TRANSCRIPTOME ANALYSIS OF POWDERY MILDEW INFECTION IN *Hevea Brasiliensis*

KAMERKAR URWASHI PUSHPAK

UNIVERSITI SAINS MALAYSIA

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TRANSCRIPTOME ANALYSIS OF POWDERY MILDEW INFECTION IN *Hevea Brasiliensis*

by

KAMERKAR URWASHI PUSHPAK

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LIST OF UNITS

mM	milliMolar
M	Molar
min	min
mL	milliliter
µL	microliter
ng	Nano gram
g	Gram
h	hr
mg	milligram
$A_{260/280}$	Absorbance at 260nm and 280nm
nm	nanometer

LIST OF ABBREVIATIONS

A3SS	Alternative 3' splice site
A5SS	Alternative 5' splice site
ABA	Abscisic acid
AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
AS	Alternative splicing
BLAST	Basic local alignment search tool
cDNA	Complementary deoxyribonucleic acid
CTAB	Cetyl Trimethylammonium Bromide
DAG	Directed acyclic graph
DEG	Differentially expressed genes
DEPC	Diethyl Pyro carbonate
ERF	Ethylene response factors
ET	Ethylene
ETI	Effector triggered immunity
ETS	Effector triggered susceptibility
FDR	False discovery rate
FPKM	Fragments Per Kilo Base of transcript sequence per Million base pairs sequenced
GO	Gene Ontology
H1	RRIM 929 healthy
H2	RRIM 2025 healthy
HCl	Hydrochloric acid
HMDS	Hexamethyldisilazane
HR	Hypersensitive response
I1	RRIM 929 infected

I2	RRIM 2025 infected
IAN	Instituto Agronomico do Norte
IGV	Integrated genome viewer
InDel	Insertion-Deletion mutation
IRRDB	International Rubber Research and Development Board
ITS	Internal Transcribed Spacer
JA	Jasmonic acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
LRR	Leucine rich repeat
LTC	Latex-Timber Clones
MAPK	Mitogen activated protein kinase
MAS	Marker assisted selection
MgCl2	Magnesium chloride
MRB	Malaysian Rubber Board
mRNA	Messenger ribonucleic acid
MXE	Mutually exclusive exons
Na2EDTA	Sodium ethylenediaminetetraacetic acid
NaCl	Sodium chloride
NB-LRR	Nucleotide binding leucine rich repeat
NBS	Nucleotide binding Site
NO	Nitric oxide
NTP	Nucleotide Phosphate
PAMP	Pathogen associated molecular markers
PB	Prang Besar
PCR	Polymerase Chain Reaction
PDI	Percent disease index
PPI	Protein-Protein Interaction

PR	Pathogen Response
PR	Pathogen response
PRR	Pattern recognition receptors
PTI	PAMP triggered immunity
QC	Quality control
qRT-PCR	Quantitative real time polymerase chain reaction
QTL	Quantitative trait loci
R gene	Resistance gene
RAPD	Random amplification of polymorphic DNA
rDNA	Ribosomal Deoxyribonucleic acid
RFLP	Restriction fragment length polymorphism
RI	Retained intron
RIN	RNA integrity number
rMATS	replicate Multivariate Analysis of Transcript Splicing
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RRIM	Rubber Research Institute of Malaysia
SA	Salicylic acid
SALB	South American leaf blight
SAM	Sequence Alignment/Map
SAR	Systemic acquired resistance
SE	Skipped exon
SEM	Scanning electron microscope
Seq	Sequence
SNP	Single nucleotide polymorphism
SSR	Simple sequence repeats
STS	Sequence tagged sites

TF	Transcription factor
TM-LRR	Transmembrane leucine rich repeat

ANALISIS TRANSCRIPTOME UNTUK JANGKITAN KULAPUK BERDEBU PADA *Hevea brasiliensis*

ABSTRAK

Konsep keimunan terhadap pelbagai fitopatogen dikaji secara meluas pada tanaman. Klon getah yang penting secara komersial dibangunkan dengan fokus utama terhadap kuantiti dan kualiti hasil lateks yang dihasilkan dari asas genetik terhad, oleh itu mudah terdedah kepada jangkitan patogen yang menghalang pengeluaran. Walaupun langkah-langkah kawalan seperti rawatan kimia tersedia ada, kemungkinan dari segi ekonomi dan kesan ekologi tidak menguntungkan. Untuk mengurangkan kerugian ekonomi, program peningkatan tanaman kini menyiasat kaedah untuk memperbaiki pemahaman yang tidak lengkap mengenai mekanisme ketahanan dan meningkatkan kekebalan yang melekat pada rakan jenis liar kultivar komersial ini terhadap pelaksanaan gen ketahanan antara spesies. Karya yang dibentangkan di sini bertujuan untuk mencirikan gen tindak balas pertahanan yang digunakan terhadap kulat Ascomycete biotrofik wajib, *Oidium heveae*, agen penyebab penyakit daun Oidium yang penting secara ekonomi di *Hevea brasiliensis*. Penguatan wilayah ITS DNA kulat mengenal pasti kulat sebagai *Oidium heveae* strain HO-73. Kajian perkembangan penyakit digunakan untuk mengesahkan RRIM 2025 sebagai strain tahan dan RRIM 929 sebagai strain rentan. Untuk kajian ini, transkripom getah diperoleh menggunakan penjujukan akhir berpasangan perpustakaan cDNA yang dihasilkan dari dua klon getah; RRIM 2025 dan RRIM 929 di bawah tekanan biotik. Kajian ini mengenal pasti 143 gen yang terlibat dalam interaksi host-patogen di *Oidium heveae* yang dijangkiti *Hevea brasiliensis* menggunakan NGS. Bahagian transkrip yang ketara (1649892) pengekodan untuk produk gen dengan fungsi yang

