoospores. DPI concentrations of 50 and 100 µM were applied at 24 h and then inoculated with $5x10^6$ zoospores/ml. Zoospores were replaced by sterile distilled water in the controls. The necrotic symptoms were detected 48 h after zoospore inoculation. In the figure, the marker pen spots located the positions of the original zoospore droplets.

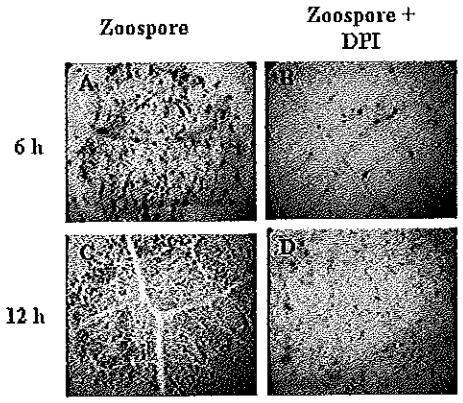

Figure 3.11 The effect of DPI on O_2^- and H_2O_2 accumulation in the *H. brasiliensis-P. palmivora* interaction. The BPM-24 leaves were pretreated with 50 µM DPI 24 h before being inoculated with $5x10^6$ zoospores/ml. O_2^- was

detected by NBT staining in the non-pretreatment with DPI (A) and DPI-pretreated tissues (B) at 6 h, and H_2O_2 accumulation was detected by DAB staining at 12 h (C and D).

4.1.4 Effect of DPI on cell death and Scp biosynthesis in the *H. brasiliensis-P. palmivora* interaction

The effect of pretreating BPM-24 leaves with DPI on cell death and Scp accumulation was also investigated in the same experiments as decribed above (in 4.1.3). Twenty-four h after zoospore application to leaves pretreated with 50 μM DPI, the size of the necroses in the tissues were clearly increased about 2-2.5 fold over the controls (Figure 3.12). Cell death as observed by the trypan blue staining was more likely to be caused by the spread of the pathogen rather than as an HR response, so presumably the HR response was suppressed after DPI pretreatment (Figure 3.13A and 3.13B). This effect was more obvious at 48 h (Figure 3.13C and 3.13D). Hence, the reduction of HR cell death, which resulted from the abolishing of the NADPH oxidase dependent O2- generation by DPI, also enhanced the spread of the pathogen. As for the Scp accumulation, this was significantly reduced by approximately 55-65%, 12 and 24 h after application of zoospores to the BPM-24 leaves pretreated with DPI, compared to the control which was not pretreated with DPI (Figure 3.14).

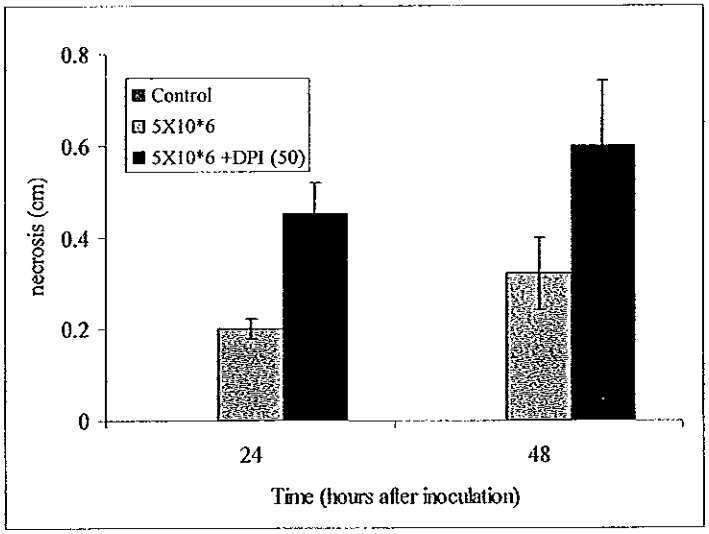

Figure 3.12 Effect of DPI on the size of the necrotic lesions. The necrotic sizes on BPM-24 leaves with/without pretreatment with 50 μM DPI were measured 24 and 48 h after inoculation with $5x10^6$ zoospores/ml.

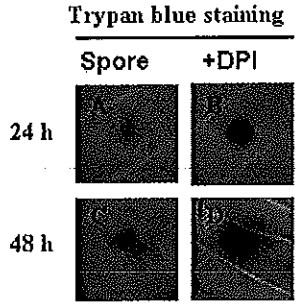

Figure 3.13 Effect of DPI on cell death. Cell death of BPM-24 leaves with/without pretreatment with 50 μM DPI were monitored by trypan blue staining at 24 (A and B) and 48 h (C and D) after inoculation with $5x10^6$ zoospores/ml.

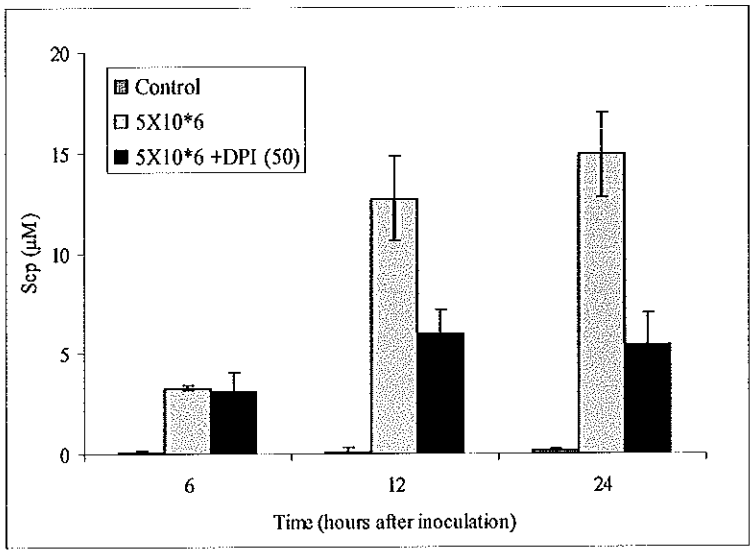

Figure 3.14 Effect of DPI on Scp production. Scp accumulation was measured at 6, 12 and 24 h in the droplets of BPM-24 leaves after inoculation with $5x10^6$ zoospores/ml inoculation. Zoospores were replaced by sterile distilled water in the controls. Each time point represents the mean ± SD of three independent experiments.

4.2 Effect of superoxide dismutase (SOD) inhibitor (diethydithiocarbamate; DDC)

4.2.1 Fungitoxicity of DDC on the growth of *P. palmivora*

Diethydithiocarbamate or DDC is a known inhibitor of the Cu/Zn SOD (Heikkila *et al.*, 1976 and Kelner and Alexander, 1986). The toxic of DDC on

mycelium growth was first tested prior to using it to test its action on ROS generation and other plant defense responses. The final concentrations in the media of tested DDC were 1, 5 and 20 mM. The diameter of the mycelium was slightly reduced after 4 days in the media containing 20 mM of DDC whereas at 1 and 5 mM, there was no observable effect on the growth (Figure 3.15).

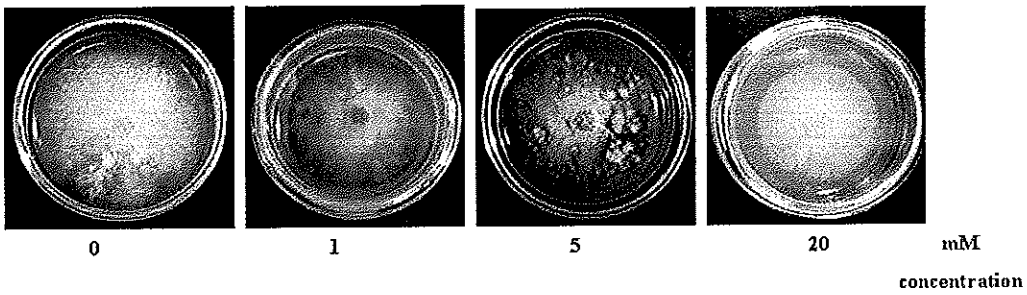

Figure 3.15 Effect of DDC concentrations on the growth inhibition of *P. palmivora* observed after 4 days.

4.2.2 Effect of DDC on *Hevea* leaf tissue damage

Any leaf tissue damage caused by DDC was also checked. The tested DDC concentrations (5 and 20 mM) showed no toxic effect on the *Hevea* tissue (Figure 3.16). The effect of 5 and 20 μM DDC on leaf necrosis after challenge with *P. palmivora* was also observed. Pretreatment with DDC produced a dose-dependent increase of the necrotic lesion (Figure 3.16). However, since it was necessary to remove the cutin or physical barrier by first treating with 0.5% Titron X-100 for 15 min before application of DDC, the toxicity of Titron X-100 was investigated. Titron X-100 had no toxic effect on the leaf tissue by itself but slightly enhanced the ability of the pathogen to penetrate the leaf tissue (comparable to the results in 4.1.2). Even 20 mM DDC had only a minor inhibitory effect on the growth of the pathogen it had no effect on tissue damage and had its biggest effect on increasing the size of the necrosis. Twenty mM of DDC was chosen for further studies.

4.2.3 Effect of DDC on ROS generation in the *H. brasiliensis-P. palmivora* interaction

BPM-24 leaves pretreated with 20 mM DDC 24 h prior to being inoculated with $5x10^6$ zoospores/ml had significantly enhanced the O_2^- accumulation

(Figure 3.17a and 3.17b). This might have been expected from its inhibition of SOD activity. The inhibition of H_2O_2 accumulation was not complete but was significantly reduced (Figure 3.17c and 3,17d). This indicated that H_2O_2 in *H. brasiliensis-P. palmivora* interaction was probably partially formed by dismutation of O_2^- and partly from other pathways.

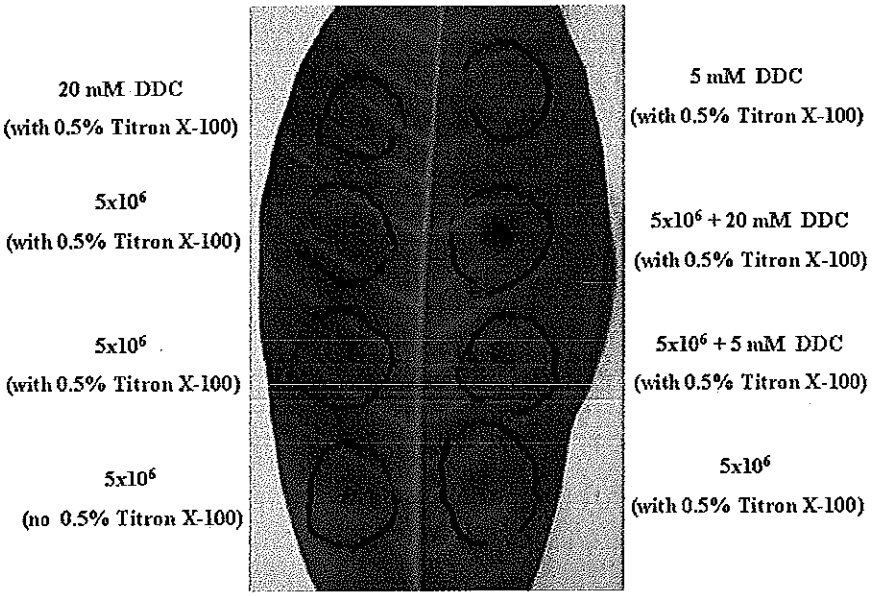

Figure 3.16 The effect of DDC on *Hevea* tissue damage and necrotic symptoms after challenging with *P. palmivora* zoospores. DDC concentrations of 5 and 20 mM were applied 24 h before inoculation with zoospores $5x10^6$ zoospores/ml. Zoospores were replaced by sterile distilled water in the controls. Necrotic symptoms were observed 24 h after zoospore inoculation. The marker pen circles locate the positions of the original zoospore droplets.

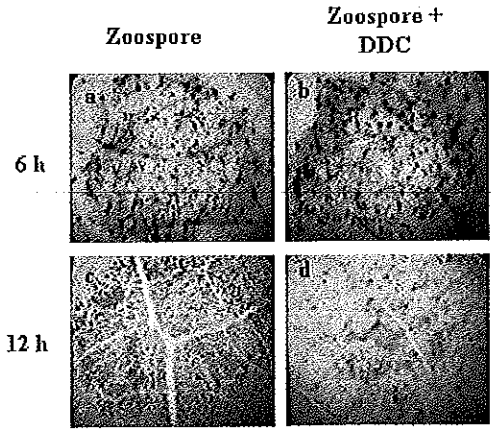

Figure 3.17 The effect of DDC on O_2^- and H_2O_2 accumulation in the *H. brasiliensis-P. palmivora* interaction. The BPM-24 leaves were pretreated with 20 mM DPI 24 h, before inoculation with $5x10^6$ zoospores/ml. H_2O_2 was detected by DAB staining in the non-pretreatment with DDC (a) and DPI-pretreated tissues (b) at 6, and H_2O_2 accumulation was detected by DAB staining at 12 h (c and d).

4.2.4 Effect of DPI on cell death and Scp biosynthesis in the *H. brasiliensis-P. palmivora* interaction

The effect of pretreatment BPM-24 leaves with DDC on cell death and Scp accumulation was also investigated in the same experiments as decribed in 4.2.3. The sizes of necrosis in the tissues at 24 h were about 2 fold larger than those from the controls, after pretreatment with 20 mM DDC and challenged by $5x10^6$ zoospores/ml (Figure 3.18). The appearance of HR cell death observed by the trypan blue staining, within 24 h without pretreatment or after DDC pretreatment, was more likely to be caused by the spread of the pathogen rather than as an HR response, so presumably the HR response was suppressed (Figure 3.19a and 3.19b). The evidence was much clearer at 48 h (Figure 3.19c and 3.19d). Thus, the reduction of HR cell death, after decreasing of H_2O_2 accumulation caused by DDC, enhanced the spread of the pathogen. Although the effect of DDC was clear in reducing HR-like cell death, the effect on Scp accumulation was very low. The decrease in the Scp accumulation, after treatment with DDC was only between 15-20% during the period tested compared to that obtained from the control (Figure 3.20). The results taken from 4.1.4 together

with 4.2.4 indicate that both O_2^- and H_2O_2 generated from the oxidative burst could be involved in necrosis and HR cell death. However, only O_2^- accumulation led to Scp production whereas H_2O_2 was not necessary for Scp induction.

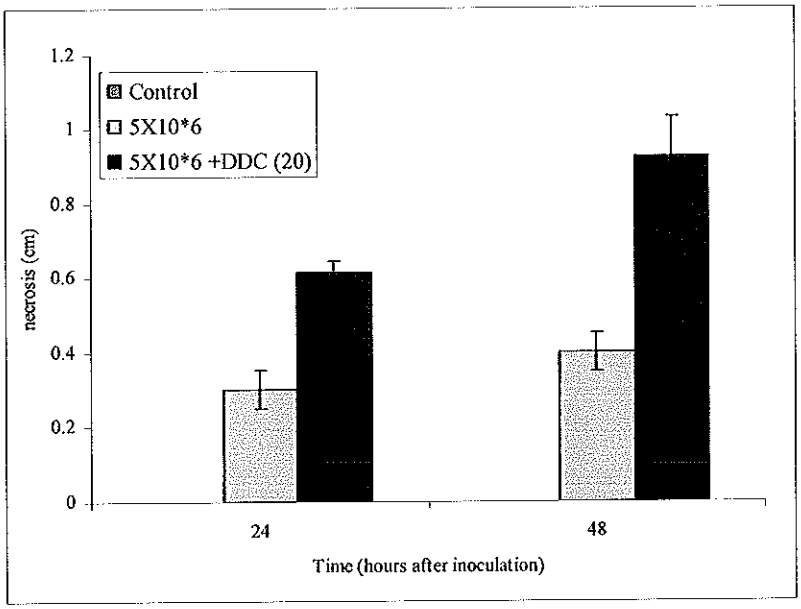

Figure 3.18 Effect of DDC on the size of the necrotic lesions. The necrotic sizes on BPM-24 leaves with/without pretreated with 20 mM DPI were detected 24 and 48 h after inoculation with $5x10^6$ zoospores/ml.

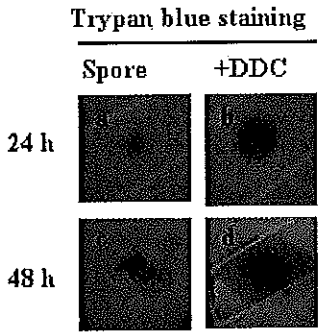

Figure 3.19 Effect of DDC on cell death. Cell death in BPM-24 leaves with/without pretreated with 20 mM DPI were monitored by trypan blue staining at 24 (a and b) and 48 h (c and d) after inoculation with $5x10^6$ zoospores/ml.

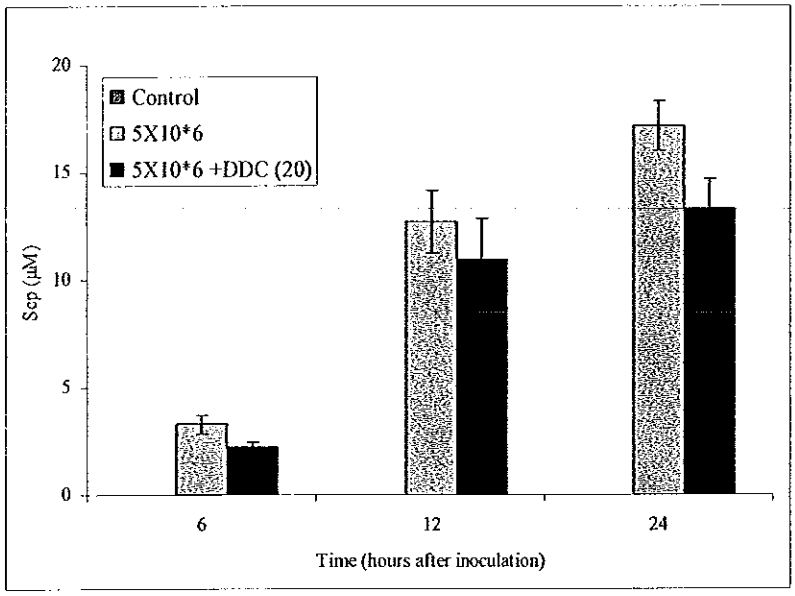

Figure 3.20 Effect of DDC on Scp production. Scp accumulation was measured at 6, 12 and 24 h in the droplets of BPM-24 leaves after zoospore $5x10^6$ zoospores/ml inoculation. Zoospores were replaced by sterile distilled water in the controls. Each time point represents the mean ± SD of three independent experiments.

Discussion

Necrotic lesions, cell death, oxidative burst and Scp accumulation were studied in the host *H. brasiliensis* and its pathogen *P. palmivora*.

