MOLECULAR WEIGHT PROFILING OF NATURAL RUBBER FROM SELECTED MALAYSIAN *Hevea brasiliensis* CLONES AND ITS RELATIONSHIP WITH AGROCLIMATIC PARAMETERS

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by

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LIST OF SYMBOLS AND ABBREVIATION

℃	Degree celcius
%	Percent
±	Plus-minus
μm	Micrometer
10×	Ten times
a.s.l	Above sea level
AcAc-CoA	Acetoacetyl-CoA
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
APP	Allylic pyrophosphate
APS	Ammonium persulfate
AR	Analytical grade
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BPM	Balai Penelitian Medan
BSA	Bovine serum albumin
$C_4H_40_6Na_2$	Sodium tartrate
ca.	Approximately
CO^2	Carbon dioxide
CoA	Coenzyme A
*c*PIP	*Cis*-1,4-polyisoprene
CPT	*Cis*-prenyl transferase
C-serum	Clear serum

Ct	Cultuurtuin
Đ	Polydispersity index
Da	Dalton
dH_2O	Distilled water
DMAPP	Dimethylallyl diphosphate
DXP	1-deoxy-ᴅ-xylulose-5-phosphate
DXS	1-deoxy-ᴅ-xylulose-5-phosphate synthase
E	East
E	Pan evaporation
eg.	Example
EST	Expressed sequence tags
FPP	Feranyl pyrophosphate
g	Gravity
GGPP	Geranylgeranyl pyrophosphate
GPC	Gel permeation chromatography
GPP	Geranyl pyrophosphate
GT	Gondang Tapen
h	Hour
HMG	*S*-3-hydroxy-3-methylglutaryl
HMG-CoA	*S*-3-hydroxy-3-methylglutaryl-CoA
HMGS	HMG- CoA synthase
HPLC	High performance liquid chromatography
HSC-70	Heat shock coagulate 70-kDa protein
IAN	Instituto Agronomico do Norte
in vacuo	In vacuum

in vivo	In plant
IPP	Isopentyl diphosphate
kDa	Kilo Dalton
kg/ha	Kilogram per hectare
kg/ha/yr	Kilogram per hectare per tapping year
kPa	Kilo Pascal
KT	Kota Tinggi
LRP	Large rubber particle
LTC	Latex timber clone
m	Meter
m/s	Meter per second
MEP	2-C-methyl-ᴅ erythritol-4-phosphate
mg	Milligram
min	Minute
mm	Millimeter
M_n	Number-average molecular weight
MP	Mevalonate diphosphate
MRB	Malaysian Rubber Board
MVA	Mevalonate
M_w	Molecular weight
MWD	Molecular weight distribution
$M_\mathrm{w}^{\mathrm{PB\ 350}}$	Weight-average molecular weight PB 350
$M_\mathrm{w}^{\mathrm{RRIM\ 3001}}$	Weight-average molecular weight RRIM 3001
$M_\mathrm{w}^{\mathrm{RRIM\ 929}}$	Weight-average molecular weight RRIM 929
$M_\mathrm{w}^{\mathrm{RRIM600}}$	Weight-average molecular weight RRIM 600

N	North
Na_2C0_3	Sodium carbonate
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
nm	Nanometer
NMR	Nuclear magnetic resonance
NR	Natural rubber
PAGE	Polyacrylamide electrophoresis
PB	Prang Besar
Pi	Inorganic phosphate
PTFE	Polytetrafluoroethylene
R	Rainfall
r	Pearson’s correlation coefficient
R^2	Regression fit
R^2_{Adjusted}	Adjusted
RAPD	Random amplification of polymorphic DNA
REF	Rubber elongation factor
R_f	Migration distance
RFLP	Restriction fragment length polymorphism
RH	Relative humidity
RI	Refractive index
RP-HLLBP	Rubber protein hectin-like protein
rpm	Rotation per minute
RRIC	Rubber Research Institute of Ceylon
RRII	Rubber Research Institute of India

RRIM	Rubber Research Institute of Malaysia
SDS	Sodium dodecyl sulfate
SE	Standard error
SPSS	Statistical Package for Social Science
SRP	Small rubber particle
SRPP	Small rubber particle protein
SSR	Simple sequence repeats
T	Temperature
TE	Tris EDTA
TEMED	Tetramethylethylenediamine
THF	Tetrahydrofuran
ThFFF	Thermal field-flow fractionation
T_{max}	Maximum temperature
T_{min}	Minimum temperature
Tukey’s HSD	Tukey’s honestly significant difference
var.	Variant
VPD	Vapour pressure deficit
WS	Wind speed
µL	Microliter

PEMPROFILAN BERAT MOLEKUL GETAH ASLI DARIPADA KLON *Hevea brasiliensis* MALAYSIA TERPILIH DAN HUBUNGANNYA DENGAN PARAMETER-PARAMETER AGROIKLIM

ABSTRAK

Getah asli atau *Hevea brasiliensis* (Willd. *ex* A, Juss.) Mull. Arg.) mempunyai berat molekul (M_w) yang tinggi dan taburan berat molekul (*Đ*) yang luas secara semulajadinya. Ini adalah sesuatu yang amat penting kerana sifat fizikal dan kemampuan pemprosesan produk getah adalah bergantung kepada ciri ini. Walau bagaimanapun, pelbagai aspek yang mungkin mempengaruhi M_w termasuklah parameter meteorologi belum dikaji dengan mendalam. Jadi, kajian ini bertujuan untuk memprofilkan M_w and MWD getah asli yang ditoreh daripada empat klon *H. brasiliensis* Malaysia (RRIM 600, RRIM 929, PB350 and RRIM 3001) berdasarkan parameter-parameter agroiklim semasa. Susu getah daripada pokok berusia sepuluh tahun dikumpulkan selama 12 bulan dari sebuah kebun getah di Malaysia. Penentuan M_w and MWD telah dilakukan dengan menggunakan kaedah kromatografi penelapan gel. Seterusnya, analisa statistik digunakan untuk mengenalpasti parameter yang menjadi faktor dan takat impak faktor tersebut ke atas perubahan M_w dan MWD. Selama tempoh eksperimen, M_w diantara klon-klon telah didapati berubah dengan ketara di mana puratanya meliputi dari 0.71 hingga 1.69 kDa. PB 350 menghasilkan purata M_w yang konsisten dalam seluruh tempoh pensampelan berbanding dengan RRIM 600, RRIM 929 and RRIM 3001. Dari aspek MWD, taburan unimodal dan dwimodal lazim telah diperhatikan di dalam semua sampel seperti yang dijangka. Pekali korelasi Pearson antara M_w klon yang dikaji dan unsur cuaca menunjukkan perkaitan sederhana pada paras keertian p=0.05. Model regresi berganda dibina

berdasarkan klon dan faktor meteorologi untuk mengetahui keupayaan unsur cuaca untuk menjelaskan perubahan dalam M_w yang telah diperhatikan. Secara umumnya, M_w didapati terjejas dengan nyata oleh suhu maksimum, min kelembapan relatif dan kelajuan angin. Suhu maksimum dan kelembapan relatif mampu menjelaskan 27% daripada perubahan M_w yang diperhatikan dalam RRIM 929 manakala 34% daripada perubahan M_w dalam RRIM 3001 dipengaruhi oleh suhu maksimum dan kelajuan angin. Untuk RRIM 600 dan PB 350, model regresi tidak dilaporkan kerana parameter agroiklim yang dikaji tidak mampu menjelaskan perubahan dalam M_w klon tersebut. Selain daripada parameter agroiklim, pengaruh jenis klon terhadap M_w pokok getah di lokasi eksperimen juga dibuktikan melalui kajian ini.