berkaitan dengan interaksi tumbuhan-patogen seperti biosintesis metabolit sekunder, biosintesis fenilpropanoid, transduksi isyarat hormon tumbuhan, spliceosome. Kejadian penyambungan alternatif (AS) dipantau untuk mengesahkan kejadian peraturan dalam perbezaan ekspresi gen yang berkaitan dengan tindak balas patogen antara klon. Transkrip gen diperkaya untuk 5 kategori AS; Skipped Exon, intron tertahan, 5'dan 3'- laman web sambungan alternatif dan ekson yang saling eksklusif. Analisis rMATS menunjukkan frekuensi kejadian AS yang tinggi dalam klon getah RRIM 2025. Kejadian exon yang dilangkau didapati mempunyai kejadian tertinggi di antara semua kejadian penyambungan alternatif yang dikenal pasti. Peraturan Transkripsi Faktor (TF) adalah yang terpenting untuk pengaturcaraan semula transkrip yang berjaya dalam imuniti. Gen yang mengekodkan keluarga TF yang didedikasikan untuk menjadi tuan rumah peraturan tindak balas imun; *bZIP*, *NAC*, *MYB*, *WRKY*, *bHLH* diperkaya dengan ketara. Gen sasaran dengan mekanisme peraturan yang sangat dijelaskan, dan TF yang dicirikan dengan baik digunakan untuk membina rangkaian Protein-Protein Interaction (PPI) bersepadu patogenesis Hevea-Oidium. Rangkaian interaksi protein dalam klon RRIM 2025 dan RRIM 929 berjaya menunjukkan interaktiviti antara produk gen PR yang dikenal pasti sebagai tindak balas terhadap jangkitan *Oidium heveae* di *Hevea brasiliensis*. Jumlah 27989 peristiwa SNP dan 10125 InDel yang berkaitan dengan gen tindak balas patogen (PR) dikenal pasti sebagai biomarker berpotensi dan dapat membantu dalam pemeriksaan molekul untuk pembiakan selektif. PCR masa nyata kuantitatif dilakukan menggunakan 6 gen sasaran yang dinyatakan dengan ketara untuk pengesahan data transkripom. Pengukuran ekspresi relatif menggunakan dua kaedah berjaya mengesahkan kestabilan dan kebolehulangan maklumat gen yang diperoleh dalam kajian ini. Gen yang dikenal pasti

dan biomarker berpotensi yang berkaitan dengannya akan menjadi asas bagi kajian seterusnya dalam ketahanan cendawan serbuk.

TRANSCRIPTOME ANALYSIS OF POWDERY MILDEW INFECTION IN *Hevea brasiliensis*

ABSTRACT

The concept of immunity against myriad phytopathogens was studied extensively in crops. The commercially important rubber clones were developed with a major focus on the quantity and quality of the latex yield generated from a limited genetic base thus were prone to pathogen infections which hamper the production. Though control measures like chemical treatments were available, the economic feasibility and ecological impact of such treatments were unfavourable. To lower the economic losses caused, crop improvement programs now investigate methods to remedy the incomplete understanding of the resistance mechanisms and augment the inherent immunity observed in wild-type counterparts of these commercial cultivars over implementation of inter-species resistance genes. The work presented here aims at characterizing the defence response genes employed against the obligate biotrophic Ascomycete fungus, *Oidium heveae*, the causal agent of economically important Oidium leaf disease in *Hevea brasiliensis*. ITS region amplification of the fungal DNA identified the fungus as *Oidium heveae* strain HO-73. The disease progression study was used to confirm RRIM 2025 as the resistant strain and RRIM 929 as susceptible strain. For this study, the Rubber transcriptomes were obtained using paired-end sequencing of cDNA libraries generated from two Rubber clones; RRIM 2025 and RRIM 929 under biotic stress. This study identified 143 genes involved in the host-pathogen interaction in *Oidium heveae* infected *Hevea brasiliensis* using NGS. Substantial portion of transcripts (1649892) coding for gene products with functions pertaining to plant-pathogen interaction like secondary metabolite biosynthesis,

phenylpropanoid biosynthesis, plant-hormone signal transduction, spliceosome, phagocytosis, and endocytosis were highly represented. Alternative splicing (AS) events were monitored to confirm the regulatory events in gene expression divergence pertaining to the pathogen response between clones. Gene transcripts were enriched for 5 AS categories; Skipped Exon, retained intron, 5' and 3' – alternative splice sites and mutually exclusive exons. rMATS analysis showed significantly high frequency of AS events in rubber clone RRIM 2025. The skipped exon event was found to have highest occurrence among all the identified alternative splicing events. Transcription Factor (TF) regulation was paramount for successful transcriptional reprogramming in immunity. Genes encoding TF families dedicated to host immune response regulation; *bZIP*, *NAC*, *MYB*, *WRKY*, *bHLH* were significantly enriched. The target genes with the highly elucidated regulation mechanism, and well characterized TFs were used to construct an integrated Protein-Protein Interaction (PPI) network of *Hevea-Oidium* pathogenesis. Protein interaction network in clones RRIM 2025 and RRIM 929 successfully showed the interactivity among the pathogen response (PR) gene products identified in response to *Oidium heveae* infection in *Hevea brasiliensis*. Total 27989 SNP and 10125 InDel events associated with PR genes were identified as potential biomarkers and could aid in molecular screening for selective breeding. Quantitative real-time PCR was performed using the six significantly expressed target genes for validation of the transcriptome data. The relative expression quantification using the two methods successfully validated the stability and reproducibility of the gene information obtained in this study. The genes identified and potential biomarkers associated with them will form the basis for subsequent studies in powdery mildew resistance.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Hevea brasiliensis was the only economically viable resource for natural rubber worldwide. It was economically important due to the ease of latex harvest as the latex producing cells (laticifers) were present at the outer layer of the bark and hence a simple incision to the bark effectively harvests the latex without killing the tree (Zhao Xiu-qian, 1986). *Hevea* has been exploited through decades for its latex production, even with the invention for synthetic rubber, the demand for natural rubber remains high due to its high-quality properties which make it desirable for use in large-scale machinery.

A regular export market for rubber was developed in Brazil in 1827; however, the introduction of the vulcanization process in 1839 boosted the beginning of commercial exploitations of rubber. Rubber domestication started with a pool of 70,000 seeds. The seedlings from Kew Garden were sent to Singapore via Ceylon in 1874. However, due to steady coffee trade, the rubber plantations were not a priority at that time until Henry Ridley, Director of Singapore Botanic Gardens, promoted the planting of rubber (LGM, 2009). He was responsible for developing the tapping system for harvesting rubber latex, economical planting procedure, and latex processing methods. By 1905, 43435 acres of rubber plantations were reported from a mere 345 acres in 1897 in Federal Malay states which further increased to 2,260,000 acres on the west coast by 1922 (LGM, 2009). In this period, the Malay peninsula accounted for 53 % of all rubber planted in India, Ceylon and other areas of South East Asia.

