COMPARATIVE GROWTH PERFORMANCE AND ACTIVITY OF DEFENSE RELATED ENZYMES AND GENE EXPRESSION IN RUBBER CLONES AGAINST *RIGIDOPORUS MICROPORUS* INFECTION

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ABSTRACT

Rigidoporus microporus is the causal agent of rubber white root disease (WRD). Control of this is commonly carried out by chemical treatment. The use of tolerant rootstocks may present an effective method to lessen its impact. The screening of rubber rootstocks for tolerance of WRD was investigated by both defense-related enzymes and gene expression, and assessment of disease symptoms. Two recommended clones in Thailand (RRIT251 and RRIT408) and RRIM623 (Malaysian clone) were screened and compared with susceptible clones (RRIM600 and BPM24) and tolerant clone (PB5/51) by inoculating young rubber seedling with *R. microporus*. The expression of *PRs* and *PAL* were analyzed at 0, 1, 3, 5, and 7 days post inoculation (dpi) along with the defense-related enzyme activities. Based on observed transcriptional level of *PRs* and *PAL* genes, CHI, PAL, and POD enzyme activities, and also evidence from WRD symptom assessment in the field, we concluded that PB 5/51 had the best tolerance with the lowest disease incidence, high PAL and POD enzyme activities, with the next best being RRIM 623 and RRIT 251, respectively. Meanwhile, BPM 24, RRIM 600, and RRIT 408 clones are moderately susceptible to WRD. The study introduces new possibilities for the control of WRD using alternative rubber rootstock clones.

Keywords *Hevea brasiliensis*, *PR* genes, *PAL* gene, chitinase, peroxidase, *Rigidoporus microporus*

INTRODUCTION

Rubber trees are the main source of commercial natural rubber that mainly used for tires, surgeons' gloves, condoms, balloons and other relatively high-value products. Nowadays, variability of climate change has been recognized as an important factor for root disease breakout such as brown root disease, red root disease and white root disease (Wattanasilakorn *et al.*, 2017). Among the root diseases, white root disease (WRD) caused by *Rigidoporus microporus* is a worldwide serious problem that can deplete the productivity of the natural latex and lead to massive death of the rubber trees (Nandris *et al.*, 1987). It was found that 95% of root diseases found in rubber tree plantation in the South of Thailand were identified as white root rot disease (Rojsojitt *et al.*, 2011). The pathogen penetrates the root, colonizes the tissues, and degrades the host's cell structure. Rhizomorphs and infection sites remain below ground and a decaying tree do not show visible aerial symptoms, until the roots are severely damaged, followed by appearance of reddish brown basidiocarps on the collar of trees (Ogbebor *et al.* 2013a). In Indonesia and Malaysia, WRD attacks rubber plantations with disease severity ranging from moderate to severe (Pawirosoemardjo *et al.*, 1992; Farid *et al.*, 2009). According to Ogbebor *et al.* (2013b), the yield losses on old rubber plantations were up to 50%. In Southern

Thailand, Nissapa and Chuenchit (2011) reported that WRD in rubber plantations caused economic losses of around 4,940-96,177 dollar per hectare. Nandris *et al.* (1987) reported that root decaying pathogens caused more than 50% mortality in old stands established after forests were cleared manually; if one assumes 25 years of productive life for a rubber tree, losses came to several hundred thousand dollars per hectare. Current control approaches involve the use of chemical and biological controls, however none have proven to be fully effective. Breeders are working to develop WRD-tolerant rootstocks, which is one of the most promising avenues to a cure. Moreover, rubber tree improvement has been achieved by incorporating new sources of genetic variability to disease resistance against WRD. Therefore, the rubber germplasm accessions have to be characterized for the disease tolerant level. These programs, focusing on disease tolerant level can be speeded up and made more effective by the use of both growth performance after infection and the defense-related gene expression or enzyme activities as selection criteria.