MOLECULAR WEIGHT PROFILING OF NATURAL RUBBER FROM SELECTED MALAYSIAN *Hevea brasiliensis* CLONES AND ITS RELATIONSHIP WITH AGROCLIMATIC PARAMETERS

ABSTRACT

Natural rubber or *Hevea brasiliensis* (Willd. *ex* A, Juss.) Mull. Arg.) inherently possesses high molecular weight (M_w) and have a wide range of molecular weight distribution (MWD) which are vital since physical properties and processing ability of rubber products are influenced by it. However, numerous aspects that may regulate rubber M_w including agroclimatical parameters are poorly investigated. Hence, this study was undertaken to profile M_w and MWD of natural rubber derived from four Malaysian clones of *H. brasiliensis* (RRIM 600, RRIM 929, PB350 and RRIM 3001) based on agroclimatical parameters. Latex of ten years old rubber tree were collected in a local Malaysian plantation for a duration of 12 months using gel permeation chromatography and was statistically analysed to determine factoring parameters and extent of impact on M_w trend while observing changes in *Đ* peak. During the experimental period, the M_w were found to be highly variable ranging from an average of 0.71×10^6 Da to 1.69×10^6 Da among the clones. PB 350 yielded rubber with consistent average M_w throughout the sampling period as compared to RRIM 600, RRIM 929 and RRIM 3001. In respect to MWD, typical unimodal and bimodal distribution were prevalent in all samples as expected. Pearson's correlation coefficient between M_w of tested clones and agroclimatic elements showed moderate association at significance level of $p=0.05$. Multiple regression models were developed based on clones and agroclimatical factors to explain the variability observed in respective M_w. In general, M_W was found to be significantly affected by maximum

temperature, mean relative humidity and wind speed. Maximum temperature and relative humidity were able to explain 27% of the M_w variance observed in RRIM 929 while 34% of M_w variance in RRIM 3001 were influenced by maximum temperature and wind speed. Regression models were not reported for RRIM 600 and PB 350 as the studied agroclimatical parameters were inadequate to explain the M_w variance. Besides agroclimatical parameters, influence of clonal type on M_w of rubber trees at the experimental location was additionally established in this study.

CHAPTER 1

INTRODUCTION

1.1 Background

Many plants are capable of secreting latex, a cloudy, often sticky coloured fluid. One of the most economically crucial source of industrial latex is the Brazilian rubber tree, *Hevea brasiliensis* (Willd. *ex* A, Juss.) Mull. Arg. Latex extracted from *H. brasiliensis* is highly desirable for commercialized rubber production due to the presence of high percentage of natural rubber (30-45%). Additionally, natural rubber derived from *H. brasiliensis* exhibits superior properties including high molecular weights (M_w), elasticity and resilience (Mooibroek & Cornish, 2000).

Natural rubber produced by *H. brasiliensis* is categorized as a polymer comprising of 320 to 35 000 isoprene molecules. The polymer which is known as *cis*-1,4-polyisoprene is formed through sequential condensation of isopentenyl diphosphate (IPP) units by rubber transferase and Mg^{2+}. IPP units serve as precursor for numerous isoprenoid compound synthesis including rubber and are synthesized through cytosolic mevalonate (MVA) pathway (Kekwick, 2001). Ko et al. (2003) proved the presence of a plastidic pathway for IPP synthesis involving 1-deoxy-D-xylulose 5-phosphate/ 2-C-methyl-D-erythritol 4-phosphate (MEP) in the laticifers of *Hevea* tree which was later proved to be exclusively involved in carotenoid production (Chow et al., 2011).

Despite mass production of synthetic rubber, industrial demand for natural rubber continue to skyrocket whereby an increase of 5.5% on yearly basis to 4.6 million tonnes in 2018 has been recorded. Comparatively world's NR production was only at 4.0 million tonnes (Bich, 2018). The ever increasing gap between world supply and consumption of raw rubber has long prompted the emergence of various new *Hevea* clones in top rubber producing countries such as Malaysia, Indonesia and

Thailand whereby over 90% of natural rubber production takes place (Beilen & Poirier, 2007). Thus, clones of *H. brasiliensis* remain as one of the most extensively studied tree among latex producing plants. Characteristics of some of these clones remain undiscovered especially in the aspect of its M_w and molecular weight distribution (MWD). Further studies on these clones may contribute vital information to produce rubber with higher quality than existing raw material for commercial purposes.

1.2 Justification

Malaysia is world's fourth largest NR producer and on the domestic front, raw natural rubber accounted for 30.2% or RM 32.3 billion of Malaysia's total export in 2017 (BERNAMA, 2018). With the exception of Subramaniam (1972) research of clonal role in NR M_w, past studies detailing role of environmental factors on M_w was inactively pursued. M_w of NR should be a focus of scientific research in Malaysia for several reasons. Firstly, investigating properties of rubber such as M_w will eventually be beneficial in long term and aid in increasing Malaysian rubber export as majority of world's NR are utilized in heavy duty machinery and automobile industry (Bich, 2018). Commercially accepted rubber characterized by high M_w are preferred in these industries because of its high performance properties such as resilience and heat resistance (Mooibroek & Cornish, 2000). Therefore, continuous production of high performance NR in the country will increase export demand and subsequently Malaysia's economic standing.

Secondly, although elevated M_w is often an indicator of superior NR quality, additional cost incurred by industry through shearing and mastication of hardened rubber blocks resultant from high M_w proves to be a hindrance as well. Mastication and rubber shearing are executed to reduce overall M_w to ease subsequent processing (Chaikumpollert et al., 2011). Hence a need to control NR M_w before industrial processing has arisen. In addition to internally engineering NR biosynthesis mechanism to produce desired M_w as proposed by Cornish et al. (2000) , establishing and controlling external factors such as weather, age and clone at crop level could also be implemented to address this problem. Similar to other rubber producing countries, *Hevea* clones thrives in tropical environment with sufficient water supply such as Malaysia. Therefore, optimum Malaysian climate for rubber cultivation provides a good platform to gauge M_w variation caused by agroclimatical parameters without compromising latex input and tree growth.