Early research on rubber was carried out by the Department of Agriculture, but with the establishment of the Rubber Research Institute of Malaysia in 1925, the responsibility was shifted (LGM, 2009). The development of high latex yielding clones was emphasized, which observed a significant increase in the yield. However, due to the utilization of limited germplasm, these clones were highly prone to infection from pathogens (Priyadarshan and Clément-Demange, 2004).

Oidium heveae, *Corticium salmonicolour*, *Phytophthora heveae*, *Corynespora cassiicola,* and *Microcyclus ulei* were important rubber pathogens affecting the leaf tissue. The present study focuses on *Oidium heveae* infection causing powdery mildew infection in rubber clones which has recorded economic losses of 45 % where (Tu, 2012). The fungus was observed to attack post-wintering immature leaves and radial mat-like mycelial growth with powdery white appearance was a distinctive feature of *Oidium heveae* infection (Liyanage *et al.,* 2016a).

Conventionally this disease was controlled by dusting the leaves with sulfur throughout the refoliating season and fogging with fungicide tridemorphs (Calixin 75 EC). However, the application of chemicals for large plantations was labour-intensive, time-consuming, expensive and not ecologically feasible. Thus, it was recommended to avoid the planting of susceptible clones in areas prone to the disease (LGM, 2009).

Grafting resistant crown onto a productive trunk was effective but expensive with the possibility of failure due to breakdown of resistance by new fungal strains (Rivano *et al.,* 2012). Breeding programs later focused on introducing resistance traits when selecting parental clones. It was important to note that selective breeding insofar has been carried out solely for the improvement of latex and bark quality and their classification into latex clones, timber clones and latex-timber clones signify this purpose. Secondary trait observation and selection for these clones were based only in

the field trial records of disease incidences in the parental clones and challenge infection studies were lacking (Malaysian Rubber Board, 2009). Thus, a study prioritizing important secondary traits like disease resistance was important towards understanding the underlying mechanism and its application for crop improvement. Although it has been the focus of research in recent studies, the knowledge remains largely incomplete.

Knowledge on the patterns of genetic inheritance was crucial in the planning and execution of breeding for crop improvement (Tan and Tan, 1996). Hence, several research programs to improve resistance to South American Leaf Blight (SALB) was undertaken since 1927 in Brazil, but failed due to poor understanding of fungal epidemiology and resistance mechanism (Rivano *et al.*, 2012). Breeding of rubber clones by Rubber Research Institute of Malaysia began in 1928, but genetic analysis of the crop started in the early 1970s due to setbacks in yield improvement. Though genetic studies were conducted, they were mainly focused on latex production and vigour. Emphasis was given to leaf disease resistance only towards the late 1990s when the leaf blight disease outbreaks were more prominently and frequently observed (Tan and Tan, 1996).

Genomic analysis of resistance to important rubber pathogens like *Oidium, Corynespora, Colletotrichum* shows that with the application of general combining ability, a cohesive breeding program for rubber yield as well as disease resistance could be implemented (Clemente-Demange, 2007). The Cirad-Michelin-Brazil project (2007-2011) studied SALB resistance in rubber. The work analyzed *Hevea* x *Microcyclus* interaction, biology, and epidemiology of the fungus. Determination of different sources of resistance to SALB employed Quantitative Trait Loci (QTL) mapping and classification of candidate genes exhibiting differential expression during

infection (Garcia and Montoro 2011). The study proposed QTL mapping and Single Nucleotide Polymorphism (SNP) marker-assisted selection for future resistance breeding in *Hevea*.

With the aid of Next-Generation Sequencing (NGS) technology, ease of assembly of plant reference genomes for non-model systems has improved significantly. With this, a closer inspection of gene expression patterns and identification of marker sequences associated with known function genes has become a more straightforward process. Application of these studies in plant breeding has been widely applied for various crops including wheat, rice, capsicum, tomato (Vlk and Řepková 2017; Manivannan *et al.,* 2018; Aversano *et al.,* 2012).

Next-generation sequencing generated cost-efficient high throughput data which boosted transcriptomic studies. RNA sequence data analysis enables a comprehensive understanding of transcriptome profiles to observe gene expression patterns, their function, and regulatory mechanisms. The present study aims to identify the pathogen response genes in resistant rubber clones to understand resistance to powdery mildew pathogen, *Oidium heveae* in *Hevea brasiliensis* using a shot-gun transcriptomics approach. Considering the economic importance of this disease, understanding the *Oidium- Hevea* interaction was the focus of this study. Thus, application of Illumina Hi-Seq sequencing platform helped performed comparative transcriptome profiles analysis for two rubber clones RRIM 2025 (resistant) and RRIM 929 (susceptible) to detect the genes expressed in response to powdery mildew resistance. Differential gene expression to observe the gene expression changes between infected and healthy experimental conditions for each clone helped to determine the factors involved in resistance and susceptibility. Functional annotation using Kyoto Encyclopaedia of Genes and Genomes (KEGG) and Gene Ontology (GO)

databases helped to elucidate the roles of the genes identified during powdery mildew infection.

Additionally, alternative splicing (AS) events associated with the genes of interest lead to the characterization of the regulatory mechanism involved in resistance. RNA seq data analysis located the SNP events associated with the target genes. This work was the first to elucidate powdery mildew infection in RRIM 2025 and RRIM 929 clones. Gene data obtained using NGS and qRT-PCR could form a good baseline for future studies associated with powdery mildew infections. Potential biomarkers are identified in the present study and could useful in marker-assisted selection. Findings from this study can provide a comprehensive outline for future studies of similar nature. With the understanding of gene expression changes, regulation and functional characterization associated with powdery mildew infection future application for genome-wide association mapping and genetic linkage maps were possible.

1.2 Problem statement

The Malaysian rubber industry currently employs *Hevea* clones recommended by the Malaysian Rubber Board. The clone developed were tested based on the field trial. Currently, the research on secondary traits for these commercial clones was limited and were based on the observational records from the field trials. Research on *Hevea* pathogens is lacking or incomplete for many of the economically important diseases of rubber. Thus, the information available regarding the resistance or susceptibility towards these diseases were mostly based on the incidental infection records from field observations. Understanding the changes induced in response to pathogen infection can help elucidate the mechanism for disease resistance.