Plants protect themselves from pathogen by constitutive and inducible defense mechanisms (Ferreira *et al.*, 2007). Active or inducible mechanisms include accumulation of phytoalexins, oxida-

tive burst, systemic acquired resistance (SAR) by accumulations of pathogenesis-related (PR) proteins, and activated transcription of genes encoding enzymes in the biosynthesis of phenolic compound, such as phenylalanine ammonia lyase (PAL) (Montesinos, 2000). According to previous studies, Woraathasin *et al* (2017a) mentioned that *HbP-R1b* and *HbPR3* were potential defense genes, distinguishing between tolerant and susceptible rubber clones to WRD. Additionally, Sangsil *et al* (2016) suggested that the *HbPAL* gene may play a role in the molecular defense response of rubber tree to WRD infection and could be used as a selection criterion for disease tolerance.

Various defense responses combine several factors, such as defense-related enzymes linked to the PR proteins, and the enzyme accumulation prevents infections by pathogens. Defense-related enzymes such as PAL, POD (peroxidase) and CHI (chitinase) are reported to have significantly increased levels in resistant cultivars during pathogen invasion (Kumar *et al.*, 2017). Increased PAL enzyme activity in tomato is associated with its resistance to bacterial spot disease, and in pepper to infection by *Xanthomonas campestris* (Chandrasekaran *et al.*, 2017; Kim and Hwang, 2014). POD and SOD activities increased in pepper and tomato infected with the pathogen (Jetiyanon, 2007). CHI accumulated together with GLU upon fungal infection, and these enzymes are responsible for hydrolysis of fungal cell wall components (Xu *et al.*, 2016). Here, we report the screening of two recommended clones in Thailand (RRIT251 and RRIT408), RRIM623 (Malaysian clone) compare with susceptible clones (RRIM600 and BPM24) and tolerant clone (PB5/51) for WRD. The transcription levels of *PRs* and *PAL* gene, and activity of CHI, PAL, and POD enzymes were determined after *R. microporus* inoculation at the seedling stage. This first comprehensive characterization of WRD tolerance in rubber tree opens new perspectives of investigating the molecular mechanism of WRD resistance and screening for potential tolerant clones.

MATERIALS AND METHODS

Plant materials: The rubber seeds were germinated in a sandy bed for 15 days and replanted in polybags filled with fine-loamy soil as the medium. Rubber seedlings with similar performance were used in this research. In the study, three clonal rubber clones including a tolerant clone (PB5/51) and two susceptible clones (RRIM600 and BPM24) were used as the baseline characteristics for disease tolerant level. Three rubber clones consist of two recommended clones in Thailand (RRIT251 and

RRIT408) and RRIM623 (Malaysian clone) were screened, compared with the susceptible clones (RRIM600 and BPM24) and tolerant clone (PB5/51) by inoculating young rubber seedling with *R. microporus*.

Fungal inoculation preparation: *R. Microsporus* isolate RG15 was provided by the Pest Management Department, Prince of Songkla University. It was isolated from basidiocarps collected from an infected rubber tree. The mycelium was freshly grown on 2% w/v potato dextrose agar (PDA) media for 7 days for use as an inoculum.

Seedling inoculation with *R. microporus* for disease measurement: 4-month old rubber seedlings in plastic pots with three replicates were inoculated. Five hundred g of mushroom media containing *R. microporus* inoculum were placed near the stem and root, and mixed with top soil medium in which the rubber seedlings grown. The inoculation was conducted for eight months of the research.

Seedling inoculation with *R. microporus* for defense-gene and enzyme activity assay: The rubber seedlings with three replications were inoculated with *R. microporus* following Woraathasin method (Woraathasin *et al.*, 2017a) with some modifications. Inoculation was done by creating 30.0 mm x 3.0 mm wounds on the stem, about 1 cm from the taproot, using a sterilized surgical blade. The area ready to be inoculated was first surface sterilized with 90 % ethanol and then 30.0 mm x 5.0 mm agar with actively growing mycelia was placed on the wounds, and wrapped in plastic. Mock inoculations (negative control treatment) were carried out with sterile PDA agar. The two-month old leaves were harvested from each rubber seedling inoculated with *R. microporus* at 0, 1, 3, 5, and 7 days post inoculation (dpi), and were kept in liquid nitrogen and stored at -80°C for protein extraction.