1.3 Problem statement

Most *Hevea brasiliensis* researches in Malaysia focuses primarily on maximizing latex yield while other factors such as rubber quality has not been studied in depth. High M_w and wide distribution of the weight is an inherent characteristic of *Hevea* rubber. Therefore, the M_w and MWD of the rubber is a crucial indicator of rubber quality (Cornish, 2001b). Although high M_w is desired, extremely elevated M_w incurs additional cost during industrial processing. Hence controlling M_w and MWD at crop level is beneficial for downstream processing. Environmental influence on M_w and MWD variation has also been suggested by Kovuttikulrangsie and Sakdapipanich (2004). Unfortunately, no research was conducted to validate the hypothesis. M_w variation in *Parthenium argentatum* Gray (guayule) rubber according to season was investigated only once until date (Cantú et al., 1997). Lack of interest in M_w and MWD could be attributed to the fact latex yield were given more priority research wise in the classic case of quantity versus quality. Also, studies such as this are often neglected because of its time consuming nature and the difficulty in pinpointing the exact agroclimatical cause that is responsible for M_w and MWD variation. In order to address these concerns, the present research was undertaken. This study focuses mainly on the impact of different agroclimatical elements viz. rainfall, minimum and maximum temperature, pan evaporation, wind speed, relative humidity and vapour pressure deficit on molecular weights and MWD of rubber obtained from four selected Malaysian clones, RRIM 600, RRIM 929, PB 350 and RRIM 3001 through gel permeation chromatography. Subsequently the relationship between the parameters were evaluated statistically for significance as factors.

1.4 Objectives

1. To perform molecular weight profiling of natural rubber from four different Malaysian clones
2. To ascertain agroclimatical parameters that may influence molecular weight and its distribution among the four clones through statistical approach

CHAPTER 2

LITERATURE REVIEW

2.1 Latex and rubber producing plants

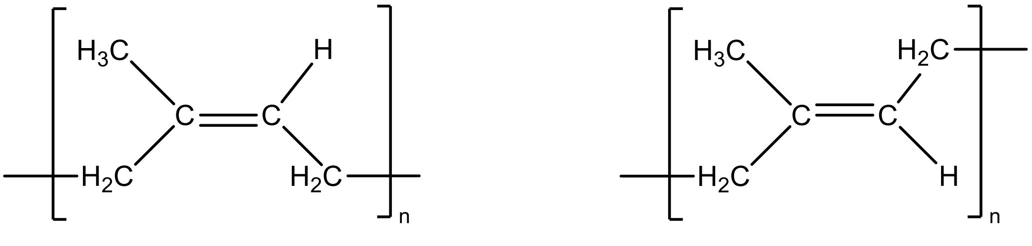
Latex, originating from the Latin word for fluid, *lac,* is defined as a milky suspension consisting of differing particles in an aqueous medium which are confined within cells of living plants known as laticifers (Jacob et al., 1993; Kekwick, 2001). Typically expressed when plants are wounded, latex oozes out to become sticky upon exposure to air and proceed to coagulate to seal the damaged area, a mechanism that mimics the role of blood in higher animals (Agrawal & Konno, 2009).

The primary function of latex in plants is still debatable (Schultes, 1993) . Agrawal and Konno (2009) suggests defense against herbivory predation as main function of latex due to elevated amount of defense chemicals and proteins observed in latex of countless species. Most of these biologically active components provide toxicity and nutrient depriving elements to deter potential predators while rubber contribute to stickiness that may slow down advancing entomological predators. Efficient mobility of latex to injury sites followed by effectiveness in warding off mandibulate bearing insects such as caterpillars adds strength to this theory (Agrawal & Konno, 2009).

Latex is found to be prevalent in ca.10% of all angiosperm species and in at least in few species of ferns (*Regnellidium* sp.), gymnosperms (*Gnetum* sp.), succulent saprophytes and fungi (Tanaka et al., 1994; Kekwick, 2001; Agrawal & Konno, 2009) . More specifically, some 40 000 species from 40 families are found to exude latex as reviewed by Agrawal and Konno (2009). Although most latex are whitish in appearance such as those exuded by *Hevea* sp. and *Ficus* sp., some taxa express yellow or orange coloured latex exemplified by plants from family Papaveraceae and

Cannabis sp. Meanwhile, some *Morus* sp. and *Nerium oleander* produces clear latex (Kekwick, 2001).

Latex is the primary source of naturally occurring biopolymer, natural rubber (NR). NR consists of isoprene monomers that exist in two forms, *trans*-1,4-polyisoprene and *cis*-1,4-polyisoprene (Figure 2.1). The latter form of NR is synthesized in at least 2 500 higher plant species belonging to 300 genera and seven families namely Apocyanaceae, Asclepiadaceae, Asteraceae, Euphorbiaceae, Moraceae, Papaveraceae and Sapotaceae (Priyadarshan, 2003). Prominent NR producing species includes natural rubber (*Hevea brasiliensis*) from Euphorbiaceae, guayule (*Parthenium argentatum* Grey), Russian dandelion (*Taraxacum kok-saghyz*), sunflower (*Helianthus* sp.) from Asteraceae, various fig tree species (*Ficus* sp.) and castilla (*Castilla* sp.) from Moraceae; and milkweeds (Asclepias) from Apocyanaceae (Mooibroek & Cornish, 2000; Beilen & Poirier, 2007). In addition to angiosperms, several fungi genera such as *Lactarius* (Tanaka et al., 1994; Ohya et al., 1997), *Russala*, *Hygrophorus* and *Peziza* also express NR (Venkatachalam et al., 2013). Of these species, *H. brasiliensis* has the highest rubber content at 30-50% followed by *T. kok-saghyz* at 30% and *P. argentatum* at 3-12%. Furthermore, all three species have high molecular weight (>1000 kDa) which is essential for rubber quality determination (Beilen & Poirier, 2007).



cis-1,4-polyisoprene *trans*-1,4-polyisoprene

Figure 2.1 Structure of *cis*-1,4-polyisoprene and *trans*-1,4-polyisoprene (Tanaka, 1989)

Even though many angiosperm families are able to synthesise *cis*-1,4-polyisoprene rubber, none can surpass potential found within Euphorbiaceae. Species such as the notable *Hevea brasiliensis* in genus *Hevea*, *Manihot* spp. and numerous spurge species including *Euphorbia lactifua* (Gnecco et al., 1997), *E. heterophylla* (Mekkriengkrai et al., 2004), *E. characias* (Spano et al., 2012) and *E. tirucalli* (Hastilestari et al., 2013) are known rubber producer. The sole source of commercial natural rubber currently, *H. brasiliensis* is irreplaceable and is being continuously traded as an important commodity worldwide thanks to extensive usage in multiple industries.

