Screening of Rubber Rootstocks for the White Root Disease Tolerance with Defense-related Genes and Enzymes

Afdholiatus Syafaah

A Thesis Submitted in Fulfillment of the Requirements for the Degree of Master of Science in Natural Rubber Production, Technology and Management (International Program)
Prince of Songkla University
2019

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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Author	Miss Afdholiatus Syafaah
Major Program	Natural Rubber Production, Technology and Management (International Program)
Academic Year	2018

ABSTRACT

The aims of this study were to determine the gene expression of three *PR* protein genes (*PR1a*, *PR3* and *PR5*), *PAL* gene, the defense-related enzyme activities such as chitinase (CHI), peroxidase (POD) and phenylalanine ammnia lyase (PAL), and to screen cultivated rubber rootstocks for white root disease tolerance. The screening of rubber rootstocks tolerance with the white root disease caused by *R. microporus* was investigated by both defense-related genes and defense-related enzymes as well, and the disease assessment symptoms. The six cultivated rubber seedlings collected from different area in Southern Thailand were used in this experiment. The expression of *PRs* and *PAL* genes were analysed at 0, 1, 3, 5, and 7 days post inoculation (dpi) as well as the defense-related enzyme activities. The disease symptom was monthly observed since 3 to 9 months post inoculation. The transcript level of *PR1a, PR3,* and *PR5* genes were generally induced in tolerant rubber clones, but the pattern of the transcriptional change was different among those six cultivated rubbers when inoculated with *R. microporus*. Among those parameters studied expression level of *PRs* and *PAL* genes, CHI, PAL and POD enzyme activities are proven as evidence of the white root disease symptom assessment in the field, we concluded that RRIM 600 was moderate susceptible clone to white root disease as shown high disease index (37.8%) and low of percent of survival seedlings (88.9%) followed by RRIT 408 and BPM 24. PB 5/51 was the most tolerance one with the low disease incidence, high PAL and POD enzyme activities, followed by RRIM 623.

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LIST OF ABBREVIATIONS AND SYMBOLS

α	=	Alpha
β	=	Beta
μg	=	Microgram
μm	=	Micrometer
μmol	=	Micromolar
μM	=	Micromolar
BSA	=	bovine serum albumin
bp	=	basepair
^{o}C	=	degree celcius
cDNA	=	complementary deoxyribonucleic acid
CHI	=	Chitinase
cm	=	centimeter
C_T	=	Threshold
DEPC	=	Diethylpyrocarbonate
DNA	=	deoxyribonucleic acid
DNS	=	dinitrosalicylic acid
dpi	=	days post inoculation
EDTA	=	ethyldiaminetetraacetic acid
g	=	earth's gravitational force
g	=	gram
h	=	hour
H_2O_2	=	hydrogen peroxide
HCl	=	hydrocloric acid
M	=	Molar
mpi	=	months post inoculation
mg	=	milligram
mgP	=	milligram protein
mL	=	milliliter
mm	=	millimeter

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

min	=	minute
mmol	=	milimol
mM	=	milimolar
mRNA	=	messenger ribonucleic acid
N	=	Normal
ng	=	nanogram
nm	=	nanometer
PAL	=	phenylalanine ammonia-lyase
PDA	=	potato dextrose agar
pH	=	potential of hydrogen ion
POD	=	peroxidase
PR	=	pathogen-related
qRT-PCR	=	quantitative real-time polymerase chain reaction
RNA	=	ribonucleic acid
rpm	=	revolution per minute
s	=	second
T_m	=	melting curve
U	=	Unit
UV	=	ultraviolet
v/v	=	volume by volume
WRD	=	White root disease
w/v	=	weight by volume

CHAPTER 1
INTRODUCTION

1.1 Rationale of the study

The white root disease (WRD) is the most important root disease in rubber plantation. It caused by the basidiomycete pathogen, *Rigidoporus microporus.* The pathogen penetrates the root, colonize the tissues, and degrade the host's cell structure (Omorusi, 2012). In Indonesia, white root disease attacks the rubber plantation both in wet or dry condition in the different disease severity in each area, moderate to severe disease severity (Pawirosoemardjo *et al*., 1992). Farid *et al.* (2009) reported that rubber plantations in Malaysia are very susceptible to WRD attacked by *R. microporus*. In Africa, Nandris *et al.* (1988) reported that WRD had reached up to 50 % in old rubber plantation in Ivory Coast, while Ogbebor *et al.* (2013) stated that the yield from old rubber plantation was loss up to 50 %. In Sri Lanka, around 5-10% of rubber cultivated areas were infected by WRD (Liyanage, 1983; Jayasinghe *et al.,* 1995). In the Southern Thailand, Nissapa and Chuenchit (2011) reported that white root disease attacked the rubber plantation causing economic losses around 24,600-478,930 baht per rai. The disease can infect the rubber tree as early as one year old.

To avoid the pathogen invasion, plants protect themselves by constitutive and inducible defense mechanism. The plant development and productivity depend on their ability to adapt stress condition whether abiotic caused by unfavourable environment or biotic infection caused by fungi, insect, or bacterial invasion. The plant resistance is determined by a particularly collaboration between constitutive and inducible defense mechanism (Ferreira *et al*., 2007). The active or inducible mechanism involves accumulation of phytoalexins, oxidative burst, systemic acquired resistance (SAR) by accumulation of pathogenesis-related (PR) proteins, and enhancing transcription of genes encoding enzyme in biosynthesis of phenolic compound, such as phenylalanine ammonia-lyase (PAL) (Montesinos, 2000).

This research studied the gene expression profile of three *PR*s and *PAL* genes in *Hevea brasiliensis* inoculated with *R. microporus.* The expression profiles of

PRs and *PAL* genes, chitinase, phenylalanine ammonia lyase, and peroxidase enzyme activities were also determined in rubber clones after *R. microporus* inoculation at the seedling stage. The symptom of rubber seedlings infected with *R. microporus* was also investigated. Data from this experiment can be used to develop the rubber plant tolerant with *R. microporus.*

1.2 Hypothesis of the study

Differences might be found for gene expression level (*PRs* and *PAL*) and enzyme activities (chitinase, phenylalanine ammonia lyase, and peroxidase) of white root disease in different clones of rubber tree. The differences in transcription and/or translation levels might be distinguish between tolerance and susceptible clones. From this reason selection of candidate clones for white root disease tolerance can be scored on seedling stage and can be shorten the breeding program.

1.3 Literature review

1.3.1 *Hevea brasiliensis*

Rubber trees, as known as para rubber, are originate from Amazon forest in Brazil. They discovered by Spanish explorers in the 15th century. In 1876, approximately 70,000 seeds have been collected by Wickham, a naturalist from Rio Tapajoz (Amazon) and those seeds were transported to Kew Botanic Garden. Around 4% of those seeds were germinated and 1911 unselected seedling were sent to the Botanic Garden, Ceylon (now Sri Lanka) during 1876. In June 1877, 22 unspecific seedlings were transported from Kew to Singapore, and distributed in Malaya. They were used as tappable plant material trees by Ridley in 1888. According to this history, it was believed that rubber trees in Southeast Asia were derived from Wickham's collection from the banks of Tapajoz (Priyadarshan, 2017).

Nowadays, bud grafted is very popular for rubber tree propagation of which combines between rootstock-scion. In Thailand, the rootstock is carried out from any early-introduced clones (Wattanasilkorn *et al*., 2012), which does not have any specific criteria. Most of rubber plantations in the South of Thailand are RRIM 600. According to Crop Protection Research Institute (2011) cited by Wattanasilakorn

et al. (2012), RRIM 600 is very sensitive to fungal disease including *Phytophthora*, leaf fall, and root disease.

Rubber clones have been produced and developed from narrow genetic diversity causing resist some of common diseases (Mohammed *et al.*, 2014). The most important rubber diseases cause economic loss in rubber plantation were south american leaf blight (SALB) caused by *Microcylus ulei* which its host plant originates from the Amazon area (Lieberei, 2007), leaf fall disease caused by *Corynespora* and *Colletotrichum*, and WRD caused by *R. microporus* (Pawirosoemardjo *et al.*, 1992; Farid *et al.*, 2009).

1.3.2 White root disease

<u>Mechanism of white root disease infection</u>

According to Ryvarden and Gilbertson (1993), the taxonomy of *R. microporus* belongs to:

Kingdom : Fungi
Phylum : Basidiomycota
Class : Basidiomycetes
Order : Polyporales
Family : Meripilaceae
Genus : Rigidoporus
Spesies : *Rigidoporus microporus*

White root disease (WRD) caused by *R. microporus* attacks several tropical crops species, including rubber tree (Pawirosoemardjo *et al.*, 1992; Farid *et al.*, 2009). The white-flattened mycelia fungus reunites its hyphae into rhizomorphs that produced from basidiocarps on dead woody substrate. In favourable conditions, the rhizomorphs from an infected woody debris buried can attack healthy roots by roots contacting of neighboring tree (Nandris *et al.*, 1987; Omorusi, 2012). After rhizomorphs contact the roots, the rhizomorphs penetrate the tap roots enter deeply in the soil. The *R. microporus* can be identified by lignin breakdown to decay the infected wood (Nandris *et al.*, 1987). The mycelial growth rate estimated around 2.5m on the infected roots of *Hevea* (Nandris *et al.*, 1987). In the further infected rubber

tree symptom, the foliage discolour for green to yellowish-brown, seemed to be bearish and downward curved leaves and finally drop. The branches die back, untimely flowering and fruit production, and the disease accumulate on the collar tree (Sujeewa *et al*., 2013).

Mycelium of white root disease is varied depends on the soil types which varies in different area and isolation period (Ubogu, 2013; Dalimunthe *et al*., 2017). Dalimunthe *et al*. (2017) stated that the WRD grows well on high humidity, well aeration soil, and high amount of organic matter content.

The cycle of white root disease is shown in Figure 1 and characteristic of *R. microporus* is shown in Figure 2.

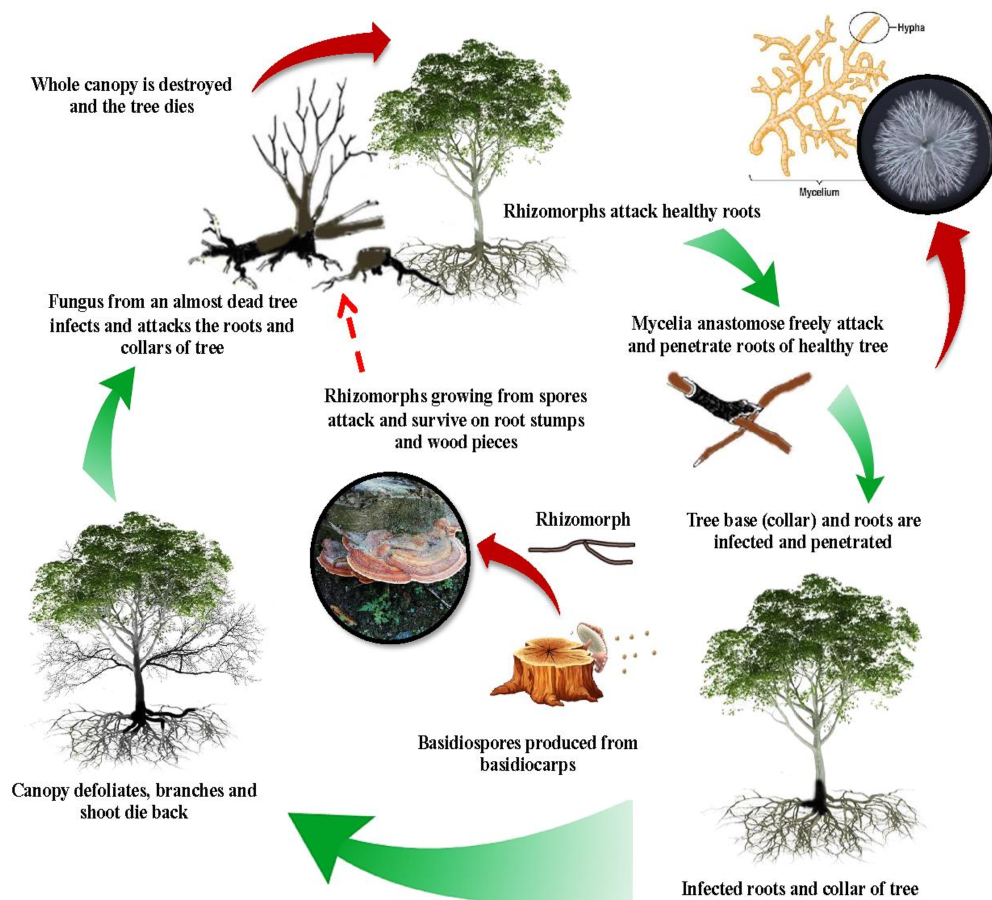

Figure 1 The cycle of white root disease.

Source : Woraathasin (2017)

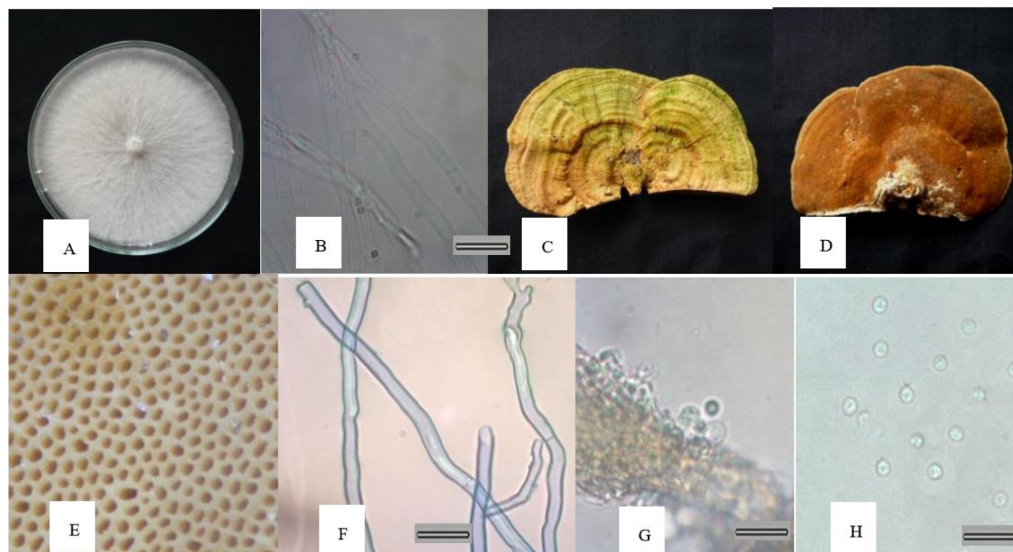

Figure 2 The characteristic of *R. microporus*: white mycelia grown on PDA media at 6 days (A), hypha (B), upper surface of fruiting body (C), lower surface of fruiting body (D), pores at lower surface (E), monomitic, the generative hypha (F), hymenium (G) and basidiospores (H).