Field records showed that specific clones exhibit resistance or tolerance as well as susceptibility. This disparity in response to infection can be observed at a genetic level using transcriptomic analysis. Since the *Hevea* clones were developed using a limited gene pool, the transcriptomic disparity will help analyse the resistance or susceptibility within the clones. The transcriptome analysis will help identify the genes responsible for resistance or susceptibility in clones which will further highlight the molecular mechanisms underlying this phenomenon. It was important to note that the expression of majority genes depend on stimulus and only the housekeeping genes required for regular functioning of the cellular system were expressed constitutively. Resistance genes were induced when the host system detects pathogenic molecular patterns. The induction of these genes helps launch a defence cascade pathway. To observe these changes at the genetic level, NGS technology was used to identify the transcripts expressed in response to infection. The clones selected for the present study will be challenge inoculated using the pathogen of interest for this study.

1.3 Hypothesis

1. The rubber clones show varying levels of resistance to *Oidium* leaf disease
2. The resistant and susceptible rubber clones exhibit differing transcriptome profiles
3. The difference in pathogen response can be detected using Transcriptomic approach

1.4 Objectives

1. To generate transcriptome profiles of *O. heveae* infected and healthy *H. brasiliensis* clones RRIM 2025 and RRIM 929.
2. To identify pathogen response (PR) genes in *H. brasiliensis* clones using comparative transcriptome profile analysis.
3. To identify the regulatory events and biomarkers associated with PR genes.
4. To validate the gene expression quantification of PR genes using real-time quantitative polymerase chain reaction (q RT-PCR) based method.

CHAPTER 2

LITERATURE REVIEW

2.1 Natural Rubber

Natural rubber was a hydrocarbon polymer composed of isoprene units. It was a high molecular weight secondary metabolite with chemical structure cis 1,4-polyisoprene. Natural rubber was produced in the specialized cells within the secondary phloem of the tree (Priyadarshan and Goncalves, 2003). *Hevea brasiliensis* was a sole commercial source of natural rubber. Currently, there were over 2500 rubber-producing plants from various taxa in the plant kingdom. However, since most of these plants have unfavourable characteristics such as low yield or low molecular weight of the polymer, *H. brasiliensis* was the exclusive species producing commercially viable natural rubber (Oktavia *et al.*, 2017).

The natural rubber industry has developed drastically during the last 100 years due to the varied applications of rubber. Products made from natural rubber ranges from tires, engineering components to gloves used in the medical profession (Sethuraj and Mathew 1992). The raw natural rubber has grades depending on its quality and commercial application. The natural rubber has unique characteristics such as great tensile strength, resistance to heat, electricity, adhesive nature, abrasion resistance, malleability (Kurian and Mathew, 2011). These properties make it a desirable raw material for various products.

Natural rubber was a major component within the colloidal liquid called latex produced in specialized cells called the laticifers. Laticifers were present in almost all tissues; however, for commercial harvesting, the bark tissue has special significance.

The precise, systematic manual incision on the bark to harvest latex was achieved via a process known as tapping (Kurian and Mathew, 2011; Narayanan and Mydin, 2012).

2.2 Hevea brasiliensis

2.2.1 Classification

Hevea brasiliensis was a monoecious, perennial tree classified under family Euphorbiaceae. Around ten species were distinguished based on their latex yield in genus *Hevea*, including *H. brasiliensis, H. benthamiana, H. camargoana, H. camporum, H. guianensis, H. microphylla, H. nitida, H. pauciflora, H. rigidifolia,* and *H. spruceana.* However, only *H. brasiliensis, H. guianensis and H. benthamiana* latex yields are economically important, since the latex from other species was of low quality as it contains high resin and low rubber content (Priyadarshan, 2017a). It was noteworthy that non-commercial *H. brasiliensis* species exhibit traits such as higher wood quality, biotic resistance and dwarf height which have better applications in plant breeding (Men *et al.,* 2018).

2.2.2 Morphology

The tree was about 30-40 m tall and reaches a height of 15 m when cultivated. Cylindrical trunk shape was common to all clones with varying bark quality (smooth to corky) and colour (light to dark brown) (Priyadarshan, 2017a; Schultes, 1990). Branching type varies considerably between conical and fan-shaped branching patterns which are commonly observed. Leaves were trifoliate and alternately located at the apical shoot, petioles were long and have apical glands. Leaflets were elliptical and pinnately veined with an entire margin. *H. brasiliensis* have inconspicuous flushes and wintering (complete defoliation before re-foliation) as an escape mechanism to

reduce the severity of the attack by *O. heveae* secondary leaf fall disease (Priyadarshan, 2017a; Schultes, 1990; Sethuraj and Mathew, 1992). Flowers were located on the base of a new bloom.

Flowers were bell-shaped with bright yellow petals. The female flowers were larger than male flowers. The male flowers were located laterally at the base of the inflorescence. Whereas the female flowers were located at the apex. The anthers were sessile and spirally arranged within the staminal column. Structurally each female flower has a basal green disk with 3-celled ovary terminating in 3 sessile sticky stigmas. Fruits in all species were trilobed capsules usually containing three seeds, sub-globose, apiculate, ellipsoidal, strongly ligneous, thick, not contorted at dehiscence, with a diameter of 3-5 cm, and turn light brown on maturation. Each carpel contains one ovoid seed. The fruit was explosively dehiscent. The woody valves twist on drying out and throw the seeds far out. Seeds were rounded in transverse cross-section, ventrally compressed but not angled, 28 mm long and 20 mm in breadth, basally grey-brown with dark brown mottling with a fresh weight of 5gm (Priyadarshan, 2017b; Sethuraj and Mathew, 1992).

2.2.3 Habitat

Rubber trees flourish in the lowland tropical areas between 6°N and 6°S. Cultivation trials in areas like the Sao Paolo region in Brazil, Mexico and Guangdong province in China proved successful. An optimum daytime temperature of shows the best growth rate in rubber trees. Plantations at altitudes above 400-500 m should be avoided as the low ambient temperatures hamper the girth increment, delay tapping, and reduce latex yield. Consistent rainy days in the morning were detrimental to the tapping schedule. Due to its superior root system, rubber trees can tolerate a drought

period of 2 to 3 months. However, the dry period longer than a month can result in complete defoliation. Wind velocity was also an important factor as strong winds cause trunk and branch snapping, impairing the tapping process thereby resulting in low yields (Chandrashekar *et al.*, 1994; Priyadarshan 2017a; Sethuraj and Mathew 1992; Umar *et al.*, 2017; H. Zhang *et al.*, 2012).