2.2 Genus *Hevea*

2.2.1 Botanical classification

Genus Hevea is a taxa under the subfamily Crotonoideae (Wurdack et al., 2005; Angiosperm Phylogeny Group, 2009). The native South American genus was first described by Fusee Aublet in 1775 for the type species of *H. guianensis* and later the name *Siphon* was proposed by L. C Richard in 1779 as an equivalent to *Hevea* as reviewed by Schultes (1970). Another name, *Caoutchoua elastica* was suggested by H. F. Gmelin in 1781 in place of *H. guianensis*. However, only the term *Hevea* and *Siphon* has been extensively used in taxonomical studies thereafter in describing this taxon. Several species such as *H. brasiliensis*, *H. pauciflora*, *H. spruceana* and *H. rigidifolia* was previously classified under Siphon, only to be reassigned into *Hevea* by J Muller-Argoviensis in 1865 (Schultes, 1970).

Number in the taxa recognized changed over the years. Mueller-Argoviensis first suggested 11 species, followed by an increase to 24 by Huber in 1906. The taxa count then decreased to finally eight species as suggested by Seibert in 1947 and rose to the taxa known today. This general trend in taxa number decrease was primarily caused by reduction of previously species rank specimen into varieties or forms (Schultes, 1970).

Until the first description of *H. brasiliensis* in 1824, *H. guianensis* was the only species associated with *Hevea* (Schultes, 1970; Wycherley, 1992). This was followed by the chronological first description of *H. pauciflora* (1854), *H. spruceana* (1854), *H. rigidifolia* (1854), *H. benthamiana* (1965), *H. nitida* (1874), *H. microphylla* (1905), *H. camporum* (1925) (Schultes, 1970) and the most recently discovered *H. camargoana* by Murca Pires in 1971 . After numerous infrageneric revision to the taxa, *Hevea* now formally harbors ten species within its genus. The species are listed in chronological order as follows: *H. guianensis*, *H. brasiliensis*, *H. pauciflora*, *H. spruceana*, *H. rigidifolia*, *H. benthamiana*, *H. nitida*, *H. microphylla*, *H. camporum* and *H. camargoana* (Wycherley, 1992; Priyadarshan, 2003).

2.2.2 Ecological niche and distribution

Primary geographical niche of this taxa is in the Amazonian Basin and its adjacent region in the highlands. This translates to a geographic range that spans across nine different countries as the following: Ecuador, Brazil, Venezuela, Peru, Surinam, Colombia, Guyana, French Guyana and Bolivia. Trees predominantly occupies low lying area which extends until the foothills of Guayana Shield in the south and Upper Orinoco in Venezuela. Soils in these lowlands are typically kaolinistic and gley type soil with low fertility and humus content while a mix of different soils are found in the higher altitude. Climate wise, *Hevea* taxa are generally confined to regions with humid equatorial, tropical monsoonal and its in-between transitional climate (Wycherley, 1992). The epicenter for *Hevea* genetic diversity is in the Rio Negro and its surrounding region. Out of ten, seven species and two important varieties comprising of *H. benthamiana, H. guianensis* and its var. *lutea, H. microphylla, H. nitida, H. pauciflora* and its var. *coriacea, H. rigidifolia* and *H. spruceana* occurs within the Rio Negro region. *H. brasiliensis* occurs at Salimoes and lower Rio Madeira, the sourthern boundary of Rio Negro. *Hevea camporum* and *H. camargoana* are the only two species which grow outside this area (Wycherley, 1992).

2.2.3 Taxa in *Hevea*

Among the ten species of *Hevea*, all are found in Brazil. In Colombia, seven species have been discovered while four occurs in Peru and Venezuela and two in the Guayanas and Bolivia. Habitat range and ecological preference, morphological variation and economical exploitation of all species are shown in Table 2.1. Distribution range of species differs accordingly with some taxa such as *H. guianensis* inhabits areas throughout genus range while *H. rigidifolia* and *H. camporum* are restricted to a smaller area comparatively. Ecological wise, most species either prefers flooded swamps or well drained soils while several others inhabit arid savannahs (Wycherley, 1992).

In general, growth forms vary greatly from species to species, from tall trees reaching height of 30-35 m (*H. guianensis*) to diminutive shrubs that are ca.2 m tall (*H. nitida* var. *toxicodendriodes*). Despite the occurrence of ten species, only three have been utilized for rubber production in which *H. brasiliensis* being the most extensively exploited commodity in the genus (Purseglove, 1968). Other potential species were not cultivated due to high percentage of resins exemplified by *H. pauciflora*, *H, spruceana*, and *H. rigidifolia*. Meanwhile, some exudes very watery latex which lacks in rubber quantity (Purseglove, 1968; Wycherley, 1992).

Table 2.1 Species and varieties in genus *Hevea* – habitat range and preference, morphological variation and extent of exploitation (Wycherley, 1992; Priyadarshan, 2003)

Species	**Distribution range**	**Ecological habitat**	**Growth form variation**	**Exploitation**
***Hevea guianensis* Aublet** var. *lutea* var. *marginata*	Wide, throughout genus's geographic range	Moist, well drained soil	Medium sized tree, short shoots, partially erect mature leaflets	Yes. But not cultivated.
Hevea brasiliensis (Willd. ex A. de. Juss.) Muell. Arg	Southern Amazon river	Infrequently flooded, well drained soil	Tall trees	Yes and widely cultivated
Hevea pauciflora (Spr. ex. Bth.) Muell. Arg var. *coriace*	Discontinuous distribution at northern and western Amazon river	Sandy or rocky, adequately drained soil	Small to medium tree, short shoots and hardy leaves	No. High resin content
***Hevea spruceana* (Bth.) Muell. Arg**	Along Amazon riverbank, lower Madeira, Rio Negro and other prominent tributaries.	Muddy banks and flooded island soils	Medium tree, short shoots, occasionally swollen trunk	No. High resin content
***Hevea benthamiana* Muell. Arg**	Northern and western Amazon basin	Flooded swamp forest	Medium tree, frequent occurrence of swollen trunk	Yes. For hybridization with H. brasiliensis
***Hevea rigidifolia* (Spr. ex Bth.) Muell. Arg.**	Endemic to Upper Rio Negro and Rio Vaupes	Dry, adequately drained rocky soils	Small tree, tough leaves	No. High resin and multiple variety
Hevea nitida var. *toxicodendroides* **Mart. ex. Muell. Arg.**	Rio Vaupes and Colombian Amazon regions	Sandstone mesas underneath quartizitic soils	Shrubs	No. Rare and small
***Hevea microphylla* Ule**	Rare and confined within middle and upper Rio Negro and Rio Guainía	Flooded sandy or lateritic soils	Small tree, slender crown and swollen trunk	No. Rare and high resin content
***Hevea camporum* Ducke**	Endemic to tributaries of Rio Madeira and Rio Tapajos	Arid savannahs	Shrubs	No. Rare and small
***Hevea camargoana* Pires**	Marajo island, Amazon river delta	Savannah and seasonal muddy swamps	Small to medium trees	No.