1 Necrosis and cell death

Cell death detected by trypan blue staining, in all treatments of two *Hevea* cultivars, BPM-24 and RRIM600, was similar but larger in size than that of the visible necrosis (Figure 3.2 and 3.3). The trypan blue method identified 2 different cell death response patterns. First, cell death caused directly by the spreading of pathogen after the leaves were inoculated with a large number of zoospores, this was observed as a light blue color. Secondly, an HR-like cell death detected as a dark blue color occurred in circumstances when the spread of the pathogen in the leaves was restricted. In both cases cell death occurred in a time- and zoospore dose-dependent manner (Figure 3.1, 3.2 and 3.3). HR-like cell death appeared 24-48 h after zoospore application and this

changed to the disease-like cell death between 48-72 h. However, when compared at 24 h, the RRIM600 susceptible cultivar required $5x10^5$ zoospores/ml while the BPM-24 resistant leaves required $5x10^6$ zoospores/ml, for the same response, a 10 fold difference in the degree of resistance.

2 The generation of reactive oxygen species (ROS) or oxidative burst

Various enzyme systems have been suggested to be the source of ROS during a plant defense response. A membrane-bound NADPH oxidase seems to have the best credentials (Doke, 1985; Levine *et al.*, 1994; Auh and Murphy, 1995; Dwyer *et al.*, 1996). However xanthine oxidases and cell wall peroxidases have also been implicated in the production of $HO_2^{\cdot}/O_2^{-}$ (for reviews see Mahalingam and Fedoroff, 2003; Laloi *et al.*, 2004). Using the inhibitor of NADPH oxidase, diphenylene iodonium (DPI), could be one strategy to establish whether O_2^{-} was produced via this enzyme in this system (*H. brasiliensis-P. palmivora*). Pretreatments of resistant BPM-24 leaves with 50 µM DPI abolished O_2^{-} generation indicating that NADPH oxidase is the source of O_2^{-} production in our model.

The origins of the H_2O_2 was also explored. Results showed that H_2O_2 accumulated as the O_2^{-} disappeared. Many reports have suggested that the membrane-bound NADPH oxidase produces O_2^{-} and then H_2O_2 is rapidly formed through the enzyme superoxide dismutase (SOD) Doke, 1985; Auh and Murphy, 1995; Ros-Barceló *et al.*, 2002). When BPM-24 was pretreated with Diethyldithiocarbamate (DDC), a chelator of Cu ions and an inhibitor of the Cu/Zn SOD (Heikkila *et al.*, 1976; Kelner and Alexander, 1986), a significant but not complete reduction of H_2O_2 accumulation occurred. This confirms that H_2O_2 is at least partially produced by the dismutation of O_2^{-} through SOD. However, there is probably more than one route to produce H_2O_2 in *Hevea* leaves after *P. palmivora* infection. Other sources could be the cell wall cationic peroxidases (Bolwell *et al.*, 1995) and amine-oxidases (Goldberg *et al.*, 1985). Allan and Fluhr (1997) and Bolwell *et al.* (2002) have suggested that the ROS can be produced either intra- or extracellular depending on the eliciting agent as well as the tissue and the organism. The reasons for these differences are not yet well-understood. Nevertheless, it is already clear that different ROS-producing enzymes

are activated by different input signals and those signals are communicated by different routes (for reviews see Mahalingam and Fedoroff, 2003).

Based on these results of the *H. brasiliensis-P. palmivora* interaction, O_2^- generated by NADPH oxidase preceded the production of SOD activity-induced H_2O_2. Moreover, the intensity of O_2^- and H_2O_2 accumulation after treatment with optimum zoospore numbers were much greater in the resistant BPM-24 than that in the susceptible RRIM600 cultivar so this correlated to the resistance of the tested cultivars.

3 The relationships between oxidative burst, cell death and scopoletin (Scp) accumulation

This work provided evidence for establishing the connection of oxidative burst and other defense responses including cell death and Scp synthesis in the *H. brasiliensis-P. palmivora* model.

3.1 Oxidative burst and cell death

During an HR, an oxidative burst coincides with the induction of cell death at the site of the pathogen attack. This localized cell death limits the spread of the invading pathogen (for review see Van Breusegem *et al.*, 2001). Levine *et al.*, (1994) were the first authors to demonstrate that ROS can also act as a signal to induce plant cell death in soybean cell suspensions. They suggested that a short pulse of H_2O_2 was sufficient to activate a hypersensitive cell death and this H_2O_2-induced cell death could be blocked by cycloheximide and protease inhibitors. There are now many reports have suggested that ROS can induce cell death and some have indicated that the oxidative burst is the prime trigger of the HR (Grant *et al.*, 2000; Jabs *et al.*, 1996; Levine *et al.*, 1994). However this has been contradicted by the work of Sasabe *et al.* (2000), Tada *et al.* (2004) and Mur *et al.* (2005) who all show that ROS could not elicit hypersensitive cell death.

In this present work, cell death was examined in parallel with the oxidative burst using BPM-24 leaves inoculated with $5x10^6$ zoospores/ml. HR cell death, indicated by a small area of trypan blue stained cells, was clearly detected at 24 h and this was followed by the maximum accumulation of O_2^- and H_2O_2 at 6 h and 12 h after inoculation, respectively. Forty-eight h after inoculation, disease-like cell death

started to appear at the infection site but HR-like cell death was still observed in neighboring cells and the O_2^- at the infection site was quickly disappearing at this time. In contrast, H_2O_2 was continuing to accumulate and correlated with the occurrence of cell death. Hence, in this case the oxidative burst preceded and may be involved in induction of HR-cell death.

3.2 Oxidative burst and Scp synthesis

H. brasiliensis can produce an antimicrobial phytoalexin called scopoletin (Scp) in response to stress (Giesemann *et al.*, 1986). Scp was generated in *Hevea* after treatment with *Microcyclus ulei*, *Corynespora cassiicola* and *P. palmivora* (Garcia *et al.*, 1995, Breton *et al.*, 1997, Churngchow and Rattarasarn, 2001). We had previously shown that Scp was produced in *Hevea* leaves as well as in seeds and calli after zoospore and palmivorein treatments (accepted paper being in process). Thus, in this work, the link between the oxidative burst and Scp accumulation in *Hevea* leaves treated with zoospores of *P. palmivora* was investigated since ROS has also been implicated as a signal required for the expression of phytoalexins (Lamb and Dixon, 1997). For example, Guo *et al.* (1998) have shown that the ROS produced in soybean cells in response to an avirulent *Pseudomonas syringae* pv. *glycinea* is necessary but not sufficient for the accumulation of a phytoalexin glyceollin. In our case Scp was measured in the droplets of zoospores on the resistant BPM-24 leaves with sterile distilled water used as a control.

Scp was first visible as a bright blue fluorescence under UV-light in the tissues as well as in the droplets after 3-4 h of zoospore application. The Scp level increased significantly at 12 h, reached a peak at 24 h and then declined. The synthesis of Scp followed the peak accumulation of O_2^- at 6 h after inoculation and coincided with the induction of H_2O_2. The decrease of Scp at later time (48 h) was possibly caused by peroxidase enzymes as revealed by the induction of Scp POD activity (accepted paper being published) and also the presence of H_2O_2. This is supported by the suggestion that Scp is an antioxidant that can be readily oxidized by POD and H_2O_2 (Levine *et al.*, 1994; Chong *et al.*, 1999; Dorey *et al.*, 1999). In addition at 48 h, the lesion was being converted to the disease-like cell death indicating that the colonized tissue had ceased to synthesize phytoalexin as suggested by Dorey *et al.* (1999). These data indicate that the oxidative burst occurred prior to Scp accumulation.

4 Effect of the inhibitors of enzymes involved in ROS generation

Nowadays, there are still conflicting views about which ROS molecule, O_2^- or H_2O_2 or both, are important signals for inducing cell death and Scp production.

4.1 Effect on cell death

The ROS inhibitors DPI and DDC were used to verify which ROS molecule is more likely to be an inducer of cell death. In BPM-24 leaves pretreated with inhibitors 24 h prior to inoculating with zoospores, HR cell death was reduced, followed by an increase of lesion size and spreading of the pathogen. Furthermore, when added together there was no evidence for any synergistic effect (data not shown). These results indicated that O_2^-, mediated from an NADPH oxidase, and H_2O_2, formed by dismutation of O_2^- by the action of SOD, were necessary to induce the pathway of HR cell death in our *H. brasiliensis-P. palmivora* system. Other supporting data has been reported by Able *et al.* (2000), they suggested that $HO_2^{\cdot}/O_2^-$ generation is a critical factor leading to the HR in tobacco cells challenged by avirulent zoospores of *P. nicotianae* (*Pn*) whereas H_2O_2 generation (that occurs largely, but not completely, via an $HO_2^{\cdot}/O_2^-$-dependent pathway) appears to play a minor role in the induction of the HR. Likewise, in a study of the lesion-stimulating disease1 (*lsd1*) mutant of *Arabidopsis*, O_2^- and not H_2O_2, initiated a runaway cell death phenotype, again indicating that O_2^- seemed to be the critical signal in the cell death process (Jabs *et al.*, 1996). Conversely, Mellersh and associates (2002), who studied the cowpea–*Erysiphe cichoracearum* interaction, reported that H_2O_2 but not O_2^- played a crucial role in HR cell death and enhanced resistance by inhibiting the penetration of nonhost fungi.

4.2 Effect on Scp production

To confirm the role of ROS in eliciting the accumulation of Scp, ROS inhibitors DPI and DDC were again used. Scp accumulation showed a clear decline after DPI pretreatment (65% reduced) and a smaller reduction (20% reduced) after DDC pretreatment. This indicated that O_2^- was essential for Scp production and H_2O_2 might also have a role. These results are similar to those of Jabs and coworkers (1997) who demonstrated that an elicitor induced O_2^- is required for and can induce the production of phytoalexins in parsley cell suspensions. In potato tuber issue, also Perrone *et al.* (2003) reported that O_2^-, was necessary for the accumulation of

phytoalexin by tobacco cells after treatment with avirulent races of *P. nictotianae* and a non-pathogen of tobacco, *P. palmivora*.

Conclusion

In conclusion, in the *H. brasiliensis-P. palmivora* model, O_2^- was generated via NADPH oxidase precede H_2O_2 which was dismutated from O_2^- by the action of the enzyme superoxide dismutase (SOD) and partly generated by other pathways. Moreover, the intensity of O_2^- and H_2O_2 accumulation after treatment with optimum zoospore numbers were much greater in the resistant BPM-24 than that in the susceptible RRIM600 cultivar and this correlated to the resistance of the tested cultivars.

Both O_2^- and H_2O_2 were involved in the control of HR cell death and pathogen spreading whereas O_2^-, rather than H_2O_2 was essential for Scp production. This model seems to be suitable for further studies on the roles of the oxidative burst in plant-pathogen interactions. However, other methods for detecting the localization of O_2^- and H_2O_2 and detection for restricting the pathogen spread *in situ* of *Hevea* tissues should also be investigated. The role of the oxidative burst on other responses such as the expression of genes involved in the defense responses, and certain types of cell death by detecting the hallmarks for apoptosis such as DNA laddering should be further investigated.

CHAPTER 4

ELICITATION ACTIVITY OF DIFFERENT ELICITORS AND MODE OF ACTION OF β-1,3-GLUCANS IN THE ELICITATION OF PLANT DEFENSE RESPONSES AGAINST PATHOGENS

Introduction

Nowadays, there is much interest in investigating and developing plant crop protection strategies. The aims are to protect plants from pathogens, reduce production costs and to find a protection way to protect crop which will be friendly to the farmer and the environment. Using elicitors from plant or pathogen or natural products, which can trigger plant defense responses, is now one of the favorite tools to induce protection. The ideal elicitor should be effective to induce strong defense responses and also induce resistance in a field test.

Plants are subjected to attack by a wide variety of microbial pathogens and insect herbivores. In response, plants express numerous defense mechanisms, many of which are induced by pathogen attack. Appropriate regulation of defense responses is important for plant fitness, as activation of defense responses has deleterious effects on plant growth. The molecular mechanisms underlying activation of plant defense responses are complex. Responses often begin after plants recognize an invading pathogen or pathogen associated molecular patterns (PAMPs). This is also associated with activation of a salicylic acid (SA)-dependent signaling pathway that leads to expression of certain pathogenesis-related (PR) proteins thought to contribute to resistance. Some other plant defense responses are controlled by mechanisms dependent on ethylene (ET) and/or jasmonates (JA). SA, JA, and ET signaling pathways interact extensively. SA and JA are mutually inhibitory for the expression of many genes.

Plant pathogens are often divided into biotrophs and necrotrophs, according to their lifestyles. Biotrophs feed on living host tissue, whereas necrotrophs kill host tissue and feed on the remains. The SA-dependent defense responses are considered effective mainly against biotrophic pathogens that feed on living tissues, such as the oomycete *Peronospora parasitica (Pp)* and bacterial *Pseudomonas*

syringae whereas the JA-dependent defense responses are effective against necrotropic pathogens (Glazebrook, 2005).

Oligosaccharins are one group of PAMPs that occur naturally as complex carbohydrates with biological regulatory functions (Albersheim *et al.*, 1983). In plants and animals, they act as molecular signals that regulate growth, development, and survival in the environment (Côté and Hahn, 1994; Bakkers *et al.*, 1999; Alban and Franz, 2001; Shibuya and Minami, 2001; Ménard *et al.*, 2004). Oligosaccharins active in plants can be derived from cell wall structures of microbes, plants, or marine macroalgaes. Only a few are fully characterized in terms of their structure and spectrum of biological activities. Laminarin (Lam) is a reserve carbohydrate of the brown algae *Laminaria digitata*, its structure consists of β-1,3 glucan backbone with an average degree of polymerization of 25 glucose units and with 1-3 single β-glucose branches at position 6 (Read *et al.*,1996; Lepagnol-Descamps *et al.*, 1998). Many reports have shown that Lam can act as the elicitor to induce plant defense responses, for example, fungal β-glucans are efficient elicitors to induce the defense responses in different plant species (Côté and Hahn, 1994; Ebel, 1998; Shibuya and Minami, 2001). Lam also stimulates defense responses in the cell suspensions of tobacco (Klarzynski *et al.*, 2000), gravevine (Aziz *et al.*, 2003), alfalfa (Cardinale *et al.*, 2000) and rice (Inui *et al.*, 1997). Ménard, *et al* (2004) showed that Lam induces ET- but not SA-dependent defense responses whereas sulfated laminarin (PS3), becomes more efficient. PS3 can induce both ET- and SA-signaling pathway in tobacco and *Arabidopsis thaliana*. *Arabidopsis* and tobacco responded similarly to Lam and PS3 treatment. In plant treated with mixture of Lam and PS3, there was no synergy for the SA-dependent acidic PR1 and the ET-dependent basic PR5 (Ménard *et al.*, 2005).

From our observations, Lam has a negative effect on the SA-dependent pathway induced by PS3 and on the reduction of resistance to such groups of pathogen that can be controlled by SA-dependent defense responses. Because there is much evidence showing that JA antagonizes SA signaling (Vidal *et al.*, 1997; Niki *et al.*, 1998; Petersen *et al.*, 2000; Kachroo *et al.*, 2001; Kloek *et al.*, 2001; Li *et al.*, 2004) so we hypothesized Lam can suppress the SA signaling pathway through JA.

Objectives

1. To test whether TTF5P has an elicitation activity to induce plant defense responses in tobacco and to identify the active component in TTF5P
2. To elucidate the mode of action of β-1,3-glucans in the elicitation of plant defense responses in tobacco and *Arabidopsis thaliana*
3. To study the suppression effect of laminarin (Lam) on the salicylic acid (SA)-dependent defense responses induced by sulfated laminarin (PS3)
4. To examine the elicitation activities of β-1,3-glucans (Lam, PS3 and Lam+PS3) in *Arabidopsis* resistance against the pathogens (*Pseudomonas syringae* and *Peronospora parasitica*)

Materials and Methods

1 Plant materials and treatment

1.1 Tobacco (*Nicotiana tabacum*) plants

Tobacco plants, *N. tabacum* cv Samsun H (seeds obtained from Altadis, Institut du Tabac, Bergerac, France), *N. tabacum* cv Xanthi nc were grown in a greenhouse under controlled conditions. After 2-3 months, plants were placed 2-3 days before any treatments in a growth chamber at 22 ± 1 ^{0}C with a photoperiod of 16 h (Figure 4.1).

1.2 *Arabidopsis thaliana*

Arabidopsis thaliana plants used throughout these experiments were of the ecotype Col-0, *coi1-16* mutant and *sid2/NahG* transgenic were. Plants were grown with a 12-h light period at 20 ^{0}C. The 6-week-old plants were transferred to a growth chamber at 22 ± 1 ^{0}C with a photoperiod of 16 h 2-3 days prior being used in for the experiments (Figure 4.2).

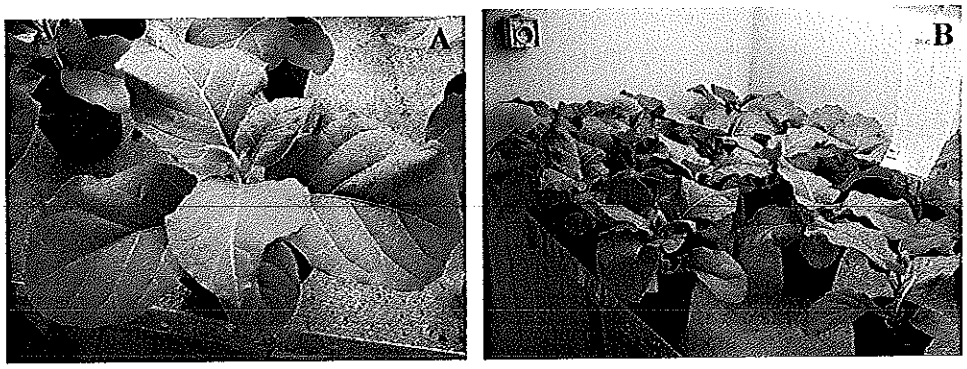

Figure 4.1 Two-month-old Tobacco plants.
(A) Two-three-month-old tobacco plants ready for testing.
(B) Tobacco plants maintained in a controlled growth chamber.