Source : Soytong and Kaewchai (2014)

Controlling of the white root disease

There are so many ways to control the white root disease, such as using clearing process of the old rubber tree land, removing or destroying the stumps or debris to minimize the inoculum resources in the area by burning the old rubber stumps. The other control methods developed by some researches are planting the antagonistic plants as botanical agent Prasetyo and Aeny (2014), biological control (Ubogu, 2013; Prasetyo and Aeny, 2014; Soytong and Kaewchai, 2014) and chemical agent (Rodesuchit *et al.*, 2012) such as fungicide application. Root exudate of *Alpinia galangal* and *Sansiviera auranthii* used as botanical agents are reported can reduce the *R. microporus* growth (Prasetyo and Aeny, 2014). Rotiorinol, a bioactive compound from *Chaetomium cupreum* inhibited white root disease in rubber tree from 60-80% in both powder and oil rotiorinol extracted (Soytong and Kaewchai, 2014). Other biological control, such as *Trichoderma harzianum* was found as antagonistic against *R. microporus* (Jayasuriya and Thennakoon, 2007). The chemical

agent, such as ammonium sulphate and urea inhibited the white root disease spreads in rubber budded (Soytong and Kaewchai, 2014).

1.3.3 Plant defense mechanism and root infection strategy

Some pathogenic microbes, such as oomycete, produce zoospore and penetrate on host plant cells. Some fungus produce extracellular enzymes such as chitinase, pectinase that break down the plant cell wall. Root system has passive defense mechanism by transferring apoplastic barrier surrounded by ‘Casparin Strip’ composing by lignin. In addition, other physical barrier protection, root separates their border cells by detach infected root cap, leave the unharmed apical root system, and secrete antimicrobial metabolite and protein compound such as phenolic and pathogenesis-related protein that accumulate in the surrounding infected tissue and suppress the growth of pathogenic microorganism (de Coninck *et al*., 2015).

When plant roots infected with pathogen, gather with favourable condition such as wet and cool soil condition, pathogen enters the xylem vessels through damping-off in the seedling, the pathogens produce hyphae and/or conidia. In plant organ, roots, initiatively detach defense-related compound into rhizosphere. In the other hand, in basal defense mechanism, the pathogen is recognized by the plant via microbe-associated molecular patterns (MAMPs). Infected plant activates membrane-bound pattern recognition receptor (PRRs) and releases plant-derived danger-or damage-associated molecular pattern (DAMPs), leads to MAMP-triggered immunity (MTI). MTI can be found on leaves and roots of *Arabidopsis*. MTI leads to activate inducible response upon recognition of pathogenic microorganism by producing secondary metabolism likes protein. It is regulated by phytohormons, such as jasmonic acid (JA), salicylic acid (SA), and ethylene (ET). JA is mainly involved in biotrophic pathogen, SA is involved in necrotrophic pathogen, and ET is involved in herbivory attack. The MTI also activates defense signal pathogenesis-related (PR) protein, produces reactive oxygen species (ROS) that clearly associated with the onset of a hypersensitive response (HR) (de Coninck *et al*., 2015; Ali *et al*., 2018).

In the other hand, pathogenic microorganism successfully suppressed MTI by effector-triggered susceptibility (ETS) by producing effectors that mask MAMPs. Some pathogen successfully produced hyphae or hautoria due to effector

come into the plant cell. Plant recognize effector (Avr gene) mediated by R gene leads to effector-triggered immunity (ETI) resulting in a hypersensitive response (HR) as known as programmed cell death (de Coninck *et al.*, 2015).

1.3.4 Pathogenesis-related protein

Pathogenesis-related protein (PR) protein is defined as intra-and extracellular localized proteins that accumulate in plant tissue caused by pathogen invasion or elicitor treatment (Bowles, 1990), associated with SAR against infection caused by fungi, bacteria, and viruses (van Loon and van Strien, 1999). A PR protein refers to protein that newly expressed during infection (van Loon *et al*., 1999) and detected in infected tissues (Datta and Muthukrisnan, 1999). Recent information reported that the PRs protein were grouped into 17 families (Table 1) (van Loon and van Strien, 1999; Ferreira *et al*., 2007; Ebrahim *et al*., 2011).

PR proteins also expressed in healthy plants. The *PR1*, *PR2*, *PR5*, and *PR16* genes were highly expressed in adult mature rice leaves (Hou *et al*., 2012). The *PR3s* were transcripted at rice plant organs, including meristem, root, leaf, and shoot (Nakazaki *et al*., 2006). The *PR1* is also varied expressed on healthy root, leaf, and flower compared with treated plant with pathogen or other stress (Mitsuhara *et al*., 2008), whereas the transcription level of *HbPR1b* was expressed higher than *HbPR3* in healthy rubber tissue (Woraathasin *et al*., 2017a). Those studies indicated that the PR proteins play an important rule on growth and development.

Environmental stresses both abiotic and biotic stresses were reported induced the PR proteins in many crops (Ali *et al*., 2018). A number of researchers have reported that a pepper gene encoded *PR1b* is strongly exhibited by ethephon treatment and wounding (Sarowar *et al*., 2005). *PgPR10-4* gene is induced by H_2O_2 molecules stresses (Kim *et al*., 2014) and *PgPR6* gene is strongly responsive to environmental stresses such as heavy metals, chilling, salt, and mannitol stresses (Myagmarjav *et al*., 2017).

The PR proteins have chitinase (CHI), β–glucanase (GLU), or lysozyme activity. The CHI has been grouped into four PR protein families (*PR3*, *PR4*, *PR8*, and *PR11*). One of GLU has been identified as *PR2* and/or thaumatin-like *PR5* family (van Loon, 1997). Mitsuhara *et al.* (2008) selected 12 putatively active

genes from *PR1* family genes and they reported that all of these genes were upregulated upon compatible rice-blast fungus interaction with different infection period. In another research, the expression of *HbPR1* in rubber clones was down-regulated 10-fold in RRIM 612 infected by WRD (Oghenekaro *et al*., 2016). Kim *et al*. (2009) stated that *PR5* gene as known as a vacuolar secreted protein in ginseng was elevated by abiotic and/or biotic stresses such as salt tress, heavy metal, and pathogen infection. The detail of important PR proteins are shown in the Table 1.

Table 1 Classification of pathogenesis-related proteins

Families	Type number	Properties
PR-1	Tobacco PR-1 a	Antifungal
PR-2	Tobacco PR-2	β-1,3-glucanase
PR-3	Tobacco P, Q	Chitinase type I, II, IV, V, VI, VII
PR-4	Tobacco 'R'	Chitinase type I, II
PR-5	Tobacco S	Thaumatin-like
PR-6	Tomato Inhibitor I	Proteinase-inhibitor
PR-7	Tomato P69	Endoproteinase
PR-8	Cucumber chitinase	Chitinase type III
PR-9	Tobacco 'lignin forming peroxidase'	Peroxidase
PR-10	Parsley 'PR1'	Ribonuclease like
PR-11	Tobacco 'class V' chitinase	Chitinase, type I
PR-12	Radish Rs-AFP3	Defensin
PR-13	Arabidopsis THI2.1	Thionin
PR-14	Barley LTP4	Lipid-transfer protein
PR-15	Barley OxOa (germin)	Oxalate oxidase
PR-16	Barley OxOLP	Oxalate oxidase-like
PR-17	Tobacco PRp27	Unkown

Source : Ferreira *et al*. (2007)

Pathogenesis-related protein 1 (PR1)

PR1 proteins are accumulated in response to pathogen infection, phytohormones induction (salicylic acid, jasmonic acid, ethylene), or environmental stresses. PR1 is divided into two groups as acidic and basic form based on their

isoelectric points. PR1 was reported play crucial role in antifungal properties. The PR1 founded in tobacco was accumulated around 1-2% of total leaf protein (Alexander *et al*., 1993).

Acidic PR1a

The acidic PR1 (PR1a) proteins are soluble at pH 3 and denature under the acid condition. Datta and Muthukrishman (1999) reported that the PR1a protein does not encode other specific targeting peptide sequence. Six acidic PR1 (PR1a) protein were identified in different tobacco species. These six PR1a are serologically linked and categorized both at DNA and protein level and serologically connected (Pfitzner and Goodman, 1987). Matsuoka *et al*. (1987) reported that PR1a protein was also observed in the xylem and exacellular of tobacco leaves infected with tobacco mosaic virus using biochemical and immune-localization experiments. In another plant such as tomato, extracellular proteins linked to the acidic PR-1 proteins of tobacco do not need to have a low pI (Datta and Muthukrishman, 1999).

Basic PR1

The basic PR 1 (PR1b) normally contains a hydrophobic N-terminal region of 30 amino acids as a signal peptide for translocation of endoplasm reticulum (ER) (Payne *et al.*, 1989). Based on the amino acid sequence, this domain was found as an extention of 36 amino encoded in the cDNA clone, whereas the two genomic clones encode extentions of 18 amino acids (Payne *et al*., 1989; Sessa *et al*., 1995). Khunjan *et al.* (2016) reported that the *PR1* protein has been found as basic PR1 protein. It contained 692 nucleotides encoding 163 amino acid residus, had 647 bp long with pI of 8.56 and predicted molecular mass of 17 KDa.

Pathogenesis-related protein 3 (PR3)

Chitinases (poly [1,4(N-acetyl-B-D-glucosaminide] glycanhydrolase, EC 3.2.1.14) are enzymes that hydrolyze the N-acetylglucosamine monomer of chitin and those enzymes are available in plant tissues both crop and non-crop species. *PR3* group consists of various chitinase-lysozymes that belong to three distinct classes and exhibit differential CHI any lysozyme activities (Stintzi *et al*., 1993). The class of

CHI can be grouped by molecular, biochemical, and physiochemical criteria (Punja and Zhang, 1993). CHI, together with GLU could be directly against fungal cell wall (van Loon, 1997) and those enzymes may be induced slightly in the floral organs in infected leaves (Ferreira *et al*., 2007).

Pathogenesis-related protein 5 (PR5)

PR5, as known as thaumatin–like proteins (TLPs), is namely based on the structural and serological similarity with sweet tasting protein from fruit of *Thaumatococcuc danielli* (Vigers *et al*., 1992). PR5 is one of protein grouped has molecular weight range 22 to 26 KDa with 201-229 amino acid. Permatin, osmotin, and zeamatin are also groups to PR5. In rubber trees, the molecular weight of PR5 encoded 172 amino acid and classified as osmotin and thaumatin-like protein (Woraathasin *et al.*, 2017b). Isoforms of PR5 have been founded in many plant species, such as tobacco (Vigers *et al*., 1992), Arabidopsis (Nawrath and Métraux, 1992), *Panax ginseng* (Kim *et al*., 2009), wheat (Wang *et al*., 2010), and chili pepper (Mishra *et al*., 2017). PR5 protein has been induced by both stimuli stresses (wounding), phytohormones (SA, JA, ABA), and pathogen infection (Nawrath and Métraux, 1992; Zhu *et al*., 1995; Wang *et al*., 2010; Mishra *et al*., 2017).

In tobacco, PR5 proteins are divided into two classes, namely PRS and osmotin (Vigers *et al*., 1992). Osmotin-like protein was accumulated in the all potato organs, the higher accumulation were in the roots and mature flower (Zhu *et al*., 1995). Wang *et al.* (2010) stated that *TaPR5* gene was highly induced in cell walls on compatible interaction stripe rust infected wheat leaves. The result indicated that PR5 proteins have functional roles in SAR mechanism for antifungal activity.

1.3.5 Phenylalanine ammonia lyase (PAL)

The phenylapropanoid linked to the shikimate pathway produced aromatic amino acid. Phenylalanine ammonia lyase (PAL, EC 4.3.1.5) catalyses the *trans*-cinnamate from L-phenylalanine (Phe). The reaction is catalysed by phenylalanine ammonia lyase (PAL, EC 4.3.1.5) (Dixon *et al*., 2002) to produce precursors leading to plant defense activation, changing the composition of cell wall (Hahlbrock and Scheel, 1989), and ultraviolet-B light protectant (Huang *et al*., 2010).

The PAL activity is stimulated by a variety of environmental factor both biotic and abiotic stress and in plant development. Pathogen invasion induces the change of plant secondary metabolism based on the defense program. Infected plant with pathogen activates phenylpropanoid pathway, one of secondary metabolism, such as cell wall strengthen and antimicrobial synthesis. The general PAL in phenylpropanoid pathway is shown in Figure 3.

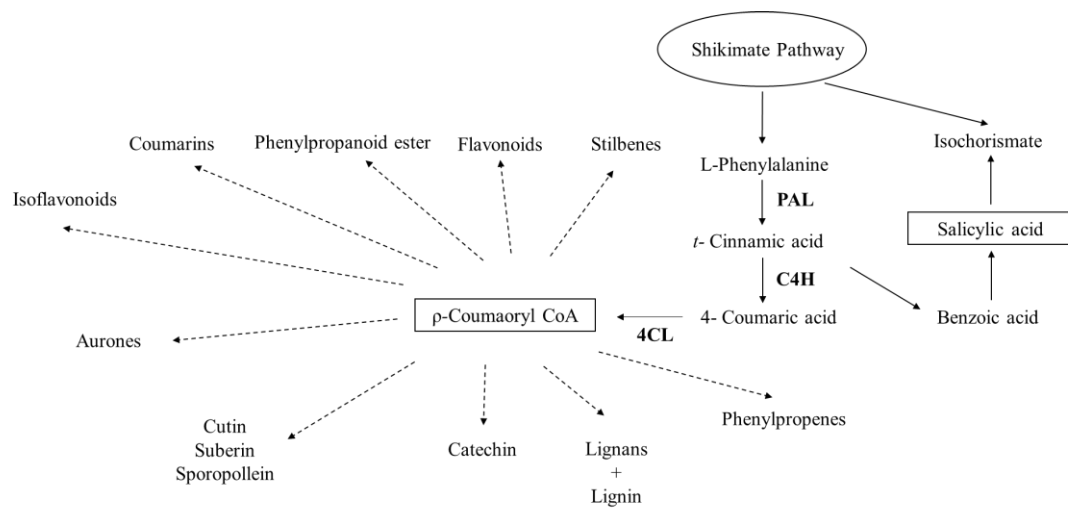

Figure 3 Phenylalanine ammonia lyase (PAL) in phenylpropanoid biosynthesis pathways. Dash line represents the multiple enzymes contribute.

Source: Adapted from Shadle *et al*. (2003); Vogt (2010); Zhang and Liu (2015)

PAL gene encoded by small family of two to four unclustered genes in tomato under normal physiology condition, wounding, have strongly induced during the hypersensitive response to tobacco mosaic virus or fungal elicitor (Pellehrini, 1994). *PAL1, PAL2, PAL3*, and *PAL4* gene families have encoded PAL genes in *A. thaliana* (Raes *et al*., 2003). In quadruple mutant of Arabidopsis had decreasing level of lignin and salicylic acid, therefore the mutant was susceptible to a virulent strain of *Pseudomonas syringae* (Huang *et al*., 2010). Eight *PAL* genes have been found in *Brachypodium* where *BdPAL1* (Bd3g49250) and *BdPAL2* (Bd3g49260) encoding in lignin biosynthesis. Invasion of *Fusarium culmorum* and *Magnaporthe oryzae* in *BdPAL* RNAi mutant of *Brachypodium* (reducted-lignin) caused increasing of necrotic lesion both leaves and roots in *BdPAL*RNAi mutant (Cass *et al*., 2015). In

cassava elicited cell, the biosynthesis pathways of phenylpropanoids involved the PAL and TAL enzyme activities (Dogbo *et al*., 2012).