2.3 *Hevea brasiliensis* (Willd. Ex. A. de. Juss. Mull. Arg.)

2.3.1 Background

Prior to the modern day uses of rubber, *H. brasiliensis* tree was predominantly utilised as a source of food particularly seeds by Amazonian Indians. Cyanide contained within seeds were reportedly removed via soaking and boiling prior to consumption during ceremonies and occasionally in periods of famine (Schultes, 1993). Waterproof fabrics, bag, balls, footwear and bottles were also some of the other articles manufactured by them using rubber. Initially the French named rubber as caoutchouc and later on known as India-rubber or simply rubber upon Priestly's discovery of rubber's ability to erase pencil mark in 1770. Gradually, rubber gained influence in the Western world and was soon converted into other products such as shoes and raincoats. However, these products were unsuccessful due to inability of raw rubber to resist extreme heat and cold where sticky and brittle conditions was observed respectively (Schultes, 1993).

A major breakthrough in rubber history took place when vulcanization, a process where rubber and sulphur were heated together, was simultaneously discovered by Goodyear and Hancock in 1839. Through vulcanization, the physical properties of rubber were retained within the temperature range of 0-100℃ as opposed to raw rubber. In addition, compounding vulcanized rubber with other chemical compound also enabled successful moulding of rubber into useful products. Discovery of vulcanized rubber and subsequent widespread usage in the automobile industry lead to a huge increase in demand for raw rubber that exceeded existing supply sourced from wild trees in Brazil (Purseglove, 1968; Schultes, 1993).

The domestication of para rubber tree, *Hevea brasiliensis* began ca. 150 years ago in an effort to complement rubber production of native Brazilian trees. The wild species was an inhabitant of the Amazonian Basin before 70 000 seeds from the vicinity was transported to the Kew Botanic Gardens by Wickham in 1876. Of these seeds, ca.2 800 germinated and 1 919 seedlings were shipped to Ceylon (Sri Lanka). In the following year, 22 seedlings from Ceylon were forwarded to Singapore Botanic Gardens of which nine were planted at the Gardens and another nine, at Kuala Kangsar, Perak, Malaya (Malaysia) in the residence of Sir Hugh Low (Wei & Bahri, 2014; Priyadarshan, 2017).

Vigorous experimental trials conducted by Ridley, the then scientific director of Singapore Botanic Gardens, in numerous aspects including tapping methods, tree growth and physiology established *H. brasiliensis* as a promising commercial source of rubber by 1900’s (Wei & Bahri, 2014). Decreased coffee prices (a major export crop for Malaya) and an increase in demand and price of rubber, prompted steady but mass planting of rubber trees in the country (Jones & Allen, 1992). By 1910, Malaysia assumed the top position in world rubber production and continued to do so until recent years whereby Thailand, Indonesia and Vietnam started producing higher rubber quantity comparatively mainly due to land conversion for non-rubber economical crops (Purseglove, 1968; Priyadarshan, 2003; Beilen & Poirier, 2007). Presently 92% of rubber for world consumption are supplied by Asian countries (ANRPC, 2016). Smaller plantations in Africa particularly those concentrated in Nigeria accounts for a small percentage of total world production (Schultes, 1993).

2.3.2 Ecology and annual growth cycle

Wild rubber trees inhabit occasionally flooded areas and well drained plateaux of the Amazonian Basin. Generally, rubber trees cultivated in nonnative regions prefers hot and humid climate accompanied by consistent rainfall distribution and temperate temperature ranging from 23℃ to 35℃. Preferred soil condition are typically moist, well drained loam with an optimum pH of 5 to 6. A large majority of rubber in Malaysia are grown on formerly low dipterocarp forest with adequate leguminous cover crops to prevent erosion and nutrient deprivation (Wycherley, 1992).

Hevea brasiliensis experiences annual seasonal growth inclusive of a ‘wintering’ period whereby heavy defoliation occurs before new foliage emerges as another growth cycle begins (Wycherley, 1992). Refoliation is soon followed by a single annual flowering season and occasionally a minor secondary flowering event transpires depending on suitability of cultivation region (Yeang, 2007). For example, in Malaysia, major flowering occurs during both March and April followed by minor event from August to September as opposed to annual flowering in south India (Priyadarshan & Clément-Demange, 2004). Upon fertilization, fruits reach full size within three months and proceeds to ripen within another three before dehiscing. Resultant seeds are often planted to provide root stocks for subsequent propagation process. A viable seed planted immediately after harvesting may germinate within three weeks of planting (Purseglove, 1968).

2.3.3 Common morphological features

An illustration of typical features observed in wild rubber trees are shown in Figure 2.3. Wild rubber tree has large spirally arranged trifoliate leaves alongside conspicuous vein structure. Each leaf consists of three leaflet attached to long petiole and varies greatly in leaf shape where irregularities are noticeable in young leaves (**3**,**6**). In general, flowers are monoecious, small and scented with the larger female flowers situated at the terminal ends of lateral and main branches accompanied by many smaller male flowers (refer **1** in Figure 2.3). Calyx are commonly bell shaped yellowed triangular lobe of five with no petals. Male flowers (**8**) are ca. 5mm long with five sessile anthers set in each of two superimposed circle positioned on a thin central column meanwhile female flower (**9**) are ca. 8mm long with three sessile stigmas and three celled ovaries (**10**). Fruits (**4**) achieve full size three months upon fertilization and ripens three months thereafter. From three lobed capsule consisting of woody endocarp (**7**) that dehisces explosively, large pale brown seeds (**2**,**5**) with irregular dark brown blotches are ejected away from parent tree (Purseglove, 1968).

2.3.4 *Hevea* clone breeding and propagation

Rubber tree breeding is a constantly advancing field. Maximising yield and subsequently dry rubber content of latex has been the ultimate objective of *Hevea* breeding endeavors since the 1920’s. Specific objectives such as high tapping intensity and high altitude and low temperature suited trees may be designed to suit a producer country’s socioeconomic and agro-climatic requirements (Varghese, 1992). Most recently, rubberwood production has been hailed as an additional aim to these breeding efforts (Priyadarshan & Clément-Demange, 2004). The main approach to achieve this objective is through creation and propagation of multiple *Hevea* clones.