Disease measurement: Evaluation of disease infection was conducted according to Soyong and Kaewchai (2014) from 3 to 8 months after inoculation with scores on 1-5 scale: 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 symptoms were assessed visually. Data represented at least three independent replicates.

The data collection as disease index (DI) was recorded every month. Data on the severity of the WRD were statistically compared 8 months after inoculation.

$$\text{Disease Index (DI)} = \frac{(1xa)+(2xb)+(3xc)+(4xd)+(5xe)}{(a+b+c+d+e)} \times \frac{100}{X}$$

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 highest infection severity category, which is 5

RNA extraction and cDNA synthesis: RNA extraction from rubber leaves was carried out based on Deng *et al.* (2012) method with modifications. A hundred mg of sample was frozen in liquid nitrogen and stored at -80°C until use. The frozen leaf tissues were ground to powder and 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. The suspension was incubated at 65°C for 5 minutes. 680 μ L of chloroform/isoamyl alcohol (24:1, v/v) was added to the suspension that was then centrifuged at 12,000 x G for 10 minutes at 4°C, twice. The supernatant was transferred to another tube, and 1/3 volume of 8 mol/L LiCl was added in the crude sample, mixed well and stored at -20°C for 4 – 16 hours. The sample was centrifuged at 12,000 x G for 20 minutes at 4°C. The crude pellet was transferred to another new tube, 100 μ L of isopropanol was directly added and the sample was stored at -20°C for 30 minutes. The pellet was centrifuged at 12,000 x G for 20 minutes at 4°C. The final pellet was washed with 70% (v/v) of ethanol, and air-dried. 1% formaldehyde agarose gel electrophoresis was used to check the integrity and purity of the isolated RNA. RNA quantification through UV-visible spectrophotometer. cDNA was synthesized by reverse transcription from 1000 ng of total RNA using transcriptome amplification kit according to the manufacturer's instructions (Thermo Scientific).

PRs and PAL gene expression analysis: PRs and PAL primers used in the study are shown in Table 1. Differential gene expression was determined by qRT-PCR. The PCR mixture was 5 μ L of SYBR green buffer, 1 μ L of 3 μ M of primer forward, 1 μ L of 3 μ M primer reverse, and 2 μ L of 50 ng cDNA template (50 ng). 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 replicate were used for this analysis. The relative gene expression was calculated from the cycle threshold (C_T) values and analyzed using two methods (Livak & Schmittgen, 2001). *18s rRNA* was used as internal reference gene for normalization (Li *et al.*, 2011).

Table 1: List of primer sequences used in qRT-PCR

Gene	Direction	Sequence (5'→3')
<i>PR1a</i>	Forward	CAG GTG GTT TGG CGC AAC TC
	Reverse	GGT CGC TGC CCA ACA AAG TT
<i>PR3</i>	Forward	TGGTCAATGTGGGCAA GCCT
	Reverse	GGTGGGTGACCATTGT CCAGT
<i>PR5</i>	Forward	TGGACCATTAGTGCTA CTCGTGGAA
	Reverse	TGCATATTCGGCCAAG GTGTTAGG
<i>PAL</i>	Forward	GGACATGCTCAAAGTT GTGG
	Reverse	TGCTGGCATTCTTCTC ATTG
<i>18s rRNA</i>	Forward	GTAAGAGGATGGTGCCG ACAAC
	Reverse	CCAAACTTCCCCAGTT ACAAGAA

Protein extraction: The protein was extracted according to Zhang *et al.* (2009) with minor modifications. 200 mg of sample leaves was crushed in liquid nitrogen in a mortar. Six ml of 0.1 M phosphate buffer pH 7 was added with 0.1 mM EDTA and 0.1 mM Ascorbic acid to the mortar and ground until fine powder remained, and the mixture was centrifuged at 12,000 rpm for 15 minutes at 4°C. Ammonium sulphate (3.366 g) was added and mixed slowly until completely dissolved, then centrifuged at 12,000 rpm for 15 minutes at 4°C. The pellet was re-dissolved with 500 μ L of 0.1 M phosphate buffer at pH 7 along 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. The absorbance was measured at 595 nm.