1.3.6 Defense-related enzymes

The compatibility interaction between host plant and pathogen lead to resistance plant or incompatible response. The various defense response leads to accumulate several factors, such as defense-related enzymes linked to PR proteins and those enzymes accumulation prevent infection by pathogen. Defense-related enzymes such as PAL, POD and CHI are reported significantly increase in resistance cultivar during pathogen invasion (Vanitha *et al*., 2009; Ge *et al*., 2014; Kumar *et al*., 2017). The defense-related enzyme can be induced by exogenous substance or elicitor treatment (Prasannath, 2017).

The enhanced of PAL enzyme activity is associated with tomato resistance to bacterial spot disease and pepper infected with *Xanthomonas campestris* (Kim and Hwang, 2014; Chandrasekaran *et al*., 2017). POD and SOD activities increased in pathogen-infected pepper and -infected tomato. This report informed that the disease defence is positively related with the amount of production of defense-related enzyme (Jetiyanon, 2007). CHI accumulated together with GLU upon fungal infection and those enzymes are responsible for hydrolysis of cell wall component such as chitin and β-1,3-glucan (Ebrahim *et al*., 2011).

1.4 Objectives

The objectives of the study are:

1. to determine the gene expression of three *PR* protein genes (*PR1a*, *PR3* and *PR5*) and *PAL* gene in rubber seedlings after inoculated with *R. microporus*
2. to determine the chitinase, peroxidase and phenylalanine ammonia lyase enzyme activity in rubber seedlings after inoculated with *R. microporus*
3. to screen cultivated rubber clones for white root disease tolerance based on defense-related gene and/or defense-related enzymes, and the WRD symptom in the field

CHAPTER 2
MATERIALS AND METHODS

2.1 Material equipments

2.1.1 Plant materials

List of Para rubber used for the experiment

Clones	Place of collection
RRIT 251	Songkhla Rubber Research Center
RRIM 600	Rubber Plantation, Songkhla
RRIM 623	Rubber Plantation, Trang
PB 5/51	The Rubber Estate Organization, Nakhon Si Thamarat
BPM 24	Songkhla Rubber Research Center
RRIT 408	Surat Thani Rubber Research Center

2.1.2 Laboratory equipments

Equipments	**Companies**
Autoclave	Tomy
Gel electrophoresis	Mupid
Gel documentation	BIO-RAD
Hot plate	WiseStir
Water bath	Grant
Centrifuge	Eppendorf
BioDrop DUO UV/VIS Spectrophotometer	BioDrop
PCR machine (DNA thermal cycle)	Biometra
pH meter	Mettler Toledo
Real-Time PCR machine (ABI 7300)	Invitrogen
Ultrapure water machine	Merck
Vortex mixer	Vortex Genie2
Spindown	Daihan Scientific Co., Ltd.
Hook	Flexlab
Laminar air flow	Mycrotech

Equipments	Companies
Micropipette	Thermoscientific
Microtube	Eppendorf
Tips 0.1-2 μL, 20-200 μL, 200-1000 μL	Eppendorf

2.1.3 Chemicals

Analytical grade

Chemicals
Absolute ethanol
Chloroform
Isoamyl alchohol
Isopropyl alchohol
Potato dextrose agar media
Tris-Base
Lithium chloride
β mercaptoethanol

Molecular biological grade

Chemicals	Companies
100 bp DNA ladder	Promega
Agarose	Merck
Ampicilin	Sigma
Deoxyribonuclease I (DNAse I)	Promega
Ethidium bromide	Merck
Taq DNA polymerase	Biolab
Trizol LS Reagent	GIBCO BRL
Universal RiboClone cDNA Synthesis System	Promega
SYBR® Green Master Mix	Bio-rad

2.2 Methods

2.2.1 Rubber rootstock seedlings preparation

The rubber seeds were germinated in the sandy bed for 15 days and directly replanted in the plastic bag filled with soil as medium for both gene expressions and enzyme activities. Other rubber seedlings were planted in the pot filled with soil as medium for diseases symptom assessment in the field.

The rubber seedlings were selected for inoculation at 4 months after planting and produced 2 whorl leaves for both laboratory and field experiments. The rubber seedlings with approximately the same diameter ranging 4-6 mm measured 1 cm above level ground were selected for inoculation.

2.2.2 Fungal inoculation

Fungal preparation and inoculation in the laboratory scale

R. microsporus isolated RG15 culture was provided by Department of Pest Management, Prince of Songkla University. It was isolated from basidiocarps from an infected rubber tree with *R. microporus.* The mycelium of the fungus was grew on 2% w/v potato dextrose agar (PDA) for 7 days and used for inoculation.

Inoculation was carried out by creating 3.0 cm x 0.3 cm wound on stem which 1 cm closed to taproot using a sterilized surgical blade. The surface area was sterilized with 70 % of ethanol. Rectangular agar (3.0 cm x 0.3 cm) containing active mycelium was placed on the wound created and wrapped using plastic. Re-inoculation was following done at 4 days post inoculation (dpi). The leaves from inoculated seedlings were kept at 0, 1, 3, 5, and 7 dpi for both RNA and protein extraction. Fungal preparation and inculation methods in the laboratory as shown as in Figure 4.

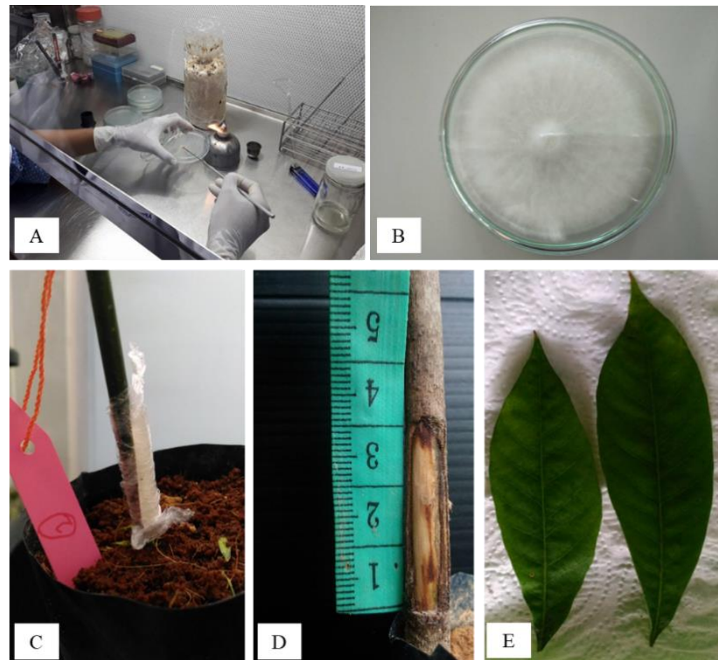

Figure 4 The preparation of fungal and inoculation method in laboratory scale: Subculture of mycelia (A), colony on PDA media at 7 days (B), PDA containing mycelia placed onto 3.0 cm x 0.3 cm wounded stem (C), inoculated stem at 7 dpi (D) and leave samples in different interval time after inoculation (E).

Fungal inoculation in the field

Selected rubber seedlings have been planted in pot, and have been waited for producing two whorl leaves. Inoculation was conducted by placing 500 g of inoculums grown on mushroom media in each pot and covered them with soil.

2.2.3 Gene expression and enzymes activity analysis

Gene expression analysis

RNA extraction and cDNA synthesis

RNA extraction from rubber leaves was carried out based on Deng *et al.* method (Deng *et al*., 2012) with modification. A hundred mg of sample was frozen in liquid nitrogen and stored at -80ºC until used. The frozen leaf tissues were ground to powder. The powder was suspended in 10 mL of CTAB, 100 mmol/L Tris-HCl (pH 8.0), 100 mmol/L EDTA, 1.4 mol/L NaCl and 5 % (v/v) β-mercaptoethanol, and the

suspension was incubated at 65ºC for 5 minutes. 680 µL of chloroform/isoamyl alcohol (24:1, v/v) was added to the suspension, and was centrifuged at 12,000 x G for 10 minutes at 4ºC, twice. The supernatant was transferred to another tube, and 1/3 volume of 8 mol/L LiCl of the crude sample was added and stored at -20ºC for 4 – 16 hours. The reaction was centrifuged at 12,000 x G for 20 minutes at 4ºC. After produce rude pellet, the rude pellet was transferred to another new tube, directly added 100 µL of isopropanol and stored at -20ºC for 30 minutes. Then the crude pellet was centrifuged at 12000 x G for 20 minutes at 4ºC. The final pellet was washed with 70% (v/v) of ethanol and air-dried. The integrity and purity of the isolated RNA was examined by 1 % formaldehyde agarose gel electrophoresis at an absorbance ratio A260/A280. cDNA was synthesized by reverse transcription from 1000 ng of total RNA using transcriptome amplification kit according to the manufacturer's institution (Thermo Scientific).

PRs and PAL gene expression analysis

PRs primers used in the study is shown in the Table 2.

Table 2 List of primer sequences used in qRT-PCR

Gene	Direction	Sequence (5'→3')
PR1a	Forward	CAG GTG GTT TGG CGC AAC TC
	Reverse	GGT CGC TGC CCA ACA AAG TT
PR3	Forward	TGGTCAATGTGGGCAAGCCT
	Reverse	GGTGGGTGACCATTGTCCAGT
PR5	Forward	TGGACCATTAGTGCTACTCGTGGAA
	Reverse	TGCATATTCGGCCAAGGTGTTAGG
PAL	Forward	GGACATGCTCAAAGTTGTGG
	Reverse	TGCTGGCATTCTTCTCATTG
18s rRNA	Forward	GTAGAGGATGGTGCCGACAAC
	Reverse	CCAAACTTCCCCAGTTACAAGAA

Differential gene expression was determined by qRT-PCR. The qRT-PCR was run in a Light Cycler at 95°C for 10 minutes, followed by 40 two-step cycles of PCR, including denaturation at 95°C for 15 s, and annealing and polymerization at 60°C for 1 min. Three independent biological replicates and two technical repeats per each biological replicated were used for this analysis. The relative gene expression was calculated from the cycle threshold (C_T) values and analyzed using two methods (Livak and Schmittgen, 2001). *18s rRNA* was used as internal reference gene for normalization (Li *et al*., 2011).

Enzyme activity assessment

<u>Protein extraction</u>

The protein was extracted according to Zhang *et al.* (2009) with minor modification. Two hundreds mg of sample leaves were crushed with liquid nitrogen in mortar. Six ml of 0.1 M phosphate buffer pH 7 added with 0.1 mM EDTA and 0.1mM Ascorbic acid was added in the mortar and ground until produce fine powder, centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant was transferred into 10 mL of fresh beaker glass. 3.366 mg of ammonium sulphate was added and mixed slowly until completely dissolved. The process was placed in cool water. The mixed solution was transferred onto the new 1.5 micro tube and centrifuged at 12,000 rpm for 15 minute at 4°C. The supernatant was discarded and the pellet was dissolved with 500 µL of 0.1 M phosphate buffer PH 7 added with 0.1 mM EDTA and 0.1 mM ascorbic acid. The protein content was determined according to Bradford method (Bradford, 1976) with BSA as the standard.

<u>Chitinase (CHI) activity assay</u>

The CHI activity was assayed according to Miller (1959). The mixed reaction buffer containing 100 µL of colloidal chitin (1% w/v), 150 µL of protein extraction, and 750µL of 1 M sodium acetate buffer pH 5.0 was incubated at 37°C for 30 minutes. One mL of DNS solution was added into the reaction mixture and was boiled at 100°C for 5 minutes. The absorbance was measured at 540 nm. DNS was used as standard curve. The CHI specific activity is expressed by µmol of N-acetyl-D-glucosamine per hour per mg of protein (µmole N-acetyl-D-glucosamine/h/mgP).

Phenylalanine ammonia-lyase activity assay

The PAL activity was determined through the convertion rate of L-phenylalanine to *trans*-cinnamic acid according to Assis *et al*. (2001) with minor modification. The PAL activity assay was measured by incubation reaction consisted of 200 μL of enzyme extract, 500 μL of 0.1 M L-phenylalanine and adjusted the final reaction up to 2 mL with 0.1 M Tris-HCl pH 8.5. The reaction mixture was mixed and incubated at 37°C for 60 min. The reaction was stopped then by adding 10 μL 6 N HCl. The absorbance of the resulting agent of *trans*-cinnamic acid was measured at 290 nm. The reaction with 0.1 M phosphate buffer pH 7 instead of enzyme extract was used as a blank. The PAL specific activity is expressed in mol of cinnamic acid per hour per mg of protein (mol/h/mgP).

Peroxidase (POD) activity assay

Peroxidase enzyme activity was assayed according to Fajardo *et al*., (1998) with minor modifications. The mixture reaction containing 10 μL of enzyme extract, 20 μL of 30% H_2O_2, 220 μL of 0.3 % (v/v) guaiacol, and adding 0.05 M of phosphate buffer pH 7 up to final volume 1,000 mL was used in this assay. The absorbance was detected at 470 nm for 60 minutes. The POD specific activity is defined as the enzyme of which could oxidase 1 μmol of guaiacol in 1 min and expressed in Unit per mg of protein (U/mgP). The POD activity was calculated following the formula:

$$\text{POD activity} = \frac{(\triangle A/\text{min}) x T.V x 10^3}{S.V \text{ x Absorptivity x } P} \text{unit.L}^{-1}$$

Where:

T.V	=	total reaction volume in mL
S.V	=	sample volume in mL
Absorptivity	=	mM absorptivity of guaiacol at 470 nm = 26.6
P	=	Path length in cm
△A/min	=	Slope from equation after absorbance measurement

2.2.4 Growth of infected rubber seedlings and white root disease symptom assessment

Growth of rubber seedlings measurement

To determine the effect of fungal inoculation among rubber clones, the height, diameter, and number of whorl of infected rubber seedlings were measured at eight months post inoculation. These infected seedlings with *R. microporus* were also compared with uninfected rubber seedlings (control). Height parameter was measured at base of plant. The diameter was measured at 5 cm above ground level.

White root disease symptom assessment

White root disease symptom assessment in the field was arranged by completely randomized design with four replications, and un-inoculated seedlings were used as control. Each treatment consisted of five to ten healthy and uniform seedlings. Each pot was inoculated with 500 g of inoculums placed in 5 cm under the soil and contacted with the taproot. The disease symptoms were observed start at one month after inoculation.

Evaluation of disease infection will be conducted according to Soytong and Kaewchai (2014) and Wattanasilakorn (2016): level 1 (healthy, green leaves), level 2 (1-25 % yellow leaves), level 3 (26-50 % yellow leaves), level 4 (51-75 % yellow leaves), and level 5 (76-100 % yellow leaves) .

The data collection as disease index (DI) was recorded every months. Data of the severity of the white root disease were statistically compared after 3 months of inoculation.

$$\text{Disease Index (DI)} = \frac{(1\text{x}a)+(2\text{x}b)+(3\text{x}c)+(4\text{x}d)+(5\text{x}e)}{(a+b+c+d+e)} \text{ x } \frac{100}{X}$$

Where,

1, 2, 3, 4, and 5 are infection categories

a, b, c, d, and e are plants that fall into the infection categories

X is the maximum disease category which is 5

The disease index was calculated from the level score as following in Figure 5.