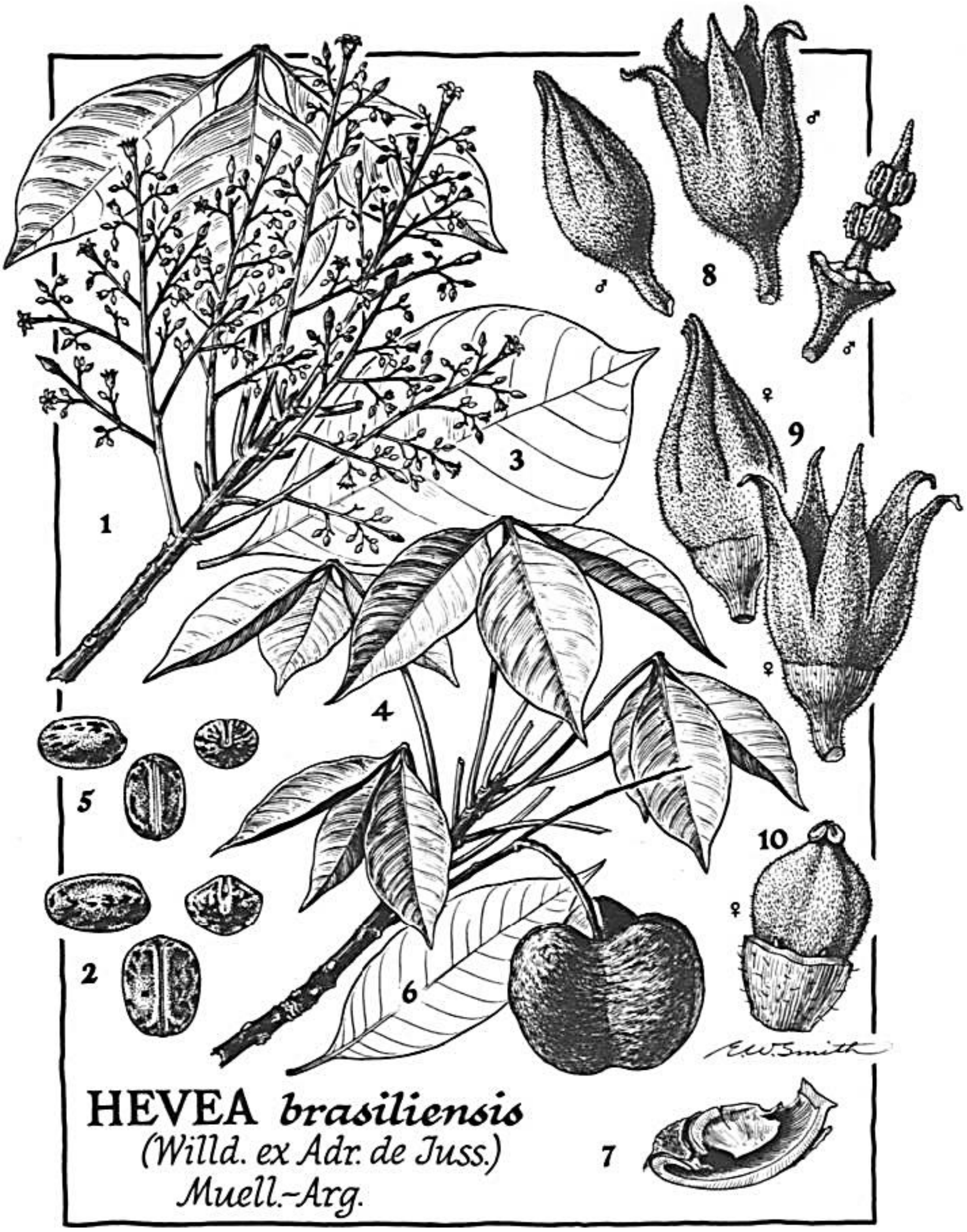


Figure 2.2 Common morphological characteristics of *Hevea brasiliensis*. **1**. Flowering branch, **2** & **5**. Seed structure, **3**. Typical large leaf with obvious venation, **4**. Fruiting branch, **6**. Typical young leaves with unusual attenuation, **7**. Woody valve of fruiting capsule, **8**. Bud and flower of staminate, **9**. Carpellate bud and flower, 10. Ovary (Schultes, 1987)

By association, additional factors or secondary attributes that may influence tree productivity levels such as stress resistance (eg. disease, wind damage, tapping panel dryness), trunk growth and seasonal yield behavior are considered during hybridisation of new clones (Priyadarshan, 2003; Priyadarshan, 2017).

P.J.S Cramer first developed bud grafting procedure in 1918 and identified first clones (Cramer's Cultuurtuin) from 33 seedlings cultivated in Penang (Priyadarshan, 2003). Cramer was aided by Van Helten who established *Hevea* vegetative propagation technique in 1915 (Marattukalam & Saraswathyamma, 1992; Priyadarshan, 2017). Bud grafting involves procuring lateral bud along a small piece of bark from one plant (scion), typically with desired characteristics such as potential clone and attaching to another plant (stock). Point of attachment are commonly secured using transparent polythene film strips until bud patch is strongly attached. Once grafted bud patch is attached firmly onto stock plant, portion of plant above the newly grafted bud will be trimmed. Hence, a two-part plant is formed, essentially comprising of root derived from stock plant and shoots from scion plant whereby plant shoot will display identical features of the scion donor. Despite numerous vegetation techniques based on seeds, roots and micropropagation, bud grafting is still the preferred commercial propagation technique for *Hevea* clones (Marattukalam & Saraswathyamma, 1992).

The clones from Cramer's Cultuurtuin (Ct3, Ct9 and Ct 38) planted together in the Sumatran east coast was found to yield approximately fourfold the amount of latex in comparison to unselected trees. This incidence eventually encouraged increased production of important primary clones in Malaysia by Major Gough and subsequently newer recombinant clones were produced once older clones has reached productivity plateau in the 1930s (Priyadarshan, 2003). Ever since, tremendous progress in *Hevea* yield improvement was observed. By 1950s, elite modern clones were producing up to 1600 kg/ha of latex compared to 496 kg/ha by unselected trees in the 1920s. Further development was observed in recent years where yield reaching 2500 kg/ha was made possible with the introduction of PB, RRIM, RRII, BPM and numerous Chinese clones. Over the years, selective breeding of *Hevea* clones had produced notable high yielding clones including RRIM 600, PB 235, PB260, RRIC 100 and BPM 24. Progenitor of these modern recombinant clones can be traced back to at least 16 primary clones such as Tjir 1, GT 1, PB 86 and PB 56 (Priyadarshan, 2017).