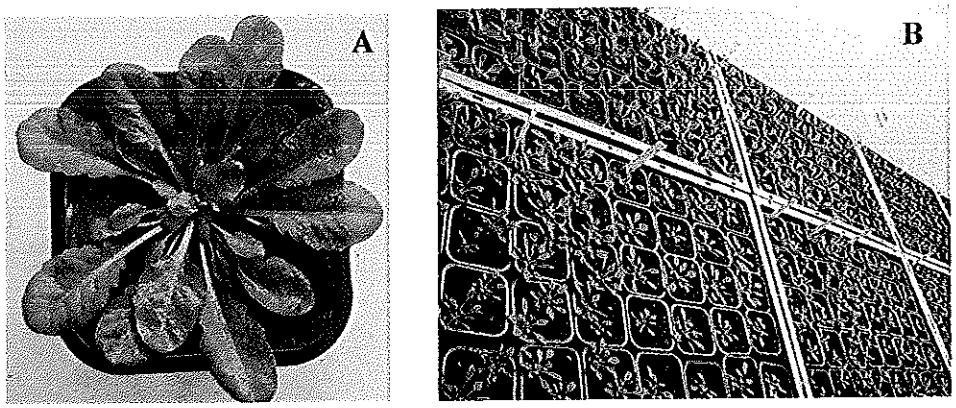

Figure 4.2 *Arabidopsis* plants
(A) Six-week-old *Arabidopsis* ready for testing.
(B) Two-three-week-old *Arabidopsis* maintained in a controlled growth chamber.

2 Elicitors

2.1 Laminarin

Laminarin (Lam), extracted and purified from the marine brown algae *Laminaria digitata* by Laboratoires Goëmar (Klarrzynski *et al.*, 2000), was kindly provided from Dr. Serge Kauffmann, IBMP, France. It was prepared according to the method reported in Ménard et al. (2004). *L. digitata* sporophytes were harvested in Brittany, extracted with hot water (100 kg of fresh algae in 200 l, 70 ^{0}C) for 2 h, and

the aqueous extracts were fractionated by ultrafiltration, using 1-m^2 membranes with a cutoff of 300 kDa (TAMI Industries, Nyons, France) and a flow rate of approximately 9.8 l/h. The ultrafiltrate was then filtered with a membrane cutoff of 1 kDa and the resulting retentiveness was desalted and lyophilized. Yields typically amounted to 10% of the kelp (brown algae) dry weight. The average molecular mass of the Lam samples was 5,300 g/mol, as measured by molecular size chromatography coupled with a refractometric detector. Lam consists of a β-1,3 glucan backbone with an average degree of polymerization (DP) of 25 and one or two C6 glucose branches. Purity, size, and structure were further analyzed by natural abundance ^{13}C NMR spectroscopy and HPAEC-PAD (Lépagnol-Descamps *et al.*, 1998)

2.2 Sulfated laminarin (PS3)

Sulfation of laminarin resulted in a laminarin sulfate fraction called PS3that was also provided from Dr. Serge Kauffmann, IBMP, France. PS3 was prepared according to the method reported in Ménard *et al.* (2004). One g of laminarin was dissolved in a mixture of 20 ml of dimethylformide (DMF) and 2.8 ml of pyridine, and the mixture was then heated to 60 ^{0}C. Sulfation was performed by continuous addition of 5.9 g of SO_3-pyridine complex for 2 h at 60 ^{0}C. Stirring was continued for 2 h. After cooling to room temperature, the supernatant was removed by pipetting from the slippery brown precipitate formed during the reaction. The latter precipitate was dissolved in a 2.5 M NaOH solution, and the resulting solution was then poured onto 99% EtOH to obtain a final concentration equal to 90% ethanol. Twelve after h stirring at 4 ^{0}C, the white precipitate was separated by centrifugation and dissolved in distilled water. The solution of PS3 was dialyzed (molecular weight cut off 1000 Da) for 7 days at room temperature against flowing deionized water. The solution was then frozen at -70 ^{0}C and freeze-dried. The apparent molecular mass of PS3 is 18 kDa as determined by gel permeation chromatography using neutral pullulans as standard. According to the methylation analysis, the C6-OH of PS3 is nearly completely sulfated, and the substitution of both C2-OH and C4-OH amounts to ~70%.

2.3 TTF5P

TTF5P, a product of Tribo-technologies, is currently used in agriculture as a fertilizer. It is a mixture of amino acids, of polysaccharides produced by algae and of

phosphite. The composition of TTF5P is algae extract, NPK and amino acids (AA). It was kindly provided from Dr. Serge Kauffmann, IBMP, France.

2.4 β-aminobutyric acid (BABA)

β-aminobutyric acid (BABA) (Sigma) was dissolved in distilled water to get various concentrations.

3 Treatment procedures

3.1 For tobacco plant

3.1.1 Infiltration method

Tobacco plant treatments were performed by infiltration of aqueous solution of elicitors into the mesophyll of fully developed leaves. The leaves were stabbed with a needle to generate a small hole and then the elicitors were infiltrated with a needleless syringe. Each leaf can be divided into 6 individual positions, and each position was treated with an elicitor (about 200 µl/spot). The sterile water was used as control. The infiltrated tissue was immediately delineated with a felt-tip marker after infiltration when necessary. All treated plants were kept in the growing chamber at 22 ± 1 ^{0}C with a photoperiod of 16 h. The leaf tissues were collected 1 and 3 days after infiltration for RNA and protein extraction, respectively.

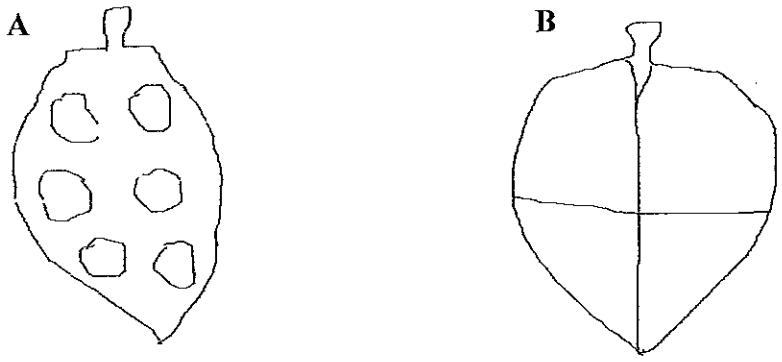

Figure 4.3 Scheme of infiltration and spray model. The elicitors and water as control were infiltrated into the mesophyll of fully developed tobacco leaves (A) or sprayed on tobacco leaves (B).

3.1.2 Spray method

The tobacco leaves can be divided into 2-4 parts by marking with felt-tip marker before treatment. The aqueous solutions of elicitors were sprayed onto an adaxial surface of the leaves. The sterile water was used as control. Similarly to the infiltration method, all treated plants were kept in the growing chamber at 22 ± 1 ^{0}C with a photoperiod of 16 h. The leaf tissues were collected 1 and 3 days after infiltration for RNA and protein extraction, respectively.

3.2 For *A. thaliana*

The elicitors were infiltrated directly (without hole generation) into the mesophyll of the fully expanded leaves of *A. thaliana* because of its thin layer which make it easier to absorb the elicitor solutions. The sterile water was used as control. The infiltrated tissue was immediately delineated with a felt-tip marker after infiltration when necessary. All treated plants were kept in the growing chamber at 22 ± 1 ^{0}C with a photoperiod of 16 h. The leaf tissues were collected 1 and 3 days after infiltration for RNA and protein extraction, respectively.

4 PR-proteins analysis by western blot

4.1 Protein extraction and preparation for electrophoresis

The elicitor infiltrated leaf area (150-200 mg of fresh weight) was kept in liquid nitrogen or stored at -80 ^{0}C until analysis. The frozen tissue was ground in the 2.5 volumes of 0.1 M sodium phosphate buffer, pH 7.5 containing β-mercaptoethanol (15 mM, or 1 µl of β -mercaptoethanol : 1 ml of buffer). The sample was centrifuged at 13000 rpm for 20 min at 4 ^{0}C. The supernatant was transferred and centrifuged again at 13000 rpm for 20 min at 4 ^{0}C. The protein sample was mixed with loading buffer (100 mM Tris-HCl, pH 6.8, 5% (v/v) β-mercaptoethanol, 30% (v/v) glycerol, 0.01% (w/v) bromophenol blue and 2% (w/v) SDS) in the ratio 4:1 of sample and loading buffer, respectively. Then the sample was denaturated by incubation at 95 ^{0}C for 5 min and stored at -20 ^{0}C until used.

4.2 Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE)

4.2.1 Preparation of SDS polyacrylamide gel

The slab gel was set up in size 7x8 cm^2, 0.5 mm thick. The composition of gel (for preparation of 2 gels) is described in table 4.1.

Table 4.1. The ingredient of the gel for SDS-PAGE

Ingredients	Stacking gel (5%)	Separating gel (12.5%)
40% Acrylamide-bisacrylamide	0.5 ml	2.48 ml
1.0 M Tris-HCl, pH 8.8	-	3.00 ml
1.0 M Tris-HCl, pH 6.8	0.5 ml	-
10% Ammonium persulfate	80 µl	150 µl
TEMED	4 µl	12 µl
Distill water	2.96 ml	2.44 ml

4.2.2 Electrophoresis

The protein samples (about 5-10 µl and 10-25 µl for the small and large comb, respectively) were applied in each lane of the slab gel. The electrophoresis was run in Tris-glycine containing 0.1% (w/v) SDS buffer (migration buffer) with 100 V or 30 mA at 25 ^{0}C until the bromphenol blue dye move to 0.5 cm from the bottom of the gel. The electrophoresed gel was then electro-transferred to the membrane for further steps.

4.3 Electro-transferring from gel to membrane

The electro-transferring were performed in the transfer buffer (1 M Tris-glycine containing 20% (v/v) methanol). To transfer proteins by the capillary process, the electrophoresed gel was placed on 2 pieces of wet (by soking in the buffer) Whatman paper following by putting Immobilon P membrane (0.45 µm, Millipore), which was immersed in the buffer for 1 min, on top of the gel. Two pieces of wet Whatman paper were used to cover onto the membrane. The transferring was carried out with a constant 150 mA and 30 V and agitation at 4 ^{0}C. After 1 h 30 min to 2 h, proteins from the gel were then diffused and ascended to the membrane. The membrane was kept for immunobloting (western blot) analysis.

4.4 Immunodetection (Western blot analysis)

The membrane (from 4.3) was immersed for 5 min in 30 ml of "milk" buffer (PBS buffer containing 1.6% (w/v) milk powder (Bio-Rad) and 0.1% (v/v) Tween 20). The buffer were removed and replaced again with the new milk buffer and incubated while shaking gently at 37 ^{0}C for 1 h. The membrane was transferred to the hybridization buffer, 30 ml of milk buffer with the first antibody (antibody of PR-proteins) and left overnight at 4 ^{0}C for complete hybridization. To remove the unbound antibody, the membrane was washed 3 times (15 min each) with the milk buffer. The membrane was exposed to mix with the HRP-conjugated secondary antibody which was made up to 1:10,000 in the milk buffer (3 µl of the secondary antibody in 30 ml of buffer), shaken gently for 1 h at room temperature, and washed again with milk buffer for 5 min. Thereafter, the membrane was washed with washing buffer (PBS 1X for 4 times) 20 min each then added 800 µl of the substrate working solution and left for 1 minute. The reagents, secondary antibody and substrate, were supplied in Bio-Rad Immum-Star Chemiluminescent Kits. The membrane was put in the sealed plastic wrapper and kept in the dark for 30 min to allow the reaction to be completed. This membrane was exposed to the X-ray film with the appropriate time exposure and then put into the film-developing machine (Kodak). All steps since after the exposure to X-ray film were performed in the darkroom. The interested protein was appeared as black strips on the X-ray film.

5 RNA isolation, semi-quantitative RT-PCR and quantitative RT-PCR

5.1 RNA isolation from the leaves of tobacco and *A. thaliana*

Total RNA was extracted from leaf tissues using RNeasy Plant Mini Kit (Quigen) according to the supplied protocol from the manufacturer. The concentration of total RNA was determined by measuring the absorbance at 260 nm. The ratio of the absorbance readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb the UV, such as protein. Pure RNA has an A_{260}/A_{280} ratio of 1.7-1.9.

5.2 Semi-quantitative RT-PCR

5.2.1 RT

The first-strand cDNA synthesis was made from 2 μg of RNA. The mixture of 1 μl oligo dT and 4 μl (equally to 2 μg) of RNA was heated at 70 ^{0}C for 3 min then placed into the ice box. The cDNA was synthesized by the action of enzyme SuperScript™ III Reverse Transcriptase (Invitrogen) in the reaction mixture containing 2 μl of 5X first-strand buffer, 1 μl dNTP mixture, 1 μl DDT, 0.5 μl enzyme and 0.5 μl DEPC water, then mixed with the mixture of heated RNA and oligo dT. The RT was performed at 42 ^{0}C for 1 h following by 72 ^{0}C for 10 min and held at 4 ^{0}C.

5.2.2 PCR

The synthesized cDNA (from 5.2.1) was used as a matrix for PCR step. A PCR was performed in the reaction mixture; 2.5 μl 10X PCR buffer (Invitrogen), 0.75 μl $MgCl_2$, 0.5 μl dNTP mixture, 0.1 μl *Taq* DNA polymerase (Invitrogen), 1 μl of 100 μM forward primer and 1 of 100 μM reverse primer. The specific genes were amplified using gene specific primers designed from coding sequences of each gene using MacVector software (Table 4.2 and 4.3 for tobacco and *Arabidopsis*, respectively). The PCR was performed with denaturing, annealing and extension temperatures of 94 ^{0}C for 1 min, 55 ^{0}C for 1 min and 72 ^{0}C for 1 min, respectively, and repeated for 25-30 cycles. The PCR products were separated on 1.2% agarose gel with TAE buffer at 100 V and visualized after ethidium bromide staining. Quantification of the PCR products was made in-gel using the Bio-Rad GelDoc apparatus together with the Bio-Rad Quantity One software. Control reactions to normalize RT-PCR amplification were run with the *EF1α*-specific primers and four serial dilutions of each first-strand cDNA. PCRs were performed for 28 cycles and resulted in amplification that was linearily related to the RNA amounts.

Table 4.2. The primers of tobacco genes used in semiquantitaitve RT-PCR

Gene	Primer Sequence (5' → 3')
PR-1	
Forward	GATGCCCATAACACAGCTCG
Reverse	TTTACAGCTCCAGTTCTTCAGAGG
PR-2	
Forward	CTGCCCTTGTACTTGTTGGG
Reverse	TCCAGGTTTCTTTGGAGTTCC
PR-5	
Forward	TGGGGTAAAACCACCAAACACC
Reverse	CCACCAGGGCAAGTAAAAGTGC
OMT	
Forward	TTGATGTTGGAGGTGGTC
Reverse	TCTGGTTTCACTGGTAA
CCoAOMT	
Forward	ATTGGTGTTTTTACTGGTTACT
Reverse	GCCTTGTTGAGTTCCAATACG
EF1α	
Forward	TCACATCAACATTGTGGTCATTGGC
Reverse	TTGATCTGGTCAAGAGCCTCAAG

Table 4.3. The primers of *A. thaliana* genes used in semiquantitaitve RT-PCR

Gene	Primer Sequence (5' → 3')
PR-1	
Forward	CTACGCAGAACAACTAAGAGGCAAC
Reverse	TTGGCACATCCGAGTCTCACTG
PR-2	
Forward	TCCCTTGCTCGTGAATCTCTACC
Reverse	ACAGTCCCCAAAACTTCTCATACG
PR-5	
Forward	CGATTGCGGCAAAGATTTC
Reverse	ACAAGTGAAGGTGCTCGTTTCG
PDF1.2	
Forward	ATGGCTAAGTTTGCTTCC
Reverse	TTTGGGCTGCGATTGAAGTGGTCC
PR-4	
Forward	GTTCTCCGACCAACAACTGTCAG
Reverse	GCTACATCCAAATCCAAGCCTCC
VSP2	
Forward	CTCTTCCTCACCTTTG
Reverse	TCACGAGA
EF1α	
Forward	TTGCTAAGTTTGCTTCC
Reverse	TCACTTCGCACCCTTCTTGACG

5.3 Quantitative-PCR (Q-PCR)

The Q-PCR was performed using the SYBR Green protocol (Eurogentec) with the reaction mixture contains 10 μM primers and 1 μl of RT reaction product (from 5.2.1) in a total of 20 μl per reaction. The specific primers of interested genes, designed from the coding sequences of each gene using MacVector software, are listed in Table 4.4. PCR-cycling conditions comprised an initial polymerase activation step at 95 ^{0}C for 10 min, followed by 45 cycles at 95 ^{0}C for 20 sec, 60 ^{0}C for 30 sec

and 70 ^{0}C for 15 sec. After each run, a dissociation (melt) curve was designed to confirm the specificity of the product and avoid production of primer-dimers. Quantification was detected and made using the Bio-Rad apparatus together with the Bio-Rad iCycer software. The gene for β-tubulin (*β-TUB*) was used as a house keeping gene and a control. Calculation of relative quantification was done by comparative CT method.

Table 4.4. The primers of *A. thaliana* genes used in Q-PCR.

Gene	**Primer Sequence (5' → 3')**
β-tubulin	
Forward	5' GTGGAGCCTTACAACGCTACTT 3'
Reverse	5' GACAGCAAGTCACACCAGACAT 3'
PR1	
Forward	5' AAAACTTAGCCTGGGGTAGCGG 3'
Reverse	5' CCACCATTGTTACACCTCACTTTG 3'
PR4 (HEL)	
Forward	5' AACAATGCGGTCGTCAAGGC 3'
Reverse	5' AAGCACTCACGGCTCTCAAATCCC 3'
VSP2	
Forward	5' GAAGCTGCTGGCGTGACCTA 3'
Reverse	5' CCCAGGGGTATCCTCAACCA 3'
PDF1.2	
Forward	5' AGAAGTTGTGCGAGAAGCCAAG 3'
Reverse	5' GTGTGCTGGGAAGACATAGTTGC 3'

6 Salicylic acid (SA) analysis

6.1 Extraction of SA

The leaf tissue from an infiltrated area about 150-200 mg was frozen in liquid nitrogen or stored at -80 ^{0}C until analysis. The samples were ground in liquid nitrogen, 0.5 ml of 90% (v/v) methanol contained ^{14}C labeled SA added, vortexed and kept in the ice for 20 min then centrifuged at 13,000 rpm for 15 min at 4 ^{0}C. The supernatant was transferred to a new tube (1.5 ml) and the pellet was re-extracted with

0.4 ml of 100% methanol and centrifuged again at 13,000 rpm for 15 min at 4 ^{0}C. Then pooled the supernatants, from the above two steps, together and stored at -20 ^{0}C. One to two h later, the samples were centrifuged at 13,000 rpm for 15 min at 4 ^{0}C to remove the chlorophyll. The supernatant was transferred to a new tube (2.2 ml), vacuum dried with the speed vac machine (at room temperature) and frozen overnight at -20 ^{0}C or -80 ^{0}C. Thereafter, 666 ml of 4 N HCl (500 ml of water + 166 ml of 12 N HCl) was added to each sample and all samples were incubated and mixed with a mixing frequency of 1300 rpm (Eppendorf Thermomixer comfort, Hamburg, Germany) for 30 min at 80 ^{0}C. For extraction, 1 ml of ether was added into the supernatants, and samples were gently vortexed. The organic phase was transferred into a new tube (2.2 ml), then 1 ml of ether was subsequently added to the residue and vortexed again for re-extraction. These two ether phases were combined, evaporated with a nitrogen evaporator until there was no liquid in the tube. Each sample was re-suspended in 300 µl of sodium acctate buffer (20 mM CH_3COONa, pH 5) containing 10% acetonitrile and vortexed prior to HPLC analysis while the yield quantification was achieved by counting the ^{14}C radioactivity.

