ประสิทธิภาพและกลไกของสิ่งกระตุ้นความต้านทานในข้าวต่อเชื้อแบคทีเรีย *Xanthomonas oryzae* pv. *oryzae* สาเหตุโรคใบไหม้

นายต่วน เล ธาน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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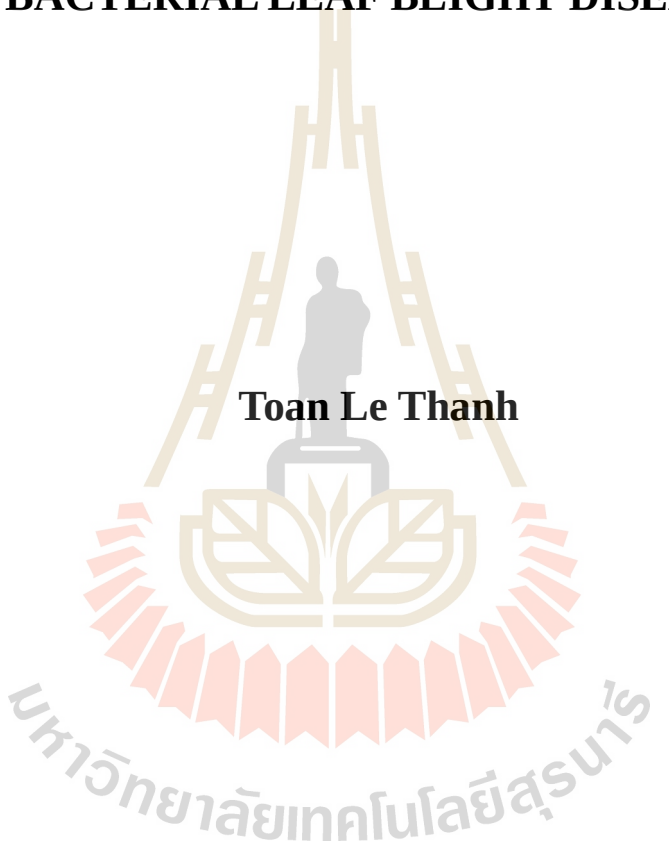
มหาวิทยาลัยเทคโนโลยีสุรนารี

EFFICACY AND MECHANISM OF INDUCERS TRIGGERING INDUCED RESISTANCE IN RICE AGAINST *Xanthomonasoryzae*pv. *oryzae* CAUSING BACTERIAL LEAF BLIGHT DISEASE

Toan Le Thanh



A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Crop Science

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EFFICACY AND MECHANISM OF INDUCERS TRIGGERING INDUCED RESISTANCE IN RICE AGAINST *Xanthomonas oryzae* pv. *oryzae* CAUSING BACTERIAL LEAF BLIGHT DISEASE

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ต่วน เล ธาน : ประสิทธิภาพและกลไกของสิ่งกระตุ้นความต้านทานในข้าวต่อเชื้อแบคทีเรีย *Xanthomonas oryzae* pv. *oryzae* สาเหตุโรคใบไหม้ (EFFICACY AND MECHANISM OF INDUCERS TRIGGERING INDUCED RESISTANCE IN RICE AGAINST *Xanthomonas oryzae* pv. *oryzae* CAUSING BACTERIAL LEAF BLIGHT DISEASE) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร. ณัฐธิญา เบือนสันเทียะ, 195 หน้า.

การทดลองนี้มีวัตถุประสงค์เพื่อ (1) ศึกษาประสิทธิภาพของสิ่งกระตุ้นในการควบคุมโรคใบไหม้ในข้าว และ (2) ศึกษากลไกการชักนำความต้านทานต่อโรคใบไหม้ในข้าว โดยนำสิ่งกระตุ้นประกอบด้วย ascorbic acid (AA), oxalic acid (OA), potassium dihydrogen phosphate (PDP), salicylic acid (SA), *Bacillus subtilis* สายพันธุ์ CaSUT007 (CaSUT007) และ *Bacillus* sp. สายพันธุ์ NT111 มาทดสอบศักยภาพในการควบคุมโรคใบไหม้ในข้าวพันธุ์ขาวดอกมะลิ ทำการประเมินอิทธิพลของสิ่งกระตุ้นต่อความงอกของเมล็ดข้าวด้วยวิธี blotting-paper จากนั้นนำสิ่งกระตุ้นที่ผ่านการทดสอบว่ามีประสิทธิภาพสูง ประกอบด้วย CaSUT007, 1.0 mM AA, 4.0 mM PDP, 0.5 mM OA และ 1.0 mM SA มาทดสอบความสามารถในการชักนำความต้านทานภายใต้สภาพเรือนทดลอง นำตัวอย่างไปศึกษาการแสดงออกของยีนด้วยวิธี real-time reverse transcription polymerase chain reaction รวมทั้งศึกษาปริมาณการสร้าง superoxide anion และ hypersensitive response ด้วยวิธี nitroblue tetrazolium staining และสังเกตการเรืองแสงสีเขียวของปฏิกิริยา HR ด้วยเทคนิค confocal microscopy ตามลำดับ นำตัวอย่างไปศึกษาการเปลี่ยนแปลงของสารชีวเคมีภายในใบข้าวโดยใช้เทคนิค Fourier Transform Infrared (FTIR) spectroscopy จากนั้นประเมินปริมาณเชื้อบนอาหาร NGA ผลการทดลองพบว่า สิ่งกระตุ้นแต่ละชนิดสามารถกระตุ้นการงอกของเมล็ดข้าวได้แตกต่างกันอย่างมีนัยสำคัญทางสถิติ สิ่งกระตุ้นที่มีชีวิตคือ CaSUT007 และ NT111 สามารถยับยั้งการเจริญของเชื้อ *Xoo* ได้ และพบว่า SA สามารถลดการเกิดโรคได้ 55.35% ซึ่งแตกต่างอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับกรรมวิธีควบคุม เมื่อนำตัวอย่างที่ถูกชักนำความต้านทานต่อเชื้อ *Xoo* ด้วย SA ไปศึกษากลไกความต้านทาน พบว่ายีนที่เกี่ยวข้องกับกลไกความต้านทาน คือ ascorbate peroxidase (*apx*) และ phenylalanine ammonia lyase (*pal*) มีการแสดงออกสูงขึ้น และเกิดขึ้นอย่างรวดเร็วกว่ากรรมวิธีควบคุม นอกจากนี้พบว่า ข้าวที่ถูกชักนำความต้านทานมีการผลิต O_2- เพิ่มขึ้น 28% ใน 3-6 ชั่วโมง และสามารถชักนำปฏิกิริยา HR บนใบข้าว คิดเป็น 110% ก่อให้เกิดอาการเซลล์ตายภายใน 48 ชั่วโมง การวิเคราะห์การเปลี่ยนแปลงของสารชีวเคมีภายในใบข้าวหลังจากปลูกเชื้อ *Xoo* เป็นเวลา 14 วัน พบว่า มีการเปลี่ยนแปลงของสารชีวเคมีภายในใบข้าวเพิ่มสูงขึ้นในช่วงคลื่นที่ 1233/1517 cm^{-1}, 1467/1517 cm^{-1} และ 1735/1517 cm^{-1} ซึ่งเป็นองค์ประกอบของลิกนินและเพคติน และยังพบ amide I β-sheet (1,629 cm^{-1}), lipid (2851 cm^{-1}และ 1735 cm^{-1}) เพิ่มสูงขึ้นกว่ากรรมวิธี

ควบคุม และยังพบว่าการใช้ สาร SA สามารถลดจำนวนประชากรเชื้อ *Xoo* ในใบข้าวอย่างมีนัยสำคัญ รวมทั้งยับยั้งกิจกรรมของยีนก่อโรคของเชื้อ *Xoo* ประกอบด้วย *hrcQ*, *rpfF* และ *rpfG* ลงอีกด้วย การแสดงออกของยีนปกป้องตนเองและสารชีวเคมีเหล่านี้ในข้าวที่ถูกชักนำด้วยสาร SA น่าจะมีความสัมพันธ์กับโครงสร้างผนังเซลล์ที่หนาขึ้นส่งผลให้ข้าวมีความต้านทานต่อการเข้าทำลายของเชื้อ *Xoo* สาเหตุโรคใบไหม้

TOAN LE THANH: EFFICACY AND MECHANISM OF INDUCERS TRIGGERING INDUCED RESISTANCE IN RICE AGAINST *Xanthomonas oryzae* pv. *oryzae* CAUSING BACTERIAL LEAF BLIGHT DISEASE . THESIS ADVISOR : ASST. PROF. NATTHIYA BUENSANTEAI, Ph.D., 195 PP.

BACTERIAL LEAF BLIGHT/INDUCER/RESISTANCE/RICE/SALICYLIC ACID/ PLANT DEFENSE MECHANISM

The objectives of this study were (1) to evaluate the efficacy of resistance inducers against bacterial leaf blight (BLB) in rice, and (2) to characterize the mechanism of induced resistance against BLB in rice. Resistance inducers including ascorbic acid (AA), oxalic acid (OA), potassium dihydrogen phosphate (PDP), salicylic acid (SA), *Bacillus subtilis* strain CaSUT007 (CaSUT007) and *Bacillus* sp. strain NT111 were evaluated for their potential in controlling rice BLB disease in rice cv. KDML105. Effects of the inducers on rice seed germination were evaluated using the blotting-paper method. Evaluation of the induced resistance in rice against *Xoo* infection was conducted under a greenhouse condition, using the most effective inducers including CaSUT007, 1.0 mM AA, 4.0 mM PDP, 0.5 mM OA and 1.0 mM SA. Expression of rice defense related genes and *Xoo* pathogenicity genes were performed by quantitative real-time reverse transcription polymerase chain reaction. Superoxide anion in the induced leaves was detected by nitroblue tetrazolium staining method. Hypersensitive response in the induced leaves was detected by observing fluorescence expression under confocal microscopy. Biochemical changes of the

induced rice leaves were examined by fourier transform infrared (FTIR) spectroscopy. *Xoo* growth in the treated rice was assessed on nutrient glucose agar plates. The results demonstrated that all of the inducers significantly stimulated germination of rice seed. Among the inducers applied as seed soak and foliar spray, SA reduced disease severity significantly about 55.35%, compared to that of the non-treated control. The defense gene expression analysis revealed that the 1 mM SA treatment showed an increased regulation of two resistance genes, ascorbate peroxidase (*apx*) and phenylalanine ammonia lyase (*pal*), in rice leaves compared to that of the non-treated plants. O_2^- production was enhanced more than 28% at 3-6 hours after inoculation (HAI), and the hypersensitive response (HR) was increased by 110% at 48 HAI in the treated rice leaves. At 14 days after inoculation, higher ratios of 1233 / 1517 cm^{-1}, 1467 / 1517 cm^{-1} and 1735 / 1517 cm^{-1} wavelengths in the treated rices were observed, suggesting alteration of monomer composition of lignin and pectin in the rice cell wall. The treated plants had more amide I β-sheet structure at the peak 1629 cm^{-1}, and some peaks such as 2851 cm^{-1} and 1735 $cm,^{-1}$ corresponding to lipids, were more intense. In addition, the population of *Xoo* in the treated rice leaves was lower than that of the non-treated ones, and the three pathogenicity genes of *Xoo*, which are *hrc*Q, *rpf*F, and *rpf*G, were expressed at a lower rate. These defense gene expression and biochemical changes of rice treated with SA could relate to the observed thickening of the cell walls, resulting in primed resistance to the BLB disease.

Academic Year 2015

Advisor's Signature_____

Co-advisor's Signature_____

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Mr. Toan Le Thanh

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LIST OF ABBREVIATIONS

AA	=	Ascorbic acid
Ais	=	Autoinducers
ANOVA	=	Analysis of variance
apx	=	Ascorbate peroxidase
ASM	=	Acibenzolar-S- methyl
ATR	=	Attenuated Total Reflectance
AzA	=	Azelaic acid
BIT	=	Sodium saccharin dihydrate
BLB	=	Bacterial leaf blight
BTH	=	Benzo[1, 2, 3] thiadiazole-7-carbothioic acid S-methyl ester
CaSUT007	=	*Bacillus subtilis* CaSUT007
CRD	=	Completely randomized design
DAI	=	Days after inoculation
DAPG	=	2,4-diacetylphloroglucinol
DAS	=	Days after sowing
DGDG	=	Digalactosyl diacylglycerol
DMRT	=	Duncan's Multiple Range Test
DSF	=	Diffusible signal factor
EC	=	Electrical conductivity
EPS	=	Extracellular polysaccharides

LIST OF ABBREVIATIONS (Continued)

ET	=	Ethylene
FTIR	=	Fourier transform infrared spectroscopy
G	=	Guaiacyl
G3P	=	Glycerol-3-phosphate
H	=	Hydroxyphenyl
H	=	Hour
HAI	=	Hours after inoculation
hpa	=	*hrp*-associated
HR	=	Hypersensitive response
hrc	=	*hrp*-conserved
hrp	=	Hypersensitive response and pathogenicity
IAA	=	Indole-3-acetic acid
INA	=	2,6-dichloroisonicotinic acid
ISR	=	Induced systemic resistance
JA	=	Jasmonic acid
kDa	=	Kilo Dalton
KDML105	=	Khao Dawk Mail 105
LPS	=	Lipopolysaccharides
MAPK	=	Mitogen activated protein kinases
MAPKK	=	MAPK kinase
MAPKKK	=	MAPKK kinase

LIST OF ABBREVIATIONS (Continued)

mg l^{-1}	=	Milligram per milliliter
µl	=	Microliter (s)
µm	=	Micrometer (s)
MGDG	=	Monogalactosyl diacylglycerol
min	=	Minute (s)
ml	=	Milliliter (s)
mM	=	Millimolar
MV	=	Midvein
NADPH	=	Nicotinamide adenine dinucleotide phosphate
NBT	=	Nitroblue tetrazolium
NIP1	=	Necrosis-inducing protein 1
nm	=	Nanometer
NO	=	Nitrite oxide
NT111	=	*Bacillus* sp. NT111
NTC	=	Non-template control
NGA	=	Nutrient glucose agar
NGB	=	Nutrient broth containing 2% glucose
O_2^-	=	Superoxide anion
OA	=	Oxalic acid
^oC	=	Degree celcius
OD	=	Optical density

LIST OF ABBREVIATIONS (Continued)

pal	=	Phenylalanine ammonia lyase
PBS	=	Phosphate-buffered saline
PCA	=	Principal Component Analysis
PDP	=	Potassium dihydrogen phosphate
PGPR	=	Plant growth-promoting rhizobacteria
PPBL	=	Plant Pathology and Biopesticide Laboratory
ppo	=	Polyphenol oxidase
PR proteins	=	Pathogenesis-related proteins
PSA	=	Peptone sucrose agar
qRT-PCR	=	Quantitative real-time reverse transcription polymerase chain reaction
QS	=	Quorum sensing
ROS	=	Reactive oxygen species
rpf	=	Regulation of pathogenicity factors
rpm	=	Round per minute
S	=	Syringyl
SA	=	Salicylic acid
SAG	=	Salicylic acid glucoside
SAMe	=	Methyl SA
SAR	=	Systemic acquired resistance
SE	=	Standard error

LIST OF ABBREVIATIONS (Continued)

SV	=	Secondary vein
T3SS	=	Type III secretion system
TF	=	Transcription factor
UHCA	=	Unsupervised hierarchical cluster analysis
WAKs	=	Wall-associated kinases
Xoo	=	*Xanthomonas oryzae* pv. *oryzae* Ishiyama

CHAPTER I

INTRODUCTION

1.1 Background of the selected topic

Rice (*Oryza sativa* L.) is one of the most important crops in the world, providing bulk of daily caloric intake for approximately 3 billion people. Asia has the largest growing area, with top production countries such as China, India, Thailand and Vietnam (Xu et al., 2013). From the 1960s, with the widespread cultivation of high-yielding and nitrogen-responsive dwarf hybrid varieties, rice diseases have become more prevalent. One of the limitation factors in rice production in Asia is baterial leaf blight disease caused by *Xanthomonas oryzae* pv. *oryzae* Ishiyama (*Xoo*) (Adhikari et al., 1995; Gnanamanickam, 2009; Bureau of Rice Research and Development, 2010). Annual rice yield losses during these recent years have been estimated at 20-75% of the worldwide rice production (Gnanamanickam, 2009; Shimono et al., 2012). The bacterium *Xoo* is able to infect rice plant at all growth and development stages (Goto, 1992; Mew et al., 1993).

In current years, farmers still regard pesticides as an essential component of disease management in order to ensure quality and quantity of crops and to feed an increasing human population (Matthews, 2006). The world pesticide consumption in 1995 was estimated to be 2.6×10^9 kg of active ingredient (Stenersen, 2004). The chemicals used are almost 50% herbicides, 25% insecticides and 21.6% fungicides; and the remainder to other products including bactericides and nematicides. Despite

the advantages farmers have gained from pesticides, numerous problems have been arisen. In a review of published works on arthropod pests, crop pathogens and weeds, Pimentel (1995) concluded that less than 0.1% of pesticides applied reaches their target organism, much of pesticides directly affects health of farmer and pollute on environment. The amount of pesticides retained on crops depends on many factors, including the formulation of pesticide used, the volume of spray applied, the type of equipment used and the quality of the spray (Matthews, 2006). A report of World Health Organization in 1990 showed that on the worldwide basis, intoxications attributed to pesticides have been estimated to be as high as 3 million cases of acute, severe poisoning annually, with 220,000 deaths (Ecobichon, 2001). In China, a report from the Chinese National Statistics Bureau showed that 48,377 pesticide poisoning cases, including 3,204 fatalities, were reported in 27 provinces in 1995 (Stenersen, 2004). The harmful effect of pesticides on environment and human health demands researches on new harmless means of disease control, such as disease-free seeds, resistant varieties, cultural methods, biological methods and induced resistance. However, the problem of pest management can not be solved by using separate methods. In current years, induced resistance has been contributed to the management of plant diseases (Tuzun and Bent, 2006; Buensanteai et al., 2009 and 2010).

Nowadays, resistance elicitors have been extensively evaluated to control plant diseases based on the induced resistance concept (Sticher et al., 1997; Vallad and Goodman, 2004; Tuzun and Bent, 2006; Walter et al., 2007; Buensanteai et al., 2009 and 2010). There are two important plant defense mechanisms including induced systemic resistance (ISR) and systemic acquired resistance (SAR). ISR relies on jasmonic acid (JA) and ethylene (ET) as signalling (Walter et al., 2007; Martinez-

Medina et al., 2013). SAR requires salicylic acid (SA) as a signal molecule and is associated with production and accumulation of defense enzymes and pathogenesis-related (PR) proteins (Malamy et al., 1990; Vidal et al., 1997; Mandal et al., 2007; Buensanteai et al., 2009 and 2010). The elicitors could be both synthetic or natural compounds and microorganisms (Steiner and Schonbeck, 1995; ; Heil, 2001; Edreva, 2004; Walter et al., 2007; El Hadrami et al., 2011). Then, the induced plant is able to resist pathogens' attack because of an enhanced ability to express defense mechanism rapidly. The induced resistance is a non-specific form of disease resistance in plants acting against a wide range of pathogens (Walter et al., 2007). Most of the reports on the induced resistance were studied with dicotyledonous plants. Considering the economic importance of monocotyledonous crop plants, the number of studies on this order is comparatively small (Steiner and Schonbeck, 1995). In rice, induction of resistance by applying biotic and abiotic inducers has been demonstrated by Smith and Metraux (1991), Steiner and Schonbeck (1995), Du et al. (2001), Nandakumar et al. (2001), Yoshioka et al. (2001), Nakashita et al. (2001, 2002a and 2002b), Babu et al. (2003), Yasuda et al. (2003), Kagale et al. (2004), Saikia et al. (2006), Taheri and Tarighi (2010), Chithrashree et al. (2011), Govindappa et al. (2011), Nahar et al. (2011), Pal et al. (2011), and Shimono et al. (2012). However, these researches have concentrated mainly on rice fungal diseases, and the mechanism that an inducer acts inside a bacterial infected plant has not been fully revealed, and whether or not it can be applied to control bacterial leaf blight disease in rice production system. The induction method would diminish the use of toxic chemicals to bacterial disease control, and can be an alternative, non-toxic and ecologically friendly approach for rice protection and hence for sustainable agriculture (Heil, 2001; Edreva, 2004).

1.2 Research objectives of this study

1.2.1 To evaluate the efficacy of resistance inducers in triggerring induced resistance in rice plant against *Xoo* causing bacterial leaf blight (Chapter III).

First, effect of inducers on rice germination and *Xoo* growth in vitro was assessed. Then, efficacy of the selected inducers on induced resistance in rice against *Xoo* was evaluated under a greenhouse condition. And expression of defense genes of rice plant on defense reaction and pathogenicity genes of *Xoo* on induced resistance by the effective inducer in rice were determined.

1.2.2 To characterize the mechanism of induced resistance in rice plants induced by the effective inducer against *Xoo* (Chapter IV).

Histopathological responses of the induced resistance in rice were examined, and the biochemical changes of lignin, pectin, amide I and lipid were studies, as well as the growth of *Xoo* in treated rice plant was assessed.

1.3 Hypotheses of this study

1.3.1 Resistance inducers could trigger induced resistance in rice plant against *Xoo*.

1.3.1.1 When properly applied, the inducers could effectively control the bacterial leaf blight disease caused by *Xoo*.

1.3.2. The mechanism of induced resistance in rice could be regulated by genes and plant biochemical component changes.

1.3.2.1 The mechanism of induced resistance in rice could be regulated by defense genes. Pathogenicity genes of *Xoo* could be inhibited in rice plant treated with an effective inducer.

1.3.2.2 The mechanism of induced resistance in rice could be expressed as histopathological reactions.

1.3.2.3 The mechanism of induced resistance in rice could lead to changes of lignin, pectin, amide I and lipid.

1.3.2.4 Growth of *Xoo* could be inhibited in treated rice plant.

1.4 Significance and designed routes of this study

14.1 Significance of this study

In current years, environmental and human health problems are the big concerns. In agriculture, use of pesticides is considered an essential factor of disease management. Although pesticides have advantages in controlling pests, their disadvantages are many, such as pesticide poisoning in human and environmental pollution. Induced resistance could be an alternative, non-toxic and ecologically friendly approach for plant protection. This method has many benefits including non-toxicity to plants and animals, non-negative effects on plant growth and development, broad spectrum of defense, low loading amount, long-lasting protection, low economical cost for farmers, and good profit for producers (Tally et al., 1999; Kuc, 2001). In addition, the method of induced resistance has gained acceptance by the public. The first commercialized chemical inducer is BTH (benzo[1, 2, 3] thiadiazole-7-carbothioic acid S-methyl ester), introduced in 1989 by Ciba-Geigy (Novartis) and was given the trade name as BION (in Europe and Viet Nam) and Actigard (in USA) (Tally et al., 1999). However, induced resistance is still a new plant disease management method and lack of commercial products in Asian pesticide market. Thus, this study examined the efficacy of inducers and mechanism of induced resistance

to contribute to the management of bacterial leaf blight disease in rice.

1.4.2 The designed routes of this study

In this study, the efficacy of abiotic inducers, i.e. ascorbic acid (AA), oxalic acid (OA), KH_2PO_4 (PDP), salicylic acid (SA), and biotic inducers i.e. *Bacillus subtilis* CaSUT007 (CaSUT007) and *Bacillus* sp. NT111 (NT111) would be compared.

The study was divided to two main parts including 8 succesive experimental studies as follows:

(1) In Chapter III, firstly, the effect of AA, OA, PDP, SA, CaSUT007 and NT111 on rice grain germination would be studied, and their effects on *Xoo* growth in vitro would be assessed. Then, their efficacies would be evaluated on inducing resistance in rice against *Xoo* under a greenhouse condition. Next, rice defense genes including *apx*, and *pal*, and *Xoo* pathogenicity genes including *hrc*Q, *rpf*F, and *rpf*G genes in rice plants treated with the effective inducer would be examined.

(2) Extending on this, in Chapter IV, the defense mechanism in rice plant treated with the effective inducer would be characterized. Firstly, histhopathological responses of induced resistance would be studied. Secondly, biochemical changes of lignin, pectin, amide I and lipid would be analysed in using FTIR spectrocopy. And the growth of *Xoo* in rice leaves treated with the effective inducer would be assessed.

1.5 Scopes and limitation of the study

1.5.1 Scopes of the study

(1) Six inducers including AA, OA, PDP, SA, CaSUT007 and NT111 were used as resistance inducers.

(2) Rice cultivar cv. KDML105 was used as a representative rice variety.

(3) Experiments were conducted under laboratory and greenhouse conditions.

1.5.2 Limitation of the study

Only the inducer that passed the initial screening was further studied on its mechanism of induced resistance in rice plant.

1.6 Expected results of the study

1.6.1 Effective inducers had ability of triggering defense in rice plants against *Xoo* causing bacterial leaf blight disease.

1.6.1.1 Inducers could effectively control the bacterial leaf blight disease caused by *Xoo*.

1.6.2 Rice genes and plant biochemical component changes could regulate the mechanism of induced resistance in rice.

1.6.2.1 Expression of rice defense genes and pathogenicity genes of *Xoo* could be altered in rice plant treated with the effective inducer.

1.6.2.2 Histopathological responses including superoxide anion and hypersensitive response would be expressed in rice plant treated with the effective inducer.

1.6.2.3 Changes of lignin, pectin, amide I and lipid coud be observed in rice plant treated with the effective inducer.

1.6.2.4 Growth of pathogen *Xoo* could be inhibited in rice plant treated with the effective inducer.

1.7 Reference

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CHAPTER II

LITERATURE REVIEW

2.1 Rice bacterial leaf blight and *Xanthomonas oryzae* pv. *oryzae*

2.1.1 Discovery and distribution

Bacterial leaf blight of rice was discovered by a farmer in Fukuoka, Japan since 1884 (Ou, 1972; Gnanamanickam et al., 1999). According to the revision of the International Code of Nomenclature of Bacteria, the Committee on Taxonomy of Phytopathogenic Bacteria of the International Society of Plant Pathology adopted the name *Xanthomonas campestris* pv. *oryzae* Dye (Mew, 1987). Until 1990, the pathogen has been named *Xanthomonas oryzae* pv. *oryzae* (Swings et al., 1990; Garrity et al., 2004).

Distribution: the disease has frequently caused damage on rice fields of Asia and parts of West Africa (Figure 2.1) (Janse, 2005; EPPO quanrantine pest, 2013).

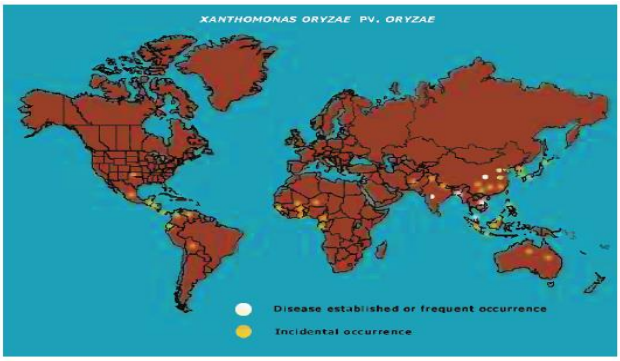


Figure 2.1 Distribution map of *Xoo* causing bacterial leaf blight of rice (*Oryza sativa*)

Source: Janse (2005)

2.1.2 Bacterial leaf blight symptoms and damages

Bacterial leaf blight can typically appear on leaves as pale-green to grey-green water-soaked streaks near the leaf tip and margins. These lesions coalesce and become yellowish-white with wavy edges (Figure 2.2a) (Nino-Liu et al., 2006). Disease symptom of inoculated bacterial leaf blight is shown in Figure 2.2b. The whole leaf may be affected, becomes whitish or greyish and then dies (Figure 2.2c). Leaf sheath of susceptible cultivars may also be attacked. Symptoms are observed easily at tillering stage, disease incidence rises with plant growth, peaking at flowering stage (Ou, 1972; Goto, 1992;Mew et al., 1993). Though leaf blight disease can occur at all growth stages, it is most common from maximum tillering till maturity (Gnanamanickam et al., 1999).