2.3 Impact of genetic diversity in rubber breeding

2.3.1 History of domestication of rubber

Domestication of rubber started in 1876 when Sir Henry Wickham transported a collection of 70,000 *H. brasiliensis* seeds to Kew Botanical Gardens. Surviving 22 seedlings from the collection gave rise to rubber plantations in Southeast Asia. However, according to Thomas (2001) modern clones originated from 1911 seed stock dispatched to Sri Lanka in 1876, from Wickham's original collection (Priyadarshan, 2017a). Twenty two seedlings from the same collection introduced in Singapore and peninsular Malaya gave rise to the rubber plantations responsible for providing the bulk of the natural rubber demand during the twentieth century (Priyadarshan, 2017b). The multiplication of the seedlings was primarily through bud grafting. The modern commercial clones and the clonal genotypes used for selective breeding were less than 10 generations removed from the wild Amazonian population. Thus, even though the commercial clones exhibit allelic diversity for genetic improvement, the genetic base of these cultivated clones was limited (Souza *et al.*, 2018; Souza *et al.*, 2015; Souza *et al.*, 2009).

2.3.2 Genetic diversity in rubber clones

Based on the in-situ hybridization studies, *H. brasiliensis* was an allotetraploid developed as a result of 5S rDNA loss during evolution. However, tetravalents observed during meiosis suggest amphidiploid nature (2n = 4x = 36) of *H. brasiliensis*. Thus, until the discovery of 2n= 18 ancestor, for practical purposes, *H. brasiliensis* will remain as an amphidiploid. Comprehensive studies employing molecular analysis will elucidate the origin details (Jain and Priyadarshan, 2009; Priyadarshan and Goncalves, 2003).

H. brasiliensis clones were divided into two distinct populations; the domesticated Wickham population and wild Amazonian accessions with low yield and relatively high resistance to diseases (Priyadarshan and Clément-Demange, 2004). Current commercial rubber clones originate from Wickham's limited collection and thus have a small genetic foundation (Lam *et al.*, 2009).

Clonal propagation and breeding process with the aim to improve yield productivity emphasized on development of high yielding latex clones. Though it helped develop clones with improved traits, it resulted in further reduction of genetic diversity. Thus, selective breeding though successful was not sustainable. One of the impacts of limited genetic diversity was the susceptibility to pathogen infections observed in commercially cultivated clones (Priyadarshan and Goncalves, 2003; Sethuraj and Mathew, 1992).

To remedy this situation, Malaysia imported seedlings belonging to *Hevea brasiliensis, Hevea benthamiana, Hevea guianensis, Hevea spruceana* and *Hevea pauciflora* in 1951-52. International Rubber Research and Development Board (IRRDB) carried out expeditions in 1981 and 1995 to enrich the available *H. brasiliensis* germplasm and to increase latex and timber productivity. This introduction

of new genetic material helped develop the RRIM 900 and 2000 series with an enhanced yield of 3000 kg/ha/annum (Oktavia *et al.,* 2017; Priyadarshan and Goncalves 2003; Priyadarshan 2016; Besse *et al.,* 1994; Lekawipat *et al.,* 2003).

2.3.3 Malaysian rubber clones

Commercially available clones for plantation in Malaysia were studied and developed by Rubber Research Institute of Malaysia (RRIM), also known as Lembaga Getah Malaysia (LGM). Based on their latex and wood production potential, the rubber clones were subdivided into two categories; latex- timber clones (LTCs) and latex clones. The clones classified as Latex-Timber clones (LTCs) exhibit high-quality latex yield and rubber wood production. Physical traits such as growth vigour and bole quality were optimal in these clones. As the name suggests, these clones were exploited for latex and timber yields. Similarly, the Latex clones exhibit high latex yield, but poor timber yield; hence they were exploited only for their latex producing capacity.

Lembaga Getah Malaysia categorizes the clones recommended for plantation into two groups; Group I clones were grouped together on the basis of yield data. The large-scale trials were conducted in different environmental conditions for desirable secondary trait observation. Though both groups represent high yielding clones, the clones from Group I were endorsed for planting at a commercial scale. Latex-Timber clones RRIM 908, RRIM 911, RRIM 921, RRIM 928, RRIM 929, RRIM, 936, PB 260, PB 350, PB 355, PB 359 and Latex clones RRIM 938, PB 280, RRIM 901, PB 366 comprise the Group I clone.

Group 2 consists of 33 clones which were further sub-divided into Group 2A and 2B to enable early selection among the newly recommended clones. Clones within

Group 2A were selected based on early yield reports based on three-year field trial data and comprises 7 Latex timber clones and 2 Latex clones. Latex-Timber clones RRIM 2001, RRIM 2002, RRIM 2007, RRIM 2009, RRIM 2015, RRIM 2016, RRIM 2019 and Latex clones RRIM 2004, RRIM 2005 were listed within the group.

Group 2B clones were those which did not show good early performance in large scale trials. Group 2B comprises of 10 latex-timber clones (RRIM 2008, RRIM2014, RRIM 2020, RRIM 2023, RRIM 2024, RRIM 2025, RRIM 2026, RRIM 2027, RRIM 2028, RRIM 2033) and 14 latex clones (RRIM 2003, RRIM 2006, RRIM 2010, RRIM 2011, RRIM 2012, RRIM 2013, RRIM 2017, RRIM 2018, RRIM 2021, RRIM 2022, RRIM 2029, RRIM 2030, RRIM 2031, RRIM 2032) (Rubber Research Institute Malaysia, 1979; Malaysian Rubber Board, 2009).

The Malaysian Rubber Board (MRB) updates planting recommendations every three years, which provides information regarding the availability, status, and performance of clones. The clones were divided into groups based on their performance in field trials (**Figure 2.1**). Group, I consist of clones that have yield reports from trials with minimum five-year on BO-I, two years on BO-II and well-characterized secondary traits. The Group I clone were further classified as Latex-Timber Clones (LTC) that have high latex, wood yield and growth vigour with straight boles and (ii) Latex clones that have high latex yield. Latex-Timber clones were recommended for both Latex and Timber quality whereas; Latex clones were unsuitable for wood production. Group 2 clones were divided into two sub-groups A and B, where Group 2A consists of new clones with minimum three-year yield data in large-scale trials. Group 2B contains newly introduced clones with good five-year yield records and secondary traits recorded from Small Scale Clone Trials.