6.2 Yield quantification

To quantify the extraction efficiency of each sample, the yield was then measured. Fifty µl of each re-suspended sample (from 6.1) was mixed with 3 ml of scintillation liquid (MP) and the radioactive count compare to the standard ^{14}C labeled SA.

6.3 HPLC analysis

For HPLC analysis, salicylic acid was analyzed by using a reverse phase C18 column (Nova pak, 3.9X150 mm). Sample and standard SA (Sigma) were injected 20 or 100 µl and 25 or 50 µl, respectively into the column. The gradient of elution was made by mixing sodium acetate buffer (20 mM, pH 5) containing 3% acetonitrile (solvent A) and distilled water (solvent B). The column was equilibrated with solvent A. SA was separated with the gradient and time schedule as in table 4.5 with the flow rate of 1 ml/minute. SA was detected by its fluorescence absorption profile with the excitation and emission wavelengths at 313 nm and 405 nm, respectively.

Table 4.5. Time schedule and gradient of elution solvent used for HPLC analysis of salicylic acid

Time (min)	Solvent A	Solvent B
0	100%	0%
10	100%	0%
20	20%	80%
30	20%	80%
32	100%	0%
40	100%	0%

7 Biological assay

To test whether the elicitors can induce the resistance against the pathogen, *A. thaliana* plants were sprayed with the elicitors 1 day prior to challenge with the pathogens.

7.1 Induced resistance against bacterial *Pseudomonas syringae*

The virulent bacterial strain, *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) strain was used in this study. The bacterial inoculum was grown at 28 ^{0}C on NYGA solid medium (5 g/1 bactopeptone, 3 g /1 yeast extract, 20 ml/1 glycerol and 15 g/1 agar) supplemented with antibiotics; 100 µg/ml Rifampicin and 25 µg/ml Kanamycin. The bacteria were transferred to NYGA broth, shaken at 150 rpm at 28 ^{0}C for 24 h then centrifuged 13,000 rpm for 3 min to harvest the bacteria. The pellet of bacteria was re-suspended in 10 mM $MgCl_2$ and diluted until the OD600 = 1, equally to 10^7 colony-forming units (CFU)/ml. The elicitor-treated plants were inoculated by pressure-infiltration of *P. syringae* at a concentration of 10^5 CFU/ml. into leaves with a needleless syringe. All treated plants were kept in the growing chamber at 22 $\pm$ 1 ^{0}C with a photoperiod of 16 h. The leaf tissues were collected 1 and 3 days after infiltration for detection of bacterial growth.

Leaf discs (0.5 am in diameter) from two different leaves from each plant were pooled and grounded in 900 µl of 10 mM $MgCl_2$ with a plastic pestle (to make to the dilution of 1:10). After grinding the tissue, they were thoroughly vortex-mixed and diluted 1:10 serially. Samples were finally plated on NYGA agar (composition as

above). Plates were placed at 28 ^{0}C for 1 and 3 days, after which the colony-forming units were counted. The bacterial growth is presented in the unit Log CFU/cm^2.

7.2 Induced resistance against *Peronospora parasitica*

Peronospora parasitica (*Pp*) isolate NOCO was maintained on living *Arabidopsis* and transferred every week onto 2- to 3-week-old *Arabidopsis* Col-0 plants by spray inoculation with a spore suspension. The elicitor-treated *A. thaliana* plants were sprayed with a suspension of 5x10^4 spores/ml of *Pp*. The inoculated plants were kept in a 100% relative humidity (RH) to ensure infection and sporulation and a 12-h-light/12-h-dark cycle with a night temperature of 19 ^{0}C. The leaf samples were observed for necrotic symptoms 10 days after inoculation and photographed.

Results

1 Eliciting activity of different elicitors on tobacco defense responses

1.1 TTF5P

TTF5P is a product from Tribo Technologies and currently used in agriculture as a fertilizer in Germany and France. It is a mixture of amino acids (AA), polysaccharides extracted from algae and phosphite. The exact composition of each component is under patent. Field trials have shown that TTF5P application to various crops leads to high levels of protection against pathogen infections as shown in Table 4.6 (these data are supported by Tribo Technologies, France).

Table 4.6. Potential activity of TTF5P to protect various crops compared to a fungicide

		Protection (%)		
Crop	**Pathogen**	**TTF5P**	**fungicide**	**comments**
strawberry	powdery mildew	98	100	no effects on pathogen
cabbage	powdery mildew	83	>83	no phytotoxicity
lettuce	bremia lactuae (mildew)	89	85	
grapevine(Bordeaux)	mildew	75	80-70	
grapevine (Dijon)	mildew	99		

1.1.1 PR-proteins expression after treating with TTF5P

TTF5P and its component including AA, algae extract (E. Algaes) and NPK (fertilizer) were applied either by infiltration into or by spraying on tobacco plants. Protein gel blotting was performed 3 days later to monitor the expression of PR-proteins in treated tobacco tissues (Figure 4.4). Acidic form of PR1, PR3 and PR5 are known to be the marker of the salicylic acid (SA)-dependent pathway whereas basic PR3 is a marker for the ethylene (ET)-dependent pathway. A strong accumulation of the acidic PR1, PR3 and PR5 proteins occurred in the tissues sprayed with TTF5P. These acidic PR-proteins seemed to increase slightly after AA treatment. No acidic PR-protein expression detected in the tissues sprayed with the separate component, E. Algaes or NPK. Thus, the inducing activity of TTF5P resulted from the combined activity of its components. Since the acidic PR-proteins detected in this experiment are the marker of the SA-dependent pathway, TTF5P was a strong inducer of the SA-dependent defense responses. TTF5P also induced the ET-dependent basic PR3. Therefore, TTF5P applied by spraying was able to induce two distinct defense signaling pathways in tobacco. After application to tobacco plants by infiltration, TTF5P and AA caused a strong expression of acidic PR-proteins, thus showing the eliciting activity in tobacco plants. However, the basic PR3 could be observed in control plants, i.e. non-treated and plants infiltrated with water, this could by explained by that the ET-dependent markers are very sensitive to different stresses including mechanical stresses.

1.1.2 Defense gene expression after spraying with TTF5P

Defense gene expression was analyzed in tobacco plants sprayed with TTF5P and its components. The tissues were collected from treated and untreated leaves 24 h after spraying and then extracted for total RNA. Semi-quantitative RT-PCR by using the specific primers was performed. This allows testing a wider spectrum of defense responses besides PR-proteins. The genes encoding the enzymes of phenylpropanoid pathway such as *O*-methyltransferase (*OMT*), caffeoyl CoA-*O*-methyltransferase (*CCoAOMT*) and sesquiterpenoid (3-hydroxy-3-methylglutaryl-Coenzyme A reductase; *HMGR*) pathways, *acidic PR1* and *basic PR2* (*glu b*) were monitored (Figure 4.5) while equal loading was checked by amplification of the *EF-1α* PCR product. The gene expression showed that TTF5P produced the strongest

expression of the tested genes much more than did the separate NPK and algae extract. However, AA also showed eliciting activity by gene expression and it was similar to that from TTF5P. Hence, TTF5P is therefore efficient in eliciting a wide range of plant defense response.

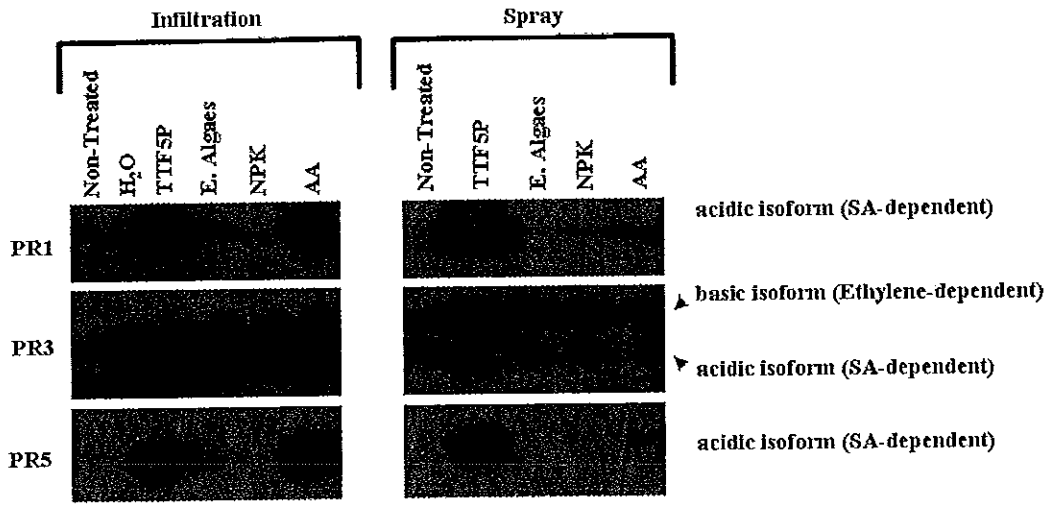

Figure 4.4 Tobacco PR-proteins expression upon treatments with TTF5P, algae extract (E. Algaes), NPK or AA. Tobacco (*N. tabacum* L cv. Samsun H) leaves were either infiltrated or sprayed with the chemicals. Proteins were extracted 3 days after treatments and submitted to protein gel blotting with specific antibodies to analyze PR1, PR3 and PR5 which are salicylic acid (SA)-dependent and basic PR3 which is the ethylene (ET)-dependent defense responses.

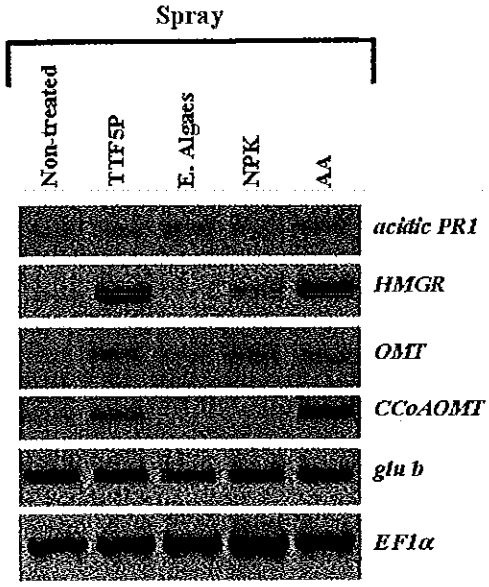

Figure 4.5 Defense gene expression in tobacco (*N. tabacum* L cv. Samsun H) sprayed with TTF5P, algae extract, NPK or AA. Tissues were harvested 24 h after treatments and total RNA was isolated and used for RT-PCR analysis. Expression of genes of *acidic PR1, HMGR, OMT, CCoAOMT* and *basic PR2* (*glu b*) was analyzed. Equal loading was monitored by amplification of the EF-1α PCR product.

1.1.3 PR-proteins expression after treating with individual and combination of TTF5P components

TTF5P, its component (AA, E. Algaes and NPK) or the combinations (NPK+AA, NPK+E. Algaes, and E. Algaes+AA+NPK) were applied by spray on the leaves of tobacco plants. In this experiment, the chemicals were sprayed on two tobacco cultivars, Samsun H and Xanthi nc to test whether different cultivars expressed different or similar responses. Three days later, the leaves were collected for protein extraction and submitted to protein gel blotting with specific antibodies to analyze PR1, PR3, PR5 and PR2 (glu b). Both cultivars reacted similarly (Figure 4.6). Again, TTF5P showed a very strong eliciting activity regarding the SA-dependent markers whereas the individual component did not induce this pathway in both tobacco cultivars. Spraying with the combinations, the results showed that NPK+AA,

and E. Algaes+AA+NPK could express eliciting activity, although it was not as strong as that with TTF5P.

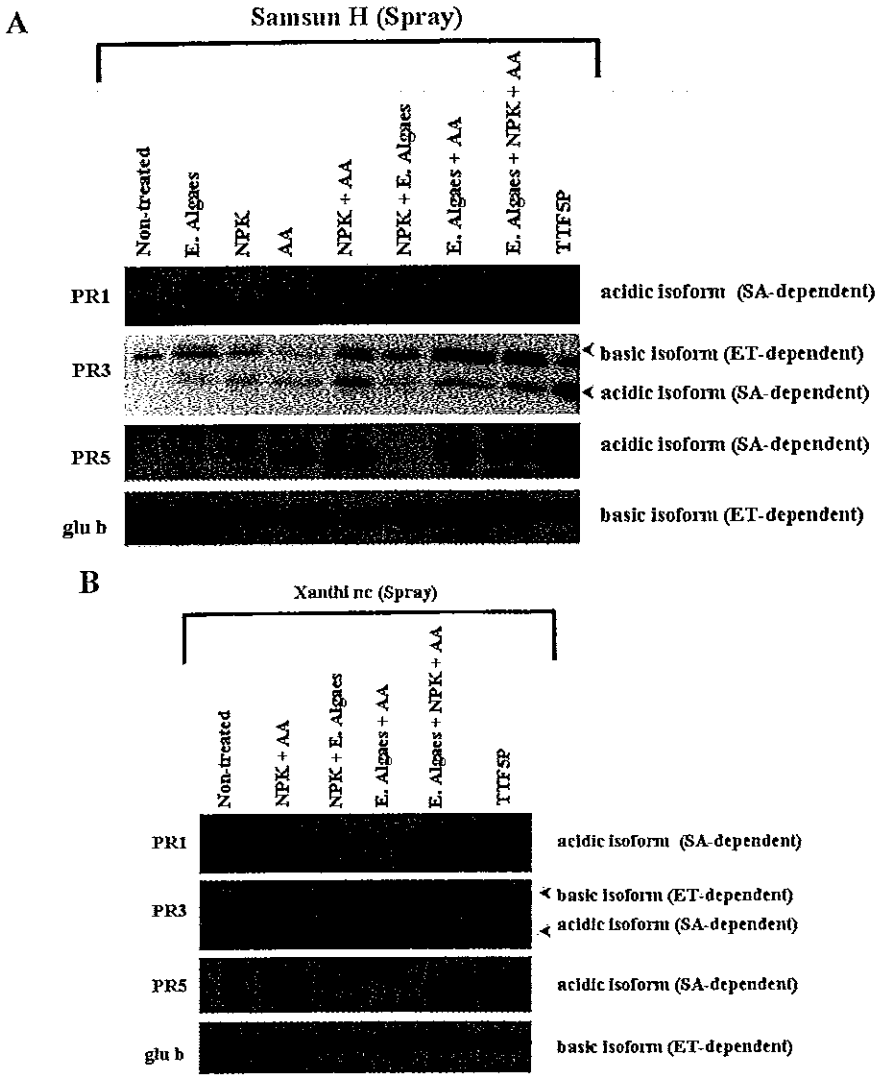

Figure 4.6 Tobacco PR-proteins expression upon treatments with TTF5P, algae extract (E. Algaes), NPK or AA and their combinations. Tobacco leaves were sprayed with each chemical, or with water for the "untreated control" leaves. Proteins were extracted 3 days after treatments and submitted to protein gel blotting with specific antibodies to analyze acidic PR1, PR3, PR5 and PR2 (glu b). Acidic PR1, PR3 and PR5 and basic PR2 (glu b) were detected in *N. tabacum* L cv. Samsun H (A) and *N. tabacum* L cv. Xanthi nc (B) leaf tissues sprayed with each products and their combinations.

1.2 PR-proteins expression after replacing algae extract by purified compounds

Since algae extract is a mixture of algal polysaccharides submitted to enzyme degradation. Laminarin (Lam) is among the polysaccharides present in the mixture. In addition, there have been many published data on the eliciting activity of Lam, a β-glucan from *Laminaria digitata* (Ménard *et al.*, 2005 and Ménard *et al.*, 2004). According to the previous results, Lam (200 µg/ml) was chosen as an appropriate elicitor. When algae extract was replaced with Lam, the eliciting activity was higher than that of TTF5P which contained the mixture of E. Algaes+AA+NPK (Figure 4.7A). Lam itself was inactive and did not cause the accumulation of PR-proteins comparable to that reported by Ménard *et al.* (2004) as similar to the eliciting activity of algae extract (Figure 4.7B).

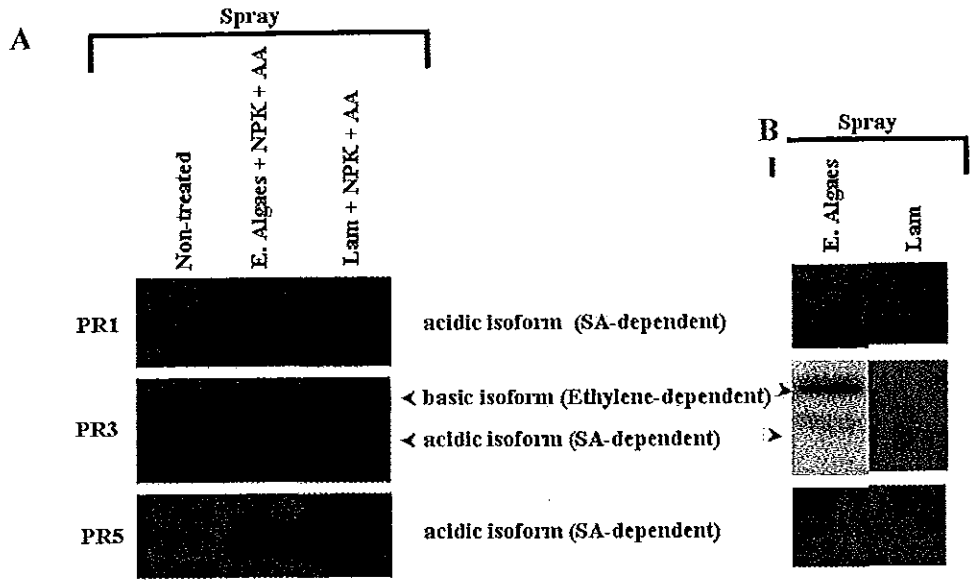

Figure 4.7 Comparison of tobacco PR-proteins expression after replacing the algae extract with Laminarin (Lam). Tobacco (*N. tabacum* L cv. Samsun H) leaves were sprayed with the mixtures of E. Algaes+NPK+AA or Lam+NPK+AA or water for the control leaves (A) and individual algae extract or Lam (B). Proteins were extracted 3 days after treatments and submitted to protein gel blotting with specific antibodies to analyze acidic PR1, PR3 and PR5 proteins.