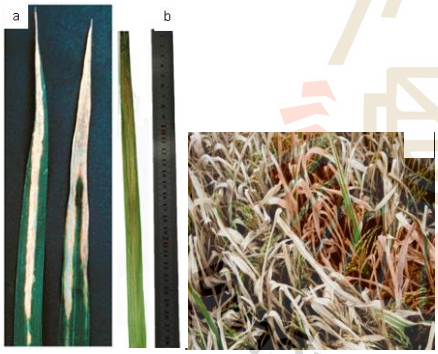


Figure 2.2 Symptoms and damages of BLB in rice plants

(a) Rice leaves with bacterial leaf blight lesions extending along the veins from the leaf tip and margins, resulting from invasion of the xylem via hydathodes by *Xoo* (Source: courtesy of Mew, 1992; as cited by Nino-Liu et al., 2006).

(b) *Xoo* was inoculated into IRBB10 rice at 2 months old at $3x10^8$ cfu ml^{-1} by leaf cutting. Photographs were taken 14 days post inoculation (Li et al., 2011).

(c) A reduction in photosynthetic area on leaves by *Xoo* attack, leading to a decrease on 1000-grain weight (IRRI, 2006).

In the tropic area, with susceptible cultivars of *O. sativa* ssp. *indica*, *Xoo* can cause two other disease syndromes with symptoms distinct from typical bacterial leaf blight as kresek and pale yellow leaf (Nyvall, 1999). Kresek syndrome, a systemic infection, leads to desiccation of leaves and death, especially in young transplanted rice plants (Reddy, 1984). The kresek syndrome was first reported as a distinct disease in Indonesia (Jabeen et al., 2012). Kresek is a seedling blight that occurs shortly after transplanting from nurseries to the field. Broken roots resulting from pulling seedlings off the seedbed serve as major entry points for *Xoo*. Later, that bacteria spread to the growing point of the seedlings by the vascular system; therefore, the base of other leaves is infected, and entire seedlings can die in 2-3 weeks. Some kresek- seedlings can survive, but have a stunted appearance with poor tiller growth and yellowish green colour (Ou, 1985; Goto, 1992). Rice seedlings less than 21 days old are the most susceptible, and temperatures between 28 °C and 34 °C favor kresek development. On the second syndrome, pale yellow leaf is observed in older rice plants or older transplanted rice seedlings and is considered a secondary effect of seedling leaf blight and wilt. Older leaves appear green and healthy, but younger leaves are always pale yellow or whitish, and their tillers do not grow fully (Ou, 1985; Mew et al., 1993).

The *Xoo* infection is affected by sunshine and dryness. The disease is mostly prevalent in areas with monthly rainfall of more than 200 mm. The temperature

for lesion development is approximately 25-30 ^{o}C. Heavy fertilizer application can affect the development of bacterial leaf blight in rice. Among three macronutrients, disease development is most favored by nitrogen (Cha, 1982). The planting density of rice also affects the severity of disease, being less severe under wider spaced conditions at constant nitrogen level (Jabeen et al., 2012). In Japan, bacterial leaf blight damage was recorded approximately 20-30% and as high as 50% (Ou, 1972). Reports from the Philippines, Indonesia and India estimated that losses due to the kresek syndrome of bacterial leaf blight, which affected transplanted seedlings, had reached 60-75%, depending on weather, location and rice variety (Ou, 1985). In Vietnam, the damage of bacterial leaf blight was 20-40% (Te and Man, 1999). In Africa, yield losses caused by *Xoo* ranged from 20% to 30% and could be as high as 50% in some areas (Verdier, 2012).

Post flowering infections have very little effect on grain yield. However, infection occurs during panicle initiation or flowering stage, grain development is severely impaired, resulting in a consequent increase in sterility (Ou, 1985; Goto, 1992; Gnanamanickam et al., 1999).

2.1.3 Morphology and physiology of *Xoo*

Xoo is a yellow, slime-producing, Gram-negative rods, round-ended bacterium. Its cells are capsulated and bacteria motile by a single polar flagellum (Figure 2.3a). Individual cells vary approximately 0.7-2.5 μm (in length) and approximately 0.4-0.7 μm (in width) (Schaad, 1988; Garrity et al., 2004; Agrios, 2005).

The bacterium is obligately aerobic and does not form spores. Optimal temperature for bacterial growth is on the range 25-30 ℃ (Bradbury, 1984). Its cells produce copious capsular extracellular polysaccharides (EPS). EPS importantly

contributes to formation of bacterial droplets or strands on infected leaves, related to rain- and wind-borne dispersal (Ou, 1972).

Its colonies on solid media containing glucose are round, convex, mucoid and yellow in colour due to production of pigment xanthomonadin-a special trait of the genus Xanthomonas (Figure 2.3b) (Schaad, 1988; Garrity et al., 2004).

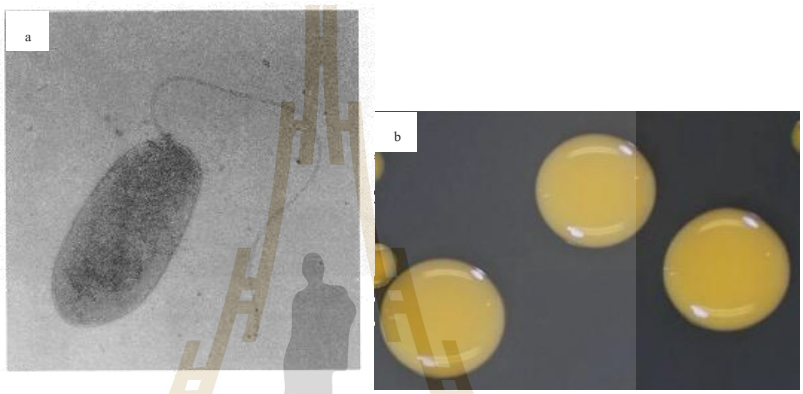


Figure 2.3 Cell morphology and colony of *Xoo*

(a) Cell morphology of *Xoo* (Guevara and Maselli, 1999);

(b) Colony of *Xoo* (Laboratory of Plant Pathology in Kyushu university, 2015).

2.1.4 Modes of pathogen infection

Bacterial pathogen, *Xoo*, typically penetrates rice leaf by hydathodes at the leaf margin (Ou, 1985). Besides, bacterial cells' drops on rice leaf surface can be suspended in guttation fluid as the leaf exudes at night and bacteria enter into the leaf by swimming (Nino-Liu et al., 2006). Moreover, *Xoo* may also gain access to xylem through wounds or openings caused by emerging roots at base of rice leaf sheath (Ou, 1985; EPPO quarantine pest, 2013). The pathogen infect through wounds easier than natural openings. In addition, new wound is more conducive to bacterial infection than

the old one (Gnanamanickam et al., 1999). After going inside leaves, bacteria multiply in intercellular spaces, then enter plant cells and spread into rice plant through its xylem (Figure 2.4b) (Noda and Kaku, 1999; Nino-Liu et al., 2006). Inside xylem, *Xoo* interacts with parenchyma cells (Hilaire et al., 2001). The pathogen not only moves vertically through the leaf through primary veins but also progresses laterally through commissural veins (Figure 2.4a) (Nino-Liu et al., 2006). After a few days, bacterial cells and EPS can fill the xylem vessels, then ooze out from leaf hydathodes, forming bacterial beads or strands on leaf surface. These beads or strands are a characteristic sign of BLB disease and also a source of secondary inoculum (Mew et al., 1993; Gnanamanickam et al., 1999).

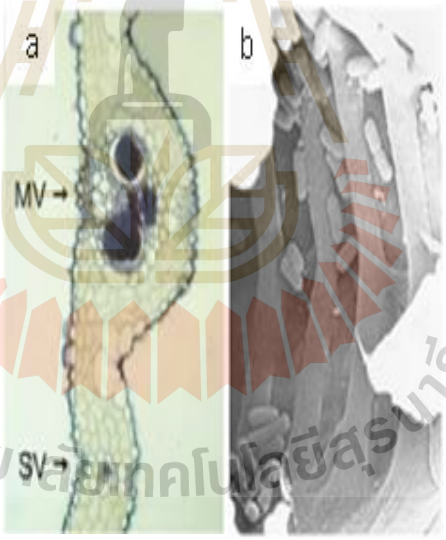


Figure 2.4 Host tissue specificity of *Xoo*

(a) Cross-section of a rice leaf infected with *Xoo*, bacterial cells were stained as dark blue to purple spots inside xylem vessels of midvein (MV) and a secondary vein (SV);

(b) Scanning electron micrograph of *Xoo* cells in a xylem vessel of a rice leaf.
Source: Photos a and b courtesy of Mew; as cited by Nino-Liu et al., 2006

2.1.5 Pathogenicity genes of *Xoo*

Xoo, grouped in Gram-negative plant pathogenic bacteria, relies on the type III secretion system (T3SS) to inject its effector proteins into host cells. The T3SS, which directly translocates effector proteins into the cytosol of host cell, is essential for bacterial pathogenicity (Figure 2.5) (Buttner and Bonas, 2009; Buttner and He, 2009; Dean, 2011).

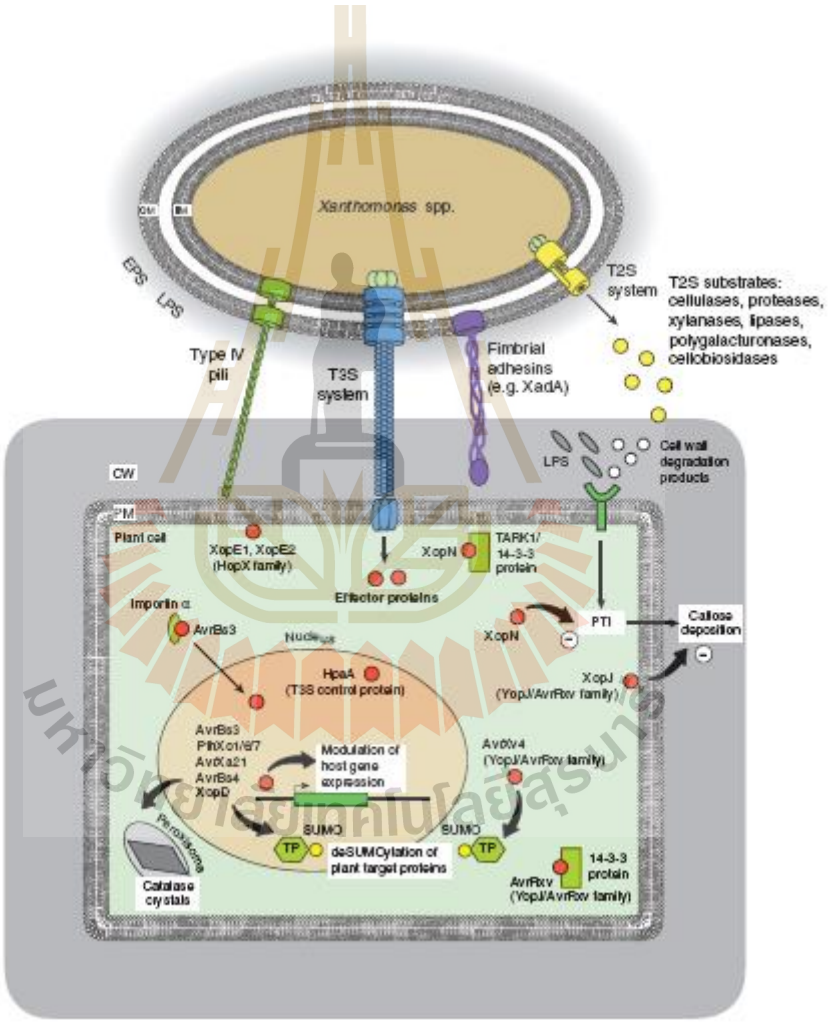


Figure 2.5 Model of known virulence factors from *Xanthomonas* spp. *Xanthomonas* spp. depend on T2S and T3S systems, adhesins, EPS and lipopolysaccharides (LPS) to successfully interact with their host plants.

Source: Buttner and Bonas (2009)

In plant pathogens, the T3SS is encoded by the chromosomal hypersensitive response and pathogenicity (*hrp*) genes (Buttner and Bonas, 2002). Those genes control the secretion and translocation of effectors that are required both for bacterial pathogenicity and induction of hypersensitive response on hosts (Alfano and Collmer, 1997; Grant et al., 2006). The *hrp* genes of Xanthomonas are highly conserved, including nine *hrp* genes, nine hrp-conserved (*hrc*) genes, and eight hrp-associated (*hp*a) genes in *Xoo* (Kim et al., 2003; Salzberg et al., 2008).

The well-conserved genes of regulation of pathogenicity factors (*rpf*), including *rpf*F and *rpf*G, are known to regulate virulence by the cell-cell communication in *Xanthomonas* (Wang et al., 2004; He et al., 2007; Subramoni et al., 2012). In *rpf* virulence regulation system, *rpf*F encodes a putative enoyl-CoA hydratase that catalyzes the synthesis of signal molecule as diffusible signal factor (DSF, cis-11-methyl-2-dodecenoic acid). Extracellular DSF is sensed by a two-component signal transduction system consisting of the sensor protein *rpf*C and response regulator *rpf*G (Barber et al., 1997; Wang et al., 2004; He et al., 2007; Ionescu et al., 2013), The DSF signal is transferred to signal cascades involved in virulence (Slater et al., 2000; He et al., 2007). In addition, *rpf*G is reported to regulate genes involved in synthesis of extracellular enzymes, extracellular polysaccharides (EPS) and biofilm formation (Slater et al., 2000).

All three genes such as *hrc*Q, *rpf*F and *rpf*G are under tight control of quorum sensing (QS) cell-to-cell communication systems (Guo et al., 2011a). The QS system produces extracellular signal molecules called autoinducers (AIs), regulates bacterial cell density and adjusts gene expression (Von Bodman et al., 2003 and 2008; Xu et al., 2015). *Xoo* produces different types of AIs, including the DSF molecule

(Jeong et al., 2008; Zhao et al., 2011). The QS system could play important roles for *Xoo* survival in the intercellular spaces of rice host tissues and cause BLB disease (Tang et al., 1991; Barber et al., 1997; Slater et al., 2000; Chatterjee and Sonti, 2002; Torres et al., 2007; Guo et al., 2011b; Zhang et al., 2013; Xu et al., 2015).

2.1.6 Sources of primary inoculum, dissemination and survival

Potential inoculum sources can infect planting materials, volunteer rice plants (Durgapal, 1985), infected straw or chaff (Devadath and Dath, 1985).

Hosts of *Xoo* are several species of wild rice including *O. sativa*, *O. rufipogon* and *O. australiensis*, and some gramineous weeds including *Leersia oryzoides*, *Zizania latifolia*, *Leptochloa* spp. and *Cyperus* spp. (Moffett and Croft, 1983).

Outbreaks of bacterial leaf blight are more likely to occur in rainy season than at other times of the year of south-east Asian and Indian Oceans (Mew et al., 1993). Wind and rain disseminate bacteria from infected rice plants and other hosts, as well as contaminate rice stubble from previous crop seasons - the most important sources of primary inoculum. Bacteria are also disseminated by irrigation water (Nyvall, 1999), as well as by humans, insects and birds (Ou, 1985; Nyvall, 1999).

2.2 Control of bacterial leaf blight disease

BLB disease management concentrates on reducing the initial inoculum and subsequent development of the pathogen *Xoo* on rice plants. These targets can be accomplished through the use of chemicals, cultural methods, disease resistant cultivars, biological controls and induced resistance.

2.2.1 Chemical control

The chemicals usually function by either killing or inhibiting multiplication

of the pathogen *Xoo* through blocking an important metabolic pathway. In the past, BLB was controlled by chemicals such as Bordeaux mixture with or without sugar, copper-soap mixture, and copper-mercury fungicides. Similarly, spraying copper oxychloride was recommended to control rice BLB disease. Besides, other organic bactericides such as dithianone, dithiocarbamate, nickel dimethyl, phenazine and phenazine N-oxide were also used. Moreover, chlorination of the field water with stable bleaching powder also effectively reduced disease severity of BLB. Dithiocarbamate fungicides were reported to inhibit the growth of *Xoo* by arresting fatty acid and lipid biosynthesis (Gnanamanickam, 2009). The use of chemicals to control bacterial diseases has been, generally, less successful than that of fungal diseases. Of the chemicals used as foliar sprays, copper compounds gave the best results. However, they seldom gave a good control of the BLB disease when environmental conditions favor development and spread of the pathogen. Bacterial strains resistant to copper fungicides, however, are quite common (Agrios, 2005). Later on, zineb, maneb or mancozeb mixed with copper compounds were used for the same purpose, especially in young plants that may be injured by the copper compounds. In addition to organic pesticides and copper compounds, antibiotics such as kasugamycin, oxytetracyclin and streptomycin have also been used against BLB diseases (Agrios, 2005; Janse, 2005). Some antibiotics could be absorbed by the plant and could systemically distribute. Formulations of streptomycin or of streptomycin and oxytetracyclin are considered the most important antibacterial antibiotics in agriculture. Unfortunately, bacterial strains resistant to antibiotics develop soon after widespread application of antibiotics. Moreover, no antibiotics is permitted on edible rice produce (Agrios, 2005). At present, flumequin, fosetyl-aluminium, 7-chloro-1-ethyl-6-fluoro-1,4-dihydro-4-exo-3-quinoline carboxylic acid and oxolinic acid can be used to control BLB disease (Janse, 2005).

Until now, an effective, economical and ecological control is yet to be introduced for BLB disease. This may be because the pathogen population is highly variable in its sensitivity to the chemicals used for disease control. The existence and development of chemical-resistant bacterial strains also pose serious problems in formulating control agents (Gnanamanickam, 2009).

2.2.2 Cultural control

A combination of cultural controls is required to combat BLB disease. Infestation of fields or infection of crops with *Xoo* should be avoided by using healthy rice seeds. Sanitation practices such as removing and burning infected straw, and decontaminating tools are very important to reduce inoculum of *Xoo* in a field, to limit spread of the bacteria. Treating rice seed with hot water at 52 °C for 20 minutes often considerably reduces the quantity of infected seeds. Adjusting fertilizing and watering could help rice plants not to extremely succulent during the period of infection (Agrios, 2005; Janse, 2005).

2.2.3. Disease resistant cultivars

Planting resistant rice cultivars has been the major method of BLB management (Reissig et al., 1986; Janse, 2005). Wild rice species represent natural source of resistance to their pathogens. Untill now, twenty-three major BLB resistance genes have been identified (Mew et al., 1992; Zhang et al., 1998). However, only two of which, namely Xa1 and Xa21, were cloned from rice (Song et al., 1995). In rice, breeding programs at IRRI and national rice improvement programs in the Philippines, Indonesia and India, resistance genes including Xa4, xa5, Xa7 and Xa21 were targeted for transfer to commercially important rice varieties (Nelson et al., 1996). Xa21 was also transferred by IRRI scientists from the wild rice species, *Oryza longistaminata*, into a

cultivated indica varieties including IR24, IR72, MH63, and IR51500 (Song et al., 1995; Datta, 2002).

In rice, single-gene resistance has been the first mean of control of BLB, but unfortunately, because of using rice plants having single-gene resistance in large-scale usage continuously, the new virulence pattern of the *Xoo* strains occurred, leading to breakdown of resistance (Mew et al., 1992). For instance, the highly resistant BLB locus, Xa21, was found to confer resistance to *Xoo* in India and the Philippines (Ikeda et al., 1990; Khush et al., 1990). In India, a sub-population of *Xoo* virulence to rice line containing gene Xa21 was isolated during a BLB epidemic that occurred in 1998 (Venkatesan and Gnanamanickam, 1999). Other studies have shown that *Xoo* virulent on gene Xa21 present in rice line in Nepal (Adhikari et al., 1999). Therefore, pyramiding of multiple resistance genes into rice varieties is one way to delay the virulence shifts (Kinoshita, 1995; Mackill and Ni, 2001). Many scientists have started to pyramid lines with different R-gene combinations. Four resistance genes including Xa4, xa5, xa13 and Xa21, were pyramided using PCR-based markers. This four-gene pyramid was found to be effective against much of the population of *Xoo* (Huang et al., 1997).

2.2.4 Biological control

The severity of losses incurred due to the BLB disease necessitates the development of strategies that are ecology-conscious and cost effective. Bacterial biocontrol agents were studied on bacterial leaf blight disease of rice including *Bacillus* spp.; *B. lentus*; *B. cereus*; *B. circulans*, and *Pseudomonas fluorescens* (Velusamy and Gnanamanickam, 2003), *B. polymyxa, B. subtilus, Burkholderia glumae, P. aeruginosa, P. fluorescens, Stenotrophomonas maltophilia, Streptomyces mutabilis* (Janse, 2005), and *Trichoderma virens* (Mukherjee et al., 2012). Key to consistent good performance by the beneficial bacteria in controlling *Xoo* appears to be

bacterial population size that is introduced by bacterial treatments and maintained throughout the critical phases of rice growth.

2.2.5 Induced resistance

Plant itself has defense systems that can be induced following infection by diverse microorganisms (biotic agents) with a variety of pathogenic traits (El Hadrami et al., 2011) or abiotic agents (Walter et al., 2007).

Besides using biotic inducers, some abiotic ones have been used to induce the resistance in rice plant against many kinds of pathogen. Exogenous SA leads to accumulation of hydrogen peroxide in rice leaves. Superoxide dismutase activity, which generates H_2O_2, did not show any change, but the H_2O_2 degrading enzyme peroxidase was inhibited (Ganesan and Thomas, 2001). Untill 2013, gene Oscyp7172 was discovered to play an important role in bacterial leaf blight resistance by regulating the diterpenoid phytoalexin biosynthesis and H_2O_2 generation (Li et al., 2013).

In another research, BLB severity in the rice plants pretreated with acibenzolar-S-methyl (ASM), a plant defense inducer, was significantly reduced, compared to the untreated control. Besides, induced rice plants increased phenolic content and pathogenesis-related (PR) proteins such as β-1,3-glucanase, chitinase and thaumatin-like protein, belongs to PR 5 group. Efficacy of induced resistance could persist for up to 3 days in induced rice plants (Babu et al., 2003).

Furthermore, some abiotic inducers from plant extracts were studied on induced resistance. The leaf extracts of *Datura metel* significantly reduced in vitro growth of *Xoo*. Its methanol extract exhibited the best control of pathogens approximately 10-35% more toxicity than aqueous one. Foliar application of leaf extracts effectively reduced the incidence of BLB diseases of rice under greenhouse condition. Pre-inoculation application of leaf extract was found better than post-

inoculation application. Induction of induced resistance as evident from the increased accumulation of PR proteins and other defense related compounds was observed in induced rice plants (Kagale et al., 2004).

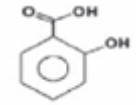
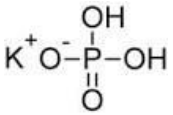
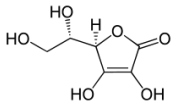
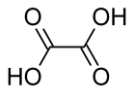
Sphaeropsidin A, the main phytotoxin produced by *Diplodia cupressi*, had a specific activity against *Xoo*. The antibacterial activity of this chemical was the presence of C-7 carbonyl group, C-13 vinyl group, double bond of ring C, tertiary C-9 hydroxy group, hemiketalic lactone functionality and primarane arrangement of the tricylic carbon skeleton (Evidente et al., 2011).

In another research on controling bacterial leaf blight in rice, Nisha et al. (2012) surveyed the efficacy of extracts of four plants including *Azardiracta indica*, *Ages mermelos*, *Cassia auriculata* and *Vitex negundo* to bacterial leaf blight disease. The extract of *Vitex negundo* induced PR protein bands at 33 kDa and 14 kDa as well as defense related enzymes including polyphenol oxidase, peroxidase and β-1,3-glucanase. The expression of high PR protein levels and enzyme activities proved that the extract of *Vitex negundo* acted as a resistance inducer to BLB disease rather than antagonism to *Xoo*.

2.3 Some characteristics of resistance inducers

Inducers are very stable molecules that induce an immune defense response in plants. They have low molecular weight and synthesized as such or released from polymeric precursors during infection (Holopainen et al., 2009). In another way, inducers for a plant refers to chemicals from various resources that can trigger physiological and morphological reactions (Mejia-Teniente et al., 2010). The inducers used in the study are listed in Table 2.1

Table 2.1 Inducers known to induce disease resistance in plants

Inducer	Structure	Plant	Disease name	Reference
Salicylic acid (SA)		Rice	Blast	Du et al. (2001); Daw et al. (2008)
		Rice	Abiotic stress	Wang et al. (2009); Li and Zhang (2012)
Potassium dihydrogen phosphate (PDP)		Rice	Blast	Mananhar et al. (1998)
Ascorbic acid (AA)		Rice	Stress	Tokunaga and Esaka (2007)
		Rice	Salt stress	Athar et al. (2008)
Oxalic acid (OA)		Rice	Blast	Du et al. (2001)
		Oil seed rape	*Sclerotinia sclerotiorum*	Toal and Jones (1999)
		Cucumber	Anthracnose	Descalzo et al. (1990)
Bacillus spp.		Rice	BLB	Velusamy and Gnanamanickam (2003); Velusamy et al., (2004 and 2006); Chithrashree et al. (2011)

2.3.1 Salicylic acid (SA)

Salicylic acid ($C_7H_6O_3$, synonym 2-hydroxybenzoic acid) is a white, odourless crystalline form, pH 2.4 at 20 ^oC, melting point approximately 158-161 ^oC. Molecular weight: 138.12 g mol^{-1}. LD50 (oral, rat) is 891 mg kg^{-1} (somnolence - general depressed activity, muscle weakness). LC50 (inhalation, rat) is 1 h, > 900 mg per m^3, LD50 (dermal, rabbit) is > 10.000 mg kg^{-1}. Toxicity to fish, LC50 of *Leuciscus idus* (Golden orfe), is 90 mg l^{-1}. Toxicity to daphnia and other aquatic invertebrates, EC50 of *Daphnia magna* (water flea), is 105 mg l^{-1}, 24 h (Sigma-Aldrich, 2012a).

SA is a phenolic derivative, distributed in a wide range of plant species. It is a natural product of phenylpropanoid metabolism. Chemically, SA contains an aromatic ring with a hydroxyl group (Figure 2.6 and Figure 2.7). It is moderately soluble in water but highly soluble in polar organic solvents. The pH of its aqueous solution is 2.4; pKa is 2.98 and log Kow is equal to 2.26. Until now, SA has been characterized in 36 plants including barley, crabgrass, rice and soybean, with the level approximately 1 mg g^{-1} fresh mass. Nonetheless, a maximum quantity of 37.19 mg g^{-1} fresh mass is detected in the leaves of rice. The direct roles of SA are related to enhancement of chlorophyll and carotenoid pigments, flower induction, modifying activity of some important enzymes, photosynthetic rate, plant growth, signal, thermogenesis and ion uptake. It has direct functions on effect of ABA on leaf abscission, ethylene biosynthesis, and stomatal movement (Figure 2.8 and Figure 2.9) (Hayat et al., 2007).