Phenylalanine ammonia-lyase (PAL) activity assay: The PAL activity was determined through the conversion rate of L-phenylalanine to trans-cinnamic acid according to Assis *et al.* (2001) with minor modifications. The PAL activity assayed was measured by incubation reaction with 200 μ L of enzyme extract, 500 μ L of 0.1 M L-phenylalanine and adjusting the final reaction volume to 2 ml with 0.1 M Tris-HCl pH 8.5. The mixture was incubated at 37°C for 30 minutes, and reaction was stopped by adding 10 μ L of 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 moles of cinnamic acid per hour per mg of protein ($\text{mol.h}^{-1}.\text{mg}^{-1}\text{protein}$).

Peroxidase (POD) activity assay: The POD 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 % H₂O₂, 220 µL of 0.3 % (v/v) guaiacol, was diluted with 0.05 M of phosphate buffer at pH 7 to final 1,000 ml volume. The absorbance was read at 470 nm for 1 minute. The POD specific activity is defined in units that could oxidase 1 µmol of guaiacol in 1 minute, and expressed in Unit per mg of protein (Unit.mg⁻¹ P). The POD specific activity was calculated following the formula:

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

where as

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

ΔA/min = Slope from equation after Absorbance measurement

Chitinase (CHI) activity assay: The CHI activity was determined by Miller (1959). The mixed reaction buffer containing 100 µL of colloidal chitin (1% w/v), 150 µL of protein extract, and 750 µL of 1M sodium acetate buffer at pH 5.0 was incubated

at 37°C for 30 minutes. 1 mL of DNS solution was added into the reaction mixture that was boiled at 100°C for 5 minutes. The absorbance was measured at 540 nm. N-acetyl-glucosamine was used to make the standard curve. The CHI specific activity is expressed in µmol of N-acetyl-glucosamine per hour per mg of protein (µmole N-acetyl-glucosamine.h⁻¹.mg⁻¹ P).

Statistical analysis: Statistical analysis of enzyme activities was carried out with R program using one-way ANOVA with significance level (p) of 0.05. Further analysis was carried out using the LSD test (Gomez and Gomez, 1984). Correlation coefficients were generated using R program. The error bars in the figures represent standard errors of means.

RESULTS AND DISCUSSION

Growth of rubber seedlings and white root disease symptom assessment: A number of studies have reported that fungal infection suppressed the growth of plant infected with the pathogen. Table 2 shows the height, diameter, and number of whorls for the six rubber clones, after 8 months of post inoculation with *R. microporus*. Based on the growth parameters of these rubber seedlings, there were significant differences, RRIM 600 and BPM 24 growths were below the others (Table 2).

Table 2 The growth of six cultivated rubber seedlings at 8 months after inoculation with *R. microporus* and without inoculation (control)

Clone	Growth parameter					
	Height (cm)		Diameter (mm)		Number of whorl	
	Inoculated	Control	Inoculated	Control	Inoculated	Control
RRIT 251	104.9±5.6ab	113.5±6.4b	12.2±0.0ab	12.0±0.6a	5.7±0.8ab	7.0±0.4a
RRIM 600	78.4±5.8b	148.4±5.6ab	7.1±0.6bc	14.4±0.4a	4.9±0.2b	7.8±0.4a
RRIM 623	111.5±9.5a	129.7±5.3b	12.9±1.4a	10.9±0.4a	5.4±0.3ab	7.2±0.4a
PB 5/51	113.5±7.9a	163.4±7.3a	10.5±0.9abc	13.3±0.4a	6.1±0.4a	7.0±0.5a
BPM 24	86.3±6.6ab	146.5±9.7a	8.2±0.9bc	12.6±0.4a	4.7±0.3b	7.2±0.6a
RRIT 408	94.4±3.6ab	131.3±29.7ab	10.9±1.0abc	10.7±0.7a	5.6±0.2ab	6.0±0.5a

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.