1.3 Establishing the important component in TTF5P

The aim of this experiment is to inquire about which component in TTF5P is most active. Appearance of PR1 was detected because it showed a clearly different expression after the chemical treatments. After the mixture of two components were sprayed on tobacco leaves, only Lam+NPK was inactive (Figure 4.8) similarly to the activity of E. algaes+NPK (without AA) shown in Figure 4.6. AA seemed to be the most important elicitor. Thus, the dilution of AA and the mixtures contained diluted AA were then monitored for their eliciting activities and compared with that of TTF5P including a 2 fold dilution (Figure 4.8). The result showed that TTF5P induced acidic PR1 expression in a dose-dependent manner. AA by itself also induced expression in a dose-dependent manner. When TTF5P diluted, meaning that all components were diluted, the PR1 reduction occurred in a similar way to the mixture when the AA was diluted. The expressed PR1 was correlated to the amount of AA in each treatment; the PR1 was decreased when AA was diluted. These data confirmed that AA should be the most important substance in TTF5P.

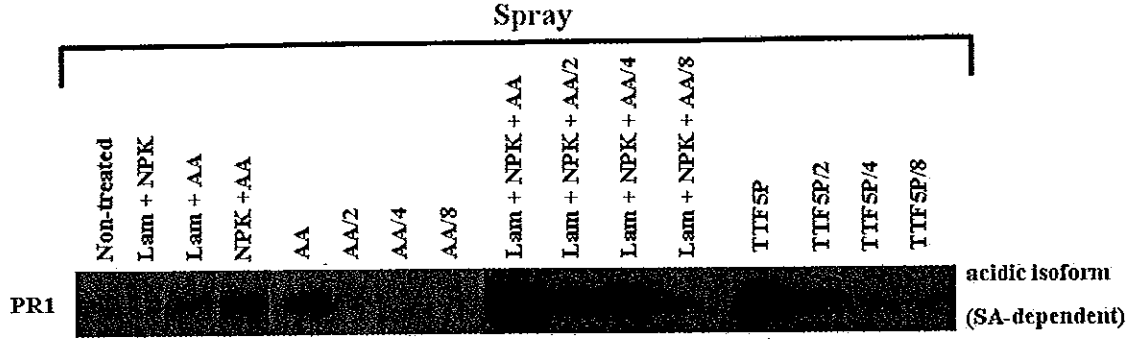

Figure 4.8 Tobacco acidic PR1 expression upon treatments with TTF5P, Laminarin (Lam), NPK or AA or their combinations and their dilutions. Tobacco (*N. tabacum* L cv. Samsun H) leaves were sprayed with each chemical, with water for the control. Proteins were extracted 3 days after treatments and submitted to protein gel blotting with specific antibodies to analyze acidic PR1.

1.4 PR-proteins expression after replacing AA with purified compounds

The nonprotein amino acid β-aminobutyric acid (BABA) was nominated as the most likely inducer to replace the AA since it is know to be a potent inducer of resistance against infection by various pathogens (Jakab *et al.*, 2001). In certain cases it exerted its function via priming of the SA-dependent defense mechanisms in Arabidopsis (Zimmerli *et al.*, 2000). In other cases, however, BABA acts as a resistant inducer activate SA-signaling pathway (Narusaka *et al.*, 2006) or do through potentiation of ABA-dependent signaling pathways (Ton and Mauch-Mani, 2004).

1.4.1 β-aminobutyric acid (BABA)

Various concentrations of BABA; 0.25, 0.5, 1 and 2 mM, were applied either by infiltration into or by spraying on tobacco plant leaves. Protein gel blotting was performed 3 days later to monitor the expression of PR-proteins in treated tobacco tissues (Figure 4.9). Interestingly, BABA applied by infiltration into tobacco leaves did not induce PR-proteins expression whereas application by spraying did. The results showed that the markers of SA-dependent signaling pathway, acidic form of PR1, PR2 and PR3 were responded to BABA in a dose-dependent manner by increasing of BABA concentrations. In addition, the obtained data was compatible with many previous reports which have shown that BABA had two distinct functions as mentioned above. In this experiment, BABA could act as the priming substance when using low amount (0.25 mM) whereas at high dose it could behave as eliciting molecule to induce the defense responses. However, 0.5 mM BABA induced a similar magnitude of PR-proteins expression as did AA (as shown in Figure 4.6).

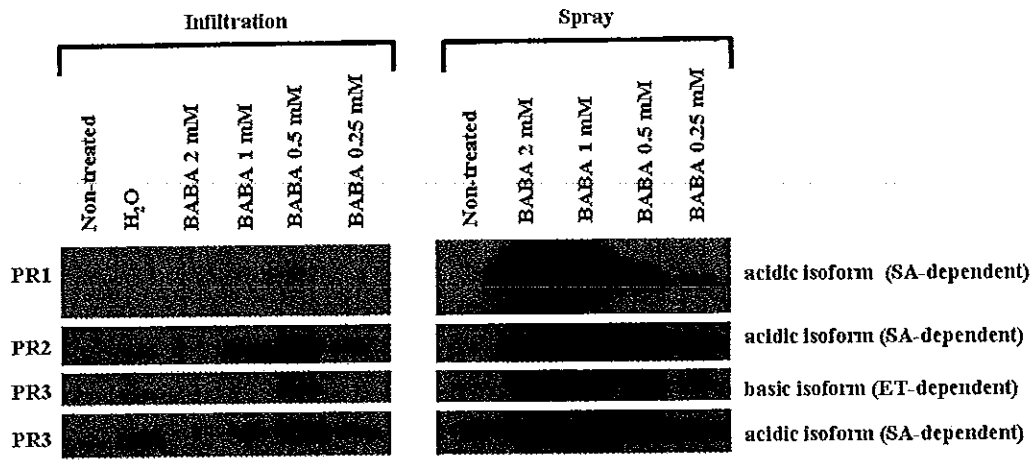

Figure 4.9 Tobacco (*N. tabacum* L cv. Samsun H) PR-proteins expression upon treatments with β-aminobutyric acid or BABA. Tobacco leaves were either infiltrated or sprayed with various doses of BABA, or water of leaf untreated. Proteins were extracted 3 days after treatments and submitted to protein gel blotting with specific antibodies to analyze acidic PR1, PR2 and PR3 proteins.

1.4.2 Replacing AA with β-aminobutyric acid (BABA)

Since 0.5 mM BABA induced a similar magnitude of PR-proteins expression as that of AA (as shown in Figure 4.6 and 4.9). Thus, 0.5 mM of BABA itself or combinations were applied by spraying onto tobacco plants. The mixture of AA+Lam+NPK was also applied to be the positive control and to compare the activity of another mixture which used BABA instead of AA. Protein was extracted 3 days later and submitted to gel blotting and monitor the acidic PR1 expression in treated tobacco tissues (Figure 4.10). An interesting point is that PR1 was reduced in the treatment of BABA+Lam, which was repeated more than 2 times, thus showing the antagonistic between these two substances. This point needed more detailed study to confirm the suppression effect. The mixture of BABA+Lam+NPK restored acidic PR1 expression revealed that NPK might inhibit the suppression effect of BABA+Lam. Moreover, BABA+Lam+NPK caused stronger PR1 expression than that induced by AA+Lam+NPK, suggesting that BABA could be more active than AA.

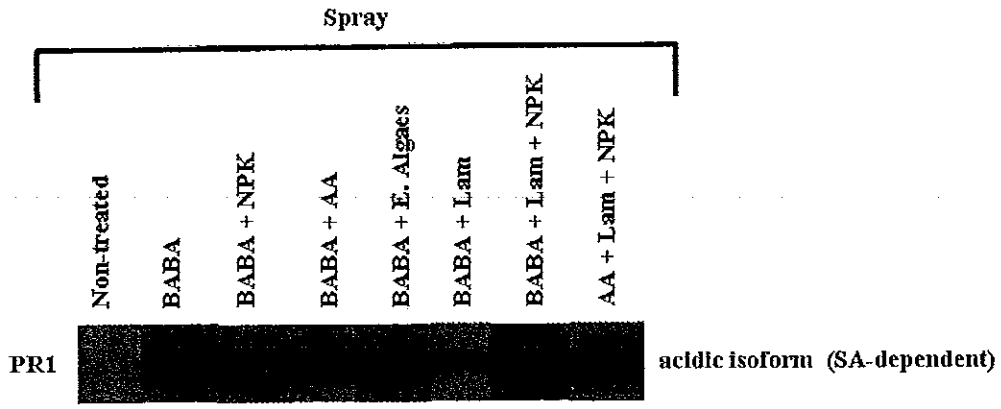

Figure 4.10 Tobacco (*N. tabacum* L cv. Samsun H) acidic PR1 expression upon spraying by BABA and the combinations of BABA and other chemicals. Tobacco leaves were sprayed with each chemical, with water of leaf untreated. Proteins were extracted 3 days after treatments and submitted to protein gel blotting with specific antibodies to analyze acidic PR1.

1.5 Suppression effect of Lam on BABA-induced acidic PR1 expression

The suppression effect of Lam was confirmed by treating the tobacco plants with the mixture of BABA+Lam. The mixtures, containing various BABA at final concentrations of 0.25, 0.5 and 1 mM in the mixtures and Lam at 200 µg/ml, were sprayed on tobacco leaves. The untreated and treated tissues were collected 3 days after treatments, extracted for proteins and submitted to gel blotting with specific antibody for the acidic PR1. The suppression effect of Lam on PR1 expression was very high in the mixture contained lowest dose of BABA whereas the repression efficacy was reduced after increasing BABA concentrations (Figure 4.11). At the highest dose of BABA used (1 mM), it seemed that there was no effect of Lam on suppression of BABA-induced PR1.

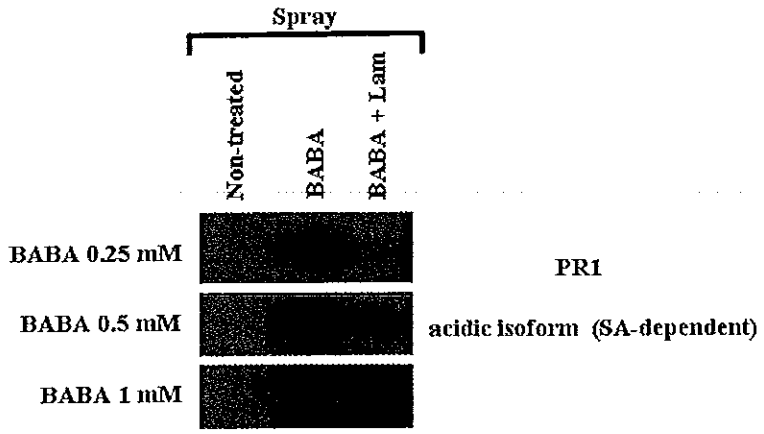

Figure 4.11 Suppression effect of Lam on BABA-induced acidic PR1 expression. Tobacco (*N. tabacum* L cv. Samsun H) leaves were sprayed with the mixtures of BABA+Lam or by water of leaf untreated. Proteins were extracted 3 days after treatments and submitted to protein gel blotting with specific antibodies to analyze acidic PR1 protein.

The suppression effect of Lam on SA-dependent signaling pathway was similar to the observation reported by Benoît Boachon, a master student who undertaking his training stage at IBPM. He found that Lam showed a suppression activity on the SA-dependent signaling pathway induced by a sulfated laminarin called PS3. I have continued studying in more details based on his finding by using Lam and PS3 and their combination (Lam+PS3). Moreover, there have been many literature reports suggesting to date about cross-talk of the signaling pathway in plant defense responses, SA was suppressed trough JA, conversely, JA was suppressed by SA.

2 Mode of action of β-1,3 glucans in the elicitation of plant defense responses against pathogens and the suppression effect of Lam on the SA-dependent signaling pathway induced by sulfated laminarin (PS3)

2.1 The defense responses induced by Lam, PS3 and Lam+PS3 in tobacco plants

2.1.1 PR-proteins expression

This experiment was performed by infiltration of tobacco leaves. This method was preferred to spraying method because all chemicals can be tested on the

same leaf and additionally the directly treated tissues were assayed. Tobacco leaves were infiltrated with Lam (200 μg/ml) or PS3 (200 μg/ml) or a mixture of Lam and PS3 (Lam+PS3, 200 μg/ml each). Leaf tissues from the control and treated were collected 3 days after treatments for protein extraction and western blot analysis. By Protein gel blotting (Figure 4.12), as expected, there was no acidic PR1 observed in Lam treated tissue, but strongly induced in PS3-treated tissue, surprisingly it was reduced in the tissue infiltrated with the mixture. However, the expression of the basic PR5 was quite similar in all treatments. Although Ménard, *et al.* (2005) had previously shown that there was no synergy between Lam and sulfated laminarin (PS3) for the SA-dependent acidic PR1 and the ET-dependent basic PR5, the results from this experiment displayed an antagonistic effect of these two elicitors.

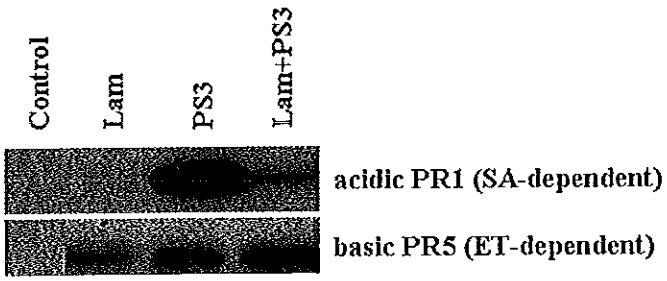

Figure 4.12 Expression of PR proteins in tobacco leaves upon treatment with Lam or PS3 or Lam+PS3. Tobacco leaves were infiltrated with Lam (200 μg/ml), PS3 (200 μg/ml) and a mixture of Lam and PS3 (Lam+PS3, 200 μg/ml each). Proteins were extracted 3 days after treatments and submitted to protein gel blotting with the specific acidic PR1 and basic PR5 proteins.

2.1.2 Defense genes expression

To test the eliciting activity of Lam, PS3 and Lam+PS3 for inducing defense response at the molecular level, tobacco leaf tissues were harvested 24 h after treatments for total RNA extraction. The expression of genes encoding both proteins in 2.1.1 was analyzed by semi-quantitative RT-PCR, while equal loading was determined by amplification of the *EF-1α* PCR product, to confirm the results of

protein analysis (Figure 4.13). The results were similar to the protein gel blotting analysis, the PCR product of *acidic PR1* was detected in PS3-treated tissue and it was suppressed after treatment with the mixture (Lam+PS3) whereas the basic *PR5* was detected in all treatments except in control. Indeed, we also monitored the gene encode enzyme of phenylpropanoid pathway, caffeoyl CoA-*O*-methyltransferase (*CCoAOMT*), it was occurred in the tissue treated with PS3 and Lam+PS3 with similar level.

Many reports have suggested that in tobacco, salicylic acid (SA) induces the expression of the acidic isoforms of PR1, PR2, PR3 and PR5 proteins (Ward *et al.*, 1991; Yalpani *et al.*, 1991; Cordelier *et al.*, 2003), and ethylene induces the expression of the basic counterparts (Brederode *et al.*, 1991; Knoester *et al.*, 1998; Ohtsubo *et al.*, 1999; Coledelier *et al.*, 2003). Thus, the results from 2.1.1 and 2.1.2 indicated that Lam suppresses the SA-dependent signaling pathway but not affects such different defenses, the ET-dependent signaling pathway, for instance. Indeed, it revealed that there was no interaction between Lam and PS3 which could reduce the elicitor activity of PS3 because only the acidic PR1 was reduced when treated with Lam+PS3 compared with PS3 alone whereas other defense markers, basic PR5 and *CCoAOMT* were not (Figure 4.13).

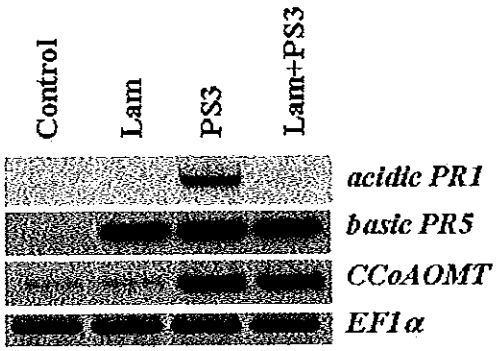

Figure 4.13 Expression of transcription markers in tobacco leaves. Tissues from tobacco plants were harvested 24 h after treatments with Lam (200 µg/ml) or PS3 (200 µg/ml) or Lam+PS3 (200 µg/ml each) and total RNA was isolated and used for semi-quantitative RT-PCR analysis. *Acidic PR1*, *basic PR5* and caffeoyl CoA-*O*-methyltransferase (*CCoAOMT*)

was monitored. Equal loading was confirmed by amplification of the *EF1α* PCR product.

2.1.3 Analysis for salicylic acid (SA) content

Furthermore, in order to determine whether the Lam could inhibit the synthesis of signal molecule, the SA amounts were also measured in the leaf tissues treated with Lam or PS3 or Lam+PS3 for 24 h. SA accumulated strongly in the PS3-treated tissue and the level was decreased significantly in the one infiltrated by Lam+PS3 whereas no considerable SA increased compared to the control in Lam-treated tissue (Figure 4.14). These results corresponded to that observed in protein and gene expression of PR1. Therefore, these support the suppression effect of Lam on the SA signaling pathway induced by PS3 in tobacco plant, but not other signaling pathways.