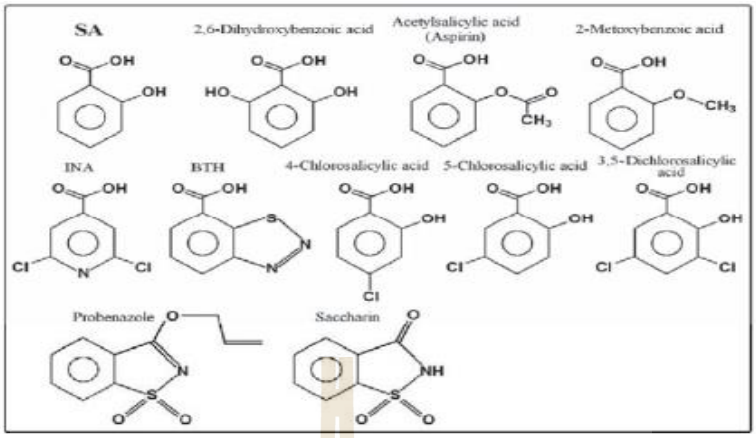


Figure 2.6 Active analogs of SA

Note: INA (2,6-dichloroisonicotinic acid); BTH (7-Carboxybenzothiadiazole)

Source: Kawano and Furuichi (2007)

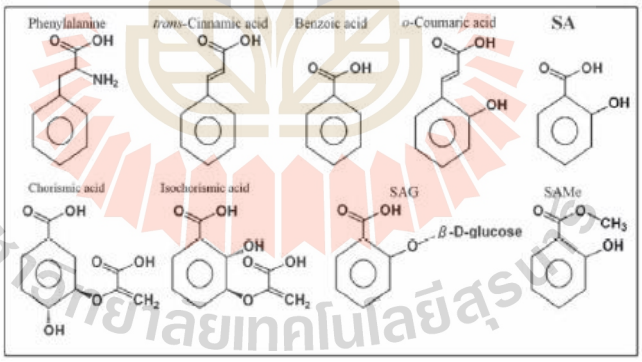


Figure 2.7 Precursors and storage forms of SA in plants

Note: SAG (salicylic acid glucoside), SAMe (methyl SA)

Source: Kawano and Furuichi (2007)

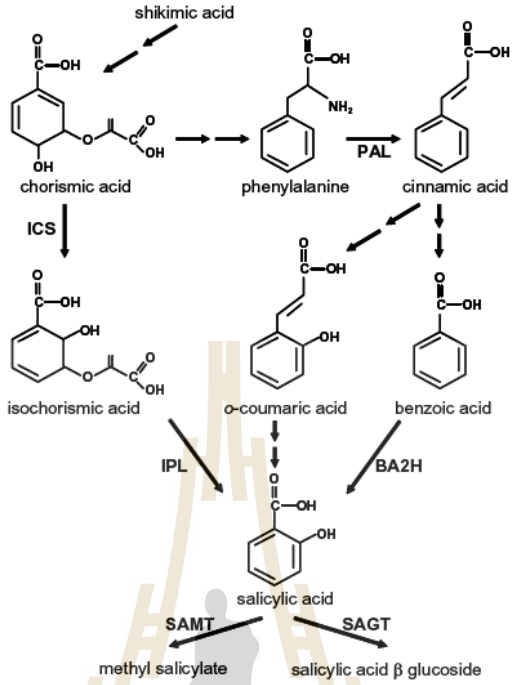


Figure 2.8 Salicylic acid biosynthesis pathways in plants

Source: Chaturvedi and Shah (2007)

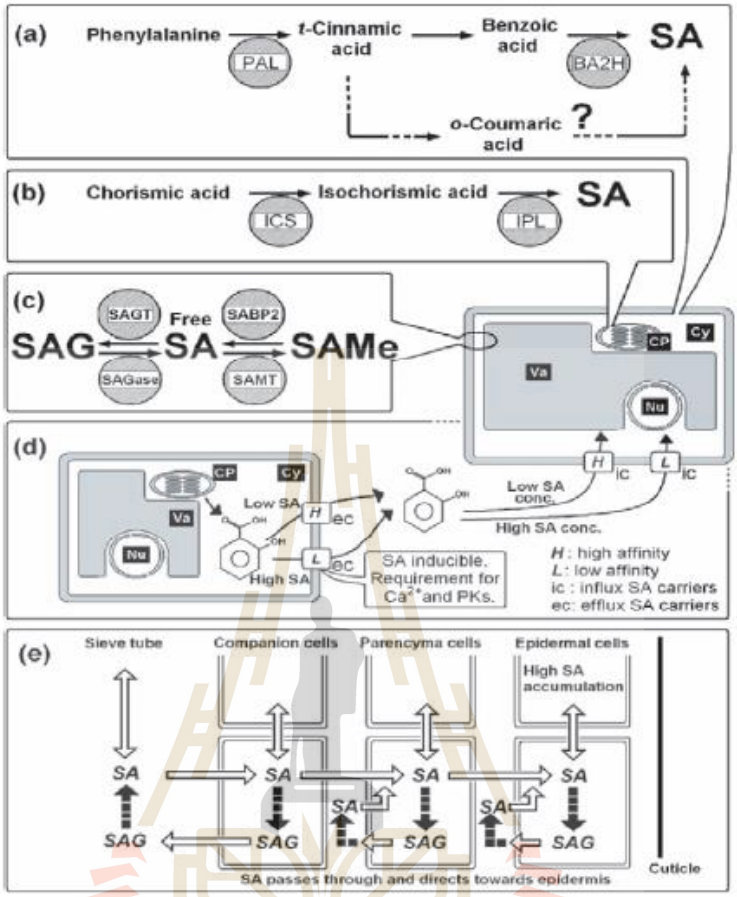


Figure 2.9 Biosynthesis and translocation of SA. (a) Typical SA biosynthetic path found in the cytosol of tobacco, cucumbers and other plants, involving PAL and BA2H as key enzymes. (b) Novel SA biosynthetic path found mainly in the chloroplasts of *Arabidopsis thaliana* involving ICS and IPL as key enzymes. (c) Inter-conversions between SA and SAG, and SA and SAMe. SAGase, SAGT, SABP2, and SAMT are the enzymes involved in SAMe-SA-SAG inter-conversions. (d) Movement of SA at cellular level. (e) Movement of SA at tissue level.

Source: Kawano and Furuichi (2007)

Researches involving SA in induced resistance in plants have been reported. Du et al. (2001) demonstrated that exogenous SA application at 1.0 mM concentration induced rice plant against blast disease approximately 27%. Similar results were obtained by Daw et al. (2008) using 8 mM SA, which induced resistance in susceptible rice plant against blast disease. Other authors (Wang et al., 2009; Li and Zhang, 2012) observed that the exogenous SA application at difference concentration of 0.5, 1 and 2 mM reduced the abiotic stress in rice plants.

2.3.2 Potassium dihydrogen phosphate (PDP)

Potassium dihydrogen phosphate (KH_2PO_4, synonym potassium phosphate, monopotassium phosphate) is a white crystalline form, melting point approximately 252.6 ^oC. Molecular weight is 136.09 g mol^{-1}. LD50 (dermal, rabbit) is > 4.640 mg kg^{-1} (Sigma-Aldrich, 2012b) (Figure 2.10).

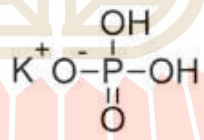


Figure 2.10 Molecular structure of KH_2PO_4

Source: http://sci9bestq3bm.wikispaces.com/Monopotassium+Phosphate (2015)

KH_2PO_4 has been used as an inducer in some plants. Spraying KH_2PO_4 at concentration from 1 to 4 mM helped tomato and potato plants resistant to many kinds of fungal diseases (Kessmann et al., 1994), as well as in corn, grapevine and cucumber (Mananhar et al., 1998).

2.3.3 Ascorbic acid (AA)

Ascorbic acid ($C_6H_8O_6$, synonym antiscorbutic factor, L-Threoascorbic acid, Vitamin C), pH 1.0-2.5 at 176 g l^{-1} at 25 ^oC, melting point 190-194 ^oC. Molecular weight is 176.12 g mol^{-1}. LD50 (oral, rat) is 11.900 mg kg^{-1} (sense organs and special senses on nose, eye, ear and taste, somnolence, diarrhoea) (Sigma-Aldrich, 2012c) (Figure 2.11).

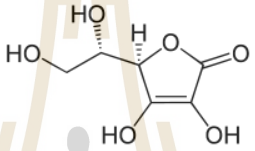


Figure 2.11 Molecular structure of L-Ascorbic acid

Source: http://en.wikipedia.org/wiki/Ascorbic_acid (2015)

This chemical has been researched on induced resistance. External addition of 20 mM AsA induced some stress-related genes and OsXIP xylase inhibitor protein (OsXIP). OsXIP was suggested to be specialized in their function and involved in defense mechanisms in rice (Tokunaga and Esaka, 2007). However, in another research on efficacy of seed preconditioning with SA and AA in increasing vigor of rice (*Oryza sativa* L.) seedling, Anwar et al. (2013) used treatment with AA at two concentrations including 10 and 20 mg l^{-1}. The results showed that a significant enhancement at 6% was recorded in seedling protease activities. Moreover, roots applied with exogenously applied AA at concentration of 50 and 100 mg l^{-1} counteracted the adverse effects of salt stress on growth of wheat by improving

photosynthesis capacity of wheat plant against salt-induced oxidative stress and maintaining ion homeostasis (Athar et al., 2008).

2.3.4 Oxalic acid (OA)

Oxalic acid ($C_2H_2O_4$, synonym 2-hydroxybenzoic acid) is a white, odourless crystalline form, pH 1.3 at 9 g l^{-1}, melting point approximately 189.5 ^{o}C. Molecular weight is 90.03 g mol^{-1}. LD50 (oral, female rat) is 1.080 mg kg^{-1}. Toxicity to fish is LC50 of *Leuciscus idus* (golden orfe) is 160 mg l^{-1}, 48 h. Toxicity to daphnia and other aquatic invertebrates, EC50 of *Daphnia magna* (water flea), is 162.3 mg l^{-1}, 48 h (Sigma-Aldrich, 2012d) (Figure 2.12).

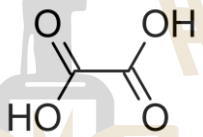


Figure 2.12 Molecular structure of oxalic acid

Source: http://commons.wikimedia.org/wiki/File:Oxalic_acid.png (2015)

Research results on induced resistance with OA have been reported. Effect of 50 concentrations of OA from 1 to 50 mM to rice seed germination of the rice variety OMCS2000 has been studied by Du et al. (2001). The results showed that all concentrations of OA did not affect rice seed germination. These authors also reported that spraying OA at concentration of 0.5, 1 and 2 mM or combination of OA and natri tetraborate reduced rice blast effectively. OA was also effective in inducing systemic resistance to *Sclerotinia sclerotiorum* in oil seed rape (Toal and Jones,

1999). In addition, 0.05 M OA also induced resistance in cucumber against anthracnose (Descalzo et al., 1990).

2.3.5 *Bacillus* spp.

Seed treatment with commercial formulation of a bioagent of *Bacillus subtilis* MBI 60 significantly increased lengths of shoot and root under in vitro conditions. Seed treatment with 2.20 x 10^8 and 2.20 x 10^9 cfu ml^{-1} significantly increased seedling emergence approximately 81-89%, compared to that of 2.20 x 10^6 and 2.20 x 10^7 cfu ml^{-1} (Kumar et al., 2011). In another research, Chithrashree et al. (2011) evaluated seven *Bacillus* spp. with the cell populations of 10^8 cfu ml^{-1} for growth promotion and induced resistance in rice against *Xoo*. The results showed that PGPR strains used as fresh solution and powdered formulations had good potential in plant growth promotion and in BLB management.

In 516 distinct rice-associated *Bacillus* strains isolated from rhizosphere samples, only 42 strains showed antagonistic ability to *Xoo* in vitro. The zone of inhibition caused by these strains varied from 1.2 to 4.0 cm in diameter. In 637 strains of fluorescent bacteria, 278 strains showed inhibition of *Xoo* in laboratory assays. Twenty seven of the antagonists produced 2,4-diacetylphloroglucinol (DAPG) and had the characteristic 745-bp fragment amplified by the PCR reaction when the sequence specific primers developed from the PhlD sequence of *P. fluorescens* Q2-67 were used (Velusamy and Gnanamanickam, 2003). One of the Bacillus strains as ALP 18 produced a heat-resistant at 121 ^oC and pronase-resistant metabolite in culture fluids. The crystalline product of this substance was produced at the rate of 1.6 mg ml^{-1}, and when amended into peptone sucrose agar (PSA), it could inhibit the growth of *Xoo*. A careful analysis of its physical properties through FTIR, 1H NMR, and 13C

NMR analyses suggested that the heat-resistant antibacterial metabolite is kanosamine. Kanosamine production has been reported earlier from other *Bacillus* spp. (Milner et al., 1996). Moreover, production of broad spectrum antibiotics such as DAPG is now known to be involved in the biological suppression of *Xoo* (Velusamy et al., 2004; 2006). DAPG is a polyketide antibiotic was known for its key role in "take-all decline" in the United States and for suppression of diseases of tobacco and other crops, including BLB in rice (Raaijmakers and Weller, 1998; Bangera and Thomashow, 1999; Velusamy et al., 2006). Since this molecule can also trigger the induction of ISR in the host at microgram levels, bacterial population size appears to be of lesser importance. This aspect, however, remains to be fully clarified. Isolation and purification of the metabolites produced by the Bacillus strains showed production of 3.9-5.5 μg ml^{-1} of IAA by the different Bacillus strains. Some of them also produced GA3-like substance (Lindow et al., 1998).

2.3.6 Cost of induced resistance on agricultural cultivation

Cost of induced resistance could be affected by rice genotype, environment and plant nutrition (Kover and Schaal, 2002; Walters et al., 2005). The activation of rice defense machinery requires a reallocation of plant resources including defense-related genes (Bowling et al., 1994; Heil and Baldwin, 2002). Following an priming treatment, possible outcomes could differently express in terms of disease resistance expression, as following: (i) defense mechanism is triggered and there is no further change in the defense expression levels following pathogen challenge, (ii) defense mechanism is triggered and there is a further increase in the defense expression levels or an activation of a different set of defense responses

following pathogen challenge, and (iii) defense mechanism is not expressed until pathogen challenge has occurred (Walters and Boyle, 2005; Walters et al., 2005).

2.4 Mechanism of defense resistance against bacterial leaf blight disease in rice

Naturally, the basic defense in plant is weak. This basic defense can occur naturally or be caused by pathogen infection, stresses or herbivore attack (Heine, 2008; Pareek et al., 2010; Ahmad and Prasad, 2012). There are two ways of creating artificially disease resistance in plants. First, R genes to a virulent isolate of a plant pathogen are identified on natural sources of organism, isolated and transfered to target cultivated plants, using biotechnological methods. The cultivated plants containing R genes show high level of disease resistance against one or several isolates of plant pathogen (Loebenstein and Carr, 2006; Tuzun and Bent, 2006; Parker, 2009). Second, the susceptible plants can be induced using resistance inducers, leading to an activation of an array of defense-related genes, leading to many layers of defense responses of susceptible plants, which is called induced resistance. The induced plants show lower level of disease resistance, but against a wide range of plant pathogens (Punja, 2004; Walters et al., 2007; Parker, 2009). Induced resistance with inducers will be researched in this study.

In case of rice bacterial infection process, pre-entry, entry and colonization are three steps (Figure 2.13) (Gnanamanickam, 1999; Jeger and Spencer, 2001; Agrios, 2005). Protection from a pathogen's initial invasion is achieved via passively physical and/or chemical barriers which are shown on the left side of Figure 2.13 (Agrios, 2005).

+ Physical barriers are mainly resulted in characteristics of plant surface, including cuticle, stomata and cell walls. Presence of secondary cell wall and size of stomatal pores affect the successful ratio with which a bacterium invades a host. Vertical orientation of leaves limit formation of moisture films of leaf surfaces, inhibit infection by pathogens reliant on water for motility (Agrios, 2005).

+ Chemical barriers including phytoanticipins, phenols, quinones, inhibiting compounds, lactones, cyanogenic glucosides, saponins, terpenoids, stilbenes and tannins, have antimicrobial activity (Agrios, 2005).

If the pathogen can overcome the initial barriers, the plant continues to create active defense reactions (Figure 2.13) (Agrios, 2005).

+ First is pathogen recognition. Firstly, OsRac GTPase is required for pathogen recognition. The Rac GTPase family belongs to the Rac superfamily of small GTPases. Members of this superfamily process GTPase activity which is used for activation of protein kinases (MAPK cascades). In plants, Rac GTPases serve diverse functions in many important cellular activities, including polar growth, cell differentiation and stress responses. In rice, seven genes encoding Rac GTPases have been characterized (Chen and Ronald, 2011). Next, MAPK cascades play important roles in transmission of extracellular signals to downstream components through protein phosphorylation. A MAPK cascade consists of three kinases such as a MAPK, a MAPK kinase (MAPKK) and a MAPKK kinase (MAPKKK). Seventeen MAPKs have been identified in rice (Chen and Ronald, 2011). Following pathogen recognition and signal transduction, defense responses are activated that protect plants from infection. These following responses include rapid and delayed active defenses.

+ Rapid active defenses happen at the membrane including fluxes of ions K^+, H^+ and Ca^{2+}; the oxidative burst triggered signals; at the cell wall including reinforcement of the cell wall. Besides, hosts employ hypersensitive cell death to prevent the spread of pathogens. Moreover**,** antibiotic compounds including phytoalexins create a toxic micro-environment in the infected cell and, hopefully, leading to prevent disease establishment (Nooden, 2004; Agrios, 2005; Walter et al., 2007).

+ Delayed active defenses including the containment of the pathogen, wound repair, the acquisition of induced resistance and the expression of pathogenesis-related (PR) proteins. Physical reactions including cork cells, gums and tyloses can protect the plant from further infection. In the case of induced resistance, SA interact with SA-binding proteins, resulting in formation of reactive oxygen species or activation of gene expression. The level of SA increases around necrotic lesions and remain high in plants having acquired resistance. The SA itself also acts as a signal which is translocated systemically throughout the plant. Besides, there are many other defense respones including induced systemic resistance which jasmonic acid and ethylene play the role key; brassinolide (Nakashita et al., 2003). These pathways of induced resistance can enhance their defense efficacy (Agrios, 2005; Walter et al., 2007). At last, there are an accumulation of phytoalexins and an expression of PR proteins. Phytoalexins can create a toxical environment to limit pathogen development. PR proteins can disrupt pathogen nutrition. Based on amino acid sequence, serological relationship and enzymatic activities, PR proteins are classified into 17 groups including PR1 to PR17. In rice, only a few groups of PR genes, including PR1, PR8 and PR10, has been reported to be induced following bacterial or fungal infections (Chen and Ronald, 2011).

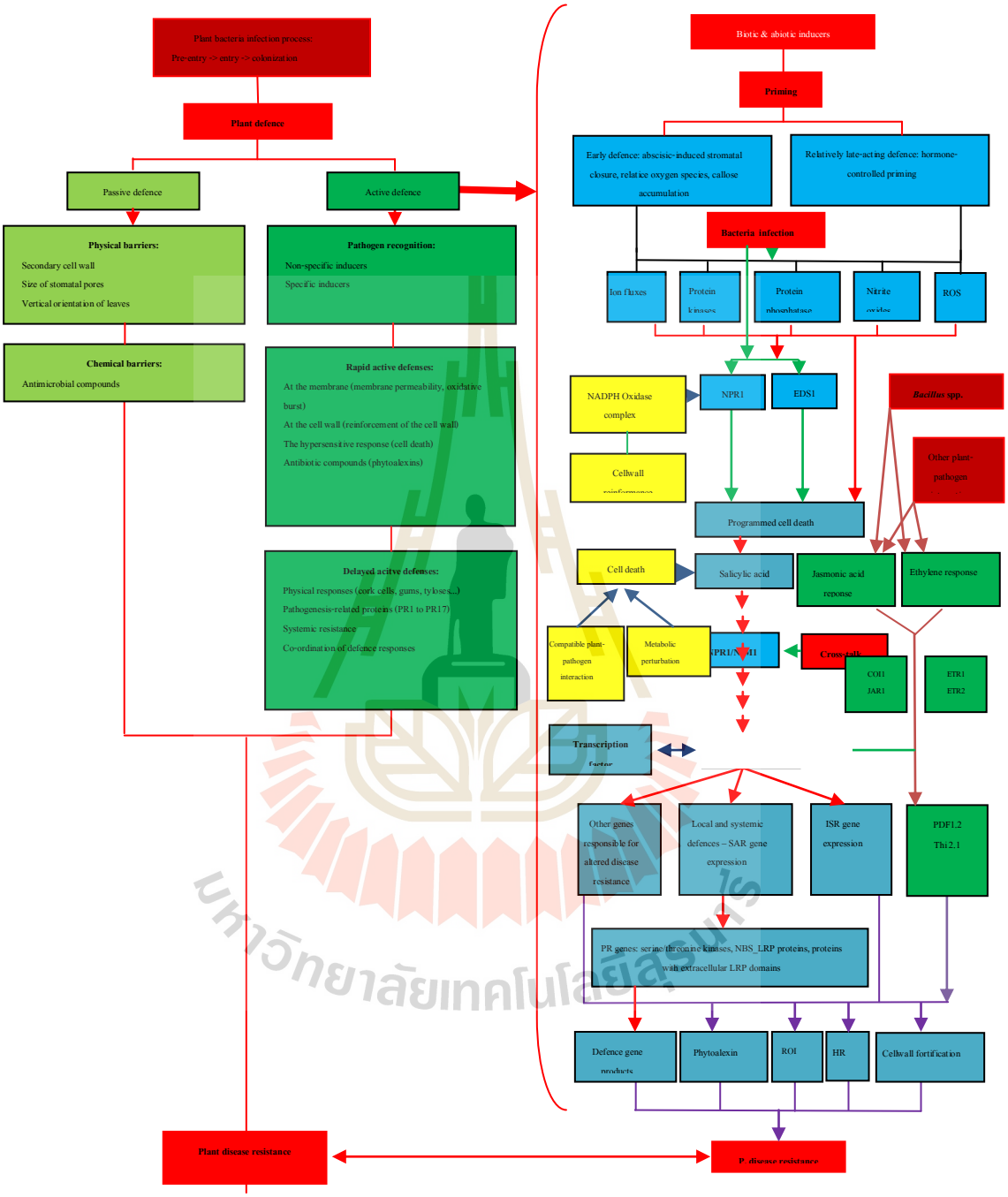


Figure 2.13 General pathway of disease resistance to bacterial pathogen in plant Modified from sources: Scheel and Wasternack (2002); Pastor et al. (2013)

The active defense can be described in details on the right side of Figure 2.13. In plant, induced defense mechanism is a complex phenomenon relating multiple defense layers that become active at different stages of pathogen attack.

Priming or elicitation competency is a phenomenon that elicits primed plants to express defense mechanism. Then, when an attack by pathogen or abiotic stress occurs, cellular defense responses of primed plants display both early or late-acting defense responses faster or stronger (Conrath et al., 2006). Late-acting defense responses are involved in hormone activity in plant including SA and absicic acid. Early acting defense responses occur within the first few hours of pathogen contact and express as an oxidative burst (Lamb and Dixon, 1997; Bestwick et al., 1998), rapid accumulation of reactive oxygen species (ROS), abcisic-induced stromatal closure (Pastor et al., 2013), dityrosine cross-linking of tyrosine-rich proteins in the wall (Bradley et al., 1992), increased levels of phenolic compounds associated with wall fortification (Nicholson and Hammerschmidt, 1992), and redistribution of phospholipase D in the plasma membrane to the site of attack (Young et al., 1995), callose synthesis and deposition of a callose-rich papilla between the cell wall and the plasma membrane (Brown et al., 1993; Pastor et al., 2013). The mechanism of early defense responses is based on the activities of ion fluxes, ROS, nitrite oxides, protein kinases and protein phosphatase. Ion fluxes are Ca^{2+} and H^{+} influx, K^{+} and Cl^{-} exflux (Figure 2.14). These ion fluxes lead to activation of a MAP kinase specific to defense response, oxidative burst and subsequent induction of defense response. ROS is not only have direct toxicity to pathogens, but also is the central component of plant defense signal transduction pathways leading to the hypersensitive response (HR), cell wall reinforcement and defense gene expression (Pastor et al., 2013) (Figure

2.15). Nitrite oxide enhances the ROS-mediated induction of hypersensitive cell death (Lamattina and Polacco, 2007; Pastor et al., 2013) (Figure 2.16). GTP-binding proteins and protein phosphorylation / dephosphorylation are involved in transfering signals from receptors to down-stream pathways in defense signal transduction (Pastor et al., 2013) (Figure 2.17). Later, infection of bacteria itself also enhances these activities (Figure 2.18). All of them lead to programmed cell death (Scheel and Wasternack, 2002).

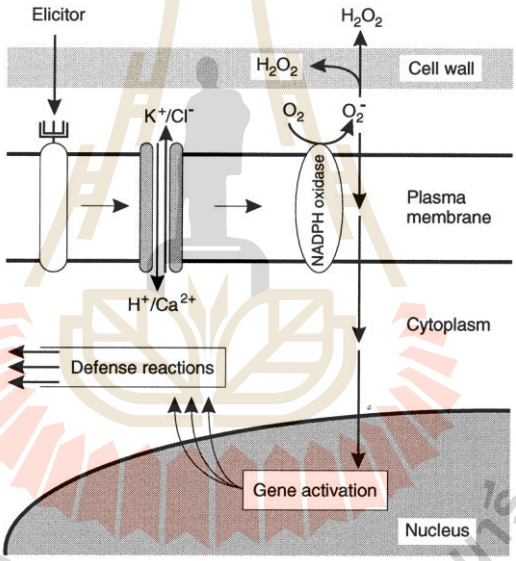


Figure 2.14 Hypothetical scheme for inducer-induced signal transduction

Source: Jabs et al. (1997)

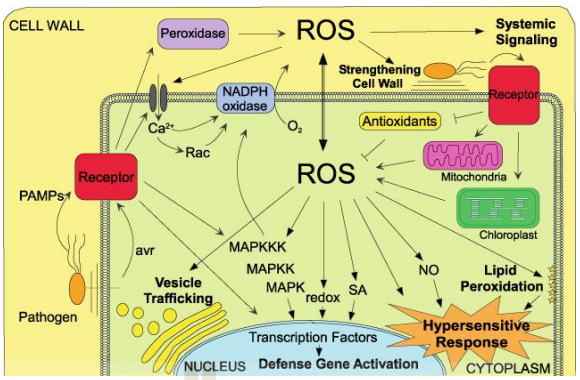


Figure 2.15 Roles of ROS in plants

Source: Graves (2012)

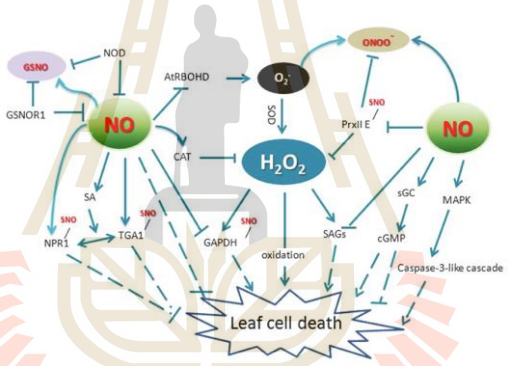


Figure 2.16 Roles of NO in leaf cell death. AtRBOHD-an NADPH oxidase; CAT - catalase; cGMP-cyclic guanosine monophosphate; GAPDH-glyceraldehyde 3-phosphate dehydrogenase; GSNO-S-nitrosoglutathione; GSNOR1- S-nitrosoglutathione reductase 1; MAPK-mitogen-activated protein kinase; NOD-NO degrading dioxygenase; NPR1- non-expression of PR protein 1; NR-nitrate reductase; PrxII E- peroxiredoxin II E; SAG-senescence-associated genes; sGC-soluble guanylate cyclase; SOD-superoxide dismutase; sGC-soluble guanylate cyclase TGA1-TGACG motif binding factor 1.