The inoculated RRIM 600 showed the least height and diameter, followed by BPM 24 and RRIT 408. On the other hand, inoculated RRIM 623, PB 5/51, and RRIT 251 showed better growth performance than the others. The growth of inoculated rubber seedlings was obviously suppressed compared to control without inoculation as shown in Fig. 1. The

height of the inoculated seedlings was clearly reduced by 7.58 to 47.20 % compared to the control, and the diameter was suppressed by 21.11 to 50.94 %. The height, diameter, and number of whorls of infected RRIM 600 were the most inhibited compared to control (by 47.20 %, 50.94 %, and 37.31 %, respectively), followed by BPM 24.

Figure 1: Morphology of six clonal varieties of rubber, cultivated to 8-month-old seedlings, in two conditions: C is the control without inoculation with pathogen, while I means inoculated with *R. microporus*.

The disease index of each rubber rootstock was measured by scoring yellowish leaves. A number of rootstocks showed the disease symptoms few months after fungal inoculation, such as yellowish leaves appeared at 3 months post inoculation (mpi) on RRIM 600, while the other cultivated rubber seedlings, RRIT 251, RRIM 623, PB 5/51 showed less yellowish leaves (disease score was less than 3). The symptoms tended to become more severe by six to nine mpi. The disease indexes of BPM 24, RRIT 408, and RRIM 600 were over 30 % (31.4, 36.7 and 37.8 % respectively) at 8 mpi, slightly higher than of other tested clones. Meanwhile, RRIT 251, PB 5/51, and RRIM 623 rootstocks showed less yellowish leaves (≤ 25.7 %), respectively. Our study revealed that PB 5/51, RRIM 623, and RRIT 251 clones are comparatively tolerant to WRD, with a low disease incidence (25%) and high defense-related enzyme activity. At the end of the investigation period, all the cultivated rubber rootstocks were alive, and even RRIM 600 was for 88.9 % definitely alive (Table 3).

Table 3: Disease index and survival rate of six rubber clones cultivated to seedlings at 9 months post inoculation with *R. Microporus*

Clone	Disease index (DI)	Survival rate (%)
RRIT 251	25.7	100.0
RRIM 600	37.8	88.9
RRIM 623	24.4	100.0
PB 5/51	25.0	100.0
BPM 24	31.4	100.0
RRIT 408	36.0	100.0

In our study, the symptoms of infected rubber trees were similar to prior reports (Farid *et al.*, 2009; Kaewchai & Soyong, 2010; Nakaew *et al.*, 2015), according to which the symptoms include 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 accumulating on lateral roots, producing fruit bodies and causing death of plant. A study conducted by (Kaewchai & Soyong, 2010) found that 5-month-old RRIM 600 showed the symptoms at 70 days after inoculation, while Wattanasilakorn *et al.* (2012) reported that yellowing of leaves in 5-month-old RRIM 600 clones appeared at 112 days, and the survival rate was below 33 % at 5.5 months after infection. They concluded that the RRIM 600 is sensitive to WRD. Different times of symptom appearance in the same clone might be due to variations in fungal virulence by the specific strain tested. Also environmental conditions might be a contributing factor. Prasannath (2017) mentioned that

soil characteristics (physical, chemical, and biological) are strongly related to WRD incidence. Moreover, the heterogeneity of seedlings in rubber root stocks, due to open pollination, may be another factor affecting disease resistance.

Defense-related gene expression: To optimize the specific primers for qRT-PCR analysis, the amplification efficacy of each primer was examined. The transcript level of *PR1a* in all six rubber clones was significantly induced at 1 dpi (Fig. 2A). The transcript level of *PR1a* in RRIM 623 and PB 5/51 was initially upregulated at 1 and 3 dpi compared to the others, and the peak level was at 3 dpi (4.7 and 2.6 fold upregulation, respectively), and subsequently downregulated at 5 and 7 dpi. In RRIT 408, the *PR1a* transcript level was early induced at 1 dpi, and then 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 was varied. These *PR1a* transcript levels reached the peak level at different times (RRIT 251 was at 5 dpi, RRIM 600 was at 3 dpi, and BPM 24 was at 7 dpi). Various transcriptional studies have reported upregulated *PR* genes in plants after fungal infection (Ali *et al.*, 2018). The gene expression of *PR1* in para rubber infected by *R. microporus* increases disease resistance (Oghenekaro *et al.*, 2016; Sangsil *et al.*, 2016; Woraathasin *et al.*, 2017a). In this study, *PR1a* expression was not significantly elevated during fungal infection, but *PR1a* gene expression in RRIM 623 and RRIT 251 was higher than in the other clones. *PR1a* gene expression was not induced in PB 5/51, which is known as a tolerant clone (Woraathasin *et al.*, 2017a).