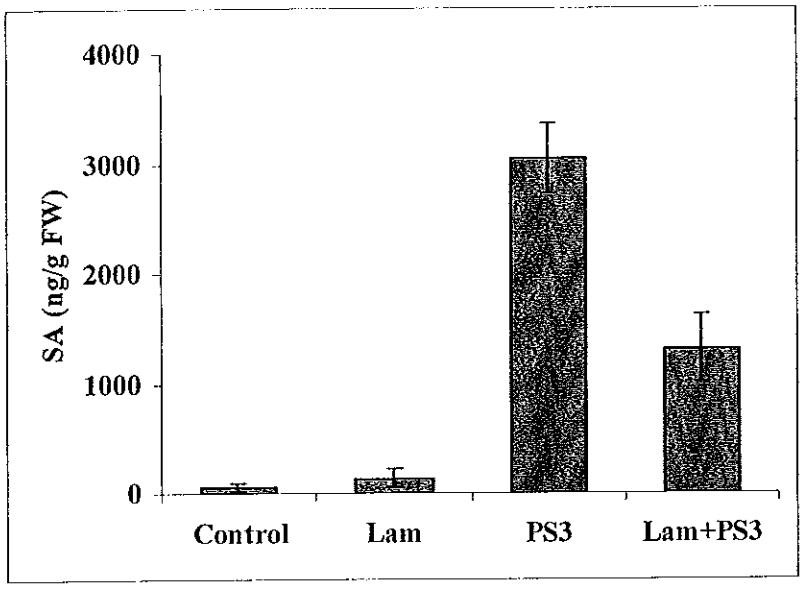

Figure 4.14 SA quantification in tobacco leaves. Tissues were harvested 24 h after infiltration with Lam (200 μg/ml) or PS3 (200 μg/ml) or Lam+PS3 (200 μg/ml). The SA content was quantified by HPLC. Experiments were individually done 3 times in a similar manner. Results are representative of a typical experiment and are expressed as the mean ± SD calculated from two HPLC runs.

2.2 The defense responses induced by Lam, PS3 and Lam+PS3 in *Arabidopsis thaliana*

The experiment in *Arabidopsis* was previously performed by Benoît Boachon using the same concentrations as used in tobacco plants; Lam (200 µg/ml), PS3 (200 µg/ml) and a mixture of Lam and PS3 (Lam+PS3, 200 µg/ml each). He showed that in wild-type Columbia-0 (Col-0) plants, the suppression effect on the *PR1* gene expression was not observed by this concentration. When the concentration of Lam was increased to 500 µg/ml whereas PS3 was decreased to 100 µg/ml and used the mixture of Lam and PS3 with these concentrations. By this condition, Boachon found the suppression effect of Lam, thus this condition was used for further studies.

2.2.1 Defense gene expression measured by quantitative RT-PCR

2.2.1.1 Defense responses in wild-type Columbia-0 (Col-0) plants

The suppression activity of Lam was tested to determine if its effect only on the SA-dependent defenses or also affected other signaling pathways in *Arabidopsis* plant. Lam (500 µg/ml), PS3 (100 µg/ml) and a mixture of Lam+PS3 (500 µg/ml, 100 µg/ml) were infiltrated in to Arabidopsis leaves. Total RNA was isolated from leaf tissues at 24 h after treatments, converted to cDNA, and subjected to quantitative RT-PCR analysis. The expression level of marker genes of different pathways, including *PR1*, *PR4/HEL*, *PDF1.2* and *VSP2* which are well recognized as markers for the SA, ethylene (ET), ethylene/jasmonic acid (ET/JA) and JA signaling pathway, respectively, were monitored. *PR1* expressed highly in the treatment of PS3, thus, PS3 could be a strong inducer of SA-signaling pathway and it was significantly decreased in Lam+PS3 treatment but no *PR1* induced after infiltration by Lam (Figure 4.15). However, in tissue infiltrated with Lam exhibited *PR4*, *PDF1.2* and *VSP2* expression corresponded to the previous result (Ménard *et al.*, 2004) that is Lam induced expression of ET-dependent pathway. PS3 also induced strongly *PR4* and *PDF1.2* expressions whereas there were not significantly reduced in the level of these two genes after treated with Lam+PS3. *VSP2* expression level was strongly induced in tissue infiltrated by Lam, revealed that Lam in an effective inducer of JA signaling pathway, and its level was reduced in Lam+PS3 treatment. There was no effect of Lam on other markers of ET and JA pathway, indicating that Lam has the negative effect on only SA pathway. These results indicated that the inhibitory effect of Lam

on SA signaling pathway in *Arabidopsis* plants which is similar to that what we found in tobacco plants (in 2.1.1 and 2.1.2). To date there have been many literatures have reported the mutually antagonistic effect of these two signal molecule, SA and JA (Vidal *et al.*, 1997; Niki *et al.*, 1998; Petersen *et al.*, 2000; Kachroo *et al.*, 2001; Kloek *et al.*, 2001; Li *et al.*, 2004), the point that JA suppress SA will be focused on. Hence, it is possible that in Lam+PS3 treatment, SA was blocked through Lam-induced JA resulting in PR1 reduction compared to that expressed in PS3-treated tissue. However, *VSP2* was decreased in the treatment of Lam+PS3, and this may be due to the inhibitory effect of the PS3-induced SA on JA.

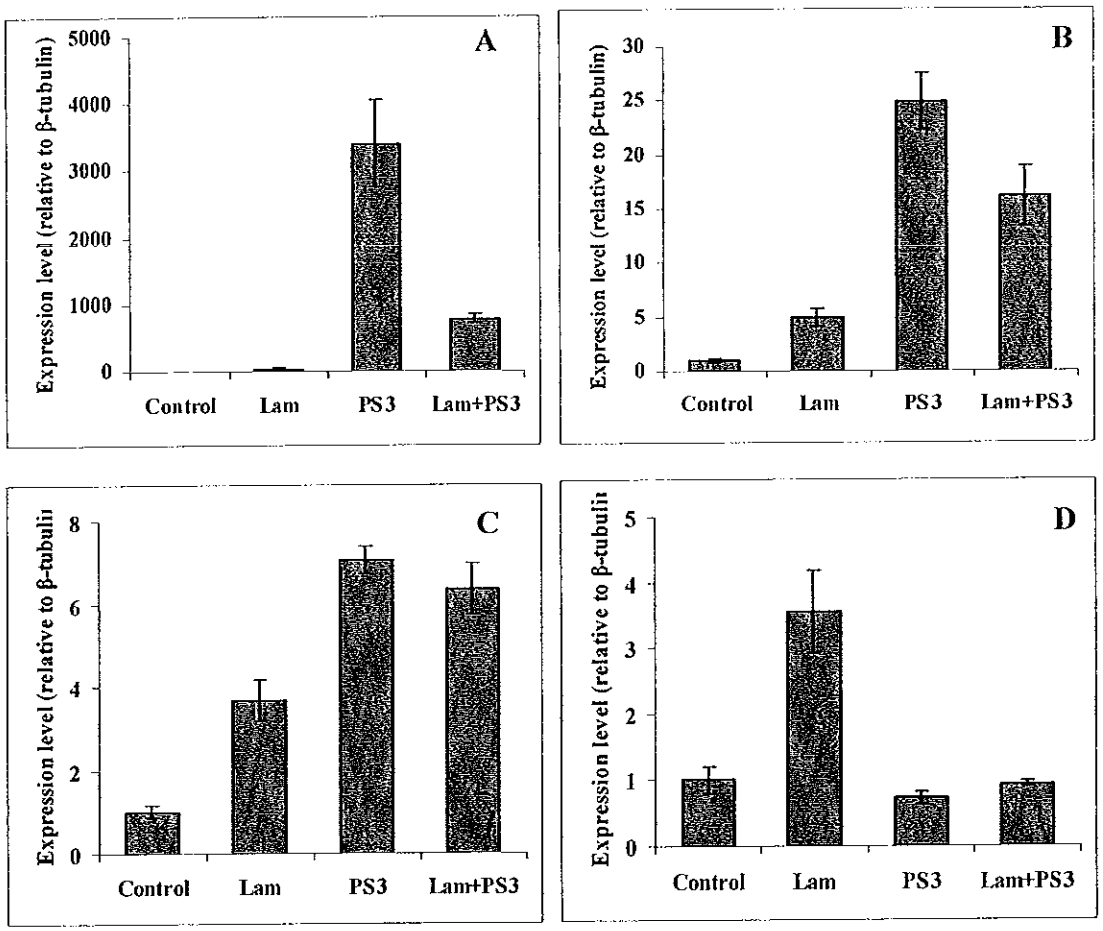

Figure 4.15 Quantification RT-PCR in *Arabidopsis* wild-type Col0 plants. Leaves of six-week-old Arabidopsis were infiltrated with Lam (500 µg/ml), PS3 (100 µg/ml) and a mixture of Lam and PS3 (Lam+PS3, 500 µg/ml and 100 µg/ml, respectively). Twenty four h after treatment, samples were collected and extracted for RNA and subjected to quantification RT-PCR

analysis. The *PR1* (A), *PR4* (B), *PDF1.2* (C) and *VSP2* (D) transcript levels in untreated and treated tissues were normalized to the expression of β-tubulin measured in the same sample and expressed logarithmically. Calculation of the relative quantification was carried out by the comparative CT method. Error bars indicate standard deviations of triplicate analysis.

2.2.1.2 Defense responses in *coi1-16* mutant plants which impaired in the perception of JA

We have hypothesized that Lam suppress SA pathway via JA. Therefore, a mutant impaired in the perception of JA, *coi1-16* was further studied to investigate our hypothesis. In the *coi1-16* mutant, the important result was that the expression level of *PR1* in Lam+PS3 treatment was much higher than those of tissues treated with Lam or PS3 meaning that *PR1* expression was restored in Lam+PS3 treatment (Figure 4.16). This revealed that if the JA signaling is defective, the SA signaling pathway could occur. There was no suppression effect of Lam when the JA signaling was blocked indicating that Lam inhibits the SA pathway through JA. As expected, the level of *VSP2* expression was very low in all treatments while *PR4* and *PDF1.2* genes in the mixture Lam+PS3 treatment were induced much greater than those after Lam or PS3 treatment.

2.2.1.3 Defense responses in *sid2/nahG* plant, *SA induction deficient2* which is impaired in the production of SA

According to the gene expression in wild-type Col-0 (2.2.1.1) when reduction of the *VSP2* level occurred after the Lam+PS3 treatment, we hypothesized that it may be resulted from the suppression effect of SA on JA. To test this hypothesis, the defense gene expression levels were examined in *sid2/nahG* plant, *SA induction deficient2* which is impaired in the production of SA and whereas *nahG* is transgenic plant line able to expresses the bacterial SA-degrading enzyme salicylate hydroxylase. This *sid2/nahG* plants could not accumulate elevated levels of SA, *PR1* gene was abolished in this line (Figure 4.17). However, *PR4* and *PDF1.2* were occurred at high level revealed the inhibitory effect of SA on JA signaling similarly with recent reports, *eds4* and *pad4* mutants that are also impaired in SA accumulation,

exhibit enhanced JA-dependent gene expression (Gupta *et al.*, 2000; Clarke *et al.*, 2000).

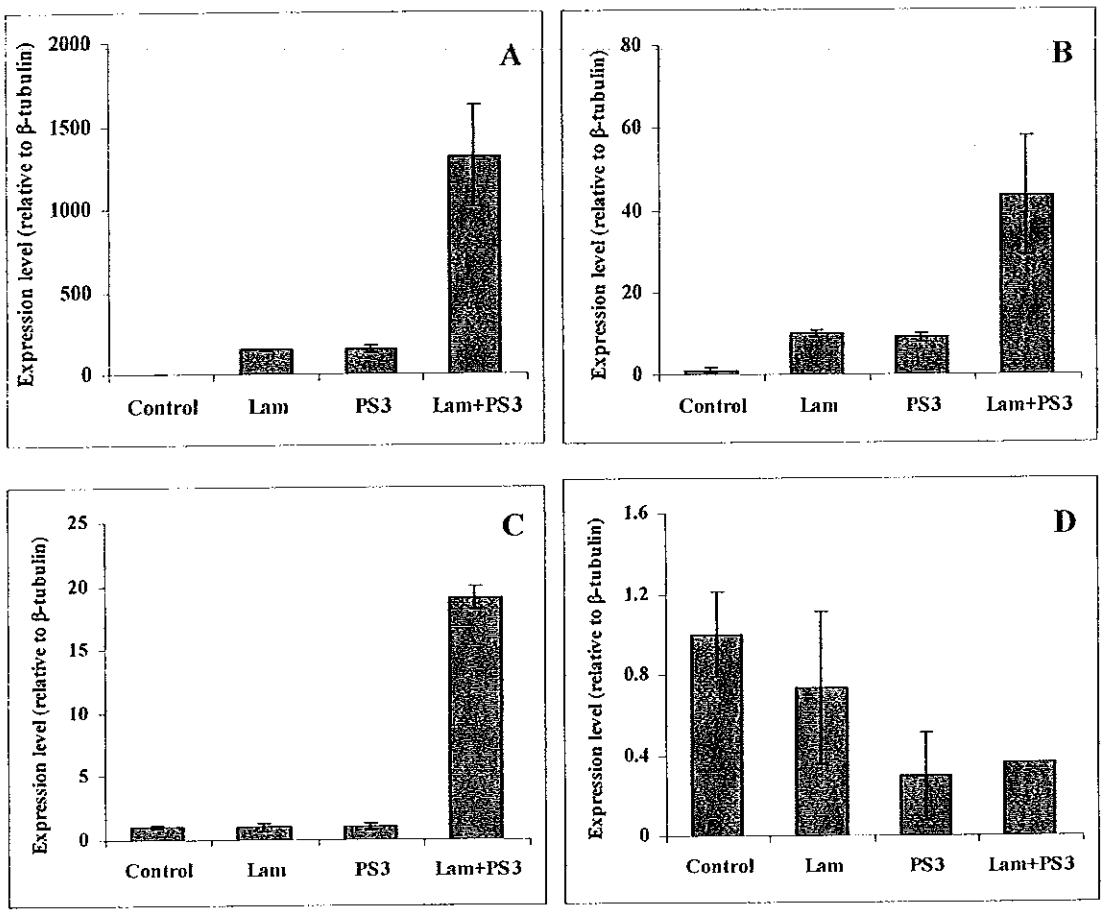

Figure 4.16 Quantification RT-PCR in *Arabidopsis* mutant defective in the JA signaling pathway, *coi1-16* plants. Leaves of six-week-old of *coi1-16* were infiltrated with Lam (500 µg/ml), PS3 (100 µg/ml) and a mixture Lam+PS3 (500 µg/ml and 100 µg/ml, respectively). Twenty four h after treatment, samples were collected and extracted for RNA and subjected to quantification RT-PCR analysis. The *PR1* (A), *PR4* (B), *PDF1.2* (C) and *VSP2* (D) transcript levels in untreated and treated tissues were normalized to the expression of β-tubulin measured in the same sample and expressed logarithmically. Calculation of the relative quantification was done by comparative CT method. Error bars indicate standard deviations of triplicate analysis.

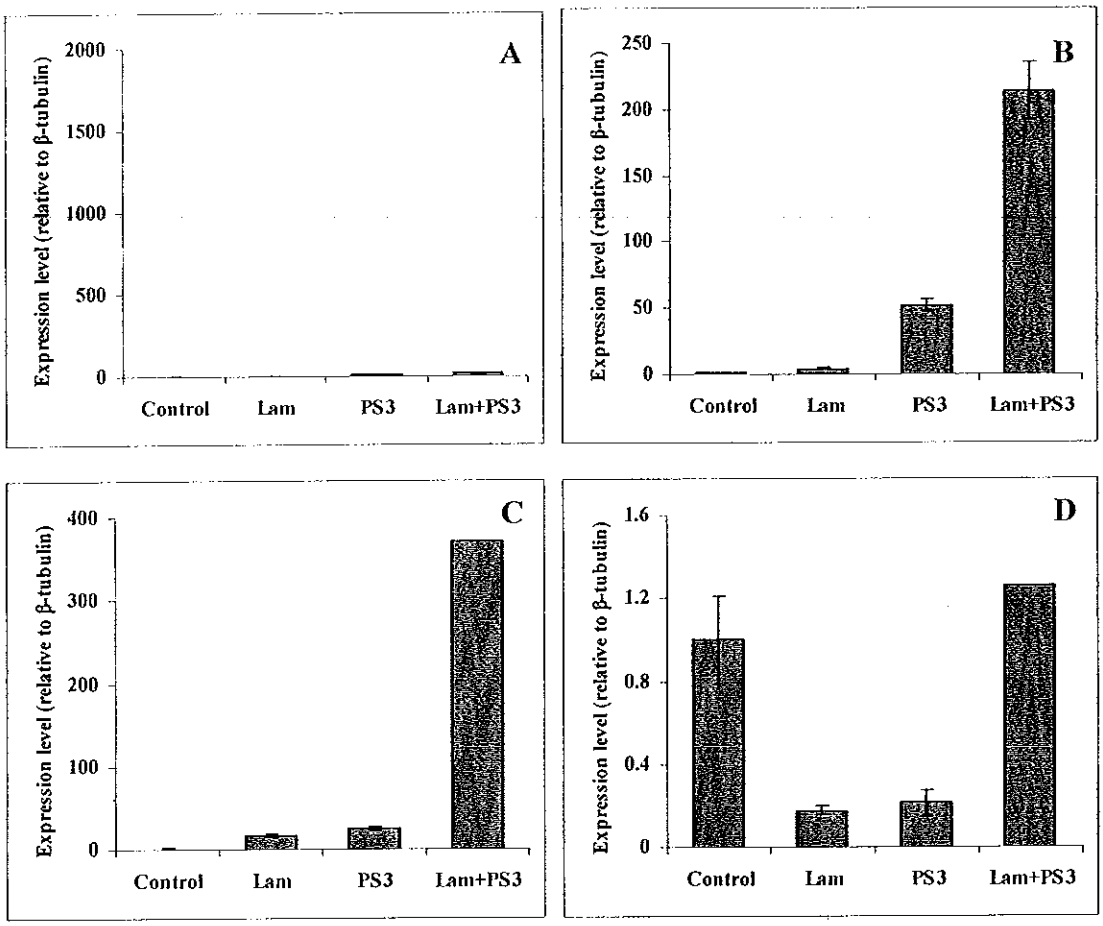

Figure 4.17 Quantification RT-PCR in Arabidopsis mutant defective in *sid2/nahG* plant, *SA induction deficient2* which is impaired in the production of SA and transgenic plant line. Leaves of six-week-old of *sid2/nahG* were infiltrated with Lam (500 µg/ml), PS3 (100 µg/ml) and a mixture Lam+PS3 (500 µg/ml and 100 µg/ml, respectively). Twenty four h after treatment, samples were collected and extracted for RNA and subjected to quantification RT-PCR analysis. The *PR1* (A), *PR4* (B), *PDF1.2* (C) and *VSP2* (D) transcript levels in untreated and treated tissues were normalized to the expression of β-tubulin measured in the same sample and expressed logarithmically. Calculation of the relative quantification was done by comparative CT method. Error bars indicate standard deviations of triplicate analysis.