Source: Wang et al. (2013)

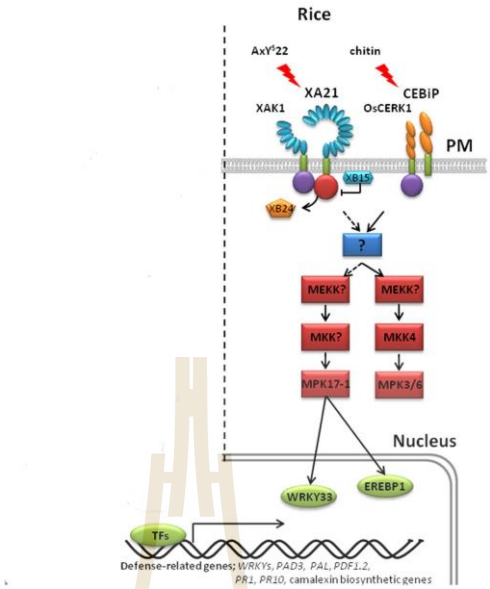


Figure 2.17 Models for pattern recognition receptor-mediated phosphorylation pathways in rice

Source: Park et al. (2012)

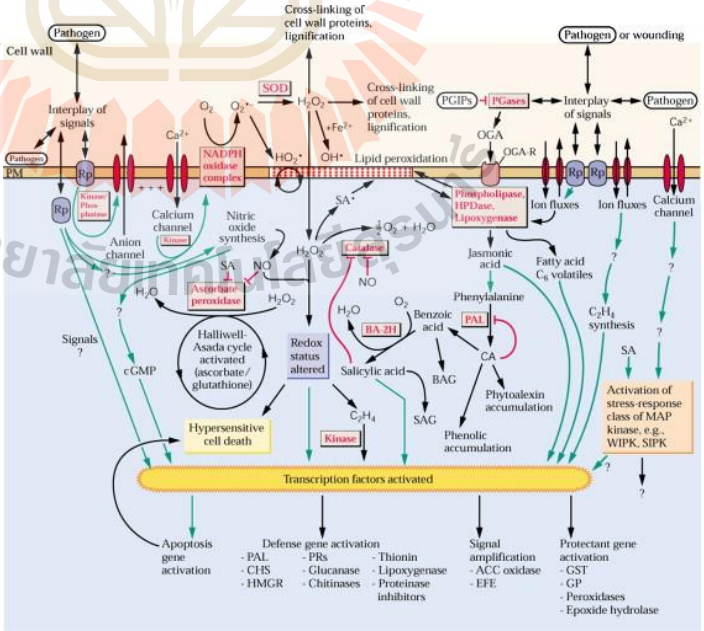


Figure 2.18 Models for inducible cell death in plant

Source: Hofiusa (2007)

The death of cell both inhibit the invasion of bacterial pathogens inside plant and stimulate a production of SA (Nooden, 2004). This kind of plant hormone can directly affect the pathogens as well as act as a signal of SAR (Walter et al., 2007). Moreover, compatible plant-pathogen interaction and metabolic perturbation can cause cell death, and then create SA (Scheel and Wasternack, 2002). In addition, beneficial bacteria including *Bacillus* sp. and other plant-pathogen interaction will create jasmonic acid response and ethylene response, lead to ISR (Walter et al., 2007) (Figure 2.19 and 2.20).

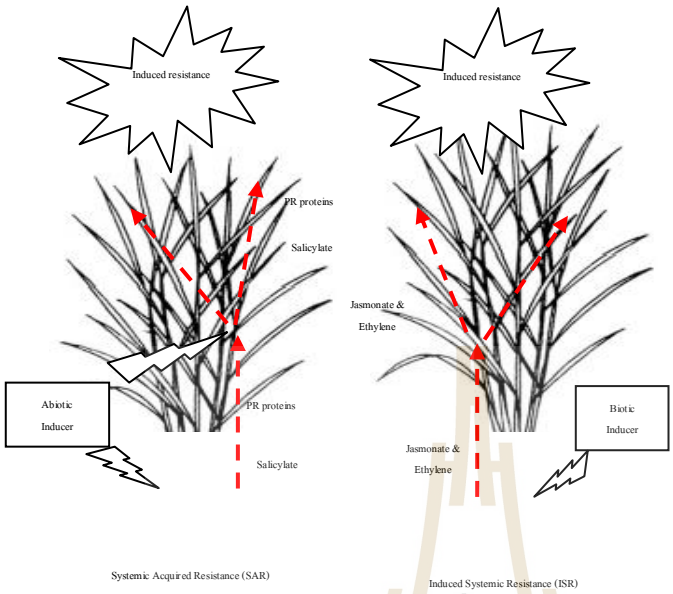


Figure 2.19 Schematic representation of systemically induced immune responses in rice. The SAR is typically activated in healthy induced tissues of locally infected plants. During pathogen infection, a mobile signal moves on the vascular system to activate defense reactions in distal plant tissues. SA is an essential signal molecule for the onset of SAR, as it is required for the activation of a large set of genes that encode pathogenesis-related proteins (PRs) with antimicrobial properties. The ISR is mainly activated upon colonization of plant roots by beneficial microorganisms. ISR is commonly regulated by JA- and ET-dependent signaling pathways and is typically not associated with the direct activation of PR genes. Instead, ISR-induced plants are primed for increased JA- and ET-dependent gene expression after pathogen attack. Both SAR and ISR have a broad resistant spectrum to plant pathogens. Modified from sources: Vallad and Goodman (2004); Pieterse et al. (2009)

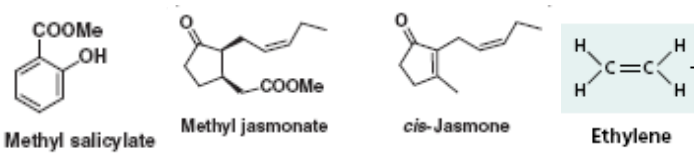


Figure 2.20 Molecular structures of salicylate, jasmonate and ethylene

Source: Taiz and Zeiger (2003); Parker (2009)

Both SAR and ISR are complex defense mechanisms that repress or stimulate together or crosstalk. The crosstalk between SAR and ISR is controlled by NPR1-dependent or -independent regulatory genes (Figure 2.21). In NPR1-independent pathway, NIM1 can be the regulatory genes. Members of several transcription factor (TF) families modulate the defense transcriptome (Eulgem, 2005; Somssich, 2006), and regulatory gene NPR1/NIM1 (Wang et al., 2006). In current days, approximately 1600 TF genes were identified in rice (Xiong et al., 2005), with more than 100 large TF families including MYB, WRKY, AP2/EREBP, bHLH, bZIP, C_2H_2 and NAC (Qu and Zhu, 2006; Tsuda and Somssich, 2015).

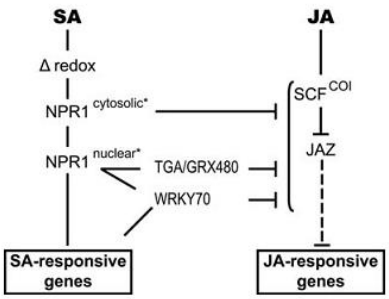


Figure 2.21 Schematic representation of crosstalk between SA and JA signaling

Source: Parker (2009)

Lastly, defense signals stimulate SAR genes, ISR genes and other resistance-related genes. These genes create phytoalexins, ROI, HR, cell wall fortification and other defense gene products that could kill or inhibit pathogen growth directly or indirectly (Figure 2.22). All of these expression are responses of induced resistance.

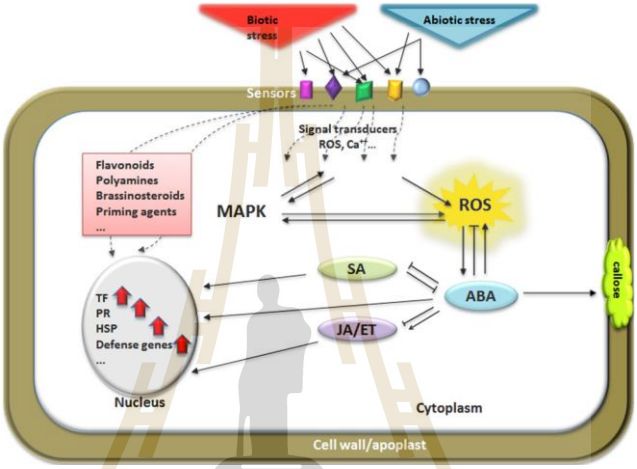


Figure 2.22 Elements possibly involved in cross-tolerance between plant pathogens and drought stress

Source: Rejeb et al. (2014)

Gene expression has been studied on induced resistance in plants with biotic or abiotic inducers. However, knowledge of gene expression and metabolic composition changes on induced resistance in plants is not many. There is no result on expression of pathogenicity genes after using inducers triggerring induced resistance against *Xoo.*

Similarly, FTIR spectroscopy has been used to analyze changes in biomass composition of phytoplankton algae (Stehfest et al., 2005), zinc and/or cadmium accumulation in *Gynura pseudochina* (L.) DC. (Panitlertumpai et al., 2013), cell wall modifications during the habituations of bean (*Phaseolus vulgaris* L.) callus cultures

to dichlobenil (Alonso-Simon et al., 2004), rapid determination of lipid accumulation in fresh water microalgae (Dean et al., 2010), the mechanism of Cd^{2+} and Pb^{2+} in mango peel waste (Iqbal et al., 2009), differentiation of plant pollens (Mularczyk-Oliwa et al., 2012), rapid detection of sugars and acids in tomatoes (Beullens et al., 2006), molecular chemistry of plant protein structure in yellow (*Brassica rapa*) and brown (*Brassica napus*) canola seed tissues (Yu, 2008), influence of arbuscular mycorrhizal fungi on zinc biogeochemistry (Kangwankraiphaisan et al., 2013). There is no result on biochemical changes study on induced resistance against *Xoo*.

In general, the methods of gene expression including defense genes and pathogenicity genes, histopathological study, and biochemical changes using FTIR spectroscopy can be applied to study mechanism of induced resistance in this thesis. The results will be novel. They will contribute to the knowledge of BLB disease resistance in rice, with the present reports of rice genetics and genomics (Brar et al., 2005; Zhang and Wing, 2013) and proteome analysis of the plant-pathogenic bacterium *Xoo* (Xu et al., 2013).

2.5 Rice cell wall

Rice cell walls are composed predominantly of polysaccharides approximately 90% of total dry weight, together with less amounts of structural glycoproteins approximately 2-10%, phenolic esters approximately 2%, ionically and covalently bound minerals approximaely 1-5%, and enzymes. In living rice tissue, water may account for up to 70-90% of the volume of a cell wall (Rose, 2003).

Two important components of polysaccharides on disease resistance are lignin and pectin (Huckelhoven, 2007). Lignins result from polymerization of phenoxy

radicals derived from three monolignols including syringyl (S), guaiacyl (G) and p-hydroxyphenyl (H). During growth of rice cell, lignification firstly occurs at the cell corners and compound middle lamella regions in a pectic-substance-rich environment of H and G units, and may be of a highly condensed type. The lignin patterns may differ depending on the cellular function of the lignified elements (Boudet, 2003). The S unit of lignin has been shown to accumulate in resistant wheat leaves during HR (Bishop et al., 2002; Menden et al., 2007). Changes in the S / G ratio are resulted from changes in the activity of phenylalanine ammonia-lyase (Chen and McClure, 2000; Gayoso et al., 2010). *Pal* genes catalyses and regulates the production of precursors for lignin biosynthesis in plant cells (Nicholson and Hammerschmidt, 1992).

Three pectic polysaccharides of cell walls are homogalacturonan, substituted galacturonans, and rhamnogalacturonans. Pectins are synthesized and deposited into cell wall in a highly methylesterified form during wall assembly and then to undergo enzyme-mediated demethylation (Rose et al., 2003). Enrichment of pectins strengthens of barrier properties of cell walls during stage of colonization by the pathogen (Cherif et al., 1991; Eckardt, 2002; Raiola et al., 2011; Volpi et al., 2011; Bethke et al., 2014).

The cell wall contains proteins that are vital for cell wall assembly during growth, development and disease responses. The cell wall proteins are enzymes including hydrolases, proteases, glycosidases, peroxidases and esterases; expansins; and wall-associated kinases (WAKs) (Johnson et al., 2003). Amide I β-sheet secondary structure of protein can be involved in the resistance of plant to pathogen (Thumanu et al., 2015). Amide I β-sheets secondary of necrosis-inducing protein 1 (NIP1) had a major role in barley against fungal pathogen *Rhynchosporium secalis*

(van't Slot et al., 2003). These authors suggested that in amide I β-sheets secondary of NIP1, cysteins of diffent domains form disulfide bonds, leading to a new stable fold structures. Change of amide I structure lead to introduced high affinity binding site for plant receptor to transduce defense signal (Green et al., 2003; van't Slot et al., 2003).

Lipids are also an important component of plant cell membranes and provide energy for rice cell metabolic activities. Lipids contribute their roles on photosynthesis, physical barriers against plant pathogens, and serve as second messengers in signal transduction mechanisms on plant growth, development, and defense mechasnism (Weber, 2002; Wang, 2004; Munnik, 2010). In 2002, Feussner and Wasternack reported that formation of oxygenated fatty acids, abbreviated as oxylipins, was a response of plant cells against abiotic and biotic stress. Oxylipins could directly act as antimicrobial compounds or indirectly stimulate defense gene expression (Farmer et al., 2003). Additionally, two groups of galactolipids had different roles during the induction of systemic resistance. Plant digalactosyl diacylglycerol (DGDG) contributed to plant nitrite oxides as well as salicylic acid biosynthesis and is required for the induction of SAR. In contrast, plant monogalactosyl diacylglycerol (MGDG) controls the biosynthesis of the SAR signals azelaic acid (AzA) and glycerol-3-phosphate (G3P) (Gao et al., 2014).

2.6 Conclusion of literature review

Induced reponses in plants have been described since early 1900s. Later on, researches of Kuc et al., Maclennan et al. and Ross on apple and tobacco in 1950s were first evidences that susceptible plants had induced responses to their pathogens after being treated by chemicals or avirulent pathogens (Kuc, 2006; Tuzun and Bent,

2006). Then, this aspect of plant protection has been researched in many plants with many layers of defense responses. Although information on induced resistance is available in literature, all layers of a general defense mechanism of a specific disease in plant has not been fullly understood.

In rice plant, researches on induced resistance in fungal diseases are much more than those in bacterial diseases. Effective inducer for induced resistance in rice against BLB disease has not been yet commercialized. In addition, the mechanism of induced resistance to BLB disease in rice is not yet well understood. Limited insight is existing in interactions between inducer - plant, inducer - plant - pathogen, and inducer-pathogen. On each interaction, defense responses can be researched on aspects such as gene expression, transcriptomics, proteomics, glycoproteomics, lipomics, metabolomics, phytoalexins analysis and plant histopathogy. Therefore, finding an effective inducer to BLB disease is essential. Then, expression of defense and pathogenicity genes, levels of superoxide anion, hypersensitive reaction, and biochemical changes are important to characterize a mechanism happening in resistance-induced rice plants againt BLB disease.

2.7 Reference

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CHAPTER III

SYSTEMIC RESISTANCE INDUCERS FOR CONTROLLING RICE BACTERIAL LEAF BLIGHT CAUSED BY *XANTHOMONAS ORYZAE* PV. *ORYZAE* IN THAILAND

(*Article in review, Journal of Plant Production Science*)

ABSTRACT

Bacterial leaf blight (BLB), caused by *Xanthomonas oryzae* pv. o*ryzae* (*Xoo*), is a major foliar disease of rice in Thailand and Southeast Asia. In this study, resistance inducers including ascorbic acid, oxalic acid, potassium dihydrogen phosphate, salicylic acid (SA), *Bacillus subtilis* strain CaSUT007 and *Bacillus* sp. strain NT111 were evaluated for their potential to protect rice from the BLB disease. Our results found that all of the inducers could stimulate germination of rice seed. The biotic inducers had antagonistic activity on *Xoo*, but the abiotic inducers had not. The efficacy of each inducer varied in controlling rice BLB depending on the rate of application. Among all inducers applied as seed soak and foliar spray, SA reduced disease severity significantly, approximately at 55.35%, compared to that of the negative control. In defense gene expression analysis, the 1 mM SA treatment showed more regulatation of two resistance genes, ascorbate peroxidase (*apx*) and phenylalanine ammonia lyase (*pal*), in rice leaves over that of the control treatment. The different accumulation of products from these defense genes were more rapid and

pronounced when SA-treated rice was infected with *Xoo*, indicating the priming and inducing resistance mechanism. In addition, pathogenicity genes of *Xoo* including *hrc*Q, *rpf*F and *rpf*G were expressed at a lower rate in the treated rice plants than that of the negative control, resulting in lower BLB disease severity in the SA-treated rice plants.

Key words: *Xanthomonas oryzae* pv. *oryzae*; inducer; rice; plant defense mechanism; defense gene; pathogenity gene

3.1 Introduction

Bacterial leaf blight (BLB), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is the most important disease of rice in Thailand and Southeast Asia (Adhikari et al., 1995; Gnanamanickam, 2009; Bureau of Rice Research and Development, 2010; Shimono et al., 2012). *Xoo* is pandemic in this area due to favorable warm and wet climatic conditions (Bureau of Rice Research and Development, 2010; del Rio and Simpson, 2014). The disease is particularly severe during the rainy season starting from early June to October when high moisture favors its development. The estimates of yield losses to rice production of this disease is approximately 20-75% (Shimono et al., 2012; Yang et al., 2015). The pathogenic bacterium can overseason in piled straw, this source of inoculum is important in rice-growing fields with alternate weedy hosts. In the tropical regions, especially Southeast Asia, environmental factors such as humidity, availability of host and alternate host plants allow *Xoo* to persist continously throughout the year (Ou, 1985; Mew et al., 2004; Webb et al., 2009). Moreover, the bacterial pathogen is mostly disseminated by irrigation water, humans and insects from infected fields to another rice paddy (Ou, 1985; Nyvall, 1999). BLB typical symptoms

are usually observed at the tillering stage, peaking at the flowering stage (Mew et al., 1993; Gnanamanickam et al., 1999). The disease is widely spread in all rice varieties in Thailand, especially in susceptible cultivars such as KDML105 (Bureau of Rice Research and Development, 2010; Win et al., 2012). Recently, conventional management of BLB has relied on cultural practice, resistance cultivar and chemical approach. Chemical control by protective bactericides and antibiotic such as copper oxychloride, copper hydroxide, copper sulfate, quarternary ammonium compound, hydroxyquinoline sulfate, streptomycin and tetracylin, has been recommended. However, the application is still problematic due to continuous rainfall during the rainy season, accumulation of chemical residues, and pathogen resistance to the chemicals (Agrios, 2005; Horst, 2008; Yuan et al., 2010; Win et al., 2012).

Nowadays, resistance inducers have been extensively evaluated to control plant diseases based on the induced resistance concept (Sticher et al., 1997; Vallad and Goodman, 2004; Tuzun and Bent, 2006; Walter, 2007; Buensanteai et al., 2009 and 2010). The induced resistance is part of the plant innate defense system that confers long-lasting protection against broad range of plant pathogens (Eikemo et al., 2003; Durrant and Dong, 2004; Iriti et al., 2004; Godard et al., 2009; Graham and Myers, 2011; Iriti et al., 2011; Perazzoli et al., 2011; Verhagen et al., 2011; Hatem et al., 2012). There are two important plant defense mechanisms including induced systemic resistance (ISR) and systemic acquired resistance (SAR). ISR relies on jasmonic acid (JA) and ethylene (ET) as signalling molecules (Walter et al., 2007; Martinez-Medina et al., 2013). SAR requires salicylic acid (SA) as a signal molecule and is associated with production and accumulation of defense enzymes and pathogenesis-related (PR) proteins (Malamy et al., 1990; Vidal et al., 1997; Mandal et al., 2007; Buensanteai et al., 2009 and 2010). The inducers could be both synthetic or natural compounds and

microorganisms. The synthetic inducers including salicylic acid (SA), ascorbic acid (AA), oxalic acid (OA), potassium dihydrogen phosphate (PDP), sodium saccharin dihydrate (BIT), 2,6-dichloroisonicotinic acid, kinetin, benzo (1,2,3)-thiadiazole-7-carbothionic acid S-methyl ester or acibenzolar-S- methyl (ASM) have been extensively evaluated against several plant diseases (Nakashita et al., 2002; Slaughter et al., 2008; Perazzoli et al., 2008; Buensanteai et al., 2009; Eschen-Lippold et al., 2010; Pieterse et al., 2012; Park et al., 2013; Prakongkha et al., 2013a and 2013b). Besides, biotic inducers such as plant growth-promoting rhizobacteria (PGPR) have been evaluated to be effective in stimulating plant growth and building up resistance against various diseases, especially in rice plant (Sathiyabama and Balabramaian, 1995; Awadalla and Mahmoud, 2005; Manjunatha et al., 2009; Chithrashree et al., 2011; El Hadrami et al, 2011; Kumar et al., 2011). Their applications have been registered on many crops and in several countries (Friedrich et al., 1996; Godard et al., 1999; Nakashita et al., 2002; Vallad and Goodman, 2004; Walter, 2007; Eschen-Lippold et al, 2010; Graham and Myers, 2011). The mechanisms of these inducers on induced resistance have been reported to be involved with cytosolic H^+ and Ca^{2+} accumulation, activation of MAP-kinases, callose deposition, oxidative burst, hypersensitive response (HR), synthesis of defense enzyme as phenylalanine ammonia lyase (PAL)/peroxidae (APX)/polyphenol oxidase (PPO), accumulation of jasmonate and salicylic acid, phytoalexins and pathogenesis related (PR) proteins, and stimulation related with plant defense response against plant pathogen infection (Kagale et al., 2004; Sible et al., 2004; Iriti and Faoro, 2009; Sana et al., 2010).

Even though there has been proof of effectiveness on using several resistance inducers for controlling crop diseases, none of them has been evaluated for rice BLB protection in Thailand and Southeast Asia. Therefore, the objective of this study was to

evaluate the efficacy of different resistance inducers in controlling the BLB disease. In addition, the plant defense gene expression consisting of L-ascorbate peroxidase (*apx*) and phenylalanine ammonia lyase (*pal*) after the inducer application was investigated. The *Xoo* pathogenicity gene expressions including that of the hypersensitive response and pathogenicity such as *hrc*Q, *rpf*F, and *rpf*G were also examined.

3.2 Materials and methods

3.3.1. Preparation of abiotic inducers

Abiotic inducers including AA (CarloErba, France), OA (CarloErba, France), PDP (CarloErba, France), SA (Acros Organics, ThermoFisher Scientific, USA) obtained from the Plant Pathology and Biopesticides Laboratory, Suranaree University of Technology, Nakhon Ratchasima, Thailand were used in the study. The concentration of each inducer was varied according to the previous reports (Du et al., 2001; Shalata and Neumann, 2001; Gilliland et al, 2003; Kashyap and Dhiman, 2009; Jayaraj et al., 2010). The solutions of these inducers including 1.0 and 3.0 mM AA, 1.0 and 4.0 mM PDP, 0.5 and 2.0 mM OA, 1.0 and 4.0 mM SA were prepared as previously described (Shalata and Neumann, 2001; Gilliland et al, 2003; Kashyap and Dhiman, 2009; Jayaraj et al., 2010). The solutions were stirred with a magnetic stirrer for 1 h to assure complete solubility. Deionized water was used as a negative control.

3.2.2 Preparation of biotic inducers and culture condition of *Xoo*

The PGPR including *B. subtilis* strain CaSUT007 (CaSUT007) and *Bacillus* sp. strain NT111 (NT111), and bacterial pathogen *Xoo* strain SUT1-121 (virulent strain), stored in nutrient broth with 10% glycerol at -80°C, were retrieved by streaking them from nutrient broth onto nutrient glucose agar (NGA) at 28 ± 2 °C for

48 h, and propagated in 500 ml of nutrient broth containing 2% glucose (NGB) for 48 h at 28 ± 2°C with constant shaking at 180 rpm. Finally, the bacterial cultures were resuspended in sterile distilled water, and density of the suspension was adjusted to $1x10^8$ cfu ml^{-1} based on optical density (OD) of 0.2 at 600 nm (Prathuangwong and Buensanteai, 2007; Buensanteai et al., 2008).

3.2.3 Effects of biotic and abiotic inducers on rice seed germination

Rice seeds *Oryza sativa* cv. KDML105 were treated with two biotic inducers and four abiotic inducers with two different concentrations as stated in 3.3.1. The rice seeds were surface-disinfected with 95% ethanol (v/v) for 2 min, followed by soaking in 1% NaOCl (v/v) for 20 min. The seeds were then washed with sterile distilled water for 5 times in order to remove the bleach. Subsequently, the rice seeds were mixed thoroughly with 2.5 ml of the inducer solutions. The seeds were put onto petri dishes containing two layers of blotting-paper, and covered with a layer of blotting-paper (Buensanteai et al., 2009; Anwar et al., 2013). After that, all petri dishes were incubated at 30 ^oC. Percentage of seed germination, root length and shoot height were recorded at 48, 72 and 96 h after incubation (HAI). The experiment consisted of 5 replications, 10 rice seeds per replication. And the experiment was repeated 3 times.

3.2.4 Antagonistic effects of the inducers on *Xoo* growth

The experiment was carried out in completely randomized design (CRD), and treatments were similar to that of the previous experiment on seed germination, with 5 replications. Antibacterial activity assay against *Xoo* was carried out by the disc diffusion method of Murray et al. (1995) and Nisha et al. (2012). Ten µl of *Xoo* suspension at density of $1x10^8$ cfu ml^{-1} was spread on each plate of the NGA medium. Subsequently, sterile Whatman no.1 paper discs, with 6 mm in diameter, were impregnated with 1 ml of each inducer and placed at the center of the petri dish, then

incubated at 25±2 ^{o}C. Diameters of the inhibition zone were measured at 48 h after incubating (Nisha et al., 2012).