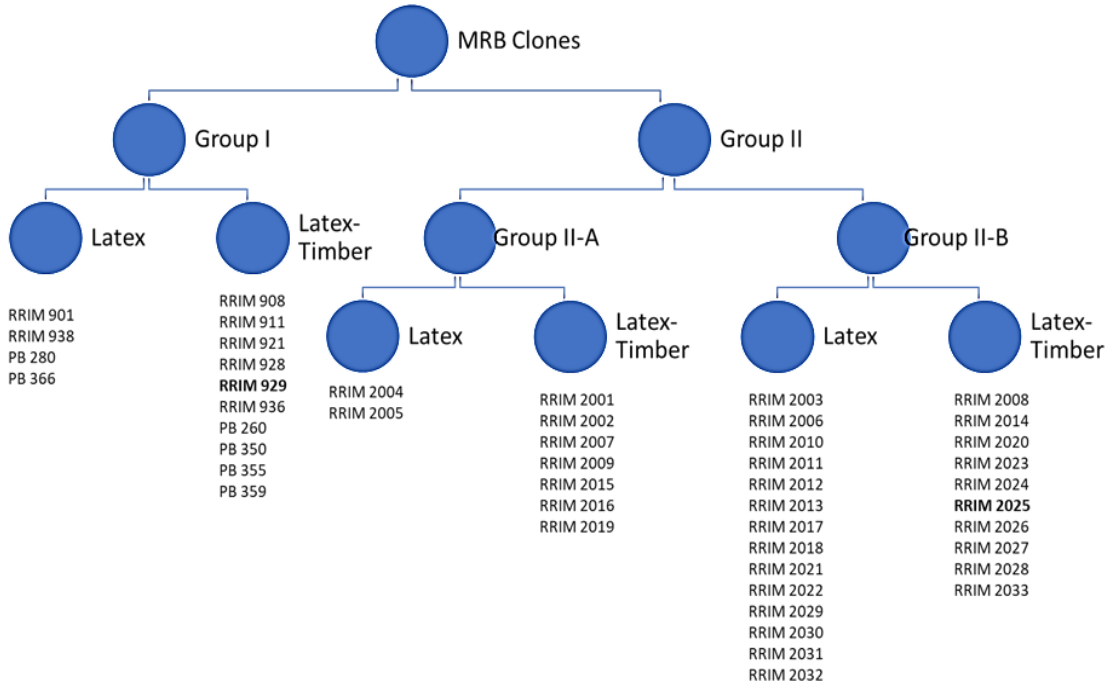

Figure 2.1 Classification Chart for MRB clones (Malaysian Rubber Board, 2009)

Based on their performance and market preference, the clones RRIM 2025 and RRIM 929 were selected for this study. The characteristics recorded by Malaysian Rubber Board clone trials were elucidated below. The two clones show similar yield records but differ in the disease tolerance potential, which will be exploited in The present studyfor understanding the resistance mechanism in rubber clones of commercial importance.

Clone RRIM 929 was a latex-timber clone in Group I with average yield records of 3143 kg/ha/year from various Large-scale clone trials. The relatively lower yields recorded as compared to the genetic potential were due to a lower number of tapping days, i.e. about 70 tapping days per year (Malaysian Rubber Board, 2009). Clone RRIM 2025 was a latex-timber clone from group II B with an average yield record of 2700 kg/ha/year extrapolated from small scale clone trials since data for

group IIB was not available. Trait comparison between RRIM 929 and RRIM 2025 was detailed in (Table 2.1 and Table 2.2).

Table 2.1 Clonal characteristics: RRIM 2025 and RRIM 929

Characteristic	**RRIM 2025**	**RRIM 929**
Parentage	IAN 873 x RRIM 803	RRIM 605 x RRIM 725
Mean Latex yield	2700 kg/ha/year	3143 kg/ha/year
Mean Timber yield	1.87m^3/tree	1.2 m^3/tree
Yield for the first two years	5	3
Resistance to wind damage	4	5
Vigour at opening	5	5
Virgin bark at opening	5	5

Here, 5 stands for Very Good; 4 represents Good; 3 represents Average; 2 represents Below Average; 1 represents Poor, and NA for data Not available (Malaysian Rubber Board 2009).

Table 2.2 Disease severity in clones

Fungal pathogens	**RRIM 929**	**RRIM 2025**
Corticium salmonicolor	N	N
Oidium heveae	M	L
Colletotrichum gloesporioides	L	M
Corynespora cassiicola	N	N
Phytophthora heveae	N/A	N

Here, N was Nil; VL was very light; L was light; M was medium, and S was severe

2.4 Powdery mildew infection in *Hevea brasiliensis*

2.4.1 Diseases of Hevea brasiliensis

Domesticated crops were under a constant threat of attack by pathogenic microbes which hampers their yield and quality. Rubber plantations have an added disadvantage of low genetic diversity which increases their vulnerability to a myriad range of phytopathogens. The various diseases of rubber can be classified based on the organ affected. The rubber tree was susceptible to several diseases and can be categorized based on the tissue (leaf, stem and branch, panel and root) affected (Sethuraj and Mathew, 1992b). Factors influencing the economic impact and severity of any disease were country-specific due to the variable climatic conditions and cultivation practices. Leaf diseases have greater importance due to the extensive cultivation of clones primarily selected for high yield at the expense of resistance to diseases (Liyanage *et al.*, 2016a; Wastie 1975; De, Liyanage, and Jacob 1992).

The International Rubber Research and Development Board survey in 1998 identified 22 diseases of great severity in rubber. The Colletotrichum leaf fall caused by *Colletotrichum gloesporioides*, Oidium leaf fall caused by *Oidium heveae*, Corynespora leaf fall caused by *Corynespora cassiicola*, Black Stripe disease caused by *Phytophthora palmivora*, pink disease caused by *Corticium salmonicolor* and white root rot disease caused by *Rigidoporus microporus* were recorded as most significant diseases of rubber in China, Malaysia, Indonesia, India, Thailand, Sri Lanka and Vietnam (De et al., 1992; Kusdiana et al., 2017; Wastie, 1975).

There were five major leaf diseases of economic importance to *H. brasiliensis*, and the causative pathogens include, *Oidium heveae*, *Colletotrichum gloesporioides*, *Phytophthora palmivora*, *Corynespora cassicola*, and *Microcyclus ulei*. Secondary

leaf fall disease caused by two fungi, *Oidium heveae,* and *Colletotrichum gloesporioides* was the most important (Yusof Azaldin and Ismail Hashim 1974; Petch 1921; Priyadarshan 2003; Wastie 1975).