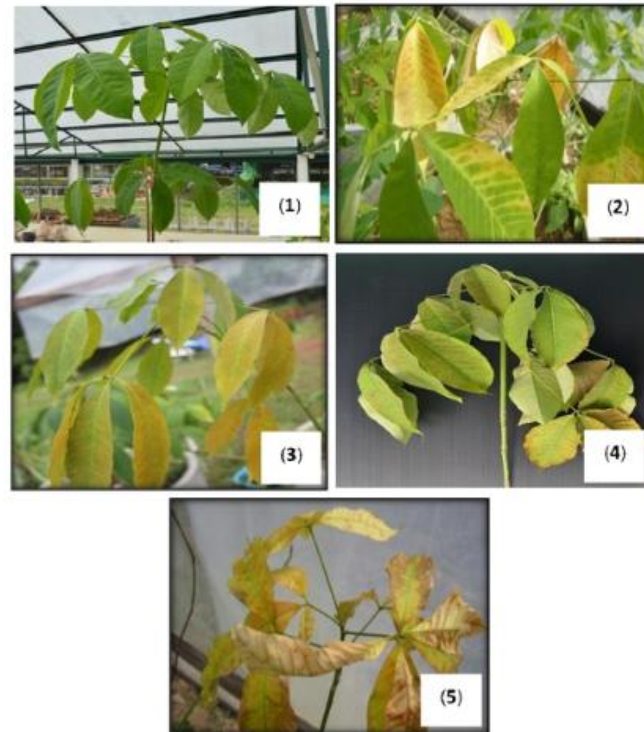

Figure 5 Development of symptoms on samplings of *H. brasiliensis.*
Source: Wattanasilakorn (2016)

2.3 Data analysis

Statistical analysis both of gene expression and enzyme activity assay were carried out with R program using one-way ANOVA with a significant level (p) of 0.05. Further analysis was carried out using LSD test. The error bars on the figures represents standard error over means. The data from the field was subjected to one-way ANOVA and further analysis was carried out using LSD test to determine the interaction between pathogens and symptom development on host.

CHAPTER 3
RESULTS

3.1 Gene expression of *PR1a, PR3, PR5,* and *PAL* genes

To optimize the specific primer for qRT-PCR analysis, the amplification efficacy of each primer was examined. The PCR product of each primer showed a single melting peak which absent of dimer primer or pseudo-primer. The *18s rRNA* was used as reference and the primer was consistently detected in the samples. The gene transcripts of *PRs* protein and *PAL* genes were monitored for seven days after inoculation. Transcript level of *PR1a* gene in six cultivated rubber seedlings infected with *R. microporus* is shown in Figure 6.

The transcript level of *PR1a* in six rubber clones were signifigantly induced at 1 dpi. The transcript level of *PR1a* in RRIM 623 and PB 5/51 were initially upregulated at 1 and 3 dpi compared to others where the maximum peak level was at 3 dpi (4.7, and 2.6 folds, respectively), and subsequently downregulated at 5 and 7 dpi, respectively. In RRIT 408, the *PR1a* transcript level was early induced at 1 dpi, and continuously decreased at 3, 5, and 7 dpi. Meanwhile the transcript level of *PR1a* gene in other clones, RRIT 251, RRIM 600 and BPM 24 were varied. *PR1a* transcript level of those three cultivated rubber seedlings were reached the maximum peak level at different time (RRIT 251 was at 5 dpi, RRIM 600 was at 3 dpi, and BPM 24 was at 7 dpi, respectively).

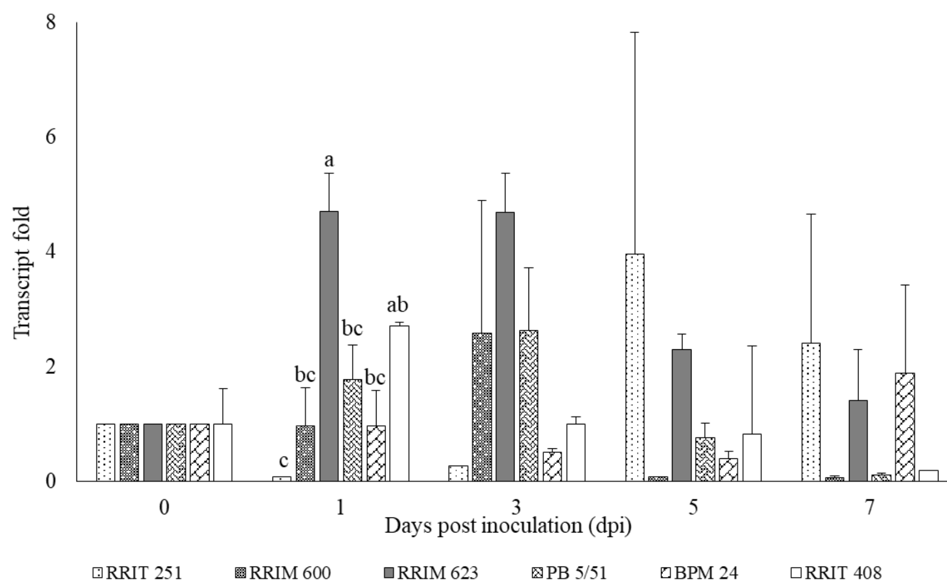

Figure 6 The *PR1a* transcript fold change of six cultivated rubber seedlings inoculated with *R. microporus.*

Different letters in each bar indicate the significantly difference (p<0.05) by LSD multiple range test. All data were presented as mean ± S.E. calculated from three independent replicates.

Transcript level of *PR3* gene in six cultivated rubber seedlings infected with *R. microporus* is shown in Figure 7. In this study, we observed the *PR3* transcript level in six inoculated rubber seedlings with *R. microporus.* The pattern of transcript fold changes were not significantly different among those six cultivated rubber seedlings after inoculated with *R. microporus*. There were steady pattern of *PR3* transcript level at those six cultivated rubber seedlings. The *PR3* gene expression in RRIM 623 and PB 5/51 were continuously elevated at 1 and 3 dpi with the maximum peak at 3 dpi, respectively, then the transcript level were decreased at 5 and 7 dpi. The *PR3* transcipt level in RRIT 251 and RRIM 600 showed the two peaks level, 1 and 7 dpi, respectively. The transcript level of BPM 24 was steady at different time intervals (1.1 to 1.89 folds, respectively). Although there were no different level of

transcription of *PR3*, in PB 5/51 infected seedlings showed the highest *PR3* transcript level at 3 dpi (5 folds, respectively).

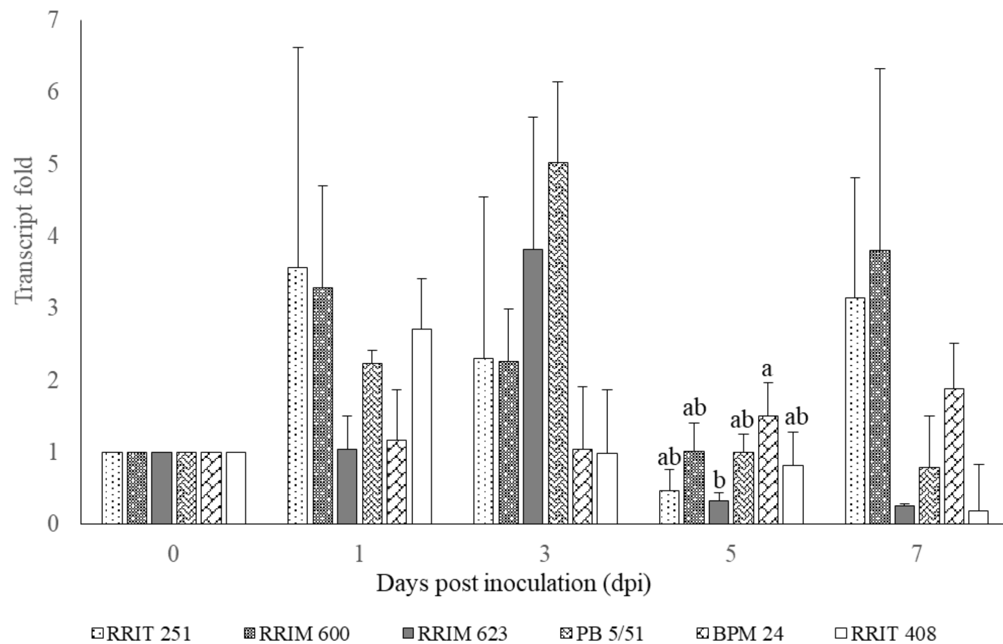

Figure 7 The *PR3* transcript fold change of six cultivated rubber seedlings inoculated with *R. microporus*.

Different letters in each bar indicate the significantly difference (p<0.05) by LSD multiple range test. All data were presented as mean ± S.E. calculated from three independent replicates.

PR5 protein as known as thaumatin-like-proteins (TLP) sometime is accumulated in plant organ after pathogen challenge by activation of salicylic acid pathway, lead to induce SAR inducible defence in plants. During inoculation time intervals, there were no significantly different of *PR5* transcript level in infected rubber seedlings during the interval time of infection. The expression of *PR5* gene in RRIM 623, PB 5/51, and RRIT 408 raised the maximum level at 3 dpi (40, 31, and 23 folds, respectively). However, transcript level of the *PR5* in those three infected seedlings were gradually downregulated at 5 dpi and dropped at 7 dpi (Figure 8). The *PR5* transcript level of RRIT 251 and RRIM 600 were similarly pattern, where the *PR5* gene was early induced at 1 dpi, continuously dropped at 3 and 5 dpi, and

increased at 7 dpi. Meanwhile, BPM 24 clone inoculated with pathogen showed the similarly level of transcription of *PR3* in remaining time observation.

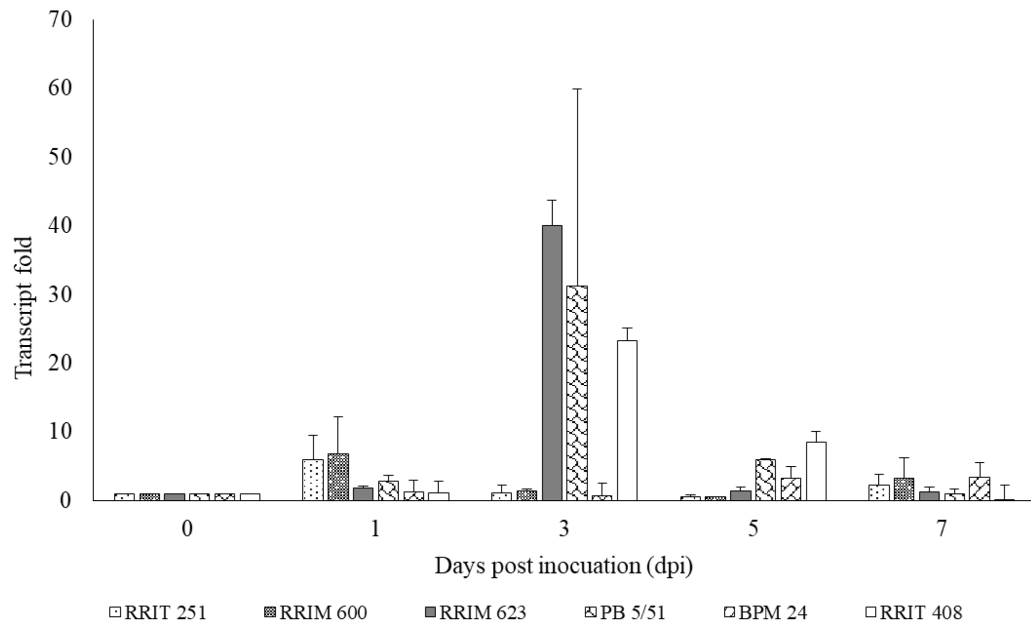

Figure 8 The *PR5* transcript fold change of six cultivated rubber seedlings inoculated with *R. microporus*.
Different letters in each bar indicate the significantly difference (p<0.05) by LSD multiple range test. All data were presented as mean ± S.E. calculated from three independent replicates.

PAL gene contributed in phenylpropanoid pathways which is initiated from L-phenylalanine to *trans*-cinnamate leading to synthesis antimicrobial and cell wall strengthening. *PAL* gene was investigated in term of plant defense response when infected with pathogen challenge. In this study, *PAL* gene was expressed in the rubber seedlings with pathogen challenge in particular time after inoculation. During inoculation time intervals, there were significantly different of *PAL* transcript level in infected rubber seedlings during the interval time of infection, at 1, 5, and 7 dpi, respectively. The result showed *PAL* gene expression on RRIM 600, RRIT 408 and RRIT 251 were downregulated at following interval time infection, except RRIM 600 was upregulated at 1 dpi and RRIT 251 at 7 dpi, respectively. The *PAL* gene was highly expressed in PB 5/51 clone with the peak level at 1 dpi (2.9 folds,

respectively), the transcript level of *PAL* in PB 5/51 was slightly decreased at different interval time infection. The BPM 24 seedlings presented the highest *PAL* transcript level at 5 dpi (3.3 folds, respectively) compared to other clones and decreased at 7 dpi (Figure 9).

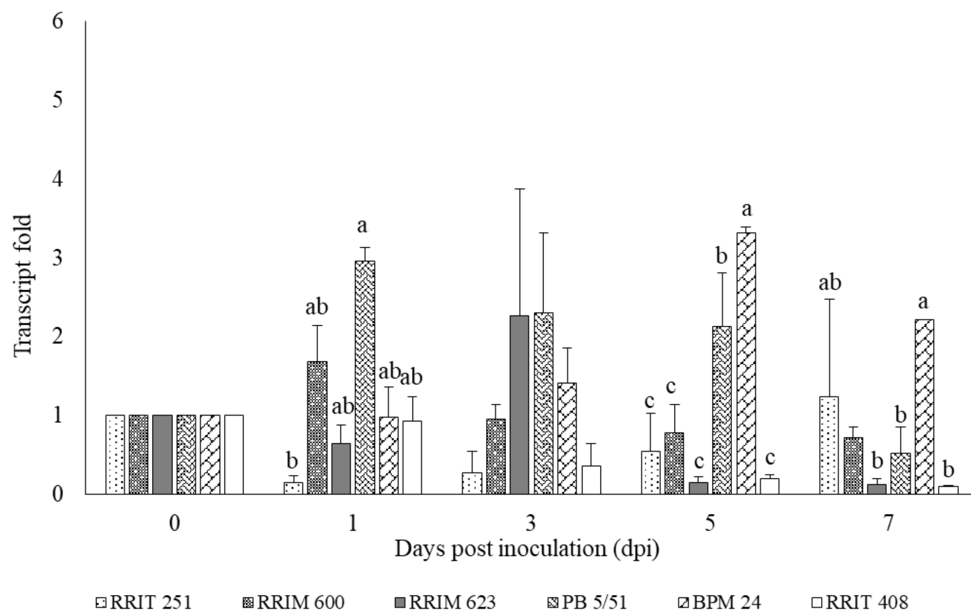

Figure 9 The *PAL* transcript fold change of six cultivated rubber seedlings inoculated with *R. microporus*.