3.2.5 Plant cultivation, induction treatment, *Xoo* inoculation and BLB disease assessment

The evaluation of induced resistance in rice againt BLB disease infection was conducted under a greenhouse condition, using the most effective inducers including CaSUT007, 1.0 mM AA, 4.0 mM PDP, 0.5 mM OA, and 1.0 mM SA. The susceptible rice seeds cv. KDML105 were surface-disinfected with 95% ethanol (v/v) for 2 min, followed by washing with sterile distilled water for 5 times to remove the alcohol residue, and soaked in water overnight. Subsequently, 10 g of the rice seeds were soaked in 50 ml of each inducer, then germinated on wet paper in the dark for 12 h (Shivalingaiah and Umesha, 2013), and two seeds were sown in a 30 cm diameter-plastic pot. Each plastic pot was filled with 5 kg of air-dried and sieved (2.0 mm) soil. The soil collected from Suranaree University of Technology farm (14^{o}59'0"N/102^{o}7'0"E) has physico-chemical characteristics including clay loam, pH 7.26, electrical conductivity (EC) at 192.5 µS cm^{-1}, available phosphate (Bray II) at 65.73 ppm, potassium (NH_4OAc) at 180 ppm, and organic matter at 1.64% (Piromyou et al., 2010). Basal fertilizers supplied for a pot were 0.2 g N kg^{-1} as urea, 52 mg P kg^{-1} as potassium dihydrogen phosphate, and 84 mg K kg^{-1} as potassium sulfate (Ning et al., 2014). Pots were kept in a greenhouse with 12 h photoperiod, 25 °C and 60-75% relative humidity. The rice plants were further induced by foliar spray with each inducer at 15, 30 and 45 days after sowing (DAS). The rice plants were watered every two days, and grown for 50 days prior to *Xoo* challenge inoculation. At 50 DAS, six mature top-leaves from each pot were randomly chosen for inoculation by cuting leaf at 3 cm from the leaf top, then inoculated with *Xoo* suspension at the concentration of

$1x10^8$ cfu ml^{-1} (Mizobuchi et al., 2002; Chithrashree et al., 2011; Xu et al., 2013). After the inoculation, the rice plants were covered with black plastic bags, and incubated in a controlled-temperature room with 100% relative humidity for 24 h (Chithrashree et al., 2011; Xu et al., 2013) before being transferred into a greenhouse.

The BLB disease severity scale was recorded at 14 and 21 days after the inoculation (DAI) (Babu et al., 2003; Govindappa et al., 2011; Xu et al., 2013), using the modified method of IRRI (1988) for assessing BLB lesions under greenhouse condition. Then, percentage of disease severity was calculated using the formula reported by Ji et al. (2008). Disease severity (DS, %) = [Sum of all numerical ratings / (Total number of leaves graded x Maximum grade)] x 100%. The experiment was carried out in CRD, with 6 treatments and 5 replications, and repeated 3 times.

3.2.6 Isolation of total RNA

The rice leaf samples were collected at 0, 12 and 24 hours after *Xoo* inoculation (HAI), frozen immediately in liquid nitrogen, and stored at -80°C. One hundred miligram of leaf sample was ground in liquid nitrogen with a mortar and pestle. The total RNA was extracted by QIAGEN RNeasy® Plant Mini Kit (QIAGEN, MD, USA), then treated with RNase-free DNaseI (Applied BioSystems, Thermo Fisher Scientific, UK) (Xin et al., 2012). Quality and quantity of the isolated RNA were checked by DeNovix DS-11 Spectrophotometer (DeNovix, USA). One hundred nanogram of each total RNA was used for quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) experiment.

3.2.7 Quantitative Realtime PCR

qRT-PCR was performed using the QuantStudio5_Real-time PCR Detection System (Applied BioSystems, Thermo Fisher Scientific, UK) to monitor the expression of rice defense related genes and *Xoo* pathogenicity genes. Sequences of the primers used are shown in Table 3.1. The primer pairs were designed based on the previous reports of Kottappalli et al. (2007), Zhang et al. (2012) and Noh et al. (2014). The constitutively expressed housekeeping genes 'actin' and '16S' were used as the reference gene (Peng et al., 2011). RNA was transcripted into cDNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Thermo Fisher Scientific, UK). PCRs were performed using the SYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, UK) in MicroAmp Optical tubes. Each PCR amplification reaction, with the total volume of 20 µL, contained 12 µL SYBR Green PCR Master Mix (2x), 1 µL of each pair of forward and reverse primers, 2 µL of first-strand cDNA and 4 µL sterile distilled water. Also, non-template control (NTC) reactions were included in all experiments. The PCR conditions consisted of denaturation at 95 ^{o}C for 10 min, followed by 40 cycles of denaturation, annealing, and extension conditions of specific primers. The cycle's conditions of primer *apx* and *pal* were 95 ^{o}C for 45 s, 55 ^{o}C for 45 s, and 72 ^{o}C for 60 s, respectively. The cycle's condition of primer *hrc*Q were 94 ^{o}C for 40 s, 58 ^{o}C for 40 s, and 72 ^{o}C for 60 s, respectively. The cycle's conditions of primer *rpf*F and *rpf*G were 95 ^{o}C for 15 s, 58 ^{o}C for 30 s, and 72 ^{o}C for 30 s, respectively. Expression of the target genes was normalized relative to the expression of the housekeeping gene such as actin and 16S to minimize sample variations (Ding et al., 2009).

Table 3.1 Primers used in the quantitative real-time PCR analysis of gene expression

Genes	Primer's sequence (5'-3')	Product size (bp)	References
apx	F: AAATACTGGAGCCTCATGGAGA	294	Kottapalli et al.
	R: AGTTCTATGCTTTGACCCTTGG		(2007)
pal	F: AGTACTTGACCGGGGAGAAGA	299	Kottapalli et al.
	R: GGCATCG TAACTTCCAAAGAAC		(2007)
*hrc*Q	F: CAGAATCATCTGTTGCATATTC	360	Zhang et al.
	R: GCCTTCAAGCGCGATGCAAC		(2013)
*rpf*F	F: GAGCTGCCACACCATCATCG	200	Noh et al.
	R: GGCGGAGTACAGATTGCCTTCT		(2014)
rpfG	F: TTTCATCACGCTCATCTCGTCGT	386	Noh et al.
	R: TCTCGAACGCATGTCTCATGTGG		(2014)
actin-1	F: GGCACCACACCTTCTACAATGAG	145	Peng et al.
	R: ACACCATCACCAGAGTC AAGCA		(2011)
16S	AGAGTTTGATCCTGGCTCAG ACGGCTACCTTGTTACGACTT	1500	Weisburg et al. (1991), Pramod et al. (2015)

The expression of gene was calculated by the ΔΔCt method (Livak and Schmittgen, 2001). Mean threshold cycle (mCt) was obtained from triplicate series of amplifications during the exponential phase. Then, mCt value of reference gene (actin and 16S) was subtracted from mCt value of the target gene (*apx*, *pal*, *hrc*Q, *rpf*F, and *rpf*G) to obtain ΔCt. Subsequently, the ΔΔCt values were calculated from corresponding ΔCt values separately for each sample, using the following formula:

ΔΔCt=[mCt target-mCt reference] - [mCt target - mCt reference]. Finally, using the ratio formula (ratio = $2^{-\Delta\Delta Ct}$), up or down-regulation of target gene was achieved in proportionate to the reference gene (Applied BioSystems, 2016). Each sample was repeated three times.

3.2.8 Statistical analysis

The data analysis was performed using SPSS 16.0 software for multiple comparison and analysis of variance (ANOVA). A value of $P \leq 0.05$ was considered to be statistically significant.

3.3 Results

3.3.1 Effect of the inducers on priming of rice seed germination

This experiment confirmed that enhanced seed germination and plant growth promotion mechanism had occurred. The results indicated that seeds pretreated with different inducers had significantly ($P \leq 0.05$) longer root length and higher shoot height, but the percentages of seed germination were not significantly different (Figure 3.1c). SA at concentration of 1 mM increased root length and shoot height approximately at 40 and 37%, respectively. Seed treatment with other inducers could also significantly increased root length and shoot height but at a lower degree than that of 1 mM SA (Figure 3.1a and 3.1b).

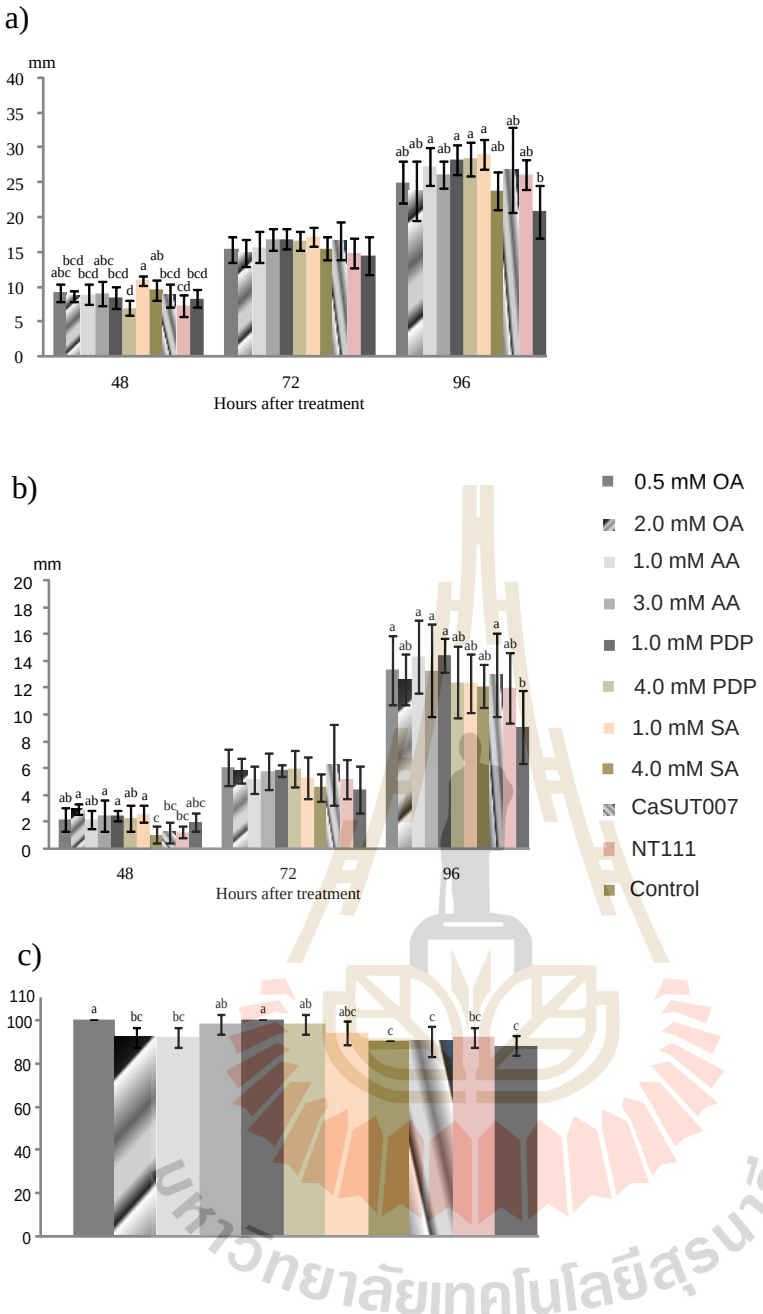

Figure 3.1 Effect of inducers on KDML105 rice seed at 48, 72 and 96 hours after treatment (HAT). a) Root length b) Shoot height c) Percentage of germination. AA = ascorbic acid, OA = oxalic acid, SA = salicylic acid, PDP = KH_2PO_4, CaSUT007 = *B. subtilis* CaSUT007. Error bars represent standard deviation for 5 replications. Values followed by the same letter are not significantly different according to Duncan's multiple range test at $P \leq 0.05$.

3.3.2 Antagonistic effect of the inducers on *Xoo* growth

This experiment showed a direct effect of the inducers on growth of *Xoo*. The results showed that all different concentration of the abiotic inducers did not significantly ($P \leq 0.05$) affect growth of *Xoo* compared to that of the control treated with distilled water. In contrast, the two biotic inducers including CaSUT007 and NT111 significantly ($P \leq 0.05$) inhibited *Xoo* growth, observed as inhibition zones of 5.50 and 4.35 mm in diameter at 24 h, respectively (Table 3.2).

Table 3.2 Diameter of inhibition zone observed after culturing of *Xoo* for 24 h on NGA medium with the inducers disc in the center

Treatment	Diameter of inhibition zone [a/] (mm)
1.0 mM ascorbic acid	0.00c
3.0 mM ascorbic acid	0.00c
1.0 mM KH_2PO_4	0.00c
4.0 mM KH_2PO_4	0.00c
0.5 mM oxalic acid	0.00c
2.0 mM oxalic acid	0.00c
1.0 mM salicylic acid	0.00c
4.0 mM salicylic acid	0.00c
B. subtilis CaSUT007	5.50 ± 1.50a
Bacillus sp. NT111	4.35 ± 1.65b
Control (water)	0.00c
F-test	*
CV (%)	10.91

[a/] Mean ± SE (standard error) followed by the same letter do not different significantly according to Duncan's multiple range test at $P \leq 0.05$.

3.3.3. Effect of the inducers on BLB disease in rice

Five inducers including 1 mM AA, 4.0 mM PDP, 0.5 mM OA, 1 mM SA and CaSUT007 were evaluated for their ability to induce resistance in KDML105 rice. The results indicated that seed and foliar treatment with the inducers significantly

reduced the severity of BLB in the rice at 14 and 21 DAI compared to that of the negative control, confirming that induction of systemic resistance had occured (Table 3.3). Disease reductions of rice plants treated with 1 mM SA were 48.11 and 55.35% at 14 and 21 DAI, respectively. Moreover, disease severities of rice plants treated with other inducers were higher than that of 1 mM SA treatment, indicating that ability of other inducers on induced resistance was lower than 1 mM SA. Application of 1 mM SA as seed and foliar treatment highly suppressed disease development after challenge inoculation of *Xoo*, proved that inducer SA at concentration of 1 mM was more effective than other inducers (Table 3.3, Figure 3.2).

Table 3.3 Efficacy of inducers on BLB disease severity in rice cv. KDML105 under greenhouse condition

Treatment	Disease severity [a/] (%)	
Seed soak and foliar spray (15, 30, 45 DAS) [b]	**14 DAI [c/]**	**21 DAI [c/]**
1.0 mM AA	28.57±3.76b	34.29±4.32b
4.0 mM PDP	27.14±3.19b	33.33±5.58b
0.5 mM OA	30.48±1.99b	37.14±2.13b
1.0 mM SA	19.52±3.10a	23.81±3.76a
CaSUT007	28.57±2.92b	34.29±5.48b
Control	37.62±6.16c	53.33±5.73c
F-test	*	*
CV (%)	15.88	17.36

Rice plants were first induced by seed soak, then further treated by foliar spray at 15, 30 and 45 days after sowing. AA = ascorbic acid, OA = oxalic acid, SA = salicylic acid, PDP = KH_2PO_4, CaSUT007 = *B. subtilis* CaSUT007.

[a/] Mean ± SE (standard error) followed by the same letter do not different significantly according to Duncan's multiple range test at $P \leq 0.05$

[b/] DAS: Days after sowing

[c/] DAI: Days after *Xoo* inoculation

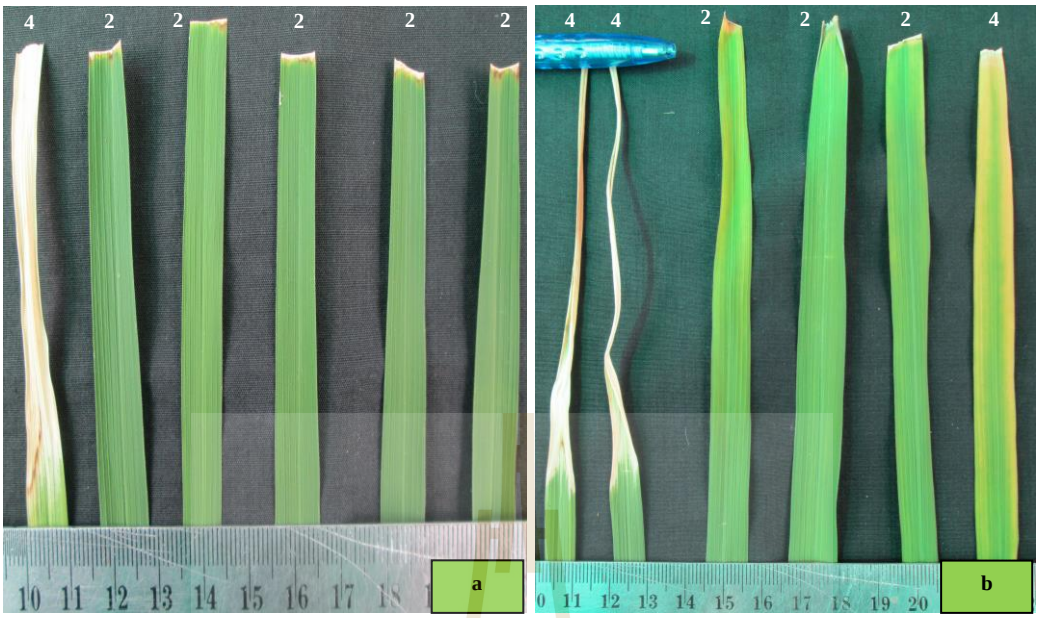

Figure 3.2 Bacterial leaf blight severities on KDML105 rice challenge inoculated with *Xoo*. (a) Seed soak and foliar spray with 1 mM SA (b) Seed soak and foliar spray with distilled water (control). The BLB disease severity scale was recorded using the modified method by IRRI (1988) for assessing BLB lesions under greenhouse condition as follows 0 = no symptom, 1 = slight discoloration at the inoculation point, 2 = lesion is less than 15 mm long, 3 = lesion is less than ¼ of the length from the inoculation point to the leaf base, 4 = lesion is between ¼ and ½ of the length, 5 = lesion is between ½ and the whole length, 6 = lesion covers the whole length, but some green area remaining and 7 = lesion covers the whole leaf.

3.3.4 Expression of the rice defense genes and *Xoo* pathogenicity genes

Application of 1 mM SA resulted in up-regulation of *apx* and *pal* gene expression compared to the control treatment. The relative expression levels of *apx*

and *pal* were significantly higher in rice plant treated with SA. *Apx* at 0 HAI was expressed approximately 52.9 fold greater than negative control at the same time point (Figure 3.3a). On *pal* gene expression, the relative expression level in rice plant treated with SA increased significantly at 12 HAI, and peaked the highest level approximately 18.9 fold at 24 HAI. Values of control samples were arbitrarily set to 1.0 (Figure 3.3b). The up-regulated *apx* and *pal* gene expression in the susceptible rice cv. KDML105 showed that the two genes played key roles in regulating induced resistance to BLB disease.

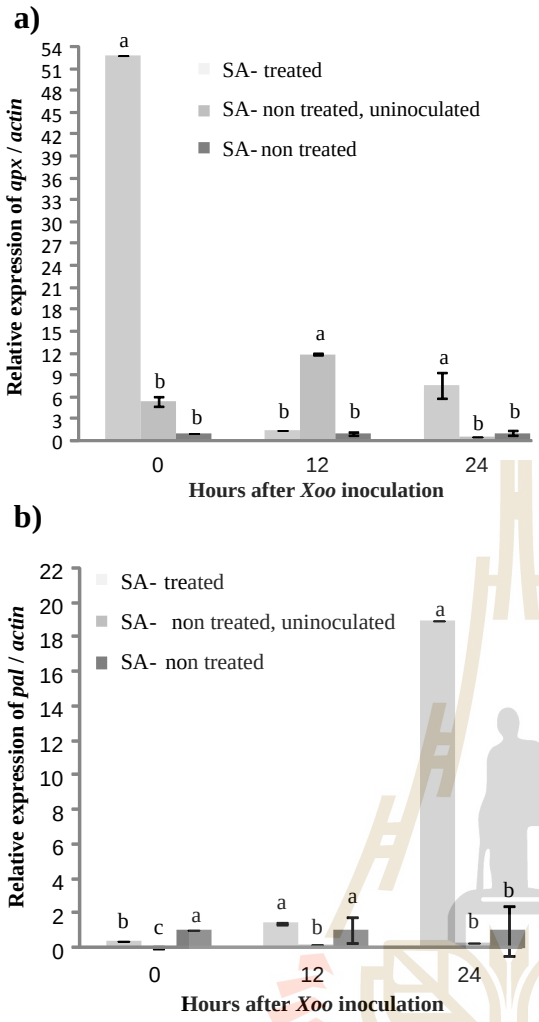

Figure 3.3 Expression pattern of (a) ascorbate peroxidase (*apx*) and (b) phenylalanine ammonilyase (*pal*) on induced resistance to BLB disease of rice under greenhouse condition. Quantification of the gene expression using Realtime-PCR and the relative expression represents the ratio of *apx* compare to the internal control actin at 0, 12 and 24 hours after *Xoo* inoculation. Data was normalized to an internal actin control, and the data delta Ct method was used to obtain the relative expression levels for the gene. Values for control samples were arbitrarily set to 1.0.

The *hrc*Q, *rpf*F, and *rpf*G are key genes in pathogenicity of *Xoo*. The gene expression level of *hrc*Q was remarkably lower in rice plant treated with 1 mM SA, with expression of 0.88 and 0.24 fold at 12 and 24 HAI, respectively (Figure 3.4a). Another gene, *rpf*F, was dramatically decreased in rice plant treated with SA at concentration of 1 mM. The activity of *rpf*F decreased approximately 0.56 and 0.001 fold at 12 and 24 HAI, respectively (Figure 3.4b). The SA-treated rice plant also showed a gradual decrease in relative epression level of *rpf*G, significantly lower than negative control. In addition, the relative expression of *rpf*G was 0.76 and 0.25 fold lower than negative control at 12 HAI and 24 HAI, respectively (Figure 3.4c). The low level of of relative expression of *hrc*Q, *rpf*F and *rpf*G in rice plant treated with SA indicated that induced resistance responses in the susceptible rice cv. KDML105 inhibited the pathogenicity genes of *Xoo*.

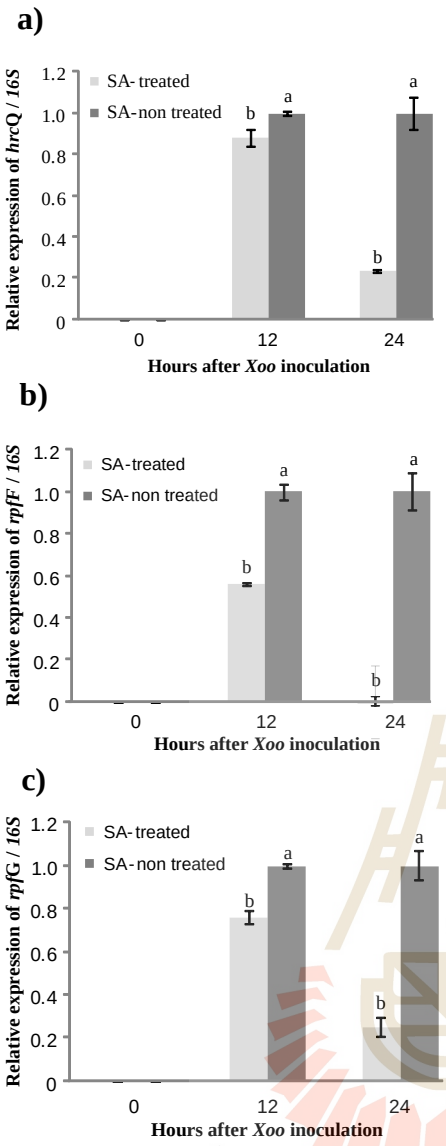

Figure 3.4 Expression pattern of *hrc*Q (a), *rpf*F (b), and *rpf*G (c) on induced resistance to BLB disease of rice under greenhouse condition. Quantification of the gene expression using Realtime-PCR and the relative expression represents the ratio of *hrc*Q compare to the internal control actin at 0, 12 and 24 hours after *Xoo* inoculation (HAI). Data was normalized to an internal actin control, and the data delta Ct method was used to obtain the relative expression levels for the gene. Values for control samples were arbitrarily set to 1.0.

3.4 Discussion

Among the 5 inducers tested, SA provided the highest protection on rice plant from the BLB disease infection. SA has been evaluated as an inducer in several economic crops, such as in tomato against *Alternaria alternata* f. sp. *lycopersici*, Potato virus X, Potato purple top phytoplasma (Esmailzadeh et al., 2008; Wu et al., 2012; Falcioni et al., 2014), in strawberry against *Colletotrichum gloeosporioides* (Zhang et al., 2016), in wheat against *Fusarium graminearum* (Makandar et al., 2012; Sorahinobar et al., 2015), in corn against Maize rough dwarf virus (Vigliocco et al., 2002), in rice against *Bipolaris oryzae*, *Magnaporthe grisea* and *Rhizoctonia solani* (Du et at., 2001; Daw et al., 2008; Shabana et al., 2008; Li et al., 2012; Usharani et al., 2013). In the current study, higher BLB disease reduction was observed in rice treated with SA at 1 mM than in the other biotic and abiotic inducers. Exogenous SA application at 1.0 mM concentration induced rice plant against blast disease approximately 27% (Du et al., 2001). Similar results were obtained by Daw et al. (2008) using 8 mM SA, which induced susceptible rice plant against blast disease. Other authors (Wang et al., 2009; Li and Zhang, 2012) observed that the exogenous SA application at different concentration including 0.5, 1 and 2 mM reduced the abiotic stress in rice plants. Certainly, SA acts an important role in induction of plant defense against several pathogens through morphological, physiological, biochemical and molecular level (Du et al., 2001; Daw et al., 2008; Esmailzadeh et al., 2008). In the case of SAR, SA plays a key role, via interaction with SA-binding proteins that can cause build-up of reactive oxygen species or activate gene expression (Kumar and Klessig, 2003). The levels of SA increase around necrotic lesions and remain high in plants having acquired resistance. Moreover, SA itself acts as the defense signal that is

translocated systemically throughout the plant (Anwar et al., 2013; Shah and Zeier, 2013). Plant defense mechanism against pathogens involves changes in gene and protein expression mediated by plant signalling transduction pathway (Daw et al., 2008; Esmailzadeh et al., 2008). These defense response pathways are triggered by plant small molecules such as SA, JA, ET and defense enzymes such as APX, PAL and LOX (Yoshioka et al., 2001; Nakashita et al., 2003; Walter et al., 2007). These genes associated with defense mechanism in plant cell were activated by early and secondary signalling transduction molecules and then trigger resistance in plant against pathogen infection (Lamb and Dixon, 1997; Walter et al., 2007).

