TISSUE CULTURE OF *Hevea brasiliensis* MÜLL. ARG. LATEX TIMBER CLONE RRIM 929

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TISSUE CULTURE OF *Hevea brasiliensis* MÜLL. ARG. LATEX TIMBER CLONE (LTC) RRIM 929

by

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LIST OF SYMBOLS AND ABBREVIATIONS

<	smaller than
%	percentage
℃	degree Celsius
µL	microliter
µm	micrometer
$\mu molm^{-2}s^{-1}$	micromole per square meter per second
2,4-D	2,4-Dichlorophenoxyacetic acid
2iP	6-(γ,γ-Dimethylallylamino)purine
$AgNO_3$	Silver nitrate
ANOVA	Analysis of variance
BA	6-Benzylaminopurine
bp	base pair
cm	centimeter
CIRAD	Centre de coopération internationale en recherche agronomique pour le développement (The French Agricultural Research Centre for International Development)
DAFe	day after flower bud emerge
g	gram
g/t/t	grams per tree per tapping
HCl	hydrochloric acid
IBA	Indole-3-butyric acid
in vitro	In glass
kb	kilobasepairs
KN	Kinetin
KOH	potassium hydroxide

LTC	Latex timber clone
m^3	cubic meter
MB	Microboutorage
mg	milligram
mg/L	milligram per litre
min	Minute
MRB	Malaysian Rubber Board
MS	Murashige and Skoog
N	Normal
NAA	1-Naphthaleneacetic acid
NB	Nitro Blue
NB	Nitro Green
NR	Natural rubber
PB	Prang Besar
PGR	Plant growth regulator
ppm	parts per million
psi	Pounds per square inch
RRIC	Rubber Research Institute of Ceylon
RRII	Rubber Research Institute of India
RRIM	Rubber Research Institute of Malaysia
SD	Standard deviation
SPSS	Statistical Package for Social Science
SSCT	Small Scale Clone Trial
SSR	Simple sequence repeat
TAE	Tris-Acetic acid-EDTA

TBE	Tris-Borate-EDTA
TDZ	Thidiazuron
Tukey’s HSD	Tukey’s honestly significant difference
V	volts
v/v	volume per volume
w/v	weight per volume
WP	Woody Plant

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KULTUR TISU *Hevea brasiliensis* MÜLL. ARG. KLON LATEKS BALAK (KLB) RRIM 929

ABSTRAK

Pokok getah *Hevea brasiliensis* Müll. Arg merupakan antara tanaman komersil penting yang ditanam dengan meluasnya di sekitar Asia Tenggara untuk memperoleh getah asli dalam bentuk lateks. Getah merupakan tanaman abadi yang sukar berakar, khususnya hanya terdapat klon tertentu sahaja yang dapat diperbaharui melalui kultur tisu. Klon yang dapat diperbaharui melalui kultur tisu akan dapat digunakan sebagai alat penambahbaikan tanaman *Hevea* yang memberangsangkan. Kebanyakan klon komersial yang sedia ada masih lagi tidak pernah diuji potensi untuk diperbaharui melalui kultur tisu, lebih banyak klon perlu dikaji. Buat pertama kalinya propagasi *in vitro* klon RRIM 929 *H. brasiliensis*, sejenis klon lateks balak (KLB) telah dikaji melalui kaedah-kaedah kultur embrio, embriogenesis somatik dan pemotongan mikro dengan menggunakan anak benih dan pokok cantuman dalam kajian ini. Kadar percambahan kultur embrio secara *in vitro* sebanyak 51% telah dicapai apabila embrio dikultur dalam medium MS yang mengandungi 5 mg/L BA and 1 mg/L IBA. Pensterilan permukaan dengan menggunakan larutan Clorox 30% selama 25 minit telah berjaya mengurangkan kadar kontaminasi kultur kepada hanya 10 %. Pucuk yang diperolehi daripada pemotongan mikro hujung pucuk dan hipokotil anak benih dapat berakar apabila dikultur dalam medium MS penuh yang mengandungi 1 mg/L IBA dan rawatan perangsang dengan menggunakan larutan berkepekatan tinggi NAA: IBA. Kesan pelbagai medium basal seperti Murashige dan Skoog (MS), medium Woody Plant (WPM), Gamborg (B5) dan medium Microbouturage (MB) terhadap pembungaan tunas askilari dari pokok cantuman telah dikaji. Medium MS dan MB yang ditambah dengan BA dan IBA yang mengandungi 3% sukrosa, memberi