2.4 *Hevea* clones in current study

2.4.1 Background

Top rubber exporting countries such as India, Malaysia, Thailand, Côte d'Ivoire Brazil and Indonesia are the leading players in clone hybridisation effort. Careful clone selection technique and recombinant breeding caused production of viable clones with desirable characteristics such as high latex yield, pathogenic and entomological resistance; and climate resilience (Priyadarshan, 2003; Priyadarshan, 2017).

In this study, Malaysian produced clones RRIM 600, RRIM 929, PB 350 and RRIM 3001 were used as experimental subjects. Malaysian clones are mostly derived based on the genetic material from the original 22 seedlings originating from Wickham's collection in 1876 (Wei & Bahri, 2014; Priyadarshan, 2017). Clones are usually grouped by Lembaga Getah Malaysia (LGM) (previously known as Rubber Research Institute Malaysia) into planting recommendation categories based on yield and performance under different environmental constraints. Class I consists of high yielding clones recommended for both commercial and smallholders planting which have undergone successful large scale trial for at least five years in differing environmental conditions. Class II comprises of new clones with promising yield for at least first three tapping years in multiple environment (Wei & Bahri, 2014).

RRIM 600 is one of the earliest high profile high yielding clone hybridized by RRIM. RRIM 600 was once recommended under Class I from 1970's to 1980's and since became prominent progenitor of many successful newer clones including IRCA 111 (Cote d' Ivoire), SCATC 7-33-97 (China) and RRIM 2001 (Malaysia) (Wei & Bahri, 2014). RRIM 929 is a latex timber clone (LTC) geared towards the production of both latex and rubberwood, introduced in the RRIM 900 series. It was grouped together with its sister clone RRIM 928 in Class II of planting recommendation from 1980 to 1997. Due to limited yield, poor growth rate and lack of good secondary characteristics, both of these clones were not recommended for large scale planting and thus was slowly phased out from production until 2003 where both clones were reintroduced in Class I of planting recommendation (Benong et al., 2007; Wei & Bahri, 2014).

PB 350 is another LTC clone originating from Prang Besar Isolated Seed Garden, hence the abbreviation PB in its name. Recommended under Class I in 2006, this clone is highly resistant to a number of disease and wind damage with good yield output (Benong et al., 2007). The latest clone to be introduced by MRB in 2009 is RRIM 3001 also known as KT/1Malaysia clone which is placed in Class II in LGM recommendation list 2013. This clone is also geared towards latex and timber production but does not grow very well on laterite soil and hilly terrains (Benong et al., 2007; Wei & Bahri, 2014). Table 2.1 highlights several important information such as parentage, latex yield and disease resistance ability of the four clones.

Table 2.2 Clone information (Priyadarshan, 2003; LGM, 2016)

Clone	Parent	Yield (kg/ha/yr)*	Disease resistance
RRIM 600	Tjir 1 × PB 86	1364	*Oidium*, *Collectotrichum*
RRIM 929	RRIM 605 × RRIM 725	1642	*Oidium*, *Collectotrichum, Corynespora*
PB 350	RRIM 600 × PB 235	1689	Pink disease, *Collectotrichum, Corynespora*, *Oidium*
RRIM 3001	IAN 873 × PB 235	2123	Pink disease, *Oidium, Collectotrichum*

*kg/ha/yr – kilogram per hectare per tapping year

2.4.2 Clonal identification

Successful clonal identification of studied tree is vital especially in the field where more than one clone may be planted together in a single area. Clones are usually identified through visual observance and if necessary, measurement of at least seven plants of the same group must be taken. Traits exclusive to a group are assessed through extent of consistency and clarity of difference beside ensuring uniformity is observed on at least 95% of test subjects (UPOV, 2009). Table 2.3 shows some of the key vegetative morphological characteristics to be considered in order to identify four *Hevea* clones in this study. Primarily, leaves and leaf clusters are observed for identification purpose. Trunk character, latex colour, foliage density and tree shape are minor features which are considered if identity could not be concluded on leaf morphology. This is due to morphological irregularities found across tree age. Hence different set of characters are evaluated at seedlings, one-year-old and five-year-old stage. In actively reproducing mature trees, seeds are a part of clonal identification character. However subtle difference between clonal seed can easily be missed by novice identifier (UPOV, 2009; LGM, 2016)

Recent advances in molecular biology added strength to existing identification process of *Hevea* clones. A number of workers have successfully identified cultivars and wild type *Hevea* tree through RAPD and RFLP method (Roy et al., 2004;

Table 2.3 Key vegetative and reproductive morphological characteristics for clonal identification (UPOV, 2009; LGM, 2016)

Organ	**Characteristics**	**RRIM 600**	**RRIM 929**	**PB350**	**RRIM 3001**
Leaf	Leaflet positions	Separated	Overlapping	Touching	Separated
	Intensity of green colouration on adaxial surface	Light	Dark	Light	Dark
	Glossiness of adaxial surface	Medium	Medium	Medium	High
	Adaxial surface texture	Moderately rough	Smooth	Smooth	Smooth
	Leaflet blade attitude relative to petiole	Semi erect	Semi drooping	Horizontal	Semi drooping
	Orientation of broadest part in leaf blade relative to leaf length	Towards apex	Towards apex	Towards apex	Middle
	Axis in longitudinal section of leaflet blade	Straight	Convex	Straight	Convex
	Margin undulation	Absent	Medium	Medium	Absent
	Shape at base	Obtuse	Cuneate	Obtuse	Cuneate
	Shape of apex	Obovate	Acuminate	Cuspidate	Acuminate
Petiole	Attitude	Semi erect	Semi erect	Semi erect	Semi erect
Seed	Length	Medium	Small	Medium	Medium
	Width	Medium	Medium	Narrow	Narrow
	Thickness	Thin	Thin	Medium	Medium
	Shape from dorsal view	Circular	Obovate	Oblong	Oblong
	Colouration	Glossy, dark brown	Faded, light brown	Glossy, dark brown	Glossy, light brown

Pethin et al., 2015). Some authors such as García et al. (2011) and Perseguini et al. (2012) employed the use of simple sequence repeats (SSR) or microsatellites markers. The former managed to construct four microsatellite markers that were able to differentiate ten out of 12 clones including GT1 and RRIM 600 while the latter combined expressed sequence tags (EST) with SSR which proved to be successful in evaluating rubber clone diversity.

2.5 *Hevea brasiliensis* latex

Hevea brasiliensis latex are essentially the cytoplasm of laticiferous cells which exist in jointed/ articulated and nonarticulated form. These vessels are found throughout all organs in the rubber tree. Commercially, latex is systematically harvested through repeated bark excision of tree trunk (Kekwick, 2001).