Different letters in each bar indicate the significantly difference ($p<0.05$) by LSD multiple range test. All data were presented as mean ± S.E. calculated from three independent replicates.

3.2 Enzyme activity assessment

Rubber seedlings treated with *R. microporus* were investigated for 7 days and the different accumulation of CHI, PAL, and POD enzyme activities were assayed. The CHI activity of six cultivated rubber is shown in Figure 10. Figure 10 showed the CHI activity among six cultivated rubber seedlings during inoculated with *R. microporus*. There were unclear CHI activity pattern among rubber seedlings-tested. In inoculated rubber seedlings, six cultivated rubber seedlings showed the different pattern of CHI activity. In normal condition (0 dpi), the CHI was active in the low activity level (0.196-0.352 μmole N-acetyl-D-glucosamine/h/mgP,

respectively). The CHI activity was induced subsequently at 3 dpi with percentage of increase of 32.5-105%, respectively except BPM 24 was reduced 9% compared to 0 dpi (normal condition). Infected BPM 24 seedlings also showed the lowest CHI activity at interval time infection among those cultivated rubber. PB 5/51 showed the highest CHI activity and the CHI activities were steady increased at 1 and 3 dpi (26 to 62 % compared to 0 dpi, respectively), and subsequently decreased at 5 and 7 dpi.

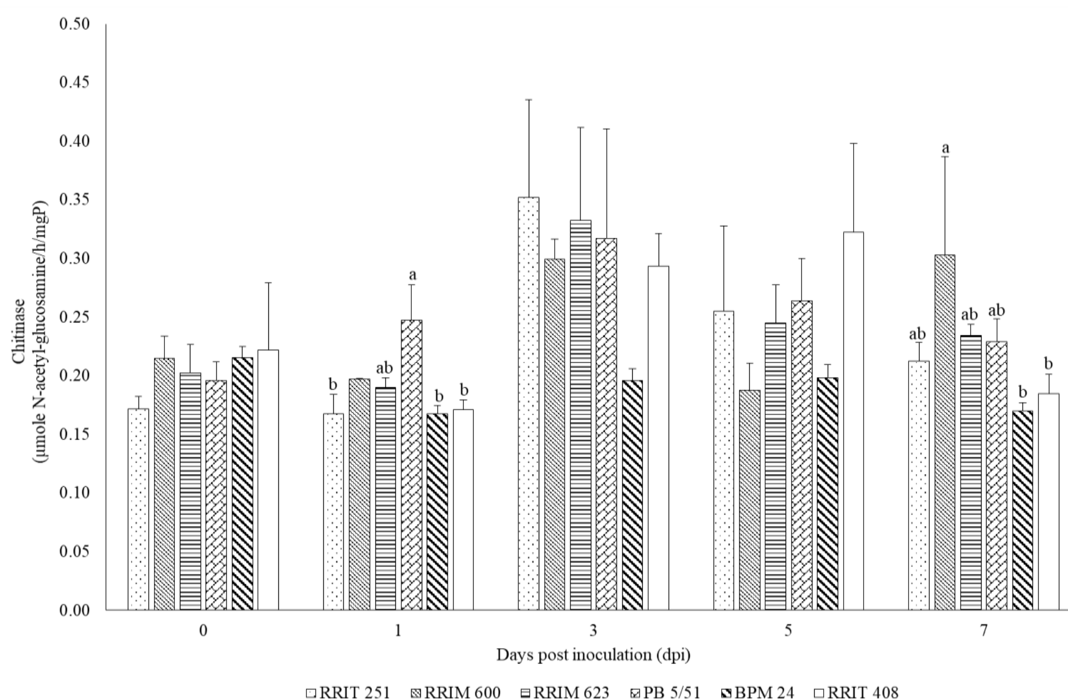

Figure 10 CHI specific activity in six cultivated rubber seedlings inoculated with *R. microporus.*

Different letters in each bar indicate the significantly difference (p<0.05) by LSD multiple range test. All data were presented as mean ± S.E. calculated from three independent replicates.

The PAL accumulation in six cultivated rubber seedlings inoculated with *R. microporus* is presented in Figure 11. During inoculation time intervals, there were significantly different of PAL activity in infected rubber seedlings at 0, 3, and 7 dpi. At 0 dpi (uninoculated seedlings), the PAL activity was 0.19-0.33 mol *trans*-cinnamic acid/h/mgP. The lowest PAL activity was in PB 5/51, whereas the highest PAL activity was in RRIM 600 and BPM 24, respectively. PB 5/51 showed the highest PAL accumulation at remaining time of observation. At the 1 dpi, mostly PAL

accumulation in each rubber clones decreased then reached the maximum level at 3 dpi. The PAL activity raised the peak level at 3 dpi (37.7 % - 401.0 % compared to 0 dpi, respectively). The highest PAL activity at 3 dpi was in PB 5/51, folowed by RRIT 251, RRIM 623, and RRIT 408 (0.990, 0.821, 0.623, and 0.611 mol *trans*-cinnamic acid/h/mgP, respectively). Moreover, those enzymes activities tend to decrease at 5 dpi in all cultivated rubber seedlings, but increased gradually at 7 dpi.

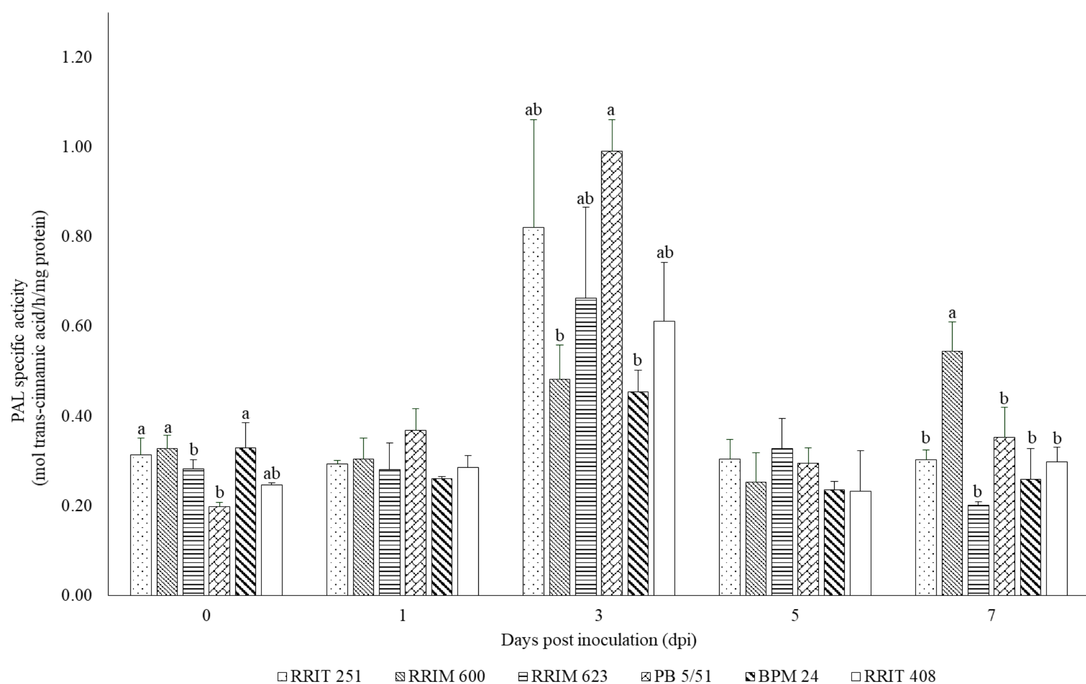

Figure 11 PAL specific activity in six cultivated rubber seedlings inoculated with *R. microporus.*

Different letters in each bar indicate the significantly difference (p<0.05) by LSD multiple range test. All data were presented as mean ± S.E. calculated from three independent replicates.

POD activity is an important key enzyme for cell wall lignification in order to respond to a number of external stresses, such as biotic stress. In this study, the POD activity was initally induced in early time after inoculation, at 1 dpi. The pattern of POD activity was similarly observed with PAL activity. The POD activity was significantly elevated and reached at maximum level at 3 dpi as presented in Figure 12. The inoculated PB 5/51-rubber seedlings with *R. microporus* showed the highest POD activity at 1, 3, 5 and 7 dpi compared to other clones. The highest POD

activity was detected at 3 dpi in PB 5/51 clone (1,314.7 Unit/mg P, respectively). The POD activity in other infected clones were around 311-601 Unit/mg P, respectively. The fungal inoculation in BPM 24 induced the POD activity in the early time interval and the PAL activity of BPM 24 clone had the lowest activity compared to the others.

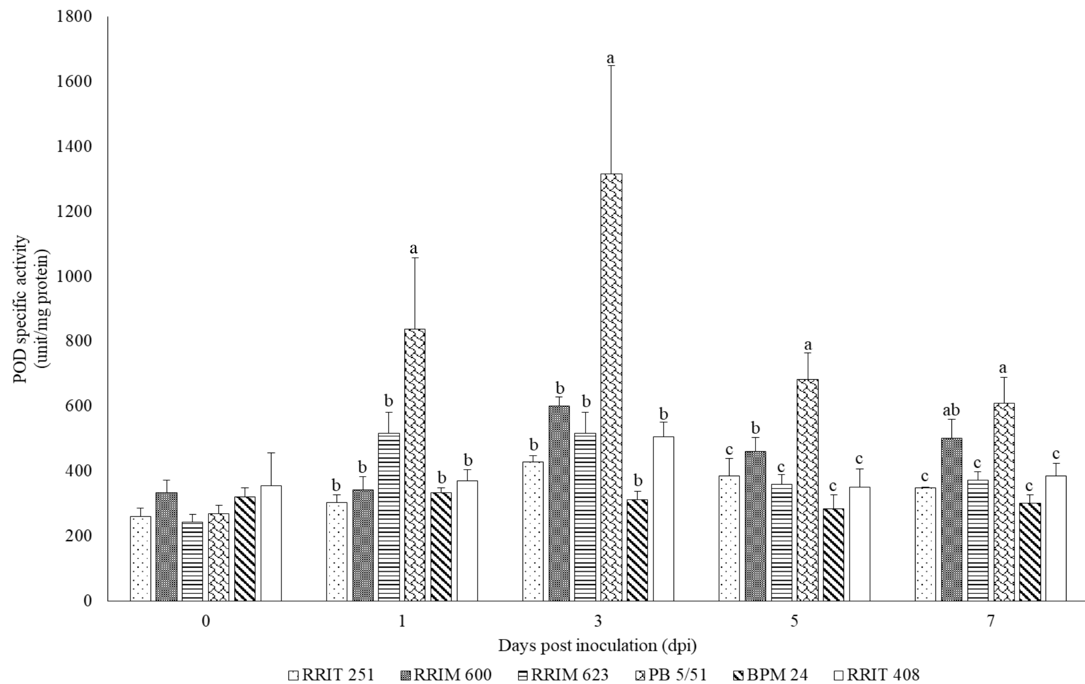

Figure 12 POD specific activity in six cultivated rubber seedlings inoculated with *R. microporus.*

Different letters in each bar indicate the significantly difference ($p<0.05$) by LSD multiple range test. All data were presented as mean ± S.E. calculated from three independent replicates.

3.3 Growth of rubber seedlings and the white root disease symptom assessment

A number of researches mentioned the fungal infection suppressed the growth of infected plant with pathogen. The growth of rubber seedlings were also measured in this trial. Table 3 showed the height, diameter, and number of whorl of six cultivated rubber seedlings after inoculated with *R. microporus* at 8 months post inocuation which represented as growth performance of infected rubber clones with *R. microporus*.

Table 3 Growth of six cultivated rubber clones (12-month-old seedlings) after inoculated with *R. microporus* at 8 months post inoculation.

Clone	Growth parameter		
	height (cm)	diameter (mm)	number of whorl
RRIT 251	104.90±5.55ab	12.24±0.02ab	5.76±0.88ab
RRIM 600	78.37±5.76b	7.07±0.61bc	4.89±0.26b
RRIM 623	111.53±9.45a	12.85±1.45a	5.44±0.34ab
PB 5/51	113.50±7.87a	10.5±0.89abc	6.13±0.39a
BPM 24	86.30±6.15ab	8.24±0.89bc	4.71±0.29b
RRIT 408	94.44±3.57ab	10.89±1.02abc	5.60±0.24ab
CV (%)	24.16	3.51	18.84
Pr>f	0.25	0.30	0.21

Different letters in each column indicate the significantly difference ($p<0.05$) by LSD multiple range test. All data were presented as mean ± S.E. calculated from three independent replicates.

Based on the growth parameter of the rubber seedlings, growth of six inoculated rubber seedlings were significantly different, the RRIM 600 and BPM 24 growth were lower than others (Table 3). The inoculated RRIM 600 showed the lowest height and diameter, followed by BPM 24 and RRIT 408. In the other hand, inoculated RRIM 623, PB 5/51, and RRIT 251 showed better growth performance than the others. Although the growth among those inoculated cultivated rubber seedlings were not significantly different, the growth were obviously suppressed compared to control (without inoculation) as shown in Figure 13. The height of the inoculated seedlings were clearly inhibited 7.58 to 47.20 % compared to the control, and the diameter was suppressed 21.11 to 50.94 %, respectively. The height, diameter, and number of whorl of infected RRIM 600 were deliberately inhibited compared to control (47.20 %, 50.94 %, and 37.31 %, respectively) followed by BPM 24.

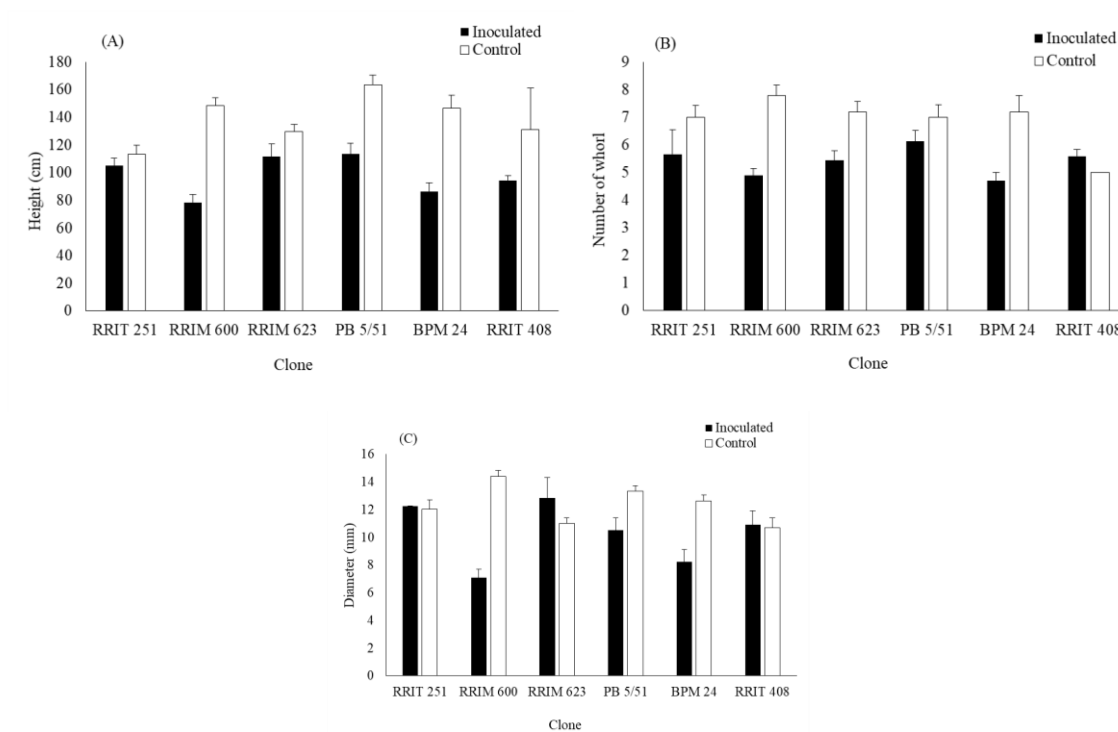

Figure 13 The growth performance between control and inoculated rubber seedlings treated with *R. microporus.* All data were measured at 8 months post inoculation, height (A), diameter (B) and number of whorl (C).