peratusan tertinggi dalam menghasilkan pucuk dari tunas aksilari. Penambahan baja Nitro Blue ke dalam medium meningkatkan peratusan kultur yang bertindak balas terhadap medium dan mengalakkan pemanjangan pucuk. Peninjauan kesesuaian kepelbagaian jenis eksplan dari pokok cantuman untuk induksi kalus telah dijalankan. Penyaringan terhadap bahagian tumbuhan yang berbeza seperti batang, daun, integumen tidak matang dari buah muda dan bunga yang tidak matang telah dijalankan dengan menggunakan medium MS yang dibekalkan dengan pengawal atur tumbersaran tumbuhan (BA, 2,4-D, KN dan NAA) dalam formulasi dan kepekatan yang optimum untuk induksi kalus yang terbaik. Kalus berjaya diinduksikan daripada kesemua eksplan dengan 3 jenis kalus yang berbeza dari segi morfologi dihasilkan. Semua eksplan yang telah diuji didapati dapat menginduksikan kalus dengan penggunaan medium MS yang ditambah dengan 1 mg/L NAA, 1 mg/L KN dan 1 mg/L 2,4-D. Antara eksplan yang diuji, hanya integumen yang tidak matang berjaya menghasilkan embrio somatik yang berjaya diperbaharui kepada anak benih apabila dikultur dalam medium MS yang mengandungi 2 mg/L BA dan 2 mg/L 2,4-D. Kesemua eksplan menunjukkan kadar induksi kalus yang tinggi dengan anter yang tidak matang menghasilkan kualiti kalus yang terbaik. Variasi somaklonal telah dikesan dalam kalus anter tidak matang yang diinduksikan dari pelbagai sitokinin (TDZ, KN, BA, Zeatin and 2iP) bersama auksin dengan menggunakan empat pasangan primer penanda SSR getah (*hmac4, hmac5, hmct1, hmct5*). Kesan asid glutamik dengan julat kepekatan antara 0.25-2.5 mg/L terhadap induksi kalus dengan menggunakan anter yang tidak matang sebagai eksplan telah dikaji. Pertambahan ketara dalam berat basah kalus (BBK) dicapai dengan penambahan BBK sebanyak 182% secara purata apabila 0.5 mg/L asid glutamik ditambah ke dalam medium MS induksi kalus. Keputusan yang didapati daripada kajian ini menyokong penggunaan

klon RRIM 929 dalam kajian perkembangan kultur tisu *Hevea* seterusnya kerana ia memang dapat diperbaharui melalui kultur tisu.

TISSUE CULTURE OF *Hevea brasiliensis* MÜLL. ARG. LATEX TIMBER CLONE RRIM 929

ABSTRACT

Rubber tree or *Hevea brasiliensis* Müll. Arg. is a commercially important crop widely cultivated in Southeast Asia for its ability to produce natural rubber in the form of latex. This perennial crop plant is known for its recalcitrance in rooting and only limited clones could be regenerated via tissue culture. Clone that could be regenerated via tissue culture will be a promising tool for *Hevea* crop improvement. Many available commercial clones have yet to be tested for their potential in regenerated via tissue culture, more clones should be explored. In this study, *H. brasiliensis* clone RRIM 929, a latex timber clone (LTC) has been studied for *in vitro* propagation via embryo culture, somatic embryogenesis and microcutting from both seedling and bud-grafted plant material for the first time. *In vitro* embryo culture of this LTC was successfully achieved with 51% germination rate when cultured in full MS medium supplemented with 5 mg/L BA and 1 mg/L IBA. Surface sterilization of seeds in 30% Clorox solution for 25 min has successfully reduced the culture contamination rate to only 10%. Shoots obtained from microcutting of seedlings-derived shoot tips and hypoctyl could be rooted on full MS medium containing 1 mg/L IBA and pulse treatment with concentrated NAA:IBA solution. The effect of different basal medium such as Murashige and Skoog (MS), Woody Plant Medium (WPM), Gamborg (B5) and Microbouturage medium (MB) were studied on the bud break of axillary bud from clonal materials. Both MS and MB basal medium were found to produce the highest percentage of shooting from the axillary bud with the supplementation of BA and IBA with 3% sucrose. Further supplementation of the medium with commercial fertilizer Nitro Blue increased the percentage of culture responding to medium and promoted