2.4.2 Oidium secondary leaf fall disease

Secondary leaf fall disease caused by *O. heveae* in *H. brasiliensis* was first reported in 1928 in West Malaysia and was observed to have reached epidemic proportions in various parts of the country (Azaldin and Hashim 1974). *Oidium* leaf fall disease was caused by powdery mildew fungus, *O. heveae*. The primary target for infection was immature leaflets formed post wintering, resulting in secondary leaf fall. The annual secondary leaf fall survey conducted by RRIM (1948-1959) stated that *O. heveae* was the dominant pathogen found on 75 % of the studied samples (Liyanage *et al.,* 2016).

2.4.3 Oidium heveae

2.4.3(a) Taxonomy and Classification

Steinman first described *O. heveae* as the causative pathogen of powdery mildew infection in *H. brasiliensis* in 1925. A study by Braun and Cook (2012), reported that *O. heveae* have catenated conidia, hyphae were hyaline, branched and septate. They also suggested that *O. heveae* was being confused with *Erysiphe quercicola,* a known powdery mildew fungus infecting other plant species (Braun and Cook, 2012). In its asexual form, the fungus appressoria were simple or lobed with a polygonal germ tube. This demonstrates that powdery mildew of rubber can be classified under genus *Oidium* and subgenus *Pseudoidium* (Limkaisang *et al.,* 2005; Boesewinkel 1980). Braun and Cook bifurcated the genus *Oidium* into 3 genera, based

on its morphology, viz., *Erysiphe* [asexual: *Pseudoidium*], *Podosphaera* [asexual: *Fiboidium*] and *Golovinomyces* [asexual: *Euoidium*] (Braun and Cook, 2012). Based on existing information about the fungal trait to form long chained conidia, *Oidium heveae* could be classified under genus *Golovinomyces* or *Podosphaera* (Liyanage *et al.,* 2016). Phylogenetic analysis using ITS sequence data suggested that *Oidium heveae* from Brazil, Thailand, Malaysia, Vietnam, China, and Sri Lanka shows similarity to asexual form of *Erysiphe quercicola* (Boesewinkel 1980; Wu *et al.,* 2019; Tam *et al.,* 2016).

Powdery mildew fungi were obligate biotrophic parasites that belong to the Phylum Ascomycota, Class Leotiomycetes, Order Erysiphales, Family Erysiphaceae. Phylogenetic analysis showed that powdery mildew forms a monophyletic clade (Bindschedler et al., 2016; Mori et al., 2000). Species belonging to the Order Erysiphales were difficult to classify. Around 700 powdery mildew species were known to infect 7600 angiosperm hosts. However, no gymnosperms host have been identified. Powdery mildew classification generated distinct scientific names for organisms with cursorily similar characteristics. Species *Erysiphe cichoracearum, Erysiphe polugoni,* and *Microsphaera penicillata* alone embody 10-20 distinct fungal species (Liyanage *et al.,* 2017; Limkaisang *et al.,* 2005; 2006).

2.4.3(b) Morphology

The powdery mildew fungal mycelia form a distinct radial mat-like pattern at the site of infection. The mycelial hyphae were septate and branched. A specialized structure called haustoria helps the fungus to absorb nutrients from the host tissue. The conidia produced were 25-44 x 13-23 µm in size, the shape was mostly ellipsoidal or barrel shaped. The conidiophore stalks form on a cylindrical structure on the hyphae

known as foot cells. Germinated spores on the host tissue develop a special structure called appressoria which aids in adherence to the host surface (Liyanage *et al.,* 2016).

2.4.3(c) Occurrence

The most favourable condition for powdery mildew infection was post wintering when new foliage produced after wintering was especially susceptible to the pathogen attack. The cool temperature and intermittent shower early in the day during the re-foliation period were ideal for fungal proliferation and dispersal (Liyanage 1982).

Reports on *O. heveae* occurrence have been recorded as early as 1937 in Malaysia. The reported incidents were highest between the months of March and April (Beeley, 1933). Re-foliation post delayed wintering period caused exposure of the new foliage to dull weather in March and April, which was favourable to the fungal growth. Plantations in the central and southern areas of Malaysia observe a good wintering period followed by good quality new foliage which was relatively free from infection due to the timely wintering. Comparatively, the plantations in the north which have late wintering remain exposed to pathogen infection (Liyanage *et al.,* 2016a; Beeley 1933).

2.4.3(d) Infection cycle

Powdery mildew fungus was obligate biotrophs, thus require a live host for growth and propagation. The mycelia and conidia appear as in a whitish dusty growth at the site of infection on the plant, hence the name powdery mildew. Schematic representation of the powdery mildew life cycle was presented in (Figure 2.2). The fungal spores germinate on the host tissue surface in the presence of optimal

environmental conditions of high relative humidity and ambient temperature 26-28°C. The spores adhere to the host tissue surface with the help of a special structure called the appressoria (Misra, 2001). The fungal hyphae developed then penetrates the host tissue to form haustorium which assist the fungus to absorb nutrient from the host tissue. The haustoria were unicellular with lobed filamentous protrusions emerging from a central globular structure (Misra, 2001). Haustoria was also known as the hub for direct plant-pathogen interaction and a possible site for immune activity (Whisson *et al.,* 2007; Jones and Dangl 2006).

The study of the powdery mildew life cycle can provide important information to help control the disease in important crops. The life cycle of the powdery mildew fungus exists in two stages; asexual and sexual. In the asexual stage, the fungus produces spores conidia, whereas the sexual stage produces ascospores. The conidia were crucial in the dispersion of the fungus to a larger area of the host and exaggerate the infection intensity whereas Ascospores play a crucial role in the survival of the fungus during the period of unavailability of the host (Tu *et al.,* 2012).