The *apx* and *pal* are the important defense genes involved in plant defense mechanism against many plant pathogens (Guo et al., 2000; Gururani et al., 2012; Koramutla et al., 2014). Accumulation of *apx* and *pal*, the key genes in the SA-dependent pathway, was rapidly expression when rice was induced by exogenous SA and challenge inoculation by *Xoo*. Therefore, we indicated that SA-mediated signalling transduction pathway in induced resistance KDML105 was activate by exogenous SA, the *apx* and *pal* played a key role in triggering the signalling transduction pathway. Biotic and abiotic inducers such as Bacillus, SA, BIT, BTH and other PGPR have been found to induce such defense enzymes in rice plants (Wendehenne et al., 1998; Pieterse et al., 2003; Ferrari et al., 2003; Chithrashree et al., 2011; Negendran et al., 2013). Besides, induction of these defense genes by exogenous application of SA and their role in plant defense against various stresses have been studied in rice plants (Chern et al., 2005; Yuan and Lin, 2007). Reports from several experiments have provided strong evidence that interaction between molecule of SA and specific plant receptor is complex processes, but are necessary on plant signal transduction. After plant-pathogen recognition, molecules of SA could stimulate and change in

conformation of plant receptor on plasma membrane such as GTP binding proteins or SA-binding protein, resulted in activating corresponding effectors such as kinases and ion channels (Klessig et al., 2000; Kumar and Klessig, 2003; Chen and Ronald, 2011). Rice contains seven genes encoding Rac GTPases which activate protein kinases (MAPK cascades) (Chen and Ronald, 2011). MAPK cascades could transmiss extracellular signals to downstream components through protein phosphorylation. Seventeen MAPKs have been identified in rice (Chen and Ronald, 2011). Activated effectors continue to transfer the signals of molecule SA to secondary messengers which could amplify the signals and send them to other reactions (Rivas and Thomas, 2005). Following pathogen recognition and signal transduction, a series of defense reactions such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, ROS production, early defense genes, systemic defense signal, late defense genes and secondary metabolite production could be activated to protect plants from pathogen infection (Sakhabudinova et al., 2003; Nooden, 2004; Vallad and Goodman, 2004; Walter et al., 2007; Koornneef et al., 2008; Parker, 2009; Pieterse et al., 2009; Graves, 2012; Anwar el al., 2013). The *apx* and *pal* are two of important genes involved in systemic resistance, especially early defense reaction. Our results revealed that these *apx* and *pal* genes in rice treated with exogenous SA expressed early and highly when compared to the negative control, and performed correlation with disease severity under greenhouse condition. In addition, Bion and dipotassium hydrogen phosphate inducers performed a higher and quicker *apx* and *pal* response in the foliar spray-treated anion plants (Kamal et al., 2008). *Apx* gene, grouped in PR9 family, has impotant roles in the hypersensitive response, polymerization of lignin from monomeric lignols and formation of papillae (Nicholson and Hammerschmidt, 1992; Bestwick et al., 1998; van Loon et al., 2006; Miller et al., 2008; Garcia-Gutierrez et al.,

2013). Similar results obtained by Chithrashree et al. (2011), and Negendran et al. (2013) proposed that higher induction of *apx* and *pal* happened after treating rice seeds with *B. subtilis* to control BLB disease. In a study of Daw et al. (2008), exogenonous SA at concentration of 8 mM induced high constitutive level of *apx* expression and resistance of rice blast. However, our results showed that the relative expression of *apx* was high at 0 HAI, dramatically dropped at 12 HAI and increased at 24 HAI. In a research of Jiang et al. (2016), these authors indicated that there are eight *apx* genes in rice plant. Their results on quantitative RT-PCR showed that while relative expression of seven *apx* genes such as *apx*2, *apx*3, *apx*4, *apx*5, *apx*6, *apx*7 and *apx*8 gradually increased from 0 HAI to 24 HAI. And the relative expression of *apx*1 gene was similar to our results, with a high expression at 0 HAI, a decrease at next time point and an increase at 24 HAI. *Pal* gene expression is aslo proposed to contribute resistance against infection of White Clover Mosaic Virus by exogenous 1 mM SA application in *Phaseolus vulgaris* (Galis et al., 2004). The role of *pal* gene of the phenylpropanoid metabolism pathway, with the product as phenylalanine ammonia lyase, plays an essential role when exogenous SA was treated on induced resistance (Malamy and Klessig, 1992). *Pal* gene is also known to play a role in the synthesis of phenolic substances, coumarins and lignins for preventing the penetration of pathogen (Metraux, 2002; Shadle et al., 2003). On the phenylpropanoid pathway, *pal* is the principal enzyme of biosynthesis of phenolics and flavonoids (Dixon and Lamb, 1990). *Pal* gene catalyses and regulates the production of precursors for lignin biosynthesis in plant cells (Nicholson and Hammerschmidt, 1992). Besides, the product of this gene was a precursor of salicylic acid-an important signal on systemic resistance (Metraux, 2002).

Xoo relies on the type III secretion system (T3SS) to inject its effector proteins into host cells, leading to either a disease or a resistance reaction. In plant pathogens, the T3SS genes are refered to hypersensitive response and pathogenicity (*hrp*) genes. The *hrp* genes of Xanthomonas are highly conserved, functioned on quorum sensing (QS) cell-to-cell communication systems, including nine *hrp* genes, nine hrp-conserved (*hrc*) genes, and eight hrp-associated (*hpa*) genes in *Xoo* (Kim et al., 2003; Salzberg et al., 2008; Guo et al., 2011). In addition, the well-conserved genes of regulation of pathogenicity factors (*rpf*), such as *rpf*F and *rpf*G, are known to regulate virulence by the cell-cell communication in *Xanthomonas* (Wang et al., 2004; He et al., 2007; Subramoni et al., 2012). On induced resistance, systemic signals as SA produced from rice plants treated by inducer could influence the pathogen's virulence machinery. Some research studies showed that exogenous SA could block biosynthesis of flagella in *E. coli* (Kunin et al., 1995), and reduce virulence of *Agrobacterium tumefaciens* (Yuan et al., 2007). To our knowledge, exogenous SA could reduce expression of pathogenicity and virulence genes in *Xoo*. This is the first study that shows the relationship between pathogenicity genes of *Xoo* and host plant on induced resistance.

3.5 Conclusion

In this chapter, the inducers stimulated the growth of shoot and root. Abiotic inducers had no direct ability to inhibit the growth of *Xoo,* but the biotic inducers had antagonistic ability on *Xoo*. Under greenhouse conditions, these inducers reduced BLB disease severity around 55.35%. Among these inducers, SA had prominent ability to reduce BLB disease severity. In addition, two resistant genes such as *apx* and *pal*

expressed earlier and higher in induced treatment than in control treatment. Besides up-regulation of defense genes, pathogenicity genes of *Xoo* such as *hrc*Q, *rpf*F and *rpf*G expressed lower in rice plant treated with 1 mM SA than control rice plant. Finally, the results suggest that 1 mM SA could be utilized for the induction of rice defense mechanism that will enable the rice plant against BLB infection. However, the mechanism of inducers acted inside a bacterial infected rice plant has not fully revealed. Thus, the future studies on the aspects of genomic expression, proteomic expression, metabolic composition changes will be researched on induced resistance to BLB disease in rice.

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CHAPTER IV

SALICYLIC ACID-INDUCED ACCUMULATION OF BIOCHEMICAL COMPONENTS ASSOCIATED WITH INDUCED RESISTANCE AGAINST *XOO*

(*Article in preparation, Journal of Plant Interaction*)

ABSTRACT

Salicylic acid (SA) is an inducer that induces systemic protection in plant against pathogen infection. In this study, seed treatment and foliar sprays with SA provided protection to rice leaves from bacterial leaf blight (BLB) disease, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Two plant defense responses, superoxide anion (O_2^-) and hypersensitive response (HR), demonstrated the capacity of SA to prime defenses. The O_2^- production was enhanced more than 28% at 3-6 hours after *Xoo* inoculation (HAI) and the HR was increased by 110% at 48 HAI in the treated rice leaves. Due to the induced defense response, the *Xoo* in treated rice grew slowly, resulting in a low population of that in the untreated rice at 14 days after inoculation (DAI). Biochemical changes in the treated rice leaves were analyzed at 14 DAI by fourier transform infrared (FTIR) spectroscopy. Higher ratios of 1233/1517 cm^{-1}, 1467/ 1517 cm^{-1} and 1735/1517 cm^{-1} wavelengths observed in treated rice suggests alteration of monomer composition of lignin and pectin in the rice cell wall. In addition, treated rice had more amide I β-sheet structure at the peak 1629 cm^{-1}. Moreover, some peaks including 2851 cm^{-1} and 1735 cm^{-1} that correspond to lipids were more intense in

treated rice leaves than that of the control one. These biochemical changes of rice treated with SA and inoculated with *Xoo* are related to primed resistance of the rice plants to the BLB disease.

Key words: bacterial leaf blight, salicylic acid, rice, defence reactions, biochemical change, FTIR

4.1 Introduction

Rice (*Oryza sativa* L.) is one of the most important crops in the world. Asia has the largest growing area, with top producing countries including China, India, Thailand and Vietnam (Xu et al., 2013). Since the 1960s, with the widespread cultivation of high-yielding and nitrogen-responsive dwarf hybrid varieties, rice diseases have become more prevalent (Khush, 1995). Among these diseases, bacterial leaf blight (BLB) disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) accounts for around 20% of annual yield loss worldwide (Shimono et al., 2012; Zhu et al., 2013).

Plants have an immune system that can be induced by biotic or abiotic inducers (Walter et al., 2007; El Hadrami, 2011; Ahmad and Prasad, 2012). Induced resistance studies were researched using both kinds of inducers in plants such as rice, grapevine, cucumber, tomato and maize. Previous studies showed that resistance can be induced by priming plants with chemical inducers such as acibenzolar-S-methyl, benzo-1,2,3-thiadiazole-7-carbothionic acid S-methyl ester (BTH), L-ascorbic acid, N-cyanomethyl-2-chloroisonicotinamide, oxalic acid, probenazole, SA, and sodium saccharin dihydrate. Also, biotic inducers such as *Bacillus subtilis, Pseudomonas* sp., and *Trichoderma virens* have been used for the management of several plant diseases (Du et al., 2001; Ganesan and Thomas, 2001; Nakashita et al., 2002; Tokunaga and

Esaka, 2007; Buensanteai et al., 2010; Chithrashree et al., 2011; Kumar et al., 2011; Nahar et al., 2011; Li and Zhang, 2012; Mukherjee et al., 2012; Anwar et al., 2013; Park et al., 2013; Takatsuji, 2014).

An important characteristic of induced resistance is the phenomenon of priming in which plants exhibit a more rapid and elevated expression of defense responses upon bacterial pathogen infection (Conrath et al., 2006). One method for priming plant defense is to apply exogenous SA to plant tissues including seeds and leaves. SA can trigger the SA-dependent signaling pathway, leading to resistance to certain pathogens. The SA-dependent pathway is associated with systemic acquired resistance (SAR) which activated upon infection (Sticher et al., 1997). In rice, exogenously supplied SA is converted to P-O-D-glucosylSA by the enzyme SA-inducible glucosyltransferase (Silverman et al., 1995), which further stimulates the production of SA (Nooden, 2004). This phytohormone can directly affect the pathogens as well as contribute to the establishment of SAR (Walter et al., 2007; Tamaoki et al., 2013). On induced resistance, systemic signals as SA produced from rice plants treated with inducer could influence the pathogen's virulence machinery. Some research studies showed that exogenous SA could block biosynthesis of flagella in *E. coli* (Kunin et al., 1995), and reduce virulence of *Agrobacterium tumefaciens* (Yuan et al., 2007). SAR stimulates the expression of resistance genes. These genes encode enzymes that contribute to production of phytoalexins, reactive oxygen intermediates, hypersensitive responses, cell wall fortification, defence enzymes, and other defence gene products that kill or inhibit pathogen growth (Thulke and Conrath, 1998; Metraux, 2002; Metwally et al., 2003; Shadle et al., 2003; Galis et al., 2004). The SA-inducible glucosyltransferase enzyme production can be maximally induced by 1 mM SA, with 7-fold increase of the enzyme activity at 6 hours after the rice being treated, in both roots and shoots

(Silverman et al., 1995). In another study, H_2O_2-degrading enzyme peroxidase was decreased, while superoxide dismutase activity did not change after treating leaves by floating in 2 or 5 mM SA (Ganesan and Thomas, 2001). However, an increased activity of peroxidase was recorded after foliar spray of 8 mM SA in rice to induce resistance to rice blast (Daw et al., 2008). These authors also recorded enhanced polyphenol oxidase activity and production of four rice phytoalexins including oryzalexin A, C, F and momilactone A following the SA treatment. Foliar spray with SA induced the production of 36 proteins with different functions, such as signal transduction, antioxidant and defence enzymes (Li et al., 2012b). The induced resistance of rice against *Xoo* by priming with SA has not yet been characterized.

Therefore, the objectives of the study were to characterize the defence responses of rice plants against BLB after being treated with SA and challenged by inoculation with *Xoo* by monitoring biochemical changes associated with known responses.

4.2 Materials and methods

4.2.1 Resistance inducer and rice cultivar

Rice (*Oryza sativa*) seeds of susceptible cultivar KDML105 and salicylic acid (SA; Acros Organics, ThermoFisher Scientific, USA) used in this study were provided by Plant Pathology and Biopesticide Laboratory (PPBL), Suranaree University of Technology, Thailand. Prior to this research, different kinds of resistance inducers at various concentrations had been screened to evaluate their effects on seed germination and BLB disease severity by the procedure of Anwar et al. (2013). Subsequently, SA at 1 mM concentration was chosen for this research because it gave the highest efficacy in controlling the BLB disease.

4.2.2 Culture condition of *Xoo*

The *Xoo* strain SUT1-121 (virulent strain) was provided by the PPBL. It was retrieved by streaking the stock culture onto nutrient glucose agar (NGA) and incubated at 28±2 °C for 48 h. After that, the bacterial culture was propagated in 500 ml of nutrient broth containing 2% glucose (NGB) for 48 h at 28±2 °C with constant shaking at 180 rpm. After the incubation, the culture was re-suspended in sterile distilled water. Finally, density of the bacterial suspension was adjusted to 1 x 10^8 cfu ml^{-1} based on its specific absorbance at 600 nm (Buensanteai et al., 2008; Nagendran et al., 2013).

4.2.3 Plant cultivation, induction treatment, and *Xoo* inoculation

The experiment was conducted with five replications. For the SA-treated treatment, the rice seeds cv. KDML105 were surface sterilized with 95% ethanol (v/v) for 2 minutes, followed by washing with sterile distilled water for 5 times to remove the alcohol residue, and soaked in water overnight. Subsequently, 10 g of the treated rice seeds were soaked in 50 ml of 1 mM SA solution, germinated on wet filter paper in a dark condition for 12 h (Shivalingaiah and Umesha, 2013), and planted in 30 cm-diameter pots containing sterile soil and organic fertilizer. The pots were kept in a greenhouse with a 12 h photoperiod at 25 °C and relative humidity approximately 60-75%. The resistance was further induced by foliar spraying with 1mM SA at 15, 30 and 45 days after sowing (DAS). At 50 DAS, six matured top-leaves of two rice plants per pot were inoculated by the cutting dip method (Govindappa et al., 2011; Xu et al., 2013). After the *Xoo* inoculation, the rice plants were covered with black plastic bags, and kept in a controlled-temperature room with relative humidity of approximately 100% at 25 ^oC for 24 h (Xu et al., 2013), and then the plants were transferred into the

greenhouse. For the SA-non treated treatment, the seeds and plants were handled similarly, but distilled water was used instead of SA.

4.2.4 Detection of superoxide anion (O_2^-) and hypersensitive response (HR) of SA-induced rice leaves

The experiment was carried out in completely randomized design (CRD) with five replications and five leaves per replication to detect superoxide anion (O_2^-). Leaf samples at approximately 3 cm from the leaf tip-cut, were collected at 3, 6, 12 and 24 hours after *Xoo* inoculation (HAI) (Li et al., 2012a). The O_2^- was detected by Nitroblue tetrazolium staining method (Wang et al., 2009). Rice leaves from both SA-treated and SA-non treated treatments were vacuum infiltrated with 20 ml of 10 mM potassium phosphate buffer, pH 7.5, containing 10 mM NaN_3 and 0.1% Nitroblue tetrazolium (NBT, 5-bromo-4-chloro-3-indolyl phosphate, Sigma) for 24 hours at room temperature. Later, the leaf samples were boiled in 95% ethanol for 60 minutes before visualizing the blue precipitates of O_2^- (Tai, 2007; Lehotai et al., 2011).

The leaf samples were collected at 48 HAI (Jha et al., 2007) to detect hypersensitive response. The leaf samples at approximately 3 cm from the leaf tip-cut, were decolorized by soaking in clear lactophenol at 65 ºC for 30 minutes, then boiled in 70% ethanol for 30 minutes (De Neergaard, 1997). These samples were mounted on microscope slides with 50% glycerol in phosphate-buffered saline (PBS) and observed with a Confocal microscope (Nikon, NIS Element C, Japan), using a green filter having excitation wavelength range 450-500 nm and detection range 515-565 nm and 10X objective lens (Reimers and Leach, 1991; Jha et al., 2007).

4.2.5 Rapid detection of biochemical changes of SA-induced rice leaves by fourier transform infrared (FTIR) spectroscopy

The experiment was conducted with five replications, one leaf per replication. Leaf samples of both SA-treated and non-treated rice plants were collected at 14 DAI (Stehfest et al., 2005) and dried in a hot air oven at 60 ^{o}C for 3 days, then ground by sterile pestles and mortars into the fine powder. Each powdered sample was taken at an equal weight and subsequently subjected to FTIR spectroscopy (Banerjee et al., 2010; Buensanteai et al., 2012) at the Synchrotron Light Research Institute, Thailand. The infrared spectra were recorded using FTIR spectroscope (Bruker Optics Ltd., Ettlingen, Germany). The spectra were collected in the 4000-900 cm^{-1} mid-infrared range at a spectral resolution of 4 cm^{-1} (Panitlertumpai et al., 2013). The individual spectra from each group were analyzed using principal component analysis (PCA) to distinguish different biochemical components of the samples by the Unscrambler X 10.1 software (CAMO, Norway). The Savitzky-Golay method (3rd polynomial, 9 smoothing points) was employed to perform second derivative spectra, then normalized using the extended multiplicative signal correction.

Unsupervised hierarchical cluster analysis (UHCA) was performed on the second-derivative spectra employing Ward's algorithm. This method utilized a matrix to define inter-spectral distances and calculated spectra distances as D-values (Buensanteai et al., 2012).

4.2.6 Assessment of *Xoo* growth in the rice plants

The experiment was carried out with five replications, one leaf per replication. The inoculated leaves of both SA-treated and non-treated rice plants were collected at 0, 1, 2, 7, and 14 DAI. Leaves were surface sterilized with 95% ethanol (v/v). Later, leaves were ground in sterile 10 mM $MgCl_2$. Leaf homogenate was

dilluted in sterile 10 mM $MgCl_2$ solution and the serial dilutions were plated on NGA plates. The NGA plates were incubated at 28 ^{o}C for 48 h (Hu et al., 2007; Zhao et al., 2013).

4.2.7 Statistical analysis

All experiments were repeated for 3 times, with similar results in all replications. Data were analyzed and subjected to analysis of variance (ANOVA) (SPSS software, version 16). The significance of treatments was determined by the magnitude of F value (P = 0.05). Treatment means were separated by duncan's multiple range test (DMRT).

4.3 Results

4.3.1 Levels of superoxide anion and hypersensitive response (HR) of SA treated-leaves against *Xoo*

In the rice leaves treated with SA and inoculated with *Xoo*, numerous blue spots appeared at 3 HAI and gradually decreased at 6, 12 and 24 HAI (Figure 4.1a and 4.1b). Blue spots were also observed in the inoculated rice leaves treated with distilled water, but were less numerous compared to that of the SA-treated leaves (Figure 4.1a and 4.1b). In the uninoculated leaves of both SA-treated and -non treated, blue spots were not detected regardless of the sampling times.

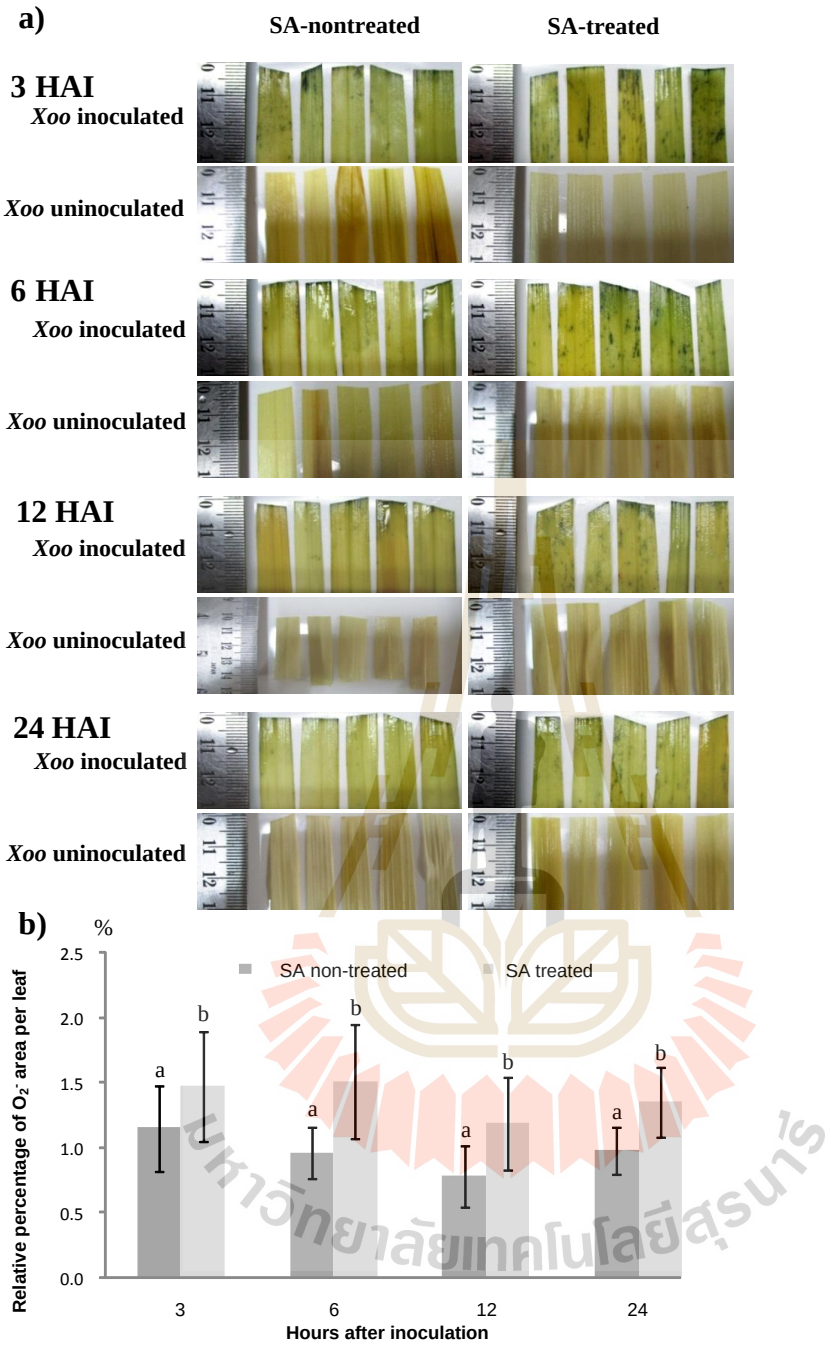

Figure 4.1 Superoxide anion levels in KDML 105 rice leaves treated with 1 mM SA and challenge inoculated with *Xoo* under a greenhouse condition assessed at 3, 6, 12 and 24 HAI. a) Dark blue spots of formazan compound resulting from the reaction of NBT with superoxide anion. b) Quantification of

superoxide anion levels using Image J software. Data are presented as mean ± SE (n=5).

At 48 HAI, HR was assessed by measuring fluorescense in the rice leaf epidemis at the inoculation point using confocal microscopy. Our results showed that the *Xoo* uninoculated leaves had no fluorescence, while the inoculated leaves showed bright fluorescence. In the *Xoo*-inoculated group, a high level of fluorescence was observed on rice leaves treated with SA (Figure 4.2a), and correlates with the high levels of the superoxide observed in Figure 4.1a. The quantification of HR had a similar pattern with that of superoxide. Rice leaves treated with SA and challenge inoculated with *Xoo* showed relatively higher fluorescence than that of rice leaves treated with distilled water and inoculated with *Xoo*, but were not statistically significant (Figure 4.2b). The HR played an important role in plant defence against bacterial infection, resulting in the appearance of necrotic lesions on leaves. Bacterial leaf blight symptoms were pale-green to grey-green water-soaked streaks, becoming whitish or greyish from the inoculation points, and developed along the leaf margins.

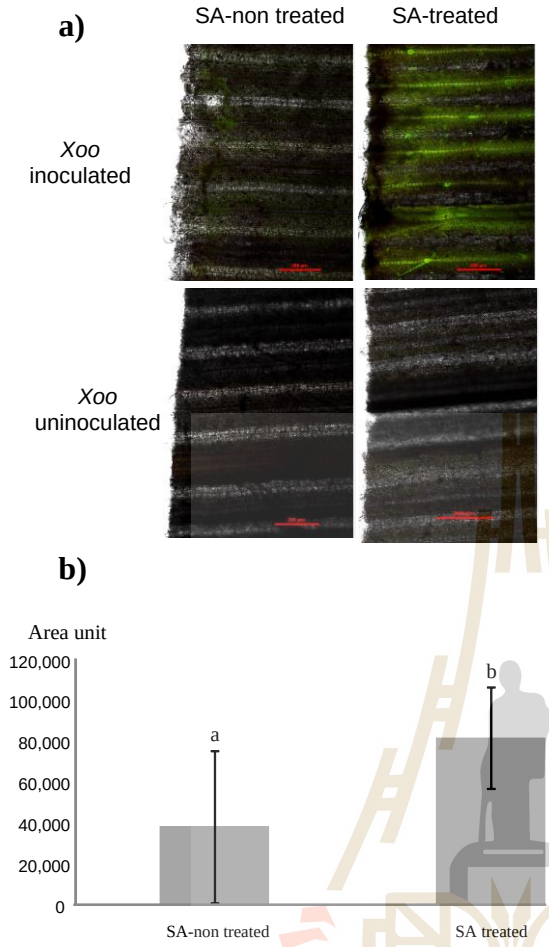

Figure 4.2 Hypersensitive response (HR) levels in KDML 105 rice leaves treated with 1 mM SA and challenge inoculated with *Xoo* at 48 HAI under a greenhouse condition. a) The HR expression was examined under a confocal microscope. b) Quantification of HR levels using Image J software. Data are presented as mean ± SE (n=5).

Our results showed that there was no blue spots of superoxide reaction and no fluorescence of HR reaction from all *Xoo* uninoculated treatments, proving that there is no histopathological defense reactions.

4.3.2 Biochemical changes of SA treated-rice leaves against *Xoo*

Salicylic acid plays an important role in the induction of plant defence against *Xoo* infection. The results showed original average spectra and second derivative average spectra of chemicals in the KDML 105 rice leaves in the range of 3000-2800 cm^{-1} and 1800-900 cm^{-1} (Fig. 4.3). The band assignments of FTIR spectra have been published in numerous publications (Table 4.1), but induced resistance has rarely been the focus of these studies. The FTIR peaks in our research were assigned to different functional groups in accordance with the wavenumbers (cm^{-1}) previously reported in the literatures.

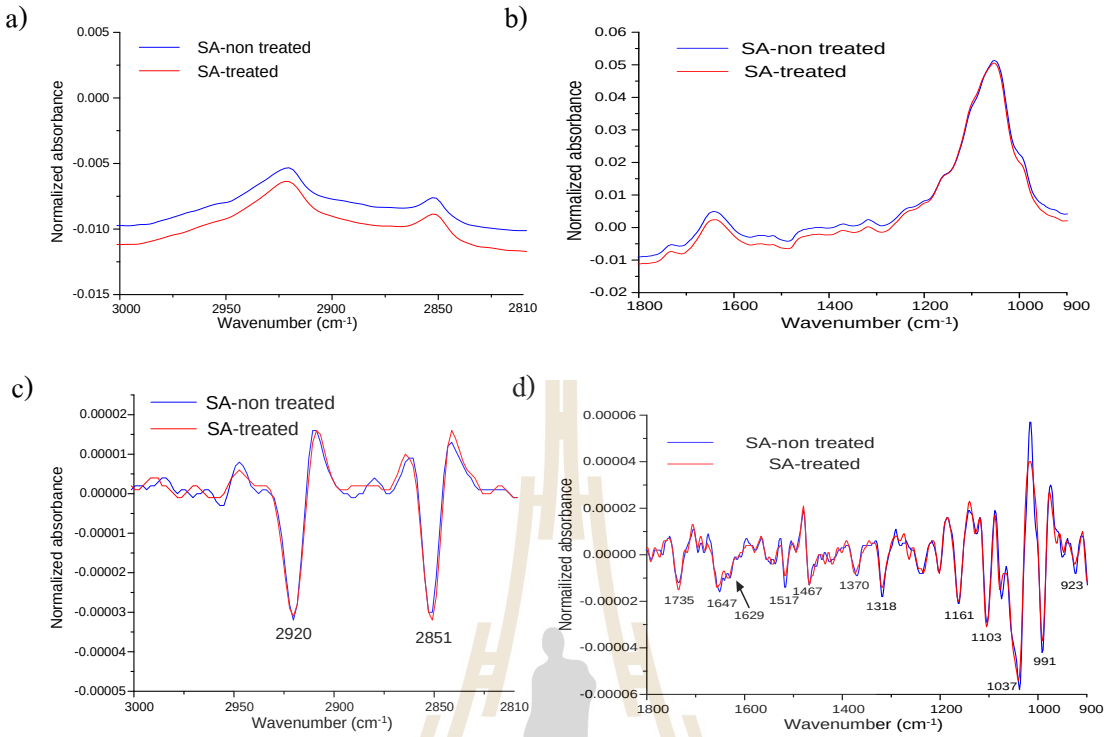

Figure 4.3 Representative original average FTIR spectra (a and b) and second derivative average spectra (c and d) in KDML 105 rice leaves treated with 1 mM SA and inoculated with *Xoo*, at 14 DAI, under a greenhouse condition. a) Representative original average spectra in the range of 3000-2810 cm^{-1}. b) Representative original average spectra in the range of 1800-900 cm^{-1}. c) Representative second derivative average spectra in the range of 3000-2810 cm^{-1}. d) Representative second derivative average spectra in the range of 1800-900 cm^{-1}. Twelve spectra per group were preprocessed by taken second derivative spectra after 9 points of smoothing and normalized with EMSC over the range.