Transcript level of *PR3* gene in the six varieties of cultivated rubber seedlings, infected with *R. microporus*, is shown in Fig. 2B. The pattern of transcript fold changes did not significantly differ between the six clonal varieties, after inoculation with *R. microporus*. There was a steady pattern of *PR3* transcript levels. The *PR3* gene expression in RRIM 623 and PB 5/51 was elevated at 1 and 3 dpi with the peak at 3 dpi, and then the transcript level decreased at 5 and 7 dpi. The *PR3* transcript level in RRIT 251 and RRIM 600 showed peaks at 1 and 7 dpi, respectively. The transcript level in BPM 24 was steady with 1.1 to 1.89 fold upregulation. Although the level of transcription of *PR3* showed no significant differences, the PB 5/51 infected seedlings showed the highest *PR3* upregulation at 3 dpi (5 fold). Commonly, *PR3* protein known as chitinase, which contributes to the defense response to fungal infection. *PR3* gene was initially induced by phytopathogens through the jasmonate-signaling pathway, leading to SAR in the plant infected by a necrotrophic pathogen (Ali *et al.*, 2018). Woraath-

asin *et al.* (2017a) reported the *HbPR3* gene was induced in both a tolerant clone (PB 5/51) and in susceptible clones (RRIM 600 and BPM 24) after infection by *R. microporus*. According to Upadhyay *et al.* (2014), interaction between *Alternaria solani* and tomato have induced *PR3* genes in early infection. The chitinase gene are also early accumulated at 2 h and was significantly increased at 4 h, 8 h, 24 h, and 48 h upon *Alternaria brassica* infection in *Brassica juncea* (Rawat *et al.*, 2017).

The expression of *PR5* gene in RRIM 623, PB 5/51, and RRIT 408 reached the maximum level at 3 dpi (40, 31, and 23 folds, respectively). However, transcript level of the *PR5* in those three infected seedlings gradually decreased by 5 dpi and further by 7 dpi (Fig. 2C). The *PR5* transcript level of RRIT 251 and RRIM 600 was similar in pattern, where the *PR5* gene was early induced at 1 dpi, continuously decreased by 3 and 5 dpi, and increased at 7 dpi. Meanwhile, the BPM 24 clone inoculated with pathogen showed a constant level of transcription of *PR3* in the observations. A number of observations have proven that the transcript level of *PR5* is clearly induced by a fungal infection (Guo *et al.*, 2016; Oghenekaro *et al.*, 2016; Wang *et al.*, 2010), but not by a bacterial infection (Liu *et al.*, 2013). The *PR5* gene was strongly upregulated in the incompatible interaction during pathogen invasion (Wang *et al.*, 2010). Oghenekaro *et al.* (2016) and Woraathasin *et al.* (2017b) agreed that *R. Microporus* infection in rubber clones significantly induced *PR5* that was highly expressed in tolerant clones. Various reports have shown that *PR1*, *PR3*, and *PR5* genes are involved in the activation of SA and/or JA pathways, as well as in the activation of SAR inducible defense program (Ali *et al.*, 2018; Wang *et al.*, 2010;). Meanwhile, according to Bonasera *et al.* (2006), *PR1*, *PR2*, *PR5*, and *PR8* genes are not markers implicating SAR in young apple shoots. Based on these reports, the *PR* genes' expression may differ by the host plant (Guo *et al.*, 2016), and those *PR* genes are diversely expressed depending on the tissue or organ (Liu *et al.*, 2013).