All data were presented as mean ± S.E. calculated from three independent replicates.

Disease index of each rubber seedlings was measured by scoring the disease symptom that presented by yellowing of leaves, level 1 presented the healthy plant, while level 5 presented the highest disease infection. Some of seedlings showed the disease symptom since few months after fungal inoculation. The disease index of BPM 24, RRIT 408, and RRIM 600 were more than 30 % (31.4, 36.7, and 37.8 %, respectively) observed at 9 mpi, slightly higher than other clones. In the other hand, RRIT 251, PB 5/51, and RRIM 623 seedlings showed the less yellowing leaves at 9 mpi (the disease index of each clone: 25.7, 25.0, and 24.4 %, respectively). Survival rate of seedlings were also slightly observed at 9 mpi. At the end of investigation conducted, all of cultivated rubber seedlings stayed alive, except RRIM 600 which

was 88.9 % alive. Disease index and survival rate of six each cultivated rubber seedlings is shown in Table 4.

Table 4 Disease index and survival rate of six cultivated rubber seedlings after inoculated with *R. microporus.*

Clone	Disease Index (%)							Survival
	Month post inoculation (mpi)							(%)
	3	4	5	6	7	8	9	(mpi)
RRIT 251	20.0	20.0	20.0	25.0	25.7	25.7	25.7	100.0
RRIM 600	24.0	28.0	26.0	28.0	31.1	35.6	37.8	88.9
RRIM 623	20.0	20.0	20.0	26.0	24.4	24.4	24.4	100.0
PB 5/51	20.0	22.2	22.2	24.4	25.0	25.0	25.0	100.0
BPM 24	20.0	20.0	22.5	25.0	28.6	31.4	31.4	100.0
RRIT 408	20.0	20.0	20.0	26.7	32.0	36.0	36.0	100.0

The WRD symptom in rubber seedlings early appeared at 3 months after inoculation. The symptom appeared on the second whorl leaves observed by changing the leaf colour from green to yellow or dried-yellow leaves. The disease symptoms were clearly observed only on few branches depend on the severity of the disease. Figure 14 exhibited the different responses among those cultivated rubber seedlings. RRIM 600 showed the yellow leaves since 3 mpi, while the other cultivated rubber seedlings, RRIT 251, RRIM 623, PB 5/51 showed less yellowish leaves (disease score was less than 3, respectively). The further symptoms slightly increased at 6 to 9 months post inoculation. The white mycelia of the fungi attacked the seedlings was clearly observed both on lateral and tap roots (Figure 15).

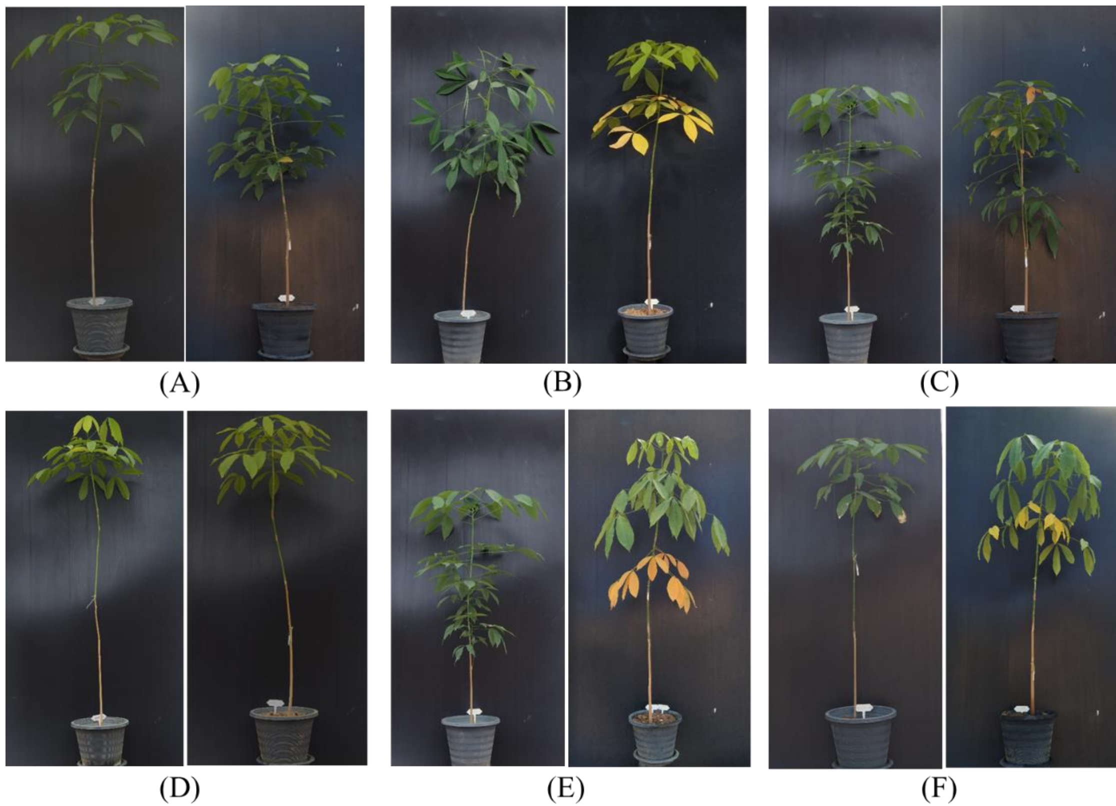

Figure 14 Morphology of 8-month-old six cultivated rubber seedlings: RRIT 251 (A), RRIM 600 (B), RRIM 623 (C), PB 5/51 (D), BPM 24 (E) and RRIT 408 (F). Left side represented as control (without inoculation with pathogen), right side represented as inoculated with *R. microporus* for 8 mpi.

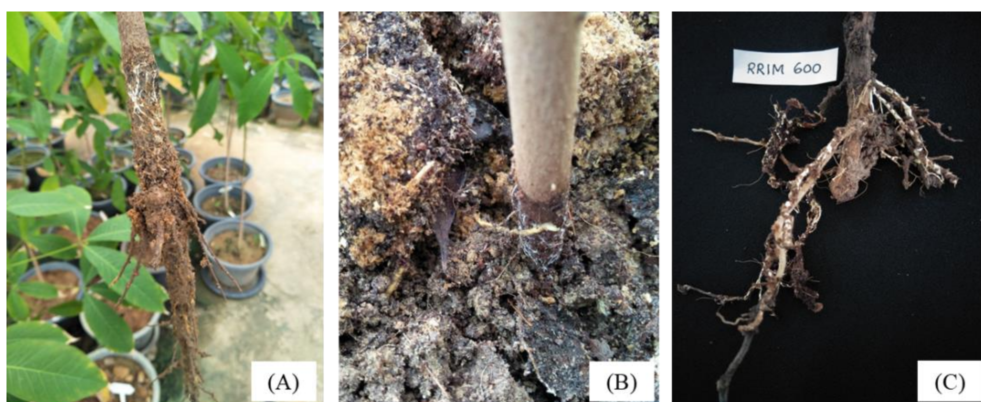

Figure 15 The rubber root inoculated with *R. microporus.* The white mycelia attack on lateral and tap roots, white mycelium on tap root of RRIT 408 (A), white mycelium on tap root of BPM 24 (B) and white mycelium attacked both lateral and tap root of RRIM 600 (C).

CHAPTER 4
DISCUSSION

4.1 *PRs* and *PAL* transcript gene expression

Pathogenesis-related (PR) proteins are initially found be acidic or basic, low molecular mass and localized in the intercellular of leaves (Ferreira *et al*., 2007). PR1 found as antifungal properties, both acidic and basic. *PR1* genes generally are found in healthy and unhealthy plant. The regulation of *PR1* gene has been clearly well explained and identified by Durrant and Dong (2004) in SAR mechanism leading to induce *PR1* gene in host plant.

Various transcriptional studies have been reported the up-regulated of *PR* genes in plants after fungal infection (Ali *et al*., 2018). Gene expression of *PR1* was upregulated early during necrotrophic pathogen infected by *Parastagonospora nodorum* in wheat (Breen *et al*., 2016) and *Colletotrichum truncatum* in pepper (Mishra *et al*., 2017), however their function is still abstruse. A number of researchers reported *PR* genes on para rubber related on pathogen infection. The gene expression of *PR1* in infected para rubber with *R. microporus* and *Neofusicoccum ribis* result in increased of disease resistance (Oghenekaro *et al*., 2016; Sangsil *et al*., 2016; Woorathasin *et al*., 2017a, 2017b). The high *PR1* expression in resistance clones also affected to decrease of necrotic lesion.

Overexpression of *PR1* in transgenic plant in signal transduction pathway leading to increase disease resistance to fungi (Mitsuhara *et al*., 2008), oomycete (Sarowar *et al*., 2005; Khunjan *et al*., 2016) and bacterial pathogen (Sarowar *et al*., 2005). Transient *PR1* gene co-expression with p19 via *Agrobacterium* co-infiltration into *N. benthamiana* has successfully detached more than half per cent of *Phytophthora palmivora* germination. It revealed that PR1 protein is one of antimicrobial as result of increased resistance (Khunjan *et al*., 2016; Breen *et al*., 2017; Ali *et al*., 2018).

In this study, *PR1a* was not significantly elevated during fungal infection, but *PR1a* gene expression of RRIM 623 and RRIT 251 showed higher expression than any other clones. *PR1a* gene expression was not induced in PB 5/51

5/51 as previously known as tolerance clone (Woraathasin *et al*., 2017a). Our result was similar reported by Bonasera *et al*. (2006) who reported that transcript level of *PR1a* sometime was not expressed during *Erwinia amylovora* infection in the apple. They assumed that this *PR1a* might be referred as a "PR-like" gene.

PR3 proteins show the CHI activity belong to six chitinase classes (class 1 to class VII except class III) (van Loon *et al*., 1994). *PR3* gene was initially accumulated or induced by phytopathogens through jasmonate-signaling pathway leading to SAR in infected plant by necrotrophic pathogen (Ali *et al*., 2018). The *PR3* gene expression was also clearly investigated in this study. The transcript level of *PR3* gene in PB 5/51 and RRIM 623 infected seedlings with *R. microporus* were induced at 1 and 3 dpi which was peaked at 3 dpi. BPM 24 showed the less of *PR3* transcript level at the interval time of infection. Meanwhile, RRIT 251 and RRIM 600 showed the lowest *PR3* transcript level at 5 dpi. Woorathasin *et al.* (2017a) reported the *HbPR3* gene was induced in both tolerant clone (PB 5/51) and susceptible clone (RRIM 600 and BPM 24) after infected with *R. microporus.* However, the highest transcript level was expressed in PB 5/51 seedlings after fungal infection. Commonly, the *PR3* gene can activate the PR3 protein as known as chitinase which contribute to the plant defense response to fungal infection.

Overexpression of chitinase genes were studied in transgenic plant in order to know the function of the gene and/or protein in disease resistance. Dong *et al*. (2017) revealed that CHI from *Eucommia ulmoides* (*EuCHIT2*) in transgenic tobacco significantly decreased the conidia growth which is further infection lead to trigger the transcriptional level of *PR1a* gene. Another studied reported by de Las Mercedes *et al*. (2006) who researched over expression of endochitinase from fungus *Trichoderma* was transformed onto tobacco leaves. The size and density of necrotic lesion in transgenic tobacco line *chit33* and *chit44* challenge with bacterial *P. syringae* were significantly decreasing compared to the control. These evidences showed that PR3 (chitinase protein) are directly and/or indirectly play a crucial key to plant defense mechanism in pathogen infection.

The *PR5* transcriptional level was investigated in various plant species, including ginseng (Kim *et al*., 2009), apple (Bonasera *et al*., 2006; Liu *et al*., 2013), wheat (Wang *et al*., 2010), cherry tomato (Guo *et al*., 2016), and rubber tree

(Oghenekaro *et al.*, 2016; Woraathasin *et al.*, 2017b). The *PR5* transcript was elevated by various external treatments or stresses, such as elicitor treatment, phytohormones induction, wounding, and/or pathogen infection. In this study, the transcript level of *PR5* gene was highly expressed in RRIM 623, RRIT 408, and PB 5/51 at 3 dpi. However, at 5 and 7 dpi, the expression level of *PR5* was not significantly different among the six cultivated rubber seedlings. A number of evidences have proved that the transcript level of *PR5* was clearly induced after fungal infection (Wang *et al.*, 2010; Guo *et al.*, 2016; Oghenekaro *et al.*, 2016), but not for bacterial infection (Liu *et al.*, 2013). The *PR5* gene was strongly upregulated in the incompatible interaction during pathogen invasion (Wang *et al.*, 2010; Mishra *et al.*, 2017). Wang *et al.* (2010) added the PR5 protein has highly shown in infected host leaves in incompatible interaction. Oghenekaro *et al.* (2016) and Woraathasin *et al.* (2017b) agreed that *R. microporus* infection in rubber clones was significantly induced and highly expressed in tolerance clones. Moreover, the *PR5* is also involved in the following biocontrol mechanism against pathogen inoculation.

Even for transgenic studies, overexpression of thaumathin-like protein (*PR5*) was reported enhance in transgenic bentgrass infected with *Sclerotinia homoeocarpa* (Fu *et al.*, 2005). *PR5* gene in transgenic wheat associated with α-1-purothionin, and β-1,3-glucanase (*PR2*), the *PR5* in transgenic wheat had enhanced resistance during *Fusarium graminearum* infection showing the lower disease severity (Mackintosh *et al.*, 2007). These results had proved that *PR5* gene also plays important rules in plant disease resistance.

Various reports showed that *PR1, PR3,* and *PR5* genes are involved in activation of SA and/or JA pathways as well as the activation of SAR inducible defense program (Niki *et al.*, 1998; Wang *et al.*, 2010; Zhang *et al.*, 2010; Ali *et al.*, 2018). Meanwhile, according to Bonasera *et al.* (2006), *PR1, PR2, PR5,* and *PR8* genes from apple are not markers for SAR in young apple shoots. Based on these reports, the *PR* genes expression may be different in difference of the host plants (Guo *et al.*, 2016) and those *PR* genes are diverse expressed depend on the tissues or organs of plant (Zhang *et al.*, 2010; Liu *et al.*, 2013).