Table 4.1 Band assignments of FTIR vibration peak (cm^{-1}) of plant rice leaf tissue based on references

Peak name	Spectral ranges	Vibration peak assignments	References
C-H stretching vibration	3000-2800	C-H Asymmetric and Symmetric stretching vibration of mainly lipid groups with the little contribution from protein,	Jouraiphy et al. (2008); Iqbal et al. (2009); Mularczyk-Oliwa et al. (2012); Sivakumar et al. (2014); Lahlali et al. (2015)
C=O esters	1740-1700	Stretching vibration of C=O ester of bond lipid, lignin, pectin or their esters	Sene et al. (1994); Kenneth and Lawrence (2005); Dokken and Davis (2007); Iqbal et al. (2009); Lahlali et al. (2015)
Amide I	1700-1600	Amide I due to C=O stretching of α-helix protein, contribution from C-N stretching (C=O stretch (80%), C-N stretch (10%), N-H bending (10%))	Sene et al. (1994); Yu et al. (2003); Ellepola et al. (2005); Jouraiphy et al. (2008); Sivakumar et al. (2014); Lahlali et al. (2015)
Amide II	1600-1500	Amide II due to N-H bending and C-N stretching of protein	Sene et al. (1994); Wetzel and LeVine

Peak name	Spectral ranges	Vibration peak assignments	References
		(N-H bend (60%), C-N stretch (40%))	(2000); Jouraiphy et al. (2008); Sivakumar et al. (2014)
C=O aromatic ring	1517	C=C aromatic ring from lignin, C-H bend	Sene et al. (1994); Wetzel and LeVine (2000); Mularczyk-Oliwa et al. (2012); Wang et al. (2012); Lahlali et al. (2015)
C-H bending	1470-1350	C-H bending from CH2 and CH3 from mainly lipids and lignin	Sene et al. (1994); Kenneth and Lawrence (2005); Kumar and Min (2011)
C-O Stretching hemicellulose and lignin	1300-1200	C-C, C-O skeletal	Alonso-Simon et al. (2004); Mularczyk-Oliwa et al. (2012)
C-C ring cellulose	1155	C-C ring from cellulose	Sene et al. (1994); Kacurakova et al. (2000); Alonso-Simon et al. (2004)
C-O-C	1103	C-O-C glycoside ether mainly	Dokken and Davis

Peak name	Spectral ranges	Vibration peak assignments	References
glycoside		hemicellulose	(2007)
C-C , C-O Stretching	1022, 1047, 1080	Stretching vibration of C-OH of alcohic groups and carboxylic acid, C-C bond of the cellulose sugar rings. Mainly C-O-C of polysaccharides	Sene et al. (1994); Kacurakova et al. (2000); Alonso-Simon et al. (2004); Iqbal et al. (2009); Suehara et al. (2012)

Second derivative spectra were applied to normalise the spectra accounting for varation in sample thickness, thus minimizing the baseline variation and providing better visual identification of bands that may overly another in the raw spectra. Second derivative spectra in the range 3000-2810 cm^{-1} between control and SA-treated rice leaves were compared in Figure 4.3c. The broad band at the peak 2851 cm^{-1}, assigned to C-H stretching vibration, was more intense in the SA treatment, compared to that of the control treatment. The comparsions between spectra of control and treated rice leaves on the range 1800-900 cm^{-1} are shown in Figure 4.3d. Biochemical changes in the SA treatment showed significantly higher spectra shifts than that of the control in three vibrational peaks as 1735 cm^{-1}, 1467 cm^{-1} and 1103 cm^{-1}. These peaks can be assigned to C=O esters (peak 1735 cm^{-1}); C-H bending (peak 1467 cm^{-1}); and C-O-C glycoside (peak 1103 cm^{-1}). In addition, alpha helix structure (1647 cm^{-1}) of amide I protein on leaves treated with 1 mM SA was changed to β-sheet

structure (1629 cm^{-1}) of amide I. In contrast, band alpha helix remained and more intense in the control rice leaf (Figure 4.3d).

The PCA technique was employed to analyze biochemical changes in the rice leaves treated with SA. The use of multivariate analysis, in particular the PCA, has proven useful in analysis of biospectroscopic data, providing two types of information: visualization of clustering of similar spectra of datasets in scores plots; and identification of variables in loadings plots. Three dimensional PCA score plot is shown in Figure 4.4a. The red points representing control treatment, although scattered widely on the right side of the graph area, can easily be distinguished from the blue points of the SA-treated one. The loading plot was used to identify variables on spectral bands that representing the correlation with the second derivative of the average spectra. The positive loading plot at 2848 cm^{-1}, 1735 cm^{-1}, 1467 cm^{-1} and 1107 cm^{-1} oppositely correlated with the negative score plot of SA treatment. The negative loading plots at 1647 cm^{-1}, 1311 cm^{-1}, 1024 cm^{-1} and 990 cm^{-1} showed an opposite correlation with the possitive score plot of the control treatment.

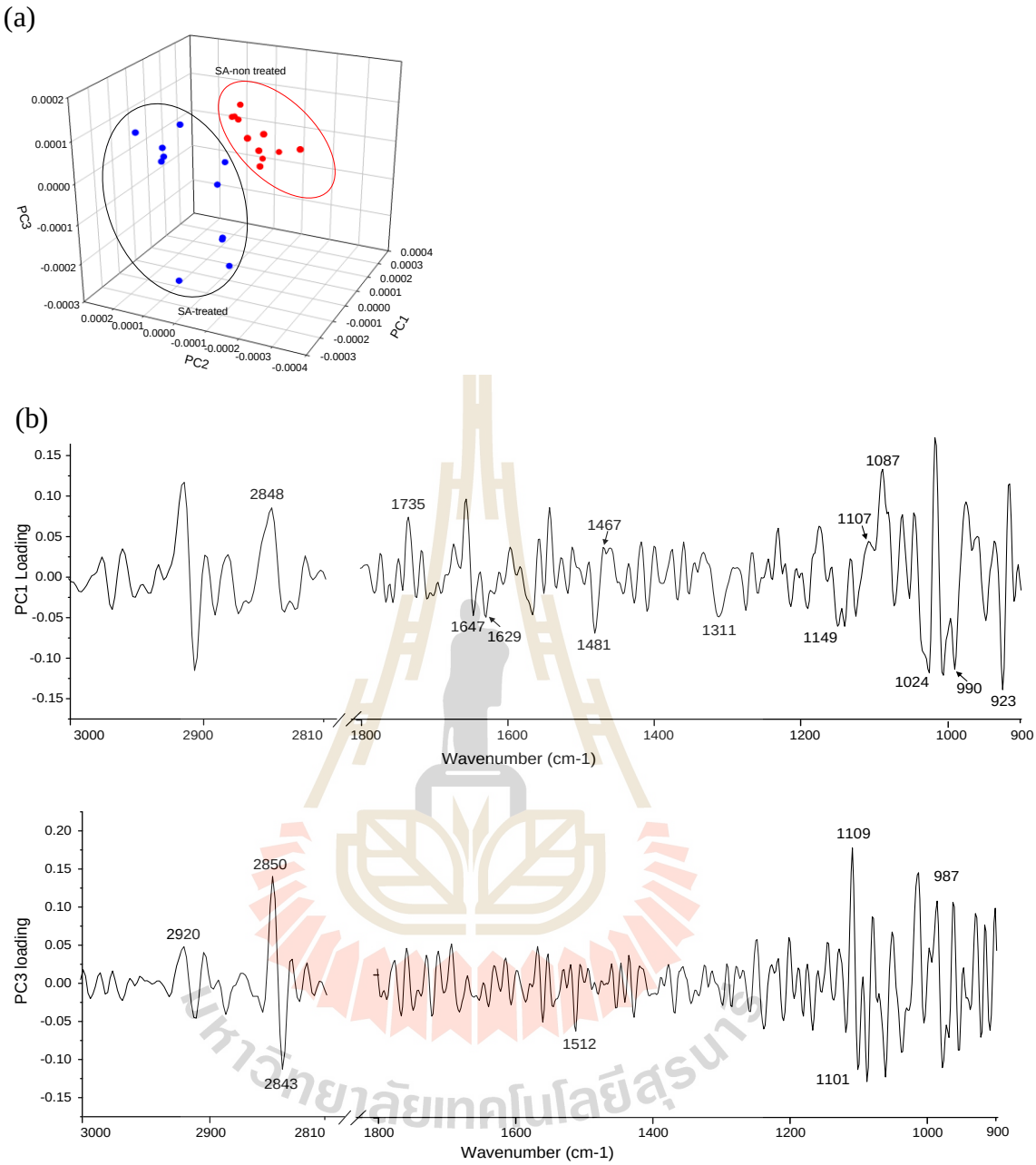

Figure 4.4 PCA analysis in KDML 105 rice leaves treated or non-treated with 1 mM SA and inoculated with *Xoo*, at 14 DAI, under a greenhouse condition. a) 3D scatter plot of score from PCA analysis. b) PC1 loading plot from PCA analysis in the range of 3000-2810 cm^{-1} and 1800-900 cm^{-1}. c) PC3 loading plot from PCA analysis in the range of 3000-2810 cm^{-1} and 1800-900 cm^{-1}.

We next investigated whether a classification procedure could provide more information about the biochemical differences of rice leaves treated with SA and then challenge inoculation with *Xoo*. To carry out such a classification, a cluster analysis was used. A dendrogram corresponding to the FTIR spectra is shown in Figure 4.5. The dendrogram displays two main branches such as A and B. The upper main branch A is separated by 1.2 units from lower main branch B. The upper main branch A is split into two sub-groups A1 and A2, separated by approximately 0.5 units. The upper main branch A contains spectra of five replications of the treatment treated with SA. The spectra within the lower main branch B are five replications of the SA-nontreated control treatment. Within this lower main branch B, the heterogenity between spectra is approximately 0.2 units.

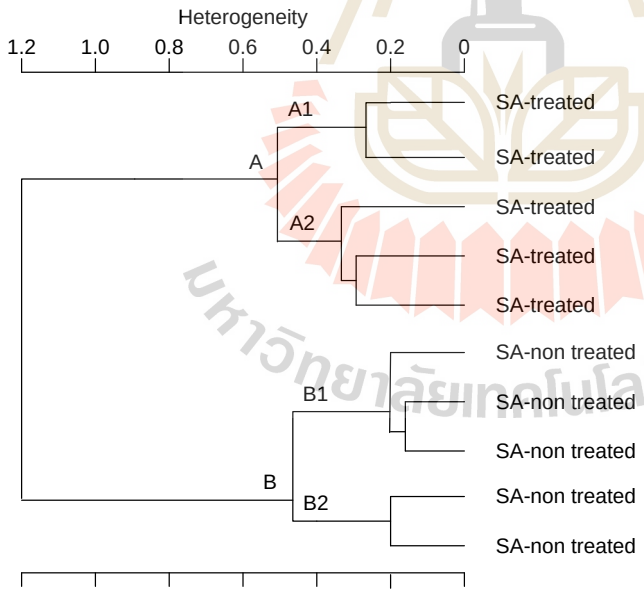

Figure 4.5 Cluster analysis of FTIR spectra in the range of 3000-2810 cm^{-1} and 1800-900 cm^{-1} in KDML 105 rice leaves treated or non treated with 1 mM SA and inoculated with *Xoo*, at 14 DAI, under the greenhouse condition.

To further specify changes of lignin and pectin in rice leaves treated with SA, ratios of 1233 / 1517 cm^{-1}, 1467 / 1517 cm^{-1} and 1735 / 1517 cm^{-1} wavelength were calculated. These biochemical component ratios indicated that SA-treated rice contained twice as much lignin and pectin compared to the control treatment as the data was statistically significant with $P \leq 0.05$ (Figure 4.6).

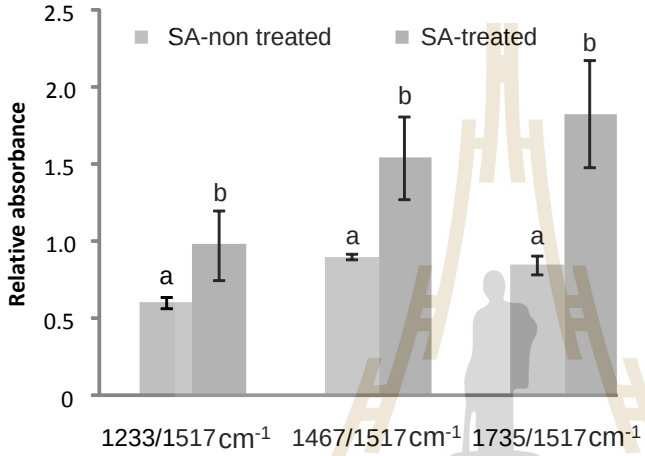

Figure 4.6 Relative absorbance ratio of some spectral peaks to the intensitive at 1517 cm^{-1} in KDML 105 rice leaves treated or non treated with 1 mM SA and inoculated with *Xoo*, at 14 DAI, under the greenhouse condition. Error bars represent standard deviation from 6 replications. Ratio of 1233/1517 cm^{-1} represents methoxyphenolic substitution in aromatic units of lignin. Ratio of 1467/1517 cm^{-1} is the ratio of syringyl to guaiacyl (S/G) of lignin. Ratio of 1735/1517 cm^{-1} is representative of an alteration in pectin synthesis.

4.3.3 Assessment of *Xoo* growth in the rice plants

Distinct difference was observed at 1 DAI between rice leaves treated or non-treated with SA. Population size of *Xoo* in rice leaves treated with SA reached maximal growth approximately $4.4x10^5$ cfu per leaf at 7 DAI, while that in rice leaves

treated with distilled water continued to multiply and reached a maximal population approximately $5.6x10^9$ cfu per leaf at 14 DAI. Maximal population size of rice leaves treated with SA was lower amount of those treated with distilled water at 14 DAI (Figure 4.7), suggesting that growth of *Xoo* was inhibited in rice leaves when treated with SA.

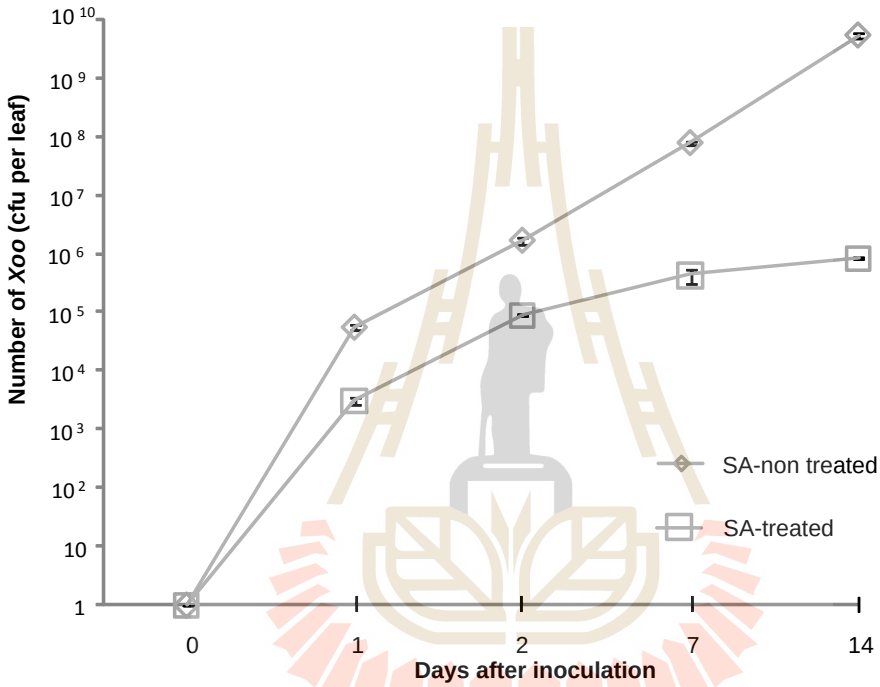

Figure 4.7 Growth curve of *Xoo* in KDML 105 rice leaves treated or non treated with 1 mM SA and inoculated with *Xoo*, under the greenhouse condition. Error bars represent standard deviation from 5 replications.

4.4. Discussion

The goal of this study was to characterize rice responses to SA inducer and *Xoo* inoculation in association with induced resistance. Resistance was assessed using analysis of O_2^-, HR, and biochemical composition changes including lignin,

pectin, amide I and lipid in rice cell, which are plant responses to pathogens (Lamattina and Polacco, 2007; Buensanteai et al., 2009; Pastor et al., 2013). Our results found that treatment of rice seeds and leaves with SA resulted in significant increases in these resistance markers in rice leaves challenged with *Xoo* inoculation compared to non infected control.

The reduction in BLB severity in rice treated with SA could result from the first resistance marker O_2^-. Nitroblue tetrazolium (NBT) staining compound reacts with O_2^- to form a dark insoluble formazan compound and precipitates in the reactive rice leaf area as dark blue spots. In the non-inoculated leaves of all treatments, regarless of the sampling times, no blue spots were detected in the leaf tissues, indicating that O_2^- in the cells was maintained at the cellular redox balance. In our study, the defence system of rice cells treated with 1 mM SA could be systemically activated and enhanced, prior to challenge inoculation with *Xoo*. Our results showed that, after inoculation with *Xoo*, rice leaves treated with SA produced 28% more O_2^- than that of the control treatment. The O_2^- increased twice as much compared to that of the control treatment at 6 HAI, then decreased gradually at 12, 24 and 48 HAI (Pietrowska et al., 2015). The pattern production of O_2^- in response to temperature stress and pathogen infection is variable in each plant (Dring, 2006) which the production of O_2^- was rapid, intense and short-lived. It is known that after the production, O_2^- is immediately released at external surface of the membrane (Auh and Murphy, 1995). The O_2^- not only has direct toxicity to pathogens, but also is the central component of the plant defence signal transduction pathways leading to the hypersensitive response (HR), cell wall reinforcement, salicylic acid synthesis and defence gene expression (Lamb and Dixon, 1997; Lehotai et al., 2011; Graves, 2012; Sharma et al., 2012; Pastor et al., 2013). The O_2^- could be converted into H_2O_2 - a

substrate for peroxidase which mediating different plant cell wall components such as cellulose, lignins, pectins and caloses (Carpin et al., 2001; Vreeburg and Fry, 2005).

Rapid rise of O_2^- leads to occurence of HR (Lamb and Dixon, 1997), the second resistance marker in our study. The accumulation of phenolic compounds, an indication of HR, was analyzed using confocal microscopy to after excitation at 488 nm (Czymmek et al., 2002). The HR can be observed on rice leaves at 36-48 HAI with *Xoo* (Jha et al., 2007). Our results showed that rice leaves treated with SA had 110% higher HR than that of the control at 48 HAI. In our study, it could be concluded that the HR was created, leading to formation of a zone of dead cells around infection zones to stop *Xoo* spread and BLB disease development with the induced effect of SA.

The differential expression of the biomarkers of O_2^- and HR after *Xoo* challenge inoculation was similar to the induction of O_2^- and HR in soybean, sweet orange and cassava by inducers including SA, chitosan, *B. amyloliquefaciens* KPS46 and *B. subtilis* CaSUT007 that were primed by exposure to lignin, pectin and amide I alteration, and lipid accumulation (Shirasu et al., 1997; Buensanteai et al., 2009; Coqueiro et al., 2015; Thumanu et al., 2015) to reinforce the plant cell wall (Banerjee et al., 2010). The resistance markers including lignin, pectin, amide I and lipid in SA-treated rice were investigated by FTIR spectroscopy. Most of peaks of FTIR spectra in our study were similar to those of the report of Sene et al. (1994) in non-inoculated rice plants.

Lignin and pectin, components of polysaccharides, are the third and fourth biomarkers that we examined in our study (Sene et al., 1994; Wang et al., 2012). Cell polysaccharides composition is a critical factor in host–pathogen interactions, resulting in improving disease responses (Vorwerk et al., 2004; Miedes et al., 2014). Polysaccharides from plant cells, when degraded into oligogalacturonides, can elicit

defence responses in plants (Shibuya and Minami, 2001). Our results showed that rice leaves treated with 1 mM SA and challenge inoculated with *Xoo* had a higher peak C-O-C glycoside at 1103 cm^{-1}, when compared to rice leaves treated with distilled water and challenge inoculation with *Xoo*. Regarding to the polysaccharides detected within the range of 1200-900 cm^{-1}, our results showed that only one peak at 1103 cm^{-1} was more intense in SA treatment while other peaks were intense in control treatment. These results are similar to the FTIR analysis of Thumanu et al. (2015) where epidemal cells of cassava leaves treated with the biotic elicitor *B. substilis* CaSUT007. This strain *B. substilis* CaSUT007 could induce resistance in cassava against casava anthracnose. Epidemal cells had initial contact with anthracnose pathogens during fungal infection. Similarly, a decrease in the amount of cellulose can lead to herbicide tolerance in Arabidopsis and a consequential structural defect of the cell wall that triggered an increased production of jasmonate (Scheible et al., 2001). Some questions remain as to why most polysaccharide peaks of treated plants in the range of polysaccharides from 1200 cm^{-1} to 900 cm^{-1} were less intense than that of the control, indicating that reduced accumulation of polysaccharide components. Those could be the alteration of materials of polysaccharides. Rice treated with SA may rearrange their polysaccharides to synthesize some specific materials for disease resistance such as lignin or pectin. Alteration of lignin monomer composition during disease resistance has been reported (Faix, 1991; Barber et al., 2000; Martin et al., 2005; Menden et al., 2007). The higher ratios of 1233/1517 cm^{-1}, 1467/1517 cm^{-1} and 1735/1517 cm^{-1} wavelengths in our results are in line with research of Martin et al. (2005), showed these ratios in resistance Dutch elm plants were higher than approximately 20-33% than those of susceptible ones. The third defence marker of lignin in rice treated with SA and challenge inoculation with *Xoo* is characterized by the ratios of 1233/1517 cm^{-}

[1] and 1467/1517 cm^{-1} wavelengths. First of all, the ratio of 1233/1517 cm^{-1} represents methoxyphenolic substitution in aromatic units of lignin (Dorado et al., 2001). In addition, changes in band intensities were shown in the ratio of 1467/1517 cm^{-1}. This correlates to the ratio of syringyl to guaiacyl (S/G) of lignin (Faix, 1991). In our study, a higher S/G ratio in leaves treated with 1 mM SA relative to the control treatment indicated an increased synthesis of syringyl monomers occurred in treated leaves. The S unit of lignin has been shown to accumulate in resistant wheat leaves during HR (Bishop et al., 2002; Menden et al., 2007). Changes in the S/G ratio can be explained by changes in the activity of phenylalanine ammonia-lyase (Chen and McClure, 2000; Gayoso et al., 2010). *Pal* catalyses and regulates the production of precursors for lignin biosynthesis in plant cells (Nicholson and Hammerschmidt, 1992). When the *Xoo* tries to penetrate the plant cell wall, the rice cell wall undergoes dynamic structural and biochemical changes (Eggert et al., 2014), including alteration in lignification (Nicholson and Hammerschmidt, 1992; Carver et al., 1998; Zhao and Dixon, 2014). Lignin is a difficult polymer for *Xoo* to degrade (Nicholson and Hammerschmidt, 1992). The increased lignification is reflected in cucumber and pepper by enhanced *apx* (Wu et al., 1997; Ray et al., 1998; Zheng et al., 2005), and in potato by *pal* (Henderson and Friend, 1979). In tomato, exogenous application of 0.01% SA enhanced resistance associated to a greater accumulation of lignin in roots upon bacterial *Ralstonia solanacearum* infection (Mandal et al., 2013). In addition, application of 0.6 mM SA enhanced pectin and lignin synthesis in soybean (Al-Hakimi, 2006).

Besides changes of lignin, alteration in pectin, the fourth biomarker in our study, also activates plant against disease. The ratio of 1735/1517 cm^{-1} is representative of an alteration in pectin synthesis (Chatjigakis et al., 1998). Enrichment

of pectins strengthens of barrier properties of cell walls during the stage of colonization by the pathogen (Cherif et al., 1991; Eckardt, 2002; Raiola et al., 2011; Volpi et al., 2011; Bethke et al., 2014).

In Gramineae species, the cell is poor in protein composition (Sene et al., 1994; Wang et al., 2012). Our results showed that amide I protein, the fifth defence marker, was changed in the range of 1700-1600 cm^{-1} wavelengths. The alpha helix structure of amide I protein was converted to amide I β-sheet structure (1629 cm^{-1}) in leaves treated with 1 mM SA, but band α- helix (1647 cm^{-1}) was more intense in the control leaf. Amide I β-sheet structure can be involved the resistance of plant to pathogen (Thumanu et al., 2015). Amide I β-sheets secondary of necrosis-inducing protein 1 (NIP1) had a major role in barley against fungal pathogen *Rhynchosporium secalis* (van't Slot et al., 2003). These authors suggested that in amide I β-sheets secondary of NIP1, cysteins of diffent domains form disulfide bonds, leading to a new stable fold structure. Change of amide I structure lead to introduce high affinity binding site for plant receptors to transduce defence signal (Green et al. 2003; van't Slot et al., 2003).

The last defence marker in rice leaves treated with SA and challenge inoculation with *Xoo* is lipid accumulation. The peak at 2851.3 cm^{-1} (CH_2 stretching vibration) and 1735 cm^{-1} (C=O esters) in leaves treated with 1 mM SA and inoculated with *Xoo* was more intense than those of leaves treated with distilled water and inoculated with *Xoo*. In 2002, Feussner and Wasternack reported that formation of oxygenated fatty acids, abbreviated as oxylipins, was a response of plant cells against abiotic and biotic stress. Oxylipins could directly act as antimicrobial compounds or indirectly stimulate defence gene expression (Farmer et al., 2003). Additionally, two groups of galactolipids had different roles during the induction of systemic resistance.