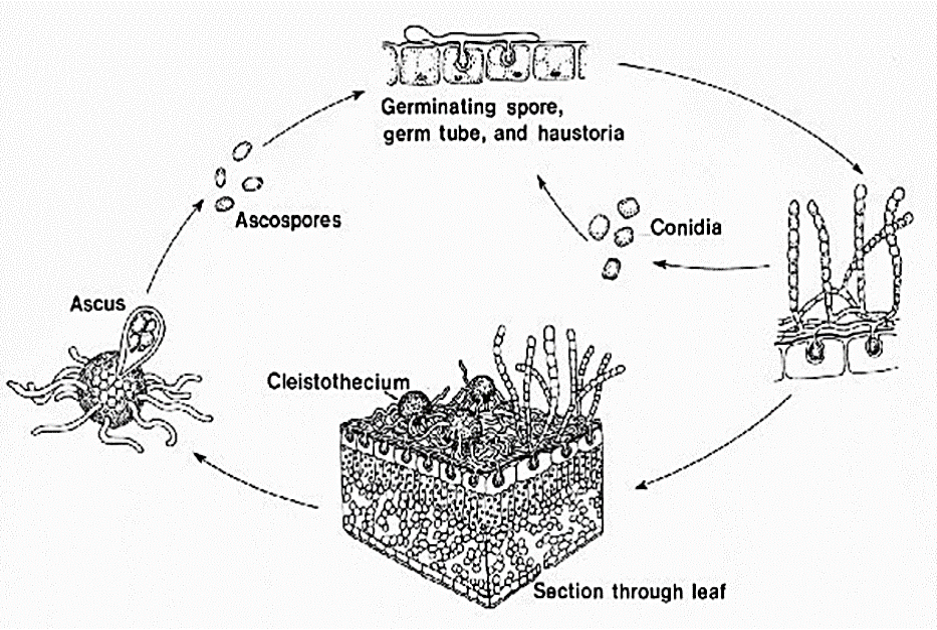

Figure 2.2 The Life cycle of Powdery mildew fungus (Misra, 2001)

Within 3 – 7 days of infection, conidia were produced in large quantities, under disease conducive conditions. Whereas ascospore production was initiated post-flowering stage of the host, in late growing season when the environmental conditions were no longer favourable for the multiplication of the fungus (Misra 2001; Liyanage *et al.,* 2016a). The ascospores survive in adverse conditions such as low temperature and humidity with the aid of ascocarps which house the ascospores. Ascocarps usually were 0.1-0.3 mm in diameter, nearly spherical and as they age turn from yellow to dark brown or black. Additionally, genetic recombination events occurring during the production of ascospores can introduce new traits such as resistance to fungicides or improve the fungal virulence.

The ascospores infect the host, thus establishing a primary infection site, which further spreads the disease at epidemic proportions. Powdery mildew mycelia in the dormant state have been observed to thrive in cold periods within the infected host buds. However, for areas with mild climatic conditions the fungus can survive in its asexual state, mycelial form on the host and the sexual stage was not observed as frequently (Misra 2001; Liyanage *et al.*, 2016a).

2.4.3(e) Symptoms and epidemiology

The powdery mildew infection was distinguished by the peculiar appearance of the mycelia and conidia on the infected host. Young leaflets were most susceptible to infection and thus were the primary target of the fungus. Infected leaves exhibit infection on lower leaf surfaces near the veins, and only superficial tissues were penetrated. The fungal hyphae grow radially forming extensive colonies, covering the entire leaf surface. The fungal colonies have a distinct powdery white appearance due to the presence of a large number of conidia. With the aid of conidia, the fungal

colonies eventually spread and cover larger areas of the host tissue. The mycelia from older colonies often turn brown or grey. The host tissue exhibiting severe infection shows hampered growth and deformities such as distortion of the leaf margin causing it to curl and cause scar patterns on seeds. (Liyanage *et al.,* 2016a; Misra, 2001; Sethuraj and Mathew, 1992; Tu *et al.,* 2012). Infected tender leaves when infected shrivel and fall off whereas, semi-mature leaves become distorted and develop necrotic spots which persist throughout the life of the leaflets (De et al., 1992; Sripathi Rao, 1975). When the leaves develop thick cuticle or when the weather conditions were unfavourable, the infection dries up, leaving yellow patches on the leaf surfaces (Pusat Penyelidikan Getah Malaysia, 1974).

The primary infecting form of the pathogen varies based on the host plant, the pathogen and climate. The infecting form could be dormant mycelia in the bud, or infected tissue or ascospores. The pathogen rapidly proliferates during the growing season when the climatic conditions were favourable. The conidia formation during this stage was profuse, and spore dispersion helps spread the infection to new host plants thus initiating new infections (Liyanage *et al.*, 2016; Misra 2001; Beest *et al.*, 2008).

In the case of ascospore mediated infection, free moisture was observed to trigger ascospore release. Powdery mildew spore germination does not need free water instead of high relative humidity can sufficiently trigger germination. This makes it difficult to manage powdery mildew infections in fields and plantations (Narayanan and Mydin, 2012).

2.4.3(f) Molecular aspect of the *Oidium – Hevea* interaction

Investigating the genetic mechanism for pathogen resistance in plants is crucial towards development of resistant varieties of crop plants. Cloning studies involving host resistance genes can elucidate the mechanism employed by the host immune system in overcoming the pathogen infection. However, the lack of such studies in *Hevea brasiliensis* was a major research gap. Differential expression studies involving rubber clones Reyan 7-33-97 and RRIC52 focused on this research gap (Li *et al.*, 2016). The study identified several genes involved in *Oidium heveae* resistance. Cell wall proteins act as the first line of defense against pathogen invasion. The cell wall glycoproteins such as the germin-like protein that have a functional role in cell wall reinforcement have been found to be induced during infection conditions in several plant systems such as *P. trichocarpa*, *H. brasiliensis* Reyan 7-33-97, Vitis *vinifera* cv. Chardonnay (Godfrey *et al.*, 2007; Zimmermann *et al.*, 2006; Li *et al.*, 2016). In the event of the pathogen successfully overcoming the cell wall defences by producing cell wall degrading enzymes, the host immune system synthesizes several pathogenesis related proteins to combat the pathogen invasion (Liyanage *et al.*, 2016; Liu *et al.*, 2016). The host response to infection was regulated by transcription factors known to modify gene expression as pathogen response mechanism. *WRKY* genes were one such extensively studied class of transcription factors (Chiang and Coaker, 2015; Conrath, 2011; Amrine et al., 2015). Varying level of regulation of the *WRKY* class genes in response to pathogen invasion has been observed to promote resistance (Pandey et al., 2010; Shen et al., 2007). Pathogenesis related proteins like glucanase, chitinase, phytoalexin were also observed to be synthesized in response to powdery mildew infection in rubber (Trognitz *et al.*, 2002). WRKY proteins, HBOH13 and HBOH14 were expressed against *Oidium heveae* in rubber at the early stages of