PAL gene has been found in infected plant by pathogen. In our study, it seemed the *PAL* transcriptional level was early expressed in PB 5/51 inoculated with

R. rigidoporus compared to other rubber clones. Meanwhile, RRIT 251 showed the lowest *PAL* transcriptional level in different interval time of infection, except at 7 dpi. *PAL* gene expression were highly expressed in rubber tolerant clone with some pathogen infection than in susceptible one (Ngobisa *et al*., 2016; Oghenekaro *et al*., 2016; Sangsil *et al*., 2016). Ngobisa *et al*. (2016) reported that infection of *Neofusicoccum ribis* on rubber leaves can induce the *PAL* gene as well as β-1,3-glucanase and chitinase, lead to the increase of resistance clone to fungal infection. Oghenekaro *et al*. (2016) stated the up-regulated *PAL* gene responses in rubber plant indicated the increase of a systemic acquired resistance by enhanced lignification against the pathogen. PB 5/51 represented as tolerant clone to white root disease, expressed higher transcriptional level of *PAL* gene during the latter stage of infection, while RRIM 600 and BPM 24 expressed lower of *PAL* gene (Sangsil *et al*., 2016). In Arabidopsis, seedlings treated with *PAL1* inhibitor 2-aminoindan-2-phosponic acid (AIP) suppressed the lignification, and made the plant completely susceptible to *Peronospora parasitica* because of the restored resistance by salicylic acid (Mauch-Mani and Slusarenko, 1996). According to Huang *et al*. (2010), *PAL* gene activity in Arabidopsis was important for basal and pathogen-induced salicylic acid accumulation.

4.2 Defense-related enzymes activity

Chitinases (poly [1,4(N-acetyl-B-D-glucosaminide] glycanhydrolase, EC 3.2.1.14) are enzymes that hydrolyze the N-acetylglucosamine monomer of chitin and those enzymes are available in plant tissues both crop and non-crop species. The different chitinase-lysozymes were grouped as various classes, namely lysozyme activities, exhibit differential chitinase, and vacoular or extracellular (Stintzi *et al*., 1993). CHI induction in plants is therefore generally non-specific and enhanced by both biotic and abiotic stresses, and is only one component of the plant response to various pathogens and stresses (Punja and Zhang, 1993). CHI and β-1,3-glucanase (GLU) delay the fungal growth through fungal cell wall synthesis disruption (Leah *et al*., 1991).

The result showed that there was different of CHI activity pattern in six cultivated rubber seedlings. However, CHI in BPM 24 seemed less activity in all

interval time of infection. Xu *et al.* (2016) reported that CHI in plant is crucial hydrolytic enzymes, which catalyses the fungal cell wall degradation. It was mentioned that CHI activity in cotton is varying in different tissue and organs. A number of researches have mentioned that CHI are important for disease resistance. Increase of CHI and GLU activities in infected grapevine leaves were evidence as respond to plant resistance treated with *Uncinula necator* (Giannakis *et al*., 1998), *Botrytis cinnera* (Renault *et al*., 1996), by wounding, or salicylic acid and ethylene treatments (Derckel *et al*., 1996). Jung *et al.* (1993) stated both GLU and CHI accumulated in sunflower-aspirin treated, and those enzymes were associated with *PR2* and *PR5* genes.

The fungal growth inhibition on fungal pathogen have been observed by different mechanism of combination between CHI and GLU mixtures (Leah *et al*., 1999). Those mechanisms are varying depend on their protein sources and the type of fungal cell wall (Selitrennikoff, 2001). In contrary, overexpression of CHI in tobacco did not increase the CHI activity, but the POD and catalase (CAT) activity increased. The plant defense enzymes (POD and CAT) help to maintain the cell wall integrity and the CHI seem be involved in both direct and indirect plant response to fungal infection (Dong *et al*., 2017).

When pathogen attacks the plant, plant continuously activates phenylpropanoid pathway, such as antimicrobial synthesis and cell wall strengthening. According to Slatnar *et al*. (2010), activity of PAL enzyme was higher induced in apple scab spot infection with *Ventyria inaequalis* than healthy peel, and the fungal infection increased the metabolism of phenolic compound. Another enzyme such as POD, also contribute into phenylpropanoid pathways and the POD enzymes play an important roles in plant defense mechanism against both necrotrophic and/or biotrophic pathogens (van Loon *et al*., 2006). POD are involved in reinforcement of the cell walls, ferulic acid cross-linking, lignification, and suberization and metabolism of ROS in plant defence responses (Hammond-Kosack and Jones, 1996; Almagro *et al*., 2018).

Inoculation of *R. microporus* in six cultivated rubber seedlings induced the PAL and POD activities at 3 dpi and PB 5/51 and the inoculation gave the highest PAL and POD activities among other cultivated rubber seedlings. In the other

hand, BPM 24 as classify as susceptible clone to WRD, showed the low of PAL and POD activities. Ma *et al*. (2018) reported the PAL, 4CL, CAD and C4H are linked to phenylpropanoid metabolism which is probably associated with apple fruit resistance to grey mould disease. Other research was conducted by Saunders and O'neill (2004), an avirulent fungal infection in alfalfa plant increases *PAL* gene expression, enhances PAL enzymatic activity and produces mediacarpin accumulation, a phytoalexin product. These results had confirmed that *PAL* gene is a key regulatory enzyme in phenylpropanoid pathway which is synthesis de novo infection site by accumulating in the tissue surrounding hypersensitive cell death (Hahlbrock ans Scheel, 1989). Riaz *et al*. (2014) added that PAL and POD activity are also a key role in wheat resistance against leaf rust infection by increase both enzyme activity in the phenylpropanoid pathway.

4.3 White root disease symptom investigation

White root disease (WRD) is one of crucial root disease founded in rubber plantation caused by *R. microporus*. The disease being affected to rubber plantation owner income due to the reducing of latex production (Nissapa and Chuenchit, 2011). The WRD symptom was investigated in 4-month-old rubber seedlings inoculated with *R. microporus*. The symptom appeared by yellowish of leaves on the second whorl leaves at three months after inoculation followed by the burned leaves, defoliation, and dead seedling because of infected root by covering the rizhomorph of the fungus on both lateral and tap roots. In our study, symptom of infected rubber tree was similar to previous reports (Farid *et al*., 2006; Kaewchai and Soytong, 2010; Wattanasilakorn *et al*., 2012; Nakaew *et al*., 2015). They mentioned the symptom was yellowing of leaves at 7-12 weeks after infection, wilting, burned leaves, and defoliation at 13-14 weeks, followed by white mycelium strands or rhizomorphs accumulation in lateral root of plants leading to produce fruit body causing dead of plant.

Our studied revealed that PB 5/51, RRIM 623, and RRIT 251 are categories as tolerance clone to WRD, due to the less disease incidence (≤ 25%, respectively) and high defense-related enzyme activity. Meanwhile, RRIT 408, BPM 24 and RRIM 600 were the most showing yellowish leaves during the fungal

infection. Apparently, based on the pathogenicity test, RRIT 408, RRIM 600 and BPM 24 are categories as moderate susceptible clone to *R. microporus* infection. It was proven by high disease incidence (31-37 %) and lower accumulation of defense-related enzyme activity. Study conducted by Kaewchai and Soytong (2010) found that the 5-month-old RRIM 600 showed the symptom at 70 days after inoculation while Wattanasilakorn *et al*. (2012) reported that yellowing leaves in 5-month-old RRIM 600 clones appeared at 112 days which had 33 % survival rate less at 5.5 months after infection. They concluded that the RRIM 600 is sensitive for white root disease infection. The difference time of symptom appearance in a same clone might be because of fungal virulence level of which affected to the plant resistance. The other thing is environmental condition may be one of reason why the symptom is differed among those clones. Prasannath (2017) mentioned the soil characters (physical, chemical, and biological characteristics) are strongly correlated with white root disease incidence. Moreover, the heterogenecity of seedling for rubber rootstocks due to open pollination may be another reason for variations observed in disease resistance.

Among those parameters: transcriptional level of *PRs* and *PAL* genes, CHI, PAL, and POD enzyme activities, and also proven evidence of white root disease symptom assessment in the field, we concluded that PB 5/51, RRIM 623, and RRIT 251 clones were category as tolerant rubber clone to the white root disease, while the BPM 24, RRIM 600, and RRIT 408 rubber were represented as moderate susceptible clone to the white root disease.

CHAPTER 5
CONCLUSION

Screening of rubber rootstocks tolerance with white root disease caused by *R. microporus* was investigated by the transcript level of *PRs* (*PR1a*, *PR3*, and *PR5*) and *PAL* genes, the defense-related enzyme activities (chitinase, phenylalanine ammonia lyase, and peroxidase), and the disease assessment symptoms. The result showed that the transcript level of *PR1a, PR3,* and *PR5* genes were generally induced in tolerant rubber clones, but the pattern of the transcriptional change was different among those six cultivated rubbers inoculated with *R. microporus*. Among those parameters, expression level of *PRs* and *PAL* genes, CHI, PAL, and POD enzyme activities are proven as evidence of the white root disease symptom assessment in the field. Studied concluded that RRIM 600 was moderate susceptible clone to white root disease as shown high disease index (37.8%) and low of percent of survival seedlings (88.9%) followed by RRIT 408 and BPM 24. PB 5/51 was the most tolerance one with the low disease incidence, high PAL and POD enzyme activities, followed by RRIM 623. Other *PRs* gene such as *PR8* and *PR11* which are known as chitinase family, *PR2* gene, and defense-related enzyme such as β-1,3-glucanase should be investigated for further researches, because both chitinase and β-1,3-glucanase are reported relate to plant defense mechanism to pathogen infection.

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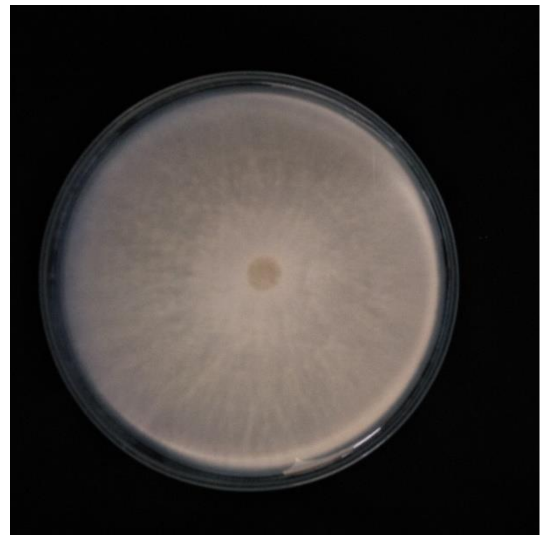
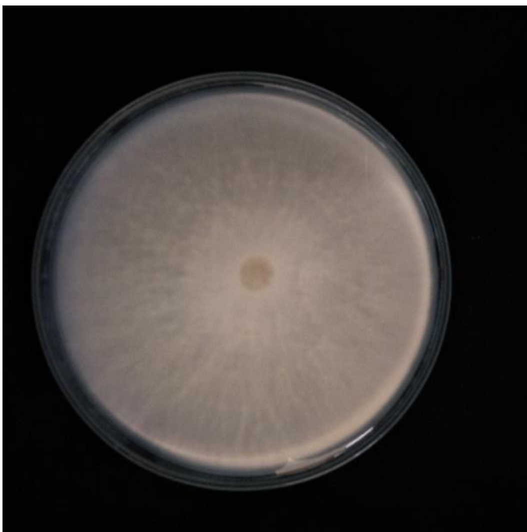
APPENDIXES