2.2.2 Salicylic acid (SA) analysis in Col0, *coi1-16* and *sid2/nahG Arabidopsis* line

As PR1 belongs to the systemic acquired resistance (SAR) class of defense-related genes that are induced in responses to elevated SA level in plants, we examined the role of SA and the SA-dependent signaling pathways in responses to Lam, PS3 and Lam+PS3 and analyzed the association between the Lam–suppressed PR1 expression and the SA level. The SA amount was measured to answer this question. In wild-type Col0, Lam showed no ability to trigger the SA production because the amount of SA in the Lam-treated tissue was slightly but not significantly induced compared to that in the control whereas the SA level in the tissue treated with PS3 was very high but was significantly reduced after treatment with Lam+PS3 (Figure 4.18). This result highlighted the negative effect of Lam on the SA biosynthesis. Additionally, in *coi1-16* mutant, SA was similarly exhibited in all treatments (Figure 4.18), supporting the point that JA can suppress SA because SA was restoring when JA was defected. As expected, we could not detect any SA in *sid2/nahG* line. Thus, from this SA level data and gene expression from 2.2.1.1 it could be implied that the *PR1* expression was correlated to SA level in *Arabidopsis* lines we tested; Col0, *coi1-16* and *sid2/nahG*. Furthermore, it indicated that the effect of Lam on SA biosynthesis consequently reduced the SA-dependent gene expression.

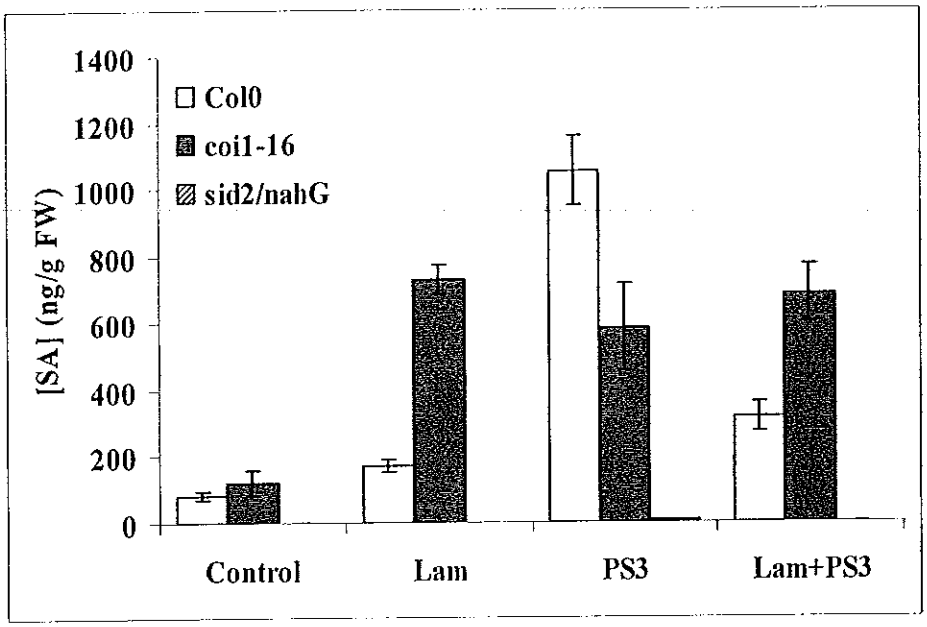

Figure 4.18 SA analysis in different lines of *Arabidopsis* plants. The leaves of six-week-old Arabidopsis; Col-0, *coi1-16* and *sid2/nahG*, were infiltrated with Lam (500 μg/ml), PS3 (100 μg/ml) or Lam+PS3 (500 μg/ml and 100 μg/ml, respectively). Twenty four h after treatment, samples were collected and extracted for RNA. The SA values represent the mean of duplicate samples ± SD. Similar results were obtained in two independent experiments.

2.2.3 Biological test: pathogen resistance by *A. thaliana*

These experiments were performed to test the eliciting activity of Lam, PS3 and Lam+PS3 in the induction of resistance against pathogens.

2.2.3.1 Induced resistance against bacterial *Pseudomonas syringae*

A. thaliana, wild-type Col-0 and the *coi1-16* mutant, were used in this experiment. Leaves were first treated by infiltration with the chemicals; Lam (500 μg/ml), PS3 (100 μg/ml) or Lam+PS3 (500 μg/ml and 100 μg/ml, respectively). Twenty-four h later, the elicitor-treated plants were challenged by pressure-infiltration with *P. syringae* pv. tomato DC3000 (*Pst* DC3000) into leaves with a needleless syringe at the concentration of 10^5 CFU/ml.. The bacterial proliferation was detected 0, 1 and 3 days after inoculation with bacteria. In the wild-type Col-0 (Figure 4.19A),

the numbers of bacteria were similar to the control and Lam-treated tissue during tested period whereas fewer bacteria were detected after PS3 treatment caused. The bacterial number was slightly higher in tissue infiltrated by Lam+PS3. Thus, PS3 which induced a strong SA-signaling pathway, showed an efficiency resistance against *Pst* DC3000 while in the mixture treatment, SA signaling was suppressed then the resistance was reduced and resulting in enhance bacterial growth. In contrary, although the growths of *Pst* DC3000 in all treatments were not significantly different, they were lower than that in control (Figure 4.19B). These results indicated that when JA was defect, the SA signal was not suppressed and restored the resistance level which was similar to that of the tissues treated with Lam or PS3 or Lam+PS3.

2.2.3.2 Induced resistance against *Peronospora parasitica*

Oomycete *Peronospora parasitica* (*Pp*) is known as obligate biotrophic pathogen. The resistance against this pathogen is mainly dependent on the SA signaling pathway. Thus the resistance response to *Pp* reveals the efficacy of the elicitor in triggering the SA dependent pathway. Leaves of *Arabidopsis* wild-type Col-0 were infiltrated with Lam (500 µg/ml), PS3 (100 µg/ml) or Lam+PS3 (500 µg/ml and 100 µg/ml, respectively). The elicitor-treated Col-0 plants were sprayed with $5x10^4$ spores/ml of *Pp* isolate NOCO, 24 h after first treatment. The results (Figure 4.20) of the inoculated leaves showed the presence of water-soaked lesions and a macerate phenotype in the control similar to the symptoms in most leaves treated with Lam. Conversely to those treated with PS3 which exhibited fewer lesions. However, the disease symptom by the Lam+PS3 treatment was moderated and less than those treated by PS3. Thus, the resistant level was reduced in Lam+PS3 treatment. Again, these results indicated the suppression effect of Lam on resistance against the biotroph *Pp*.

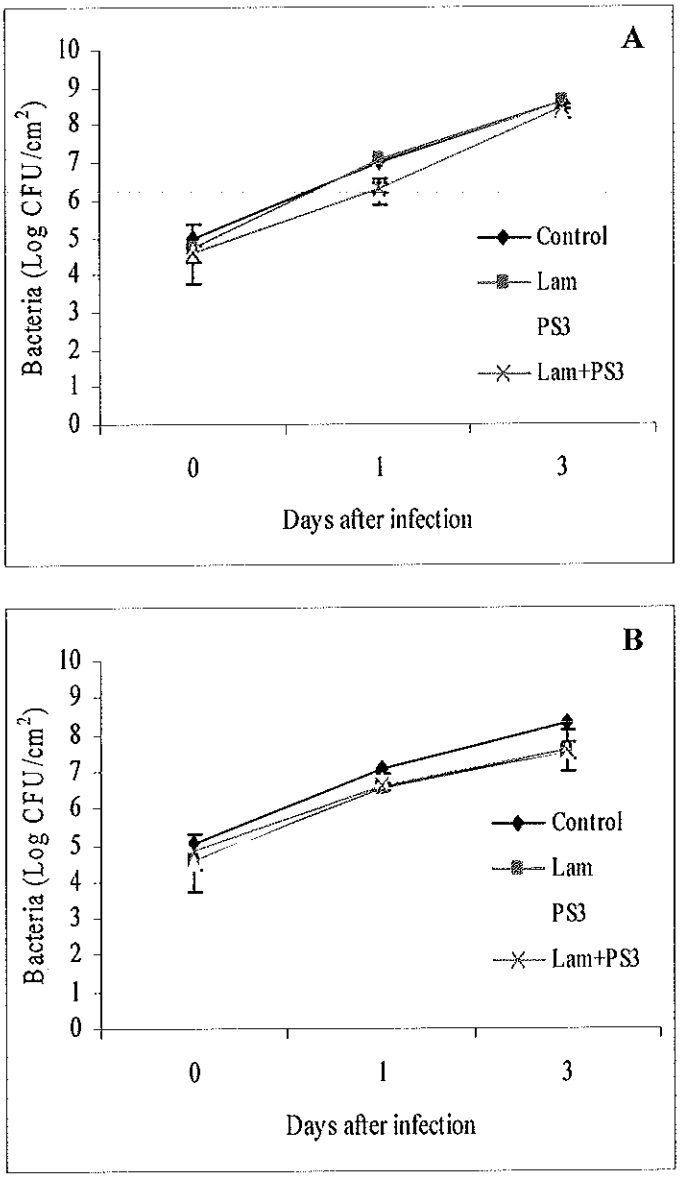

Figure 4.19 Bacterial growth of *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000). Five to Six-week-old *Arabidopsis*; wild-type Col0 (A) and *coi1-16* (B) were infiltrated with Lam (500 µg/ml), PS3 (100 µg/ml) or a mixture of Lam and PS3 (Lam+PS3, 500 µg/ml and 100 µg/ml, respectively). Twenty-four h later, all the plants were challenged by infiltration with 10^5 colony forming units (CFU)/ml *Pst* DC3000. The bacterial growth was assessed 0, 1 and 3 days after infection. Leaf discs of four infected plants were pooled for this experiment. Results are mean ± SD.

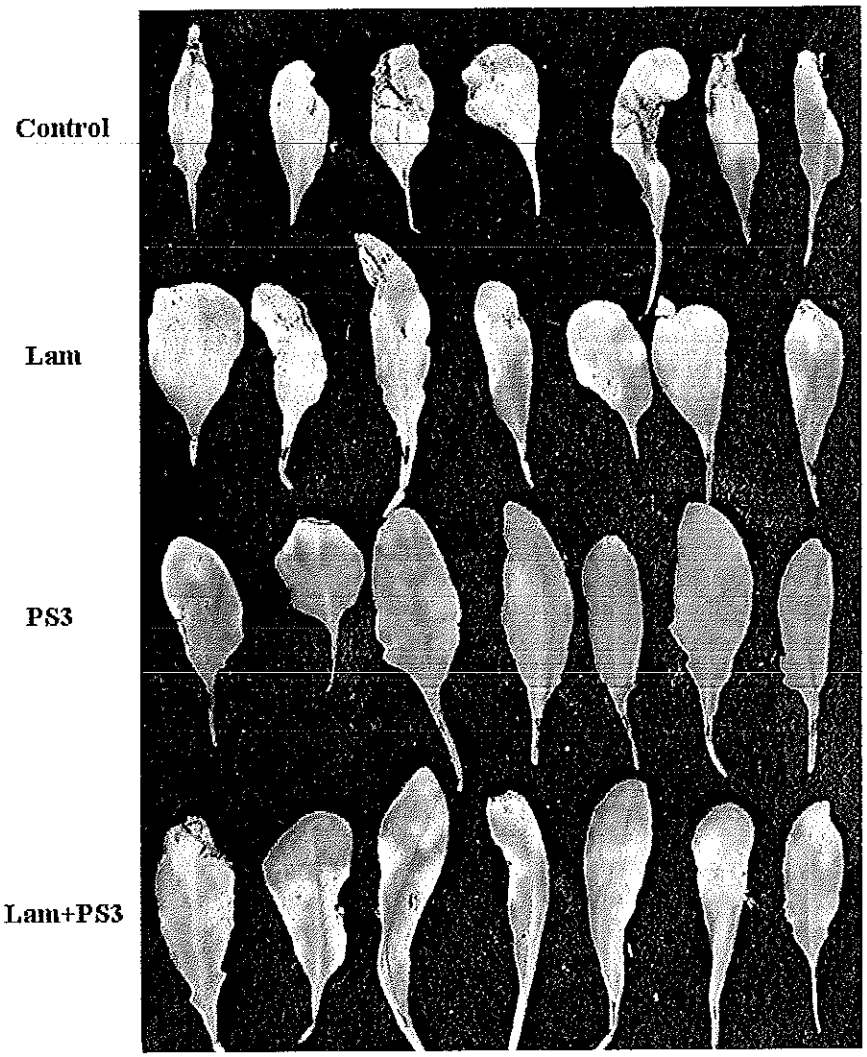

Figure 4.20 Disease symptoms caused by a *Peronospora parasitica* isolate NOCO on *Arabidopsis* wild-type Col0 leaves. Six-week-old Col0 were infiltrated with Lam (500 µg/ml), PS3 (100 µg/ml) or Lam+PS3 (500 µg/ml and 100 µg/ml, respectively). Twenty-four h later, plants were sprayed with $5x10^4$ spores/ml *Peronospora parasitica*. Pictures were taken 10 days after infection.

Discussion

1 Elicitation activity of TTF5P, laminarin and β-aminobutyric acid to induce plant defense responses

This work began because of the results of several field experiments, presented by Tribo technologies, indication that application of TTF5P to different plant species resulted in strong resistance against various pathogens. Based on this data, the hypothesis was that TTF5P induces plant defense responses. This chemical showed induced the expression of PR (pathogenesis-related)-proteins, which are well-known proteins with antimicrobial activities (Kauffmann *et al.*, 1987; Legrand *et al.*, 1987) and used as typical molecular markers of defense responses, very strongly in tobacco plants after applied by infiltration or spraying. Indeed, the tested tobacco cultivars, Samsun H and Xanthi nc showed no significant different in response to TTF5P. This observation correlates well with the resistance-inducing activity of TTF5P in field experiments. Moreover, it also induced expression of defense genes involved in phenylpropanoid pathway indicating its efficiency elicitation activity in wide range of plant defense responses. These would explain its high plant protection activities.

The separate individual components of TTF5P showed no significant elicitation activity whereas after mixing, they did induce plant defenses. Replacing the algae extract (one of TTF5P component) with laminarin (Lam), the result showed that the induced PR-protein activity was increased indicating that Lam was a more effective elicitor than the algae extract. Even though, from our results, the amino acids component (AA) appeared as a main contributor in TTF5P, we did not know the AA composition. However, we showed that β-aminobutyric acid (BABA), a potent inducer of resistance against infection by various pathogens, (Jakab *et al.*, 2001) could replaced the AA. In our experiments, BABA induced PR-proteins expression in a dose-dependent manner after applying by spray. At low BABA concentration, BABA can act as a priming agent similar to the report by Zimmerli *et al.* (2000) who found that in certain cases BABA exerted its function via priming of SA-dependent defense mechanisms in *Arabidopsis*. A primed state of the plant refer to the physiological condition in which plants are able to better or more rapidly mount defense responses, or both, to biotic or abiotic stress (Prime-A-Plant Group, 2006). Conversely, BABA acts as an inducer of resistance activating the SA-signaling pathway (Narusaka *et al.*,

2006) or do through potentiation of ABA-dependent signaling pathways (Ton and Mauch-Mani, 2004). This related to our finding that BABA at high concentration could behave as an eliciting molecule to induced defense response.

However the induction of acidic PR1 which is an SA-dependent marker was reduced in the treatment of BABA+Lam whereas other markers had not been affected suggesting the suppression effect of Lam on the SA-dependent marker. This finding was similar to the observations made by Benoît Boachon who reported that Lam showed the suppression activity on SA-dependent signaling pathway induced by sulfated laminarin called PS3. I have continued studying in more details based on his finding by using Lam and PS3 and their combination (Lam+PS3). Moreover, there have been a number of literature reports suggested the crosstalk of the signaling pathway in plant defense responses and that SA was suppressed trough JA, conversely, JA was suppressed by SA.

2 Elicitation activity of laminarin (Lam) and sulfated laminarin (PS3) to induce plant defense responses

Lam is β-glucan extracted from *Laminaria digitata* (Ménard *et al.*, 2005 and Ménard *et al.*, 2004) Its structure consists of a β-1,3 glucan backbone with an average degree of polymerization of 25 glucose units and with 1-3 single β-glucose branches at position 6 (Read *et al.*, 1996, Lepagnol-Descamps *et al.*, 1998). Many reports have shown that Lam can act as an elicitor to induce plant defense responses, for example, fungal β-glucans are efficient elicitors of defense responses in different plant species (Côté and Hahn, 1994; Ebel, 1998; Shibuya and Minami, 2001). Applied to grapevine plants, laminarin induces the accumulation of phytoalexins and the expression of a set of pathogenesis-related (PR) proteins (Aziz *et al.*, 2003).

2.1 Suppression effect of Lam on PS3 induced SA-dependent defense responses in tobacco plants

Ménard *et al.* (2004) showed that Lam induces ET- but not SA-dependent defense responses whereas sulfated laminarin called PS3 can induce both ET- and SA-signaling pathway in both tobacco and *Arabidopsis thaliana*. In tobacco, PS3 induced immunity against *Tobacco mosaic virus* (TMV) infection, with decreases in both the TMV lesion number and lesion size, whereas Lam induced only a weak

resistance and affected only lesion number. The decrease in lesion size in PS3-treated tissue is correlated with the induction of the SA signaling pathway. Increased resistance to TMV has been described in tobacco plants with high level of SA (Verberne *et al.*, 2000). Ménard *et al.* (2005) previously showed that there was no synergy between Lam and PS3 for the SA-dependent acidic PR1 and the ET-dependent basic PR5. In our results, Lam+PS3 caused a reduction of the acidic PR1 expression and salicylic acid accumulation compared to those induced by PS3. The expression of basic PR5 (both gene and protein level) and *CCoAOMT* revealed that there was no interaction between Lam and PS3 which could affect to the elicitor activity of Lam or PS3 because they were not reduced when treated with Lam+PS3 compared with PS3 alone. Therefore, Lam suppresses the PS3 induced SA-dependent signaling pathway but not affect such different defenses. The differential activation related to our results could be deduced from the recent analysis of the *pmr4 Arabidopsis* mutant. PMR4 is considered to be the main callose synthase responsible for callose deposition. The mutant is resistant to powdery mildew infection and is unable to produce a pathogen-induced callose response suggesting that callose, a linear β-1,3 glucan, or callose synthase negatively regulates the SA pathway (Nishimura *et al.*, 2003).

2.2 Lam suppresses the PS3 induced SA-dependent defense responses in *Arabidopsis* plants though jasmonic acid (JA)

Using the same concentrations that had been tested in tobacco plants; Lam (200 μg/ml), PS3 (200 μg/ml) and a mixture of Lam and PS3 (Lam+PS3, 200 μg/ml each) Benoît Boachon found that the suppression effect on PR1 expression was not observed in wild-type Columbia-0 (Col-0) *Arabidopsis* plants. He also demonstrated that the suppression effect of Lam appeared when using the mixture of increased Lam (500 μg/ml) and decreased PS3 (100 μg/ml). These observations showed that Lam can suppress the SA-defense responses but only over a limited range of concentration. If the SA-defense response was induced very strongly, Lam had no longer inhibitory effect.