PAL gene contributes in phenylpropanoid pathway initiated from L-phenylalanine to trans-cinnamate leading to synthesis of antimicrobial compounds and cell wall strengthening. The *PAL* gene was investigated as potential plant defense response, when challenged by a pathogen. In this study, *PAL* gene expression in the rubber seedlings was determined at particular times after challenge by inoculation. Between the observation time points (1, 5, and 7 dpi) there were significant differences in *PAL* transcript level. The results show that *PAL* gene expression in RRIM 600, RRIT 408 and RRIT 251 was down regulated initially by the infe-

ction, except that RRIM 600 was upregulated at 1 dpi and RRIT 251 at 7 dpi. The *PAL* gene was highly expressed in PB 5/51 clone with peak at 1 dpi (2.9 fold), while the transcript level of *PAL* in PB 5/51 slightly decreased with time. The BPM 24 seedlings presented the highest *PAL* upregulation among the clones at 5 dpi (3.3 fold), with a decrease at 7 dpi (Fig. 2D). *PAL* gene is more expressed in a tolerant rubber clone than in a susceptible one, with some pathogen infections (Ngobisa *et al.*, 2016; Oghenekaro *et al.*, 2016; Sangsil *et al.*, 2016). Our results on *PR3*, *PR5* and *PAL* genes are in agreement with those of Sayari *et al.* (2014) who evaluated the transcription level of the *PR3*, *PR5* and *PAL*

genes between resistant (Tarom) and susceptible (Khazar) rice cultivars in response to inoculation with *Rhizoctonia solani*. The results showed that *PR3*, *PR5* and *PAL* genes continued to accumulate up to 24-48 hours after inoculation in resistant genotype. The expression of those genes in susceptible genotype was not noticeable. In both roots and leaves of the resistant *Brassica napus* line, phenylpropanoid pathway genes were upregulated at more time points and at higher levels than in susceptible lines (Irani *et al.*, 2019).

Figure 2: The PR and PAL transcript levels in six rubber clones. Fold change after inoculation with *R. microporus*: PR1a (A), PR3 (B), PR5 (C) and PAL (D). All the data are presented as mean $\pm$ S.E. calculated from three independent replicates.

Activity of key enzymes related to phenylpropanoid and PR protein pathways: Rubber seedlings treated with *R. microporus* were investigated for 7 days, and the CHI, PAL, and POD enzyme activities were assayed. Fig. 3A shows the CHI activity in the six cultivated types of rubber seedlings after inoculation with *R. microporus*. The patterns of CHI activity were not consistent between the clonal varieties. In the normal condition (0 dpi), CHI had a low activity level (0.196-0.352 $\mu\text{mole N-acetyl-D-glucosamine} \cdot \text{h}^{-1} \cdot \text{mg}^{-1} \text{P}$, respectively), but was induced subsequently at 3 dpi with 32.5-105% increase in activity, except for BPM 24 in which the activity was reduced by 9%. Infected BPM 24 seedlings also showed the lowest CHI activity at all observation time points. PB 5/51 showed the highest CHI activity that steadily increased at 1 and 3 dpi (26 to 62 % compared to 0 dpi, respectively), and subsequently decreased at 5 and 7 dpi. Our results showed different CHI activity patterns in the six rubber clones. However, CHI in BPM 24 seemed less active at all observed times during infection. Xu *et al.* (2016) reported that CHI in plants is a crucial enzymes that catalyze fungal cell wall degradation. A number of studies have reported that the CHI are important for disease resistance. Increased CHI and GLU activities in infected

grapevine leaves were plant resistance responses to insults with *Uncinula necator* (Giannakis *et al.*, 1998). In addition, chitinase activity showed significant increase in roots and leaves of oil palm seedling at two weeks post infection with *G. boninense* (Naher *et al.*, 2012).