Fresh latex consists predominantly of water (58.6%) and rubber hydrocarbon (36.0%) which yields ca. 35% of dry rubber material. Through ultracytology studies, several fraction in fresh latex were fully identified by Moir (1959). In general, the topmost centrifuged fraction consists of rubber phase followed by yellowish orange layer of Frey-Wyssling particles, clear serum (C-serum) of latex cytosol and a bottom fraction encompassing lutoid particles. Rubber particles in the rubber phase consist mainly of *cis*-polyisoprene units enclosed in a layer of proteins and lipids essential for biosynthesis. Frey-Wyssling particles and C-serum are rich in natural rubber biosynthesis enzymes, hence rendering both of these fraction crucial (Sakdapipanich & Rojruthai, 2013). Frey-Wyssling particles also functions as carotenoid producer, a compound responsible for yellow tinge observed in latex of several rubber clones. Latex coagulation and regulation of laticiferous system in *Hevea* are the two main function of lutoids (Jacob et al., 1993).

Among these fractions, rubber phase has the highest volume on basis of latex amount. Besides rubber, secondary metabolites components namely alkaloids, cardenolides, terpenoids, and phenolics; various protein, amino acids and nitrogenous compounds such as protease and lectins; lipids, ash including magnesium, manganese and iron ions, inositols and other sugars are also abundant in *H. brasiliensis* latex (Hwee, 2013).

2.6 Natural rubber derived from *Hevea brasiliensis*

Commercially, *Hevea brasiliensis* NR has extensive utilization in the production of more than 40 000 products including hundreds of medical items (Cornish, 2001b) as 98% of world’s rubber demand are supplied by NR. It is an important trade commodity due to unique properties namely high molecular weight, elasticity, resilience, impact and abrasion resistance; malleability at cold temperature and effective heat disperser which are deemed necessary for high-performance applications especially in the automotive and healthcare industry (Mooibroek & Cornish, 2000). For example, heavy duty tires for various large transporters could not be manufactured without natural rubber. Similarly gloves, condoms and catheters requires rubber in latex form (Venkatachalam et al., 2013).

These exceptional properties of rubber are contributed by its structure. NR is composed of about 94% of repeating *cis*-1,4-isoprene monomers as illustrated in Figure 2.3 (Hager et al., 1979) and non-rubber constituents including proteins and lipids. NR can be fractioned into soluble (sol) and insoluble (gel) portion. Within an isoprene chain, repetitive units of *cis*-1,4-isoprene are preceded by two units of *trans*-1,4-isoprene and terminated by a fatty acid ester (Figure 2.4) (Puskas et al., 2006).

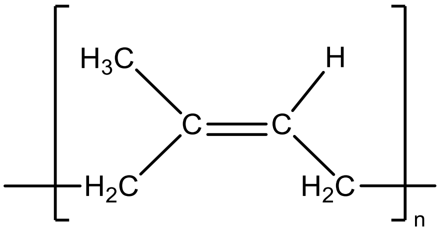


Figure 2.3 Monomer structure of natural rubber, *cis*-1,4-polyisoprene (Tanaka, 1989)

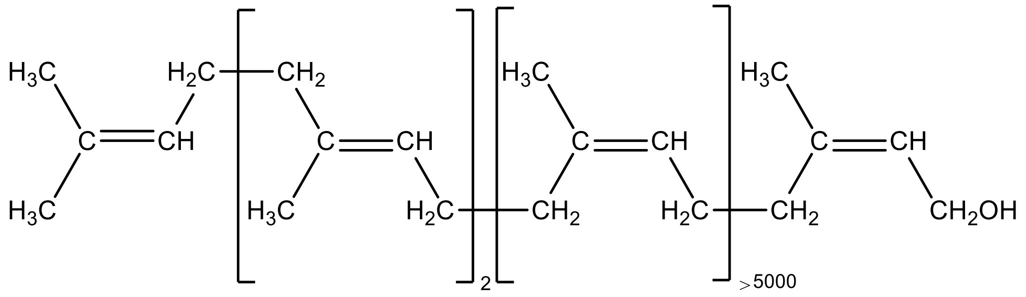


Figure 2.4 Detailed microstructure of isoprene chains in natural rubber (Tanaka, 1989; Puskas et al., 2006)

While proteins component does not influence NR characteristics, lipid especially fatty acids was found to contribute largely to major attributes seen in NR. Terminal ends of rubber molecules affects branching mechanism of NR whereby hydrogen bond formed between lipid, phosphate and carboxyl groups enables formation of branches (Tangpakdee & Tanaka, 1997). Subsequently, increased branching alters certain NR properties including tensile strength, gel content and molecular weight and molecular weight distribution which are some of the features imperative for raw NR processability (Zhou et al., 2017). Gel content in NR, for example, elevates as a result of interaction between phospholipid and proteins when kept over prolonged period resulting in a phenomenon known as storage hardening (Sakdapipanich & Rojruthai, 2013). The attributes of NR are improved significantly through vulcanization mainly to increase suitability in numerous application. Unvulcanised rubber turns sticky and soft when exposed to high temperature while cold environment results in brittle and hard rubber. In addition, lack of strength and elasticity, poor resistance to oils, solvents and free radicals are a common occurrence in NR. Sulfur crosslinkages between linear rubber chains through vulcanization enables rubber to achieve enhanced properties. Efficiency of crosslinking has been greatly promoted in recent years through addition of numerous additives to rubber mixture (Sakdapipanich & Rojruthai, 2013).

Vulcanised rubber displays tolerance at high temperature beside possessing superior tensile strength, tear strength, compression set, rebound capability and hardness in comparison to NR. An effort to emulate these properties lead to extensive research of mechanism to produce rubber synthetically (Puskas et al., 2006). Even though synthetic polyisoprene had been produced, certain NR mechanical properties including tensility and elasticity could not be reproduced due to difference in non-rubber constituents and microstructure frame (Sakdapipanich & Rojruthai, 2013). From this point onwards, natural rubber in the following text is of *H. brasiliensis* species unless stated otherwise.

2.7 Biosynthesis of NR

2.7.1 Isoprenoid production

Natural rubber (NR) synthesis is an omnipresent product of isoprenoid pathway, a common route for production of primary and secondary metabolites among different organism groups including eukaryotes, archaea and eubacteria. The basic building block of all isoprenoid compounds including NR are isopentyl diphosphate (IPP) (Figure 2.5) and its isomer dimethylallyl diphosphate (DMAPP). These precursors can be synthesized through two metabolic routes, the mevalonate (MVA) pathway and 1-deoxy-ᴅ-xylulose-5-phosphate/ 2-C-methyl-ᴅ erythritol-4-phosphate