Plant digalactosyldiacylglycerol (DGDG) contributed to plant nitrite oxides as well as salicylic acid biosynthesis and is required for the induction of systemic acquired resistance (SAR). In contrast, plant monogalactosyldiacylglycerol (MGDG) controlls the biosynthesis of the SAR signals azelaic acid (AzA) and glycerol-3-phosphate (G3P) (Gao et al., 2014)

In addition to, SA could be suppress *Xoo* growth and population. The results of Zhang et al. (2013) on putative virulence-relevant genes of *Xoo* in rice showed that *Xoo* population was approximately 10^3 to 10^4 cfu per leaf at 1 and 2 DAI. In another report on resistance of transgene Xa21 rice variety, the *Xoo* population was decreased approximately 10^4 to 10^5 cfu per leaf at 1 and 2 DAI (Peng et al., 2015). Maximum *Xoo* population in rice leaves was usually recorded at 12-15 DAI (Hu et al., 2007; Zhao et al., 2013; Chen et al., 2014). In lettuce, population size of *X. campestris* pv. *vitians* was decreased at leaves showing HR, compared to control treatment (Bull et al., 2015). Due to apoplastic water potential decreases during the process of HR, leading to inhibition of growth for bacterial pathogens (Wright and Beattie, 2004).

We also propose a simple model of systemic resistance is illustrated in this current study. After treatment, SA is bound by plant receptors on plasma membrane such as GTP binding proteins or SA-binding proteins, leading to change in receptor conformation that results in activation of kinases and ion channels (Klessig et al., 2000; Kumar and Klessig, 2003; Chen and Ronald, 2011). Rice contains seven genes encoding Rac GTPases which activate protein kinases (MAPK cascades). MAPK cascades could transmit extracellular signals to downstream components through protein phosphorylation. Seventeen MAPKs have been identified in rice (Ichimura et al., 2002; Chen and Ronald, 2011). A MAPKKK firstly phosphorylates a serine or threonine residue on a MAPKK, which in turn, activates a MAPK by the dual

phosphorylation of a threonine and tyrosine residue (Sinha et al., 2011; Cakir and Kilickaya, 2015). Activated effectors continue to transfer the signals of molecule SA to secondary messengers which could amplify the signals and send them to other reactions (Rivas and Thomas, 2005). Following signal transduction, a series of defense responses as the induction of signal transduction pathway; increasing the ion fluxes; extracellular alkalinization and cytoplasmic acidification, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation, oxidative burst, ROS production, HR, phosphorylation, and early defence reaction are activated (Koornneef et al. 2008; Kepczynska and Kro 2012; Mishra et al. 2012; Chinta et al. 2015; Al-Issawi et al. 2016). It also could create a long distant SA-signal, stimulate late defence genes and produce secondary metabolite accumulations (Koornneef et al., 2008; Mishra et al., 2012). Ion fluxes conclude Ca^{2+} and H^{+} influx, K^{+} and Cl^{-} exflux which are immediate responses of plant cells, leading to extracellular alkalinization and cytoplasmic acidification (Boller, 1995; Mishra et al., 2012; Pastor et al., 2013). Another common early significant defence event in plant cell defense responses is the oxidative burst and ROS (Albert et al., 2006). The presence of ROS may result from the transfer of one, two and three electrons, respectively, in the reduction of molecular O_2 from predominantly superoxide anion (O_2^{-}), peroxide hydrogen (H_2O_2) and hydroxyl (HO^{-}) (Mittler, 2002; Mishra et al., 2012). ROS is not only have direct toxicity to pathogens, but also is the central component of plant defense signal transduction pathways leading to the HR, cell wall reinforcement and defense gene expression (Pastor et al., 2013). The death of cell both inhibit the invasion of bacterial pathogens inside plant and stimulate a production of SA (Nooden, 2004). This kind of phytohormone can directly affect the pathogens as well as act as a signal of systemic acquired resistance (SAR) which is a SA signalling pathway (Walter et al., 2007; Prakongkha et al., 2013;

Buensanteai et al., 2014). The SA signal stimulates defence genes involved in cell wall modification, PR proteins, biosynthesis and production of secondary metabolites in plants (Stintzi et al., 1993; Taguchi et al., 2001; Mishra et al., 2012). A characteristics of plant cell wall undergoing pathogen attack in the formation of structural barriers including lignin and pectin (Cherif et al., 1991; Nicholson and Hammerschmidt, 1992; Carver et al., 1998; Quiroga et al., 2000; Eckardt, 2002; Al-Hakimi, 2006; Raiola et al., 2011; Volpi et al., 2011; Mandal et al., 2013; Bethke et al., 2014; Zhao and Dixon, 2014). Secondary metabolites including phenolic compounds, phytoalexins and compounds involved in HR are important to defense (Mishra et al., 2012). These preparation stage would help treated plants to be resistant against a broad spectrum of pathogens. After that, when the rice plant is inoculated with *Xoo*, the host could quickly recognize this infection, create stronger signal transduction pathway, ion fluxes, ROS, cell wall fortification, phytoalexins and long distance signal. All of these would stimulate defence genes to synthesize many kinds of defence products to help plant stop invasion or any secondary infection by *Xoo*. The immune responses of SA-treated plants would be faster and stronger than that of the non-treated ones.

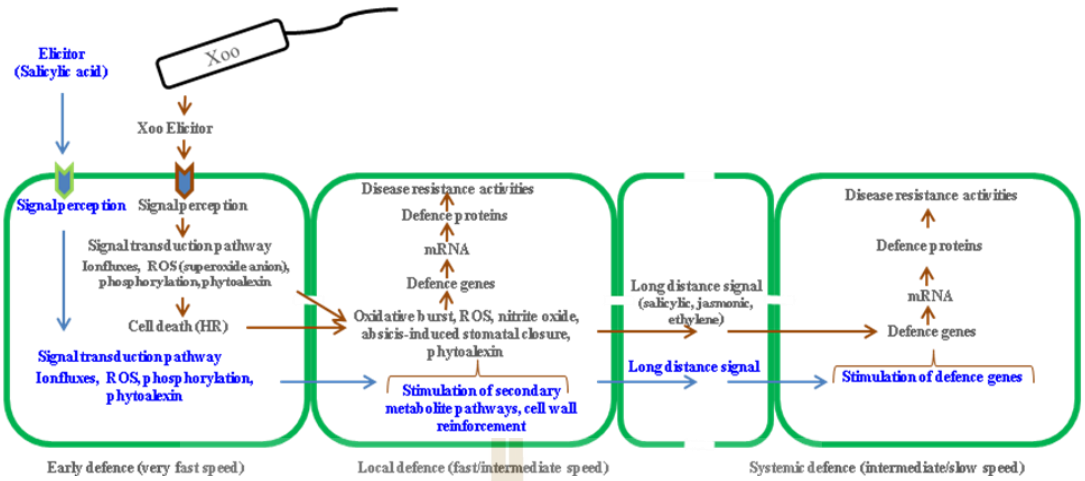

Figure 4.8 A proposed schematic model of induced resistance in rice plant against *Xoo* after being treated with SA. Briefly, the molecule of SA would be attacted by plant receptor such as GTP binding proteins or SA binding protein, leading to change in receptor conformation, resulted in activating kinases and ion channels. Activated effectors continue to transfer the signals of molecule SA to secondary messengers which could amplify the signals and send them to other reactions. Following pathogen recognition and signal transduction, a series of defense reactions as the activation of signal transduction pathway; increasing of ion fluxes; ROS; phosphorylation; phytoalexin; and activating of secondary metabolite pathway. This inducer could create long distant signal and stimulate defence genes. After activation, treated rice plants could quickly recognize this infection of *Xoo*, leading to stimulation of HR and cell death which isolates the pathogen infection. The SA-treated rice plant could fortificate cell wall, producing more phytoalexins and systemic signals. All of them would stimulate defence genes to synthesize many

kinds of defence products to help plant stop invasion or second any infection of *Xoo*.

4.5. Conclusion

The induced resistance in the susceptible rice variety KDML 105 to BLB disease by SA treatment was shown on six defence markers including the increasing production of O_2^-, HR, lipids, the alteration of prevalence of lignins and pectins, and the change of conformation of amide I. Rice cells contain diversified polysaccharide which has a particular molecular conformation interacting with neighboring molecules in a specific way to act various roles on bacterial disease resistance. The polysaccharide of cells was characterized in rice plants treated with SA and challenge inoculation with *Xoo* by using histopathological techniques and FTIR spectroscopy. Thus, this knowledge of defence mechanism induced by SA can be expanded in a species of rice that is susceptible to other pathogens. Additional studies are being conducted to further characterize the mechanism on aspects of transcriptome and proteome of inducer SA against *Xoo* in rice plant.

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CHAPTER V

OVERRALL DISCUSSION AND CONCLUSION

5.1 Overall discussion

The objectives of the study were (1) to evaluate the efficacy of inducers in inducing resistance against BLB in rice cv. KDML105 in Thailand, (2) to characterize mechanism of induced resistance against BLB in rice. Resistance inducers including AA, OA, PDP, SA, CaSUT007 and NT111 were evaluated for their potential in controlling rice BLB disease. Disease resistance in rice is led by a complex expression of signals and defense responses in plants. This defense mechanism can be induced by application of a variety of biotic and abiotic inducers. In rice plant, the research studies on induced resistance in fungal diseases are much more than those in bacterial diseases. The mechanism on induced resistance against BLB disease in rice has not yet been characterized. Additionally, an effective inducer on induced resistance in rice against BLB disease has not been commercialized. This dissertation study aimed to delineate the efficacy of some effective inducers on induced resistance against *Xoo* in Chapter III, and then, the defense responses of induced resistance in rice plant treated by 1 mM SA involved in regulation of defense genes, pathogenicity genes, superoxide anion, HR, the changes of lignin, pectin, amide I and lipid were characterized in Chapter IV.

The efficacy of inducers on BLB disease was shown in Chapter III. Different concentrations of abiotic and biotic inducers including AA, OA, PDP, SA, CaSUT007,

and NT111 show potential to protect rice cv. KDML105 from BLB disease. The efficacy of each inducer varied on controlling rice BLB depending on the rate of application. In the study, 1 mM SA provided a highest protection approximately 55.35% on rice plant from the BLB disease infection, compared to the non treated control. The SA has been evaluated as an inducer in several economic crops, such as in tomato against *Alternaria alternata* f. sp. *lycopersici*, Potato virus X, Potato purple top phytoplasma (Esmailzadeh et al., 2008; Wu et al., 2012; Falcioni et al., 2014), in strawberry against *Colletotrichum gloeosporioides* (Zhang et al., 2016), in wheat against *Fusarium graminearum* (Makandar et al., 2012; Sorahinobar et al., 2015), in corn against Maize rough dwarf virus (Vigliocco et al., 2002), in rice against *Bipolaris oryzae, Magnaporthe grisea* and *Rhizoctonia solani* (Du et at., 2001; Daw et al., 2008; Shabana et al., 2008; Li et al., 2012; Usharani et al., 2013). Exogenous SA application at 1.0 mM concentration induced rice plant against blast disease approximately 27% (Du et al., 2001). Similar results were obtained by Daw et al. (2008) using 8 mM SA, which induced susceptible rice plant against blast disease. Other authors (Wang et al., 2009; Li and Zhang, 2012) observed that the exogenous SA application at different concentration as 0.5, 1 and 2 mM reduced the abiotic stress in rice plants. Certainly, SA acts an important role in induction of plant defense against several pathogens through morphological, physiological, biochemical and molecular level (Du et al., 2001; Daw et al., 2008; Esmailzadeh et al., 2008). In the case of SAR, SA plays a key role, via interaction with SA-binding proteins that can cause build-up of reactive oxygen species or activate gene expression (Kumar and Klessig, 2003). The levels of plant endogenous SA increase around necrotic lesions and remain high in plants having acquired resistance. Moreover, SA itself acts as the defense signal that is translocated systemically throughout the plant (Anwar et al., 2013; Shah and Zeier,

2013). Plant defense mechanism against pathogens involves changes in gene and protein expression mediated by plant signalling transduction pathway (Daw et al., 2008; Esmailzadeh et al., 2008). These defense response pathways are triggered by plant small molecules such as SA, JA, ET and defense enzymes such as APX, PAL, and LOX (Yoshioka et al., 2001; Nakashita et al., 2003; Walter et al., 2007). These defense genes associated with defense enzymes in plant cell were activated by early and secondary signalling transduction molecules and then trigger resistance in plant against pathogen infection (Lamb and Dixon, 1997; Walter et al., 2007).

Induction of plant defense mechanism was confirmed by expression of defense genes and defense enzymes analysis. The 1 mM SA treatment showed up regulation of two rice defense genes including *apx* and *pal*, but inhibited expression of three *Xoo* pathogenicity genes including *hrc*Q, *rpf*F and *rpf*G. Unlike other plants, the rice plant is always endowed with very high endogenous SA levels that seldom increase upon pathogen attack (Silverman et al., 1995; Yang et al., 2004). The average level of endogenous SA in rice is approximately 1 mg/g fresh mass. And a maximum quantity of 37.19 mg/g fresh mass is detected in the leaves of rice (Hayat et al., 2007). The high endogenous SA content in rice could be an important factor contributing to diversified results of reduction of disease severity of rice plants after treated with diferent concentrations of exogenous SA (Du et al., 2001; Daw et al., 2008; Tinivella et al., 2009; Wang et al., 2009; Li and Zhang, 2012; Nagendran et al., 2013; Ibrahim, 2015). The *apx* and *pal* are the important defense genes involved in plant defense mechanism against many plant pathogens. *Apx* gene, grouped in PR9 family, has impotant roles in the HR, polymerization of lignin from monomeric lignols and formation of papillae (Nicholson and Hammerschmidt, 1992; Bestwick et al., 1998; van Loon et al., 2006; Miller et al., 2008; Garcia-Gutierres et al., 2013). *Pal* gene is known to play a role in

the synthesis of phenolic substances, coumarins, and lignins for preventing the penetration of pathogen (Metraux, 2002; Shadle et al., 2003). SA is derived from phenylalanine (Malamy and Klessig, 1992). Therefore, on the phenyl propanoid pathway, *pal* is the principal enzyme of biosynthesis of endogenous SA, phenolics and flavonoids (Dixon and Lamb, 1990; Malamy and Klessig, 1992), establishing to systemic signal of induced resistance. *Pal* gene also catalyses and regulates the production of precursors for lignin biosynthesis in plant cells (Nicholson and Hammerschmidt, 1992). On induced resistance, systemic signals as SA produced from rice plants treated by inducers could influence the pathogen's virulence machinery. Some researches showed that exogenous SA could block biosynthesis of flagella in *E. coli* (Kunin et al., 1995), reduce virulence of *Agrobacterium tumefaciens* (Yuan et al., 2007).

The mechanism of induced resistance in rice against *Xoo* by priming with highest effective inducer as SA was characterized in chapter IV, and shown on six defense markers including the increasing production of O_2^-, HR, and lipids; the alteration of prevalence of lignins and pectins; and the change of conformation of amide I. The study showed that SA- treated rice plants revealed that O_2^- production was enhanced more than 28% at 3-6 HAI, and increased HR to 110% at 48 HAI. The O_2^-, the first defense marker, not only has direct toxicity to pathogens, but also is the central component of plant defense signal transduction pathways leading to the HR, cell wall reinforcement, salicylic acid synthesis and defense gene expression (Lamb and Dixon, 1997; Lehotai et al., 2011; Graves, 2012; Sharma et al., 2012; Pastor et al., 2013). The O_2^- could be converted into H_2O_2 - a substrate for peroxidase which mediating different plant cell wall components such as cellulose, lignins, pectins and caloses (Carpin et al., 2001; Vreeburg and Fry, 2005). The second defense marker HR

was occurred, leading to formation of a zone of dead cells around infestation zones to stop *Xoo* spread and BLB disease development with the induced effect of SA. The differential expression of the biomarkers of O_2^- and HR after *Xoo* challenge inoculation was similar to the indution of those in soybean, sweet orange and cassava by inducers including SA, chitosan, *B. amyloliquefaciens* KPS46 and *B. subtilis* CaSUT007 that were primed by exposure to lignin, pectin and amide I alteration, and lipid accumulation (Shirasu et al., 1997; Buensanteai et al., 2009; Coqueiro et al., 2015; Thumanu et al., 2015) to reinforce the plant cell wall (Banerjee et al., 2010).

Further supporting evidences of induced resistance in SA-treated rice plants were shown on the biochemical changes by using FTIR spectroscopy. Biochemical component as lignin and pectin, the third and fourth biomarkers in our study, was examined (Sene et al., 1994; Wang et al., 2012). The SA-treated rice plants showed a high alteration of lignin and pectin compared with those of the non-treated one. Alteration of lignin monomer composition during disease resistance has been reported (Faix, 1991; Barber et al., 2000; Martin et al., 2005; Menden et al., 2007). The higher ratios of 1233/ 1517 cm^{-1}, 1467/1517 cm^{-1} and 1735/1517 cm^{-1} wavelengths in our results are in line with research of Martin et al. (2005), showed these ratios in resistance Dutch elm plants were higher approximately 20-33% than those of susceptible ones. The third defense marker of lignin in rice treated with SA and challenge inoculation with *Xoo* is characterized by the ratios of 1233/1517 cm^{-1} and 1467/1517 cm^{-1} wavelengths. First of all, the ratio of 1233/1517 cm^{-1} represents methoxyphenolic substitution in aromatic units of lignin (Dorado et al., 2001). In addition, changes in band intensities were shown in the ratio of 1467/1517 cm^{-1}. This is the ratio of syringyl to guaiacyl (S/G) of lignin (Faix, 1991). In our research, the higher S/G ratio in leaves treated with 1 mM SA than the control treatment indicated

that higher synthesis of S monomers occurred in treated leaves. The S unit of lignin has been shown to accumulate in resistant wheat leaves during HR response (Bishop et al., 2002; Menden et al., 2007). Changes in the S/G ratio could be explained by changes in the activity of phenylalanine ammonia-lyase (PAL) enzyme (Chen and McClure, 2000; Gayoso et al., 2010). *Pal* gene catalyses and regulates the production of precursors for lignin biosynthesis in plant cells (Nicholson and Hammerschmidt, 1992). When the *Xoo* tries to penetrate the plant cell wall, the rice cell wall occurs dynamic structural and biochemical component changes (Eggert et al., 2014), including lignification (Nicholson and Hammerschmidt, 1992; Carver et al., 1998; Zhao and Dixon, 2014). Lignin is a difficult polymer for *Xoo* to degrade (Nicholson and Hammerschmidt, 1992). The increased lignification was reflected in cucumber and pepper by enhanced *apx* (Wu et al., 1997; Ray et al., 1998; Zheng et al., 2005), and in potato by *pal* (Henderson and Friend, 1979). In tomato, exogenous application of 0.01% SA enhanced resistance associated to a greater accumulation of lignin in roots upon bacterial *Ralstonia solanacearum* infection (Mandal et al., 2013). In addition, application of 0.6 mM SA enhanced pectin and lignin in soybean (Al-Hakimi, 2006).

Besides changes of lignin, alteration in pectin, the fourth biomarker, also activates plant defense responses against disease. The ratio of 1735/1517 cm^{-1} is representative of an alteration in pectin synthesis (Chatjigakis et al., 1998). Enrichment of pectins strengthens of barrier properties of cell walls during stage of colonization by the pathogen (Cherif et al., 1991; Eckardt, 2002; Raiola et al., 2011; Volpi et al., 2011; Bethke et al., 2014).

Our results showed that amide I protein, the fifth defense marker, was changed in the range of 1700-1600 cm^{-1} wavelengths. The secondary structure of amide I protein was changed from alpha helix structure to beta sheet structure in the leaf cells. Amide I

β-sheet structure could involve the resistance of plant to pathogen (van't Slot et al., 2003; Thumanu et al., 2015). Amide I β-sheets secondary of necrosis-inducing protein 1 (NIP1) had a major role in barley against fungal pathogen *Rhynchosporium secalis* (van't Slot et al., 2003). These authors suggested that in amide I β-sheets secondary of NIP1, cysteins of diffent domains form disulfide bonds, leading to a new stable fold structures. Change of amide I structure lead to introduce high affinity binding site for plant receptors to transduce defense signal (van't Slot et al., 2003).

The last defense marker in rice leaves treated with SA and challenge inoculation with *Xoo* is lipid accumulation. The treated rice plants produced more quantity of lipid, compared to non-treated treatment. The role of lipid on disease resistance has been shown in previous studies (Feussner and Wasternack, 2002; Farmer et al., 2003; Gao et al., 2014). In 2002, Feussner and Wasternack reported that formation of oxygenated fatty acids, abbreviated as oxylipins, was an response of plant cells against abiotic and biotic stress. Oxylipins could directly act as antimicrobial compounds or indirectly stimulate defense gene expression (Farmer et al., 2003). Additionally, two groups of galactolipids had different roles during the induction of systemic resistance. Plant digalactosyldiacylglycerol (DGDG) contributes to plant nitrite oxides as well as salicylic acid biosynthesis and is required for the induction of SAR. In contrast, plant monogalactosyldiacylglycerol (MGDG) controlls the biosynthesis of the SAR signals azelaic acid (AzA) and glycerol-3-phosphate (G3P) (Gao et al., 2014).

Although further proof is needed, these apparent results on gene expression, histopthological study and biochemical changes in the defense responses of induced resistance against *Xoo* strongly suggests that an effective resistance pathway exists.

5.2 Conclussion

This study has generated a new set of tools for high-throughput analyses of rice-inducer-*Xoo* interactions and the regulation of rice defense reactions on induced resistance. These tools were employed for forward the expression of defense genes and pathogenicity genes, the histhopathology responses and the biochemical changes on the interaction of rice-inducer-*Xoo*.

5.3 Suggestion

Although the study has significantly advanced an mechanistic understanding of induced pathogen resistance against BLB disease in rice, several aspects still deserve further experimental investigation. In addition, the study has been opened up several perspectives for future research. Firstly, more studies are needed to fully disentangle the mechanism of induced resistance of SA-induced BLB resistance in rice. Furthermore, the efficacy of effective inducer on induced resistance to BLB disease in the field condition and the way of its commercial inducer formulation can be studied. These future studies will not only shed new light on how inducer induces resistance in rice but also guide novel strategies for biologically based, environmentally friendly and durable disease control in various Asian agricultural settings.

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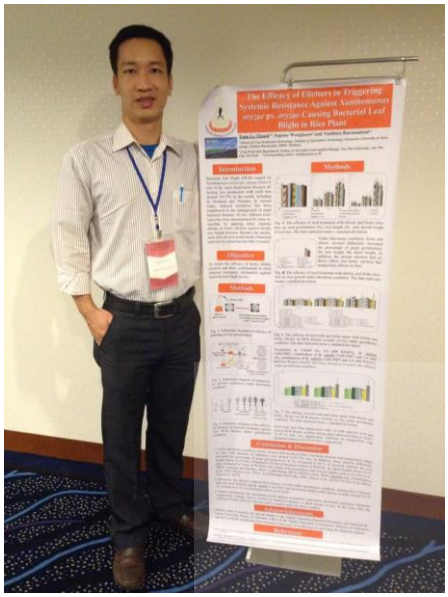
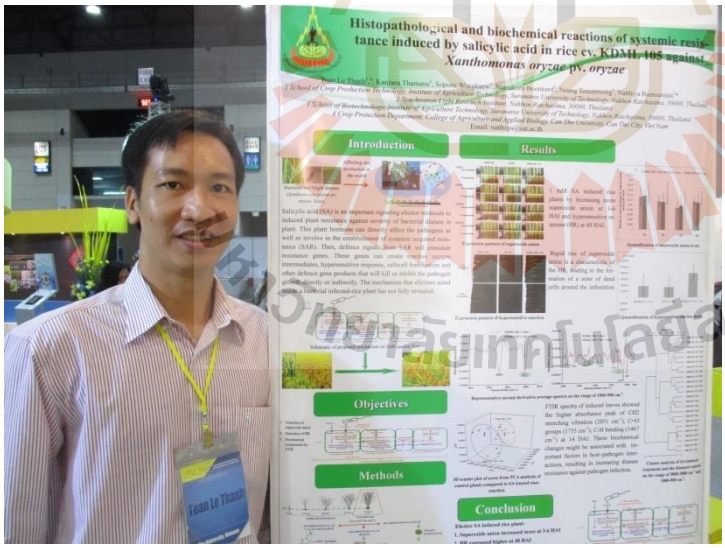
Appendix

I. ATTACHED FIGURE



Attached figure 1. The poster presentation at International Forum Agriculture, Biology and Life Sciences 2015. Sapporo, Hokkaido, Japan. June 23-25, 2015.



Attached figure 2. The poster presentation at AUM2016, Bangkok, Thailand. 25-27 Feb, 2016.

II. CHEMICALS

NGB medium

+ Beef extract	3g
+ Peptone	5g
+ Water	1L
+ Glucose	2.5g

NB medium

+ Beef extract	3g
+ Peptone	5g
+ Water	1L

NGA medium

+ Beef extract	3g
+ Peptone	5g
+ Glucose	2.5g
+ Agar	15g/L
+ Water	1L

III. Xoo strain SUT1-121

Attached table 1 Disease severity of different densities of *Xoo* SUT1-121

Treatment	Disease severity		
	7 DAI [a/]	14 DAI [a/]	21 DAI [a/]
10^5 cfu ml^{-1}	0.00±0.00a	1.43±1.30a	3.81±2.71b
10^6 cfu ml^{-1}	3.33±2.13b	6.67±1.99b	11.90±2.92c
10^7 cfu ml^{-1}	11.43±2.61c	17.14±1.99c	24.29±1.99d
10^8 cfu ml^{-1}	14.29±2.92d	28.57±1.68d	39.52±2.71e
10^9 cfu ml^{-1}	13.33±2.13cd	29.05±1.99d	41.90±2.13e
Uninoculated control	0.00± 0.00a	0.00± 0.00a	0.00± 0.00a
F-test	*	*	*
CV (%)	28.56	11.98	11.37

[a/] Mean ± SE (standard error) followed by the same letter do not different significantly according to Duncan's multiple range test at $P \leq 0.05$.

Attached table 2 Pathogenicity comparision of *Xoo* SUT1-121 to *Xoo* strain reference obtained from Rice Research Department

Xoo strain	Disease severity	
	7 DAI [a/]	14 DAI [a/]
PRE14006	21.43 b	23.81 b
PRE15004	33.81 a	37.62 a
SUT1-121	31.90 a	36.67 a
F-test	*	*
CV (%)	24.59	23.60

[a/] Mean ± SE (standard error) followed by the same letter do not different significantly according to Duncan's multiple range test at $P \leq 0.05$.

BIOGRAPHY

Mr. Toan Le Thanh was born on July, 1983 in Can Tho city, Viet Nam. He received his Bachelor of Agronomy from the Can Tho University, Vietnam in 2006. He got his second Bachelor of English Language and Literature Studies and his Master of Science in Plant Protection from the Can Tho University, Vietnam in 2010.

He started his career as a researcher in Department of Plant Protection, College of Agriculture and Applied Biology, Can Tho University, Viet Nam. In 2011, he began to be a lecturer in Department of Plant Protection, College of Agriculture and Applied Biology, Can Tho University, Viet Nam.

In 2013, he won a scholarship from the SUT-ASEAN Scholarships 2013-2016 (SUT scholarship No. 5601/927) to pursue a Doctoral Degree in Suranaree University of Technology, Nakhon Ratchsima, Thailand. He was an exchange student, Prefectural Fukui University, Fukui, Japan from Nov 2015 to Dec 2015.

Research output:

Three oral presentations: at International SUT-Agricultural Colloquium 2014, 2015 and 2016. Thailand.

Two poster presentations: International Forum Agriculture, Biology and Life Sciences 2015 at Sapporo, Hokkaido, Japan. And AUM2016 at Bangkok, Thailand.

Journal manuscripts: 1 article in review (Journal of Plant Production Science), 4 articles in preparation.