Appendix 1

1. **Fungal Preparation**

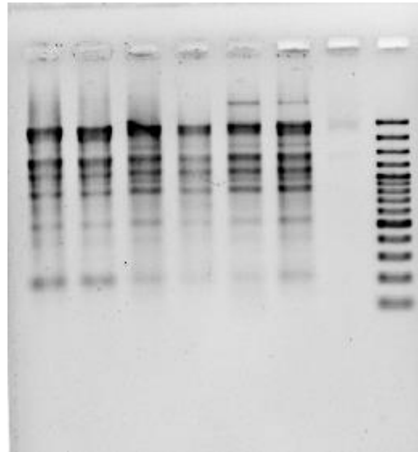


2. **Fungal inoculation for disease symptom assessment**



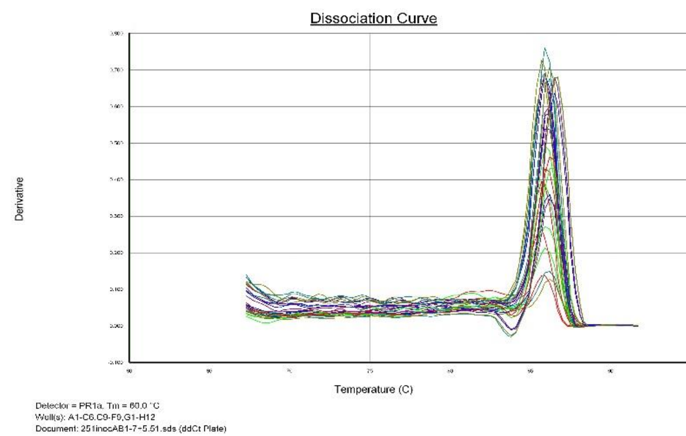
Appendix 2

1. **RNA quality checked by 1 % of agarose gel**

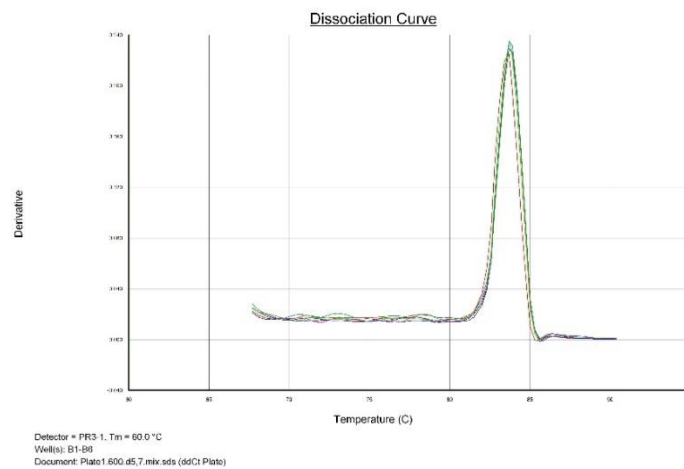


2. **Melting curve of *PRs* protein and *PAL* gene**

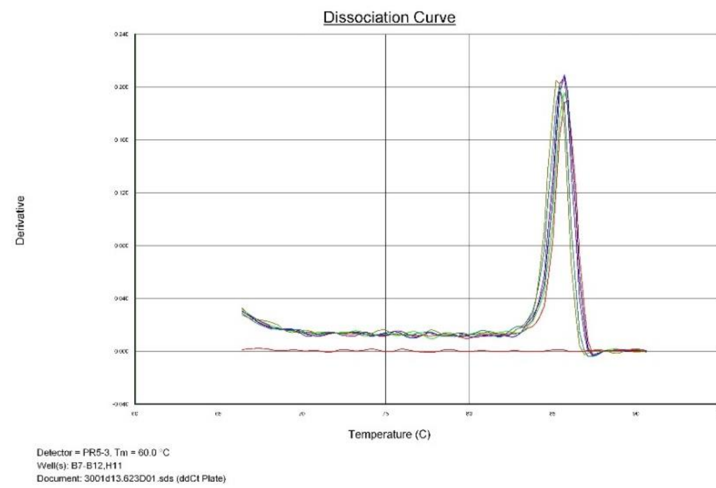
   a. *PR1a* melting curve



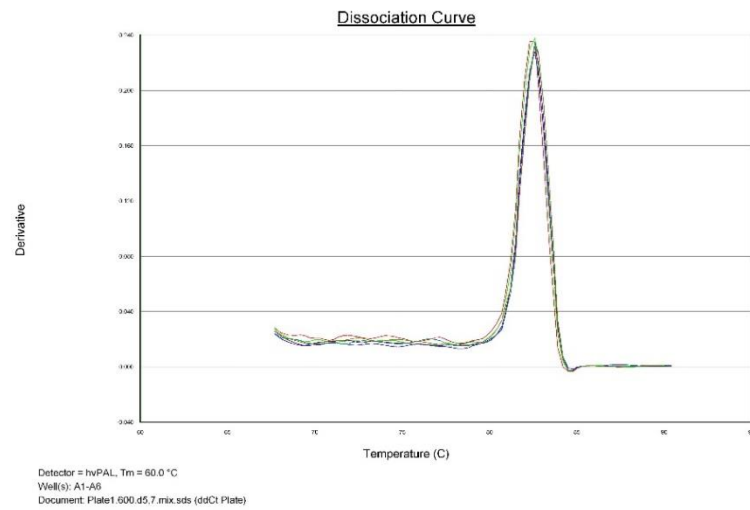
   b. *PR3* melting curve



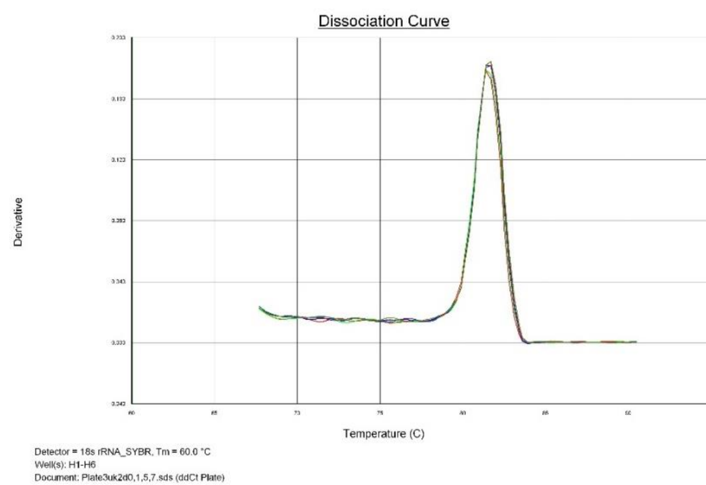
c. *PR5* melting curve



d. *PAL* melting curve



e. *18s rRNA* melting curve



Appendix 3

1. **Statistical analysis of Defense-related genes**

***PR1a* gene expression (Fold)**

Clone	0	1	3	5	7
RRIT 251	1.000	0.081±0.1c	0.273±0.1	3.962±53.8	2.412±2.2
RRIM 600	1.000	0.967±0.6bc	2.586±2.3	0.070±0.0	0.061±0.0
RRIM 623	1.000	4.701±0.7a	4.695±0.7	2.297±0.3	1.400± 0.9
PB 5/51	1.000	1.777±0.6bc	2.633±1.1	0.756±0.2	0.114± 0.0
BPM 24	1.000	0.962±1.0bc	0.503±0.1	0.390±0.1	1.878±1.5
RRIT 408	1.000	2.713±0.9ab	0.988±0.2	0.815±0.3	0.189±1.5
CV (%)		55.180	111.860	159.930	179.102
Pr>f (LSD)		0.021*	0.324	0.360	0.701

***PR3* gene expression (Fold)**

Clone	0	1	3	5	7
RRIT 251	1.000	3.567±3.1	2.300±2.2	0.462±0.3ab	3.138±1.7
RRIM 600	1.000	3.280± 1.4	2.261±0.7	1.009±0.4ab	3.805±2.5
RRIM 623	1.000	1.039±0.5	3.822±1.8	0.330±0.1b	0.256±0.0
PB 5/51	1.000	2.235±1.5	5.026±0.2	0.996±0.3ab	0.782±0.7
BPM 24	1.000	1.169±0.7	1.036±0.9	1.51±0.5a	1.880±0.6
RRIT 408	1.000	0.146±1.3	0.931±0.25	0.976±0.3ab	0.509±0.2
CV (%)		134.890	82.210	65.280	129.490
Pr>f (LSD)		0.653	0.403	0.223	0.376

***PR5* gene expression (Fold)**

Clone	0	1	3	5	7
RRIT 251	1.000	5.924±3.5	1.119±1.8	0.482±0.3	2.218±1.6
RRIM 600	1.000	6.802±5.3	1.339±0.3	0.504±0.1	3.197±2.9
RRIM 623	1.000	1.796±0.2	40.070±3.7	1.316±0.7	1.203±0.8
PB 5/51	1.000	2.781±0.8	31.214±28.7	5.973±0.1	1.015±0.7
BPM 24	1.000	1.300±0.4	0.654±0.4	3.279±1.3	3.361±2.1
RRIT 408	1.000	1.119±0.6	23.280±22.0	8.516±8.0	0.035±0.0
CV (%)		125.430	131.330	132.250	139.580
Pr>f (LSD)		0.640	0.653	0.280	0.731

***PAL* gene expression (Fold)**

Clone	0	1	3	5	7
RRIT 251	1.000	0.148±0.1b	0.281±0.3	0.554±0.5c	1.245±1.2ab
RRIM 600	1.000	1.688±0.5ab	0.959±0.2	0.782±0.4c	0.727±1.1b
RRIM 623	1.000	0.641±0.2ab	2.263±1.6	0.156±0.1c	0.129±0.1b
PB 5/51	1.000	2.958±1.8a	2.299±1.0	2.128±0.7b	0.524±0.3b
BPM 24	1.000	0.908±0.4ab	1.418±0.4	3.312±0.4a	2.218±0.0a
RRIT 408	1.000	0.935±0.3ab	0.366±0.3	0.200±0.0c	0.100±0.0b
CV (%)		95.970	118.920	50.290	90.370
Pr>f (LSD)		0.251	0.527	0.001**	0.05*

2. Statistical analysis of Defense-related enzymes

Chitinase Specific activity (micromol N-acetyl-glucosamine/h/mgP)

Clone	d0	d1	d3	d5	d7
RRIT 251	0.172±0.01	0.168±0.01b	0.352±0.08	0.255±0.07	0.212±0.01ab
RRIM 600	0.215±0.02	0.197±0.08ab	0.299±0.01	0.188±0.02	0.3030.08±a
RRIM 623	0.202±0.02	0.190±0.01ab	0.333±0.08	0.245±0.32	0.234±0.01ab
PB 5/51	0.196±0.02	0.248±0.03a	0.317±0.09	0.264±0.03	0.229±0.02ab
BPM 24	0.215±0.02	0.168±0.01b	0.196±0.01	0.198±0.01	0.170±0.01b
RRIT 408	0.222±0.06	0.171±0.01b	0.294±0.03	0.323±0.08	0.184±0.02b
CV (%)	25.032	14.886	35.662	34.424	28.950
Pr>f (LSD)	0.82	0.06	0.58	0.45	0.23

POD specific activity (Unit/mgP)

Clone	d0	d1	d3	d5	d7
RRIT 251	259.906±25	302.920±23b	427.081±19b	384.549±54bc	347.835±3c
RRIM 600	332.779±38	341.144±42b	601.141±28b	459.903±42b	500.954±59ab
RRIM 623	242.490±24	515.634±65b	515.634±65b	358.271±30bc	372.947±25c
PB 5/51	269.271±24	837.321±219a	1314.707±333a	682.243±81a	608.974±64a
BPM 24	319.659±28	333.333±16b	311.835±26b	284.649±41c	301.863±25c
RRIT 408	355.032±100	369.847±35b	505.622±44b	349.574±56bc	383.941±40bc
CV (%)	29.170	37.320	39.910	22.081	16.719
Pr>f (LSD)	0.57	0.016*	0.004**	0.002**	0.04**

PAL Specific activity (mol trans-cinnamic acid/h/mgP)

Clone	d0	d1	d3	d5	d7
RRIT 251	0.314±0.03a	0.293±0.01	0.821±0.24ab	0.304±0.04	0.302±0.02b
RRIM 600	0.328±0.03a	0.304±0.05	0.482±0.08b	0.252±0.07	0.545±0.07a
RRIM 623	0.282±0.02ab	0.280±0.06	0.663±0.20ab	0.327±0.07	0.201±0.08b
PB 5/51	0.198±0.01b	0.368±0.05	0.990±0.07a	0.295±0.03	0.352±0.07b
BPM 24	0.329±0.06a	0.260±0.00	0.453±0.05b	0.235±0.02	0.259±0.07b
RRIT 408	0.246±0.0ab	0.285±0.03	0.611±0.13ab	0.232±0.09	0.298±0.03b
CV (%)	19.15	20.13	37.89	37.16	26.45
Pr>f (LSD)	0.06	0.409	0.156	0.799	0.006**

Appendix 4

1. **Buffer preparation protocol for RNA extraction and PDA medium**

1) RNA extraction buffer 100 ml

2 % CTAB	:	2 g
100 mmol L^{-1} TRIS-HCl	:	1.576 g
100 mmol L^{-1} EDTA	:	2.92 g
1.4 mol L^{-1} NaCl	:	8.18 g
5 % v/v β mercaptoethanol	:	5 mL

Deionizer water was added to make final volume to 200 ml and sterilize using autoclave. Kept the buffer solution at room temperature for short period.

2) 8 mol L^{-1} LiCl
Added 33.9 g of LiCl to 100 ml or distilled water and sterilize using autoclave. Kept at room temperature

3) PDA medium plus antibiotic
Added 39 g of commercial PDA powder to 1 litre of distilled water, and autoclave for 15 minutes at 121°C. Waited until the PDA temperature up to 45-50°C and an appropriate amount of 0.5 % of streptomycin to each litre of medium. Storage at 4°C

2. **Buffer preparation for protein extraction and enzyme assessment**

1) 0.1 M potassium phosphate buffer pH 7, 100 mL

1 M K_2HPO_4	:	3.8 mL
1 M KH_2PO_4	:	6 mL

Deionizer water was added to make final volume to 100 ml, and adjusted the buffer to pH 7. The buffer was added with following chemical Kept the buffer at 4°C for short period.

2) Bradford reagent, 1 L

4.7 % Ethanol (from 100% stock)	:	47 mL
8.5 % Phosporic acid	:	100 mL
0.01 % coomassic brilliant blue G-250	:	0.1 g

Adjusted up to final volume 1000 mL with distilled water, filtered the reagent using Whatman #1 paper, and stored the reagent into dark bottle at 4°C.

3) 1 M Tris Base pH 8.5, 100 mL
   Tris Base 12.114 g
   Adjusted the pH to 8.5 with HCl. Brought up the volume to 100 mL with distilled water. Kept the buffer solution at 4ºC.

4) 0.1 M L-phenylalanine, 100 mL
   L-phenylalanine 1.648 g
   Brought up the volume to 100 mL with 0.1 M Tris Base pH 8. Kept at 4ºC.

5) 10 mM Trans-cinnamic acid, 10 mL
   Trans cinnamic acid 0.015 g
   Dissolve with ethanol absolute (99.99%) up to 10 mL, vortex and kept at dark bottle at 4ºC.

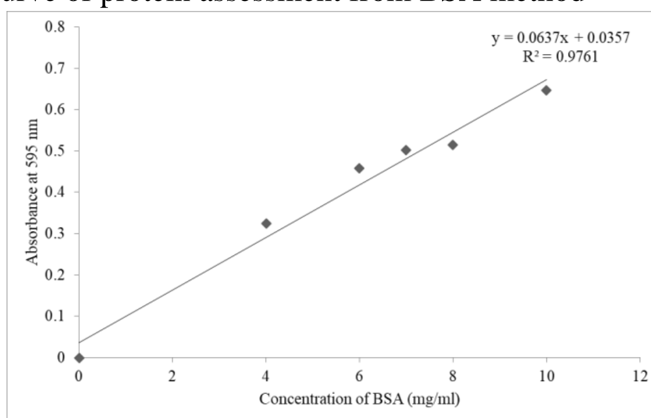
6) 0.1 M acetate buffer pH 5
   0.07 M Sodium acetate : 5.772 g

   0.03 M acetic acid : 1.778 g

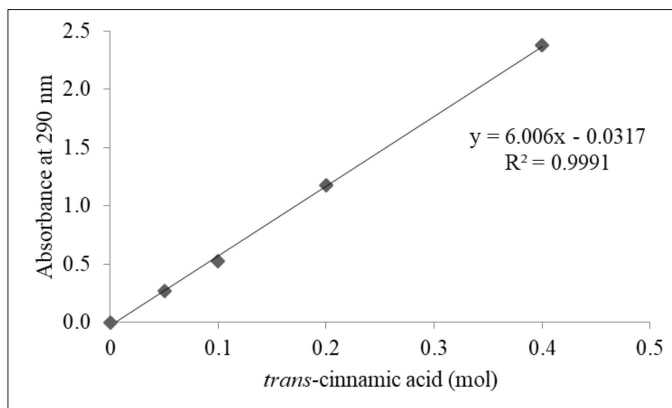
   Prepare 800 mL of distilled water in suitable container, added 5.772 g of sodium acetate to the solution, added 1.778 g of acetic acid to the solution. Adjusted the solution to desired pH to 5 using 1 N HCl. Brought up the volume to 1 L with distilled water.

3. Standard curve for cnzyme activity assessment

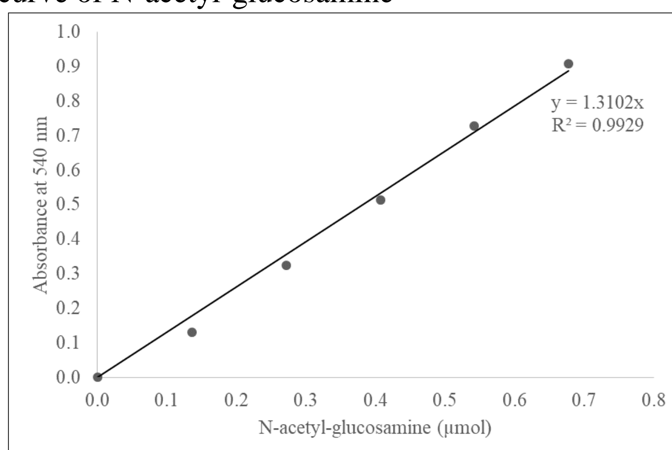
a. Standard curve of protein assessment from BSA method



b. Standard curve of *trans*-cinnamic acid



c. Standard curve of N-acetyl-glucosamine



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