We further studied the suppression effect in *Arabidopsis* plants by using these condition; Lam (500 μg/ml), PS3 (100 μg/ml) and a mixture of Lam and PS3 (Lam+PS3, 500 μg/ml and 100 μg/ml, respectively). The expression level of marker

genes including *PR1*, *PR4/HEL*, *PDF1.2* and *VSP2* which are well recognized as markers for SA, ET, ET/JA and JA signaling pathway, respectively, were monitored by quantitative RT-PCR in leaf tissues 24 h after elicitor infiltration. In Col-0 we found that only *PR1*, SA marker, level was significantly reduced in Lam+PS3 treatment whereas other signaling markers were not. This data was comparable to that obtained in tobacco plants. Interestingly, the JA marker, *VSP2*, was considerably decreased in Lam+PS3 treatment.

To date there have been many reports in the literatures on the mutually antagonistic effect of these two signal molecule, SA and JA (Vidal *et al.*, 1997; Niki *et al.*, 1998; Petersen *et al.*, 2000; Kachroo *et al.*, 2001; Kloek *et al.*, 2001; Kunkel and Brooks, 2002; Li *et al.*, 2004). Thus, it is possible that after Lam+PS3 treatment, SA was blocked through Lam-induced JA resulting in a reduction of PR1 compared to that expressed in PS3-treated tissue. We hypothesized whether Lam might suppress SA pathway via JA since coronatine stimulates the JA responses and in *Arabidopsis* JA are believed to be antagonistic to SA (Glazebrook *et al.*, 2003). Therefore, the mutant impaired in the perception of JA, *coi1-16* (*coronatine insensitive*) was further studied to elucidate our hypothesis. The result showed that PR1 expression in tissue treated with Lam and Lam+PS3 was restored in this mutant so implying that Lam suppressed SA though JA.

2.3 The negative effect of Lam on *PR1* expression is dependent on the SA level in *Arabidopsis*

As PR1 belongs to the systemic acquired resistance (SAR) class of defense-related genes that are induced in responses to elevated SA levels in plants, we examined the association between the Lam–suppressed *PR1* expression and the SA level. The SA amount was measured to answer this question. In wild-type Col0, Lam showed no ability to trigger the SA production compared to that in the control whereas the SA level in the tissue was reduced after treatment with Lam+PS3 suggested the negative effect of Lam on the SA biosynthesis. In the *coi1-16* mutant, SA was similarly exhibited in all treatments supporting the point that JA can suppress SA because SA was restoring when JA defect. Thus, it indicated that Lam affect in SA biosynthesis consequently reduced the SA-dependent gene expression.

2.4 Suppression effect of SA on JA after Lam+PS3 treatment

VSP2, a specific marker for the JA-defense response, was decreased after treatment with Lam+PS3. Because SA and its functional analogs have also been shown to prevent the expression of JA-dependent defense genes on several occasions (Pena-Cortés *et al.*, 1993; Gupta *et al.*, 2000; Kunkel and Brooks, 2002), we hypothesized that the reduction of *VSP2* may be due to the inhibitory effect of SA, induced by PS3, on JA. To test this hypothesis, the defense gene expression levels were examined in mutant impaired in the production of SA. The *sid2/nahG* plant, *SA induction deficient2* cross with transgenic plant line, which could not accumulate elevated levels of SA because of the expression of the bacterial SA-degrading enzyme salicylate hydroxylase (*nahG*), was used to verify this point. *PR4* and *PDF1.2* were detected at high level in this mutant revealed the inhibitory effect of SA on JA signaling. Similarly, there have been more recent reports that *eds4* and *pad4* mutants, that are also impaired in SA accumulation, exhibit enhanced JA-dependent gene expression (Gupta *et al.*, 2000; Clarke *et al.*, 2000).

3 Elicitation activities of Lam and PS3 to induce pathogen resistance in *A. thaliana*

3.1 Induced resistance against the bacterial *Pseudomonas syringae*

We wanted to test whether the negative effect of Lam on the SA-dependent defense response would also affect to reduce the resistance controlled by SA. SA-dependent defense responses clearly play an important role in limiting *P. syringae* growth. Supporting this fact by experiments using mutants with defects in SA signaling, including *eds1* (*enhanced disease susceptibility 1*, Aart *et al.*, 1998), *pad4* (*phytoalexin-deficient 4*, Zhou *et al.*, 1998), *eds5* (*enhanced disease susceptibility 5*, Rogers and Ausubel, 1997), *sid2* (*SA induction deficient2,* Nawrath and Métraux, 1999), and *npr1* (*nonexpressor of PR1*, Glazebrook *et al.*, 1996; Shah *et al.*, 1997), the results showed enhanced susceptibility to virulent strains and in some cases, avirulent strains. Furthermore, treatment of plants with exogenous SA or SA analogs reduces *P. syringae* growth, as does induction of SAR (Cao *et al.*, 1994; Lawton *et al.*, 1996).

Thus, in wild type Col-0, if Lam suppresses SA-defense responses, Lam itself did not induce resistance to *P. syringae* pv. tomato DC3000 (*Pst* DC3000) virulent strain, additionally, the resistance should be reduced after treatment with Lam+PS3. Our outcomes also showed similar results to the hypothesis that indicates Lam suppresses SA-dependent defenses consequence by reducing the resistance to pathogen. Conversely, in *coi1-16* mutant, the growth of *Pst* DC3000 in all treatments were not significantly different but reduced than that in control suggesting that when JA was impaired, SA signal was not repressed resulting in restoration of resistance to this bacterial pathogen. These confirmed that SA was suppressed by JA. Kloek *et al.* (2001) also used *Pst* DC3000 to exploit the inhibition of SA signaling by JA. Resistance of *coi1-16* plants to *Pst* DC3000 is associated with elevated levels of SA and enhanced expression of SA-regulated genes, indicating that coronatine contributes to virulence by activating JA signaling, thereby repressing SA-dependent defense mechanisms that limit *P. syringae* growth.

3.2 Induced resistance against *Peronospora parasitica* (*Pp*)

SA-dependent defense responses are considered effective mainly against biotrophic pathogens that feed on living tissues, such as the oomycete *Peronospora parasitica* (*Pp*) (Glazebrook, 2005). Accordingly, impaired SA production leads to increased susceptibility to various pathogens. For example, SA production is significantly reduced in *sid2* plants, resulting in increased susceptibility to both virulent and avirulent forms of *Pp* (Nawrath and Métraux, 1999). Consistent with the idea that SA signaling is effective against *Pp*, SAR is effective against this pathogen. Artificial induction of SAR by treatment with SA or SA analogs results in resistance to normally compatible isolates (Ryals *et al.*, 1997; Uknes *et al.*, 1992). Induction of SAR by infection with an avirulent pathogen is also effective (Nawrath and Métraux, 1999). Mutations in *eds5* (Nawrath and Métraux, 1999), *sid2* (Nawrath and Métraux, 1999), and *npr1* (Cao *et al.*, 1997; Delaney *et al.*, 1995; Ryals *et al.*, 1997) all show strong loss-of-induced-resistance phenotypes (Zhang *et al*, 2003). This illustrates that responses under the control of this pathway are required for SAR against *Pp*. Mutants with constitutively active defense signaling and with constitutively elevated levels of SA are resistant to *Pp* (Bowling *et al.*, 1997; 1994; Petersen *et al.*, 2000).

Therefore, the resistance against this pathogen can reveal the effectiveness of the tested elicitor in our model, Lam, PS3 and Lam+PS3, in triggering SA-dependent pathway. The results in the Col-0 wild type showed that Lam could induce resistance slightly resulting in similar symptom as appeared in control whereas PS3 exhibited very strong resistance. However, the resistance was reduced after treatment with Lam+PS3 and this correlated to the *PR1* expression and SA level. These consistent data indicated that Lam has a suppression effect on the SA-dependent defense responses and resistance against the pathogen.

Conclusion

TTF5P induced expression of PR-proteins and the defense genes involved in the phenylpropanoid pathway in laboratory experiments indicating its efficient elicitation activity for a wide range of plant defense responses. This would explain its high efficiency to protect the plants in infield tests. Since the composition of TTF5P components; NPK, amino acid (AA) and algae extract, are not known, the replacing its components by purified chemicals such as replacing algae extract with laminarin (Lam) and AA with nonprotein β-aminobutyric acid (BABA) had been performed. Accordingly, the elicitation activity was increased. Surprisingly, Lam showed suppression activity on BABA-induced PR1 expression. This finding was compatible with the demonstration by Benoît Boachon who found the reduction of the salicylic acid (SA)-responsive defense responses (*PR1* gene expression) after adding Lam into its sulfated form (PS3). This is very interesting because it is the first incident showing the suppression activity of Lam (one of β-1,3-glucans) on the SA-dependent pathway. This finding concept has been continued. The results from the *Arabidopsis* wild type Col-0 and a mutant impaired in the jasmonic acid (JA) pathway indicated that Lam suppresses the SA-dependent defense response include inhibition of elevated SA levels, reduction of *PR1* gene expression and reduced resistance against the bacterial *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) and *Peronospora parasitica* via JA. Moreover, the wild type Col-0 and the mutant *sid2/nahG* which defective in the SA signaling pathway and cannot accumulate elevated SA showed that JA is suppressed vise versa though SA. The level of JA is, however, the important

key to confirm that SA was suppressed via JA and the JA measurement is still being conducted. In order to understand how Lam suppresses SA in more details further studies will be required using other mutants impaired or blocked in other parts of the SA and JA signaling pathways. Indeed, other signaling pathways involved in plant defense responses such as ethylene and abscisic acid should also be investigated in this system to confirm whether the suppression effect of Lam is focus on only the SA-dependent defense pathway.

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APPENDIX

1 Media

1.1 Potato Dextrose Agar (PDA)

Formulae of PDA (Difco)

Approximate Formula (for 1 l)

Potato Starch	4 g
Dextrose	20 g
Agar	15 g

Suspend 39 g of the powder in 1 l of distilled water, mix thoroughly and autoclave at 121 ^{0}C for 15 minutes. Cool to 50-60 ^{0}C and pour into sterile Petri dishes. Allow to solidify for a minimum of 30 minutes.

1.2 Potato Dextrose Broth (PDB)

PDB consists of same ingredients as in PDA without agar. Dissolve 39 g of the powder in 1 l of distilled water, mix thoroughly and autoclave at 121 ^{0}C for 15 minutes.

1. 3 V_8 Agar

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

V_8 juice was mixed with distilled water, $CaCO_3$ was added and gently stirred. The Media was autoclaved at 121 ^{0}C for 15 minutes prior to pour into sterile plate.

1.4 Murashige and Skoog (MS) medium

Ingredient	(g/l)
KNO_3	1900
NH_4NO_3	1650
$CaCl_2$	322.2
$MgSO_4$	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
6-benzylaminopurine (BA)	2.0
2,4-dichlorophenoxy acetic acid (2,4-D)	2.0
Phytagel agar (0.23%)	2.3
Sucrose (8%)	80 or
Sucrose (3%)	30

Medium is adjusted to pH 5.7 before autoclaving at 121 ^{0}C for 15 minutes.

2 Protein measurement by Bicinchoninic acid (BCA) method (Smith *et al.*, 1985)

Solution A;

1) BCA-Na_2	1% (w/v)
2) sodium carbonate	2% (w/v)
3) sodium tartate	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 composes of Solution A (100 parts) and Solution B (2 parts)

Standard BSA or samples (100 µl) was added to 2 ml of solution C, mixed gently and kept at 34 ^{0}C. After 30 minutes the reaction mixtures were measured the absorbance at 562 nm.

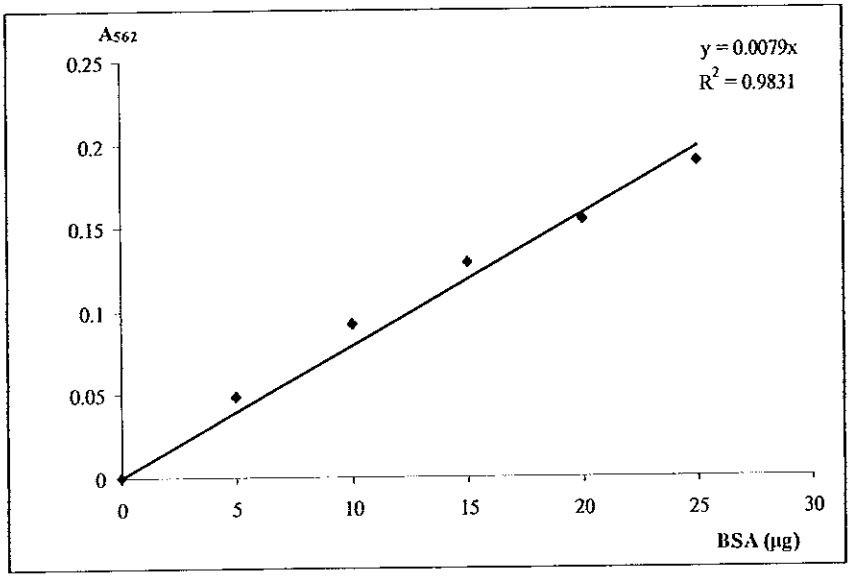

Figure A1 Standard curve of Bovine Serum Albumin (BSA) at absorbance 562 nm measured by the Bicinchoninic acid method.

3 Standard curve of scopoletin

Stock solution of 50 mM Scp was prepared by dissolving 96 mg Scp in 10 ml of 95% ethanol. The Scp stock solution was diluted to 0.5, 1, 1.5, 2 and 2.5 µM and were measured with spectrofluorometer using excitation and emission wavelength at 340 and 440 nm, respectively. The intensity of standard Scp measured by spectrofluorometer showed lineat correlation with the concentration of standard Scp (Figure A2)

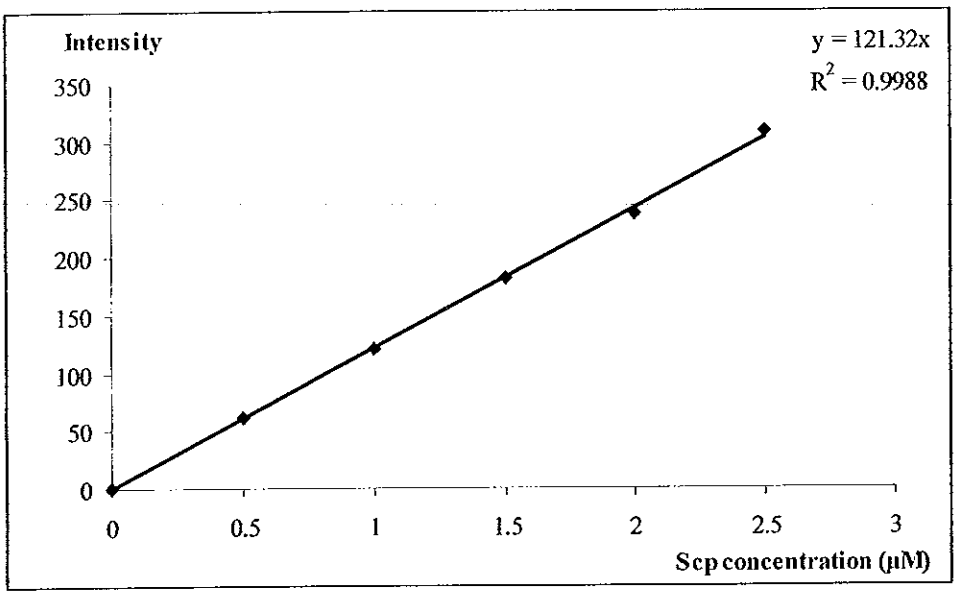

Figure A2 Standard curve of scopoletin (Scp, Sigma) by plotting the Scp concentrations versus intensity using spectrofluorophotometer (excitation and emission wavelengths of 340 nm and 440 nm, respectively).

4 Calculation for enyme peroxidase (POD) activity

Calculation

$$\text{Volume activity (U / ml)} = \frac{\Delta A_{436} / \text{min} \times 1000 \times Vt \times \text{dilution factor}}{\varepsilon \times D \times Vs}$$

Where

Vt = final volume of reaction mixture (ml)

Vs = sample volume (ml)

ε = micromolar extinction co-efficient of tetraguaiacol (cm^2/micro mol)

D = Light Path = 1 cm

1000 = derived from unit definition

5 Purity of elicitins

The purified elicitin was obtained from PDB (potato dextrose broth), vacuum filtrated, ammonium sulphate precipitation, DEAE column and Sephadex G-50

column, respectively. In this study, *Phytophthora* spp. were grown in PDB. Three weeks later, the culture medium was filtered through a Whatman filter paper No. 4, ammonium sulphate preparation and PD-10, respectively. They were monitored for the purity by the silver staining of 16.5% polyacrylamide gel (Tricine-SDS-PAGE)

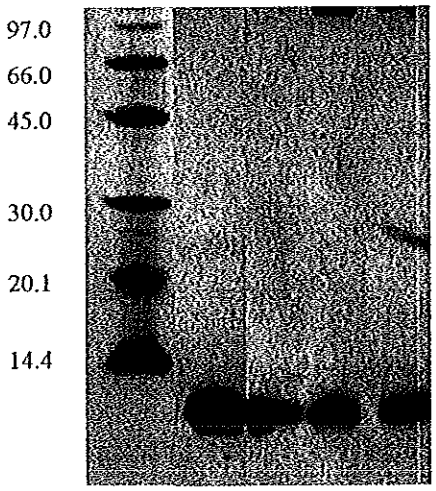

Figure A3 Purified and partial purified elicitins from culture medium of *Phytophthora* spp.; *P. palmivora Hevea* isolate [P. pal (Hevea)], *P. palmivora Durian* isolate [P. pal (Durian)] and *P. parasitica Anthurium* isolate [P. para].

Lane 1 Marker

2 Purified elicitin from P. pal (Hevea)

3 Partial purified elicitin from P. pal (Hevea)

4 Partial purified elicitin from P. pal (Durian)

5 Partial purified elicitin from P. para

VITAE

Name Miss Nion Chirapongsatonkul

Student ID 4523012

Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Biotechnology)	Prince of Songkla University	2001

Scholarship Awards during Enrolment

The Royal Golden Jubilee Ph.D. Program 4 .C. PS/44 F.1. (PHD/0225/2544)

List of Publications and Proceedings

Publications

Chirapongsatonkul, N. Nak-udom, P. and Churngchow, N. 2008. Defence Responses of Calli and Seeds of *Hevea brasiliensis* to Zoospores and the Elicitin of *Phytophthora palmivora*. J. Phytopathol (In Press).

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