The PAL activities in six cultivated rubber seedlings inoculated with *R. microporus* are presented in Fig. 3B. The PAL activity in infected rubber seedlings was clearly affected at 0, 3, and 7 dpi. At 0 dpi (uninoculated seedlings), the PAL activity was 0.19-0.33 $\text{mol trans-cinnamic acid} \cdot \text{h}^{-1} \cdot \text{mg}^{-1} \text{P}$. The lowest PAL activity was in PB 5/51, whereas the highest was in RRIM 600 and BPM 24. PB 5/51 showed the most PAL accumulation during the observations. At 1 dpi, mostly PAL activity had decreased, but then reached the maximum level at 3 dpi, peaking at 37.7 % - 401.0 % compared to 0 dpi. The highest PAL activity at 3 dpi was in PB 5/51, followed in rank order by RRIT 251, RRIM 623, and RRIT 408 (0.990, 0.821, 0.623, and 0.611 $\text{mol trans-cinnamic acid} \cdot \text{h}^{-1} \cdot \text{mg}^{-1} \text{P}$, respectively). Moreover, those enzyme activities decreased at 5 dpi in all the cultivated rubber seedlings, but increased gradually at 7 dpi. According to Slatnar *et al.* (2010), activity of PAL enzyme was induced in apple by a scab spot infection with *Venturia inae-*

qualis, over healthy peels, and the fungal infection increased metabolism of phenolic compounds. Other enzymes, such as POD, also contribute in phenylpropanoid pathways, and play an important role in plant defense against both necrotrophic and/or biotrophic pathogens (Van Loon *et al.*, 2006). The POD is involved in reinforcement of cell walls, ferulic acid cross-linking, lignification, and suberization and metabolism of ROS, in plant defense responses (Almagro *et al.*, 2008). 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. In this study, the POD activity was initially

induced after inoculation, at 1 dpi. The pattern of POD activity was similar as with PAL activity. The POD activity was significantly elevated and reached its maximum at 3 dpi, as presented in Fig. 3C. The PB 5/51-rubber seedlings inoculated with *R. microporus* showed the highest POD activity at 1, 3, 5 and 7 dpi among all the clones. The highest POD activity was detected at 3 dpi in PB 5/51 clone (1,314.7 Unit.mg⁻¹P). The POD activities in other infected clones were around 311-601 Unit.mg⁻¹P respectively. The fungal inoculation in BPM 24 induced POD activity early, but this clone had the lowest activity among all the cases.

Figure 3: Defense-related enzyme activities in six rubber clones after inoculation with *R. microporus*: Chitinase (A), Phenylalanine ammonia lyase (B), and Peroxidase (C). All the data are presented as mean $\pm$ S.E. calculated from three independent replicates.

Currently, there are rubber rootstocks with partial tolerance to WRD and significant efforts are in progress to select material with enhanced tolerance and other agronomic traits. In addition, investigations are underway to gain a better knowledge of the *Hevea-R.microporus* interaction with the objective of developing tools that could be used to speed up rubber rootstock breeding and selection programmes. Along this line, early introduced rubber clones were screened by shoot growth, root development and physiological response after inoculation with WRD and it was found that there were some clones that exhibited tentative tolerance to WRD (Wattanasilakorn *et al.*, 2012). Approximately 150 days are required from inoculation until the appearance of visible aboveground symptoms assessment in this conventional system. A decrease in time, labor, and inputs makes the laboratory assays through gene expression and enzyme activities, suitable tools for shortening the breeding program of WRD tolerance. Examining rubber gene expression profiles in contrasting disease reactions will contribute to a better understanding of the molecular interactions underlying tolerance and susceptibility to WRD and would allow the identification of new genes that could be used as markers in rubber rootstock breeding programmes. Taken together, field evaluation through visual observation and laboratory assays through gene expression and enzyme activities have led to screening of putatively tolerant rubber clones. This study represents the first report on gene expression and enzyme activities analysis for assessing the response of tolerant and susceptible rubber rootstocks to WRD. Based on observed transcriptional level of *PRs* and *PAL* genes, *CHI*, *PAL*, and *POD* enzyme activities, and also evidence from WRD symptom assessment in the field, we conclude that PB 5/51, RRIM 623, and RRIT 251 clones are rubber clones comparatively tolerant to the WRD, while the BPM 24,

RRIM 600 and RRIT 408 clones are moderately susceptible to WRD.

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