shoot elongation. The suitability of different explants from mature clonal materials for callus induction has been evaluated. Different plant parts such as stem, leaf, immature integument from young fruits and immature inflorescence were screened with MS medium supplemented with plant growth regulators (BA, 2,4-D, KN and NAA) in optimized formulation and concentration for the best callus induction. Callus were successfully induced in all explants, producing 3 different types of calli with distinct morphology. All tested explants were found to be capable of initiating callus using MS supplemented with 1 mg/L NAA, 1 mg/L KN and 1 mg/L 2,4-D. Among the explants tested, only immature integument managed to produce cotyledon stage somatic embryo which were successfully regenerated into plantlets when cultured on full MS medium supplemented with 2 mg/L BA and 2 mg/L 2,4-D**.** All explants showed high callus induction rate with best callus quality were obtained from immature anther. Somaclonal variations in immature anther-derived callus initiated by various cytokinin (TDZ, KN, BA, Zeatin and 2iP) coupled auxin was detected by SSR markers using four primer pairs from rubber (*hmac4, hmac5, hmct1, hmct5*). The effect of glutamic acid with concentration ranging from 0.25-2.5 mg/L on callus induction using immature anther as explant were investigated. Significant increase in callus fresh weight (CFW) with increment of 182 % in average CFW was achieved with the addition of 0.5 mg/L glutamic acid into MS-based callus induction medium. The results obtained in this research support the use of clone RRIM929 in further *Hevea* tissue culture development study as this clone proved to be able to regenerate via tissue culture.

CHAPTER 1

INTRODUCTION

Hevea brasiliensis Müll. Arg. commonly known as rubber tree or Pokok Getah in Malaysia is one of the most economically important crop plant in the world especially to tropical countries around the equator. *H. brasiliensis* Müll. Arg. is a perennial tropical crop tree belonging to the family of Euphorbiaceae. Originating from the native habitat of Amazonian basin in Brazil, *H. brasiliensis* was introduced to Europe and subsequently to South East Asian through Sir Henry Wickham in 1876 (Wycherley, 1969). Some 70,000 seeds (later known as Wickham's Collection) were taken from Brazil to Royal Botanic Gardens at Kew, England. These seeds with narrow genetic base had since served as the base material for the subsequent development and spread of today's millions of rubber crop in plantations across Asia and Africa (Priyadarshan, 2017a).

Natural rubber (NR) occurring as *cis*-1,4-polyisoprene is obtainable almost exclusively from *H. brasiliensis* in the form of latex and is a unique biopolymer of strategic importance. In many of its most significant applications, NR cannot be replaced by synthetic rubber alternatives (van Beilen and Poirier, 2007). Due to *H. brasiliensis*'s ability to produce latex from its specialized latex producing cell – laticifer, this crop tree has since became the economic generator for developing countries such as Malaysia and India since the dawn of automobile industry. This has made *H. brasiliensis* one of the most economically important member of the *Hevea* genus. *H. brasiliensis* has since been widely exploited worldwide for its latex production as the primary source of NR (Schultes, 1993). A breakthrough in the development of the rubber industry with the invention of the vulcanization process in

1839 sets off widespread rubber planting across countries in Southeast Asia for the tapping of latex for the production of rubber products (Mooibroek and Cornish, 2000). Since then, *H. brasiliensis* has been cultivated in over 40 countries and on more than 26 million hectares of land as reported by MRB (2016b). Malaysia is among the earliest country in the world to start with the research and developmental work on *Hevea* cultivation (Wycherley, 1959).

With the increasing demand for NR sources in tyre and other rubber industries, rubber planting alone has generated an annual GDP of RM 6 billion during the peak era of rubber plantation industry. According to ANRPC, worldwide consumption of NR increased by 5.2%, year-on-year, to 8.158 million tons during the first seven months of 2018. Demand of NR observed a growth at 5.2%, amounting to 14.017million tonnes, from 12.243 million tonnes of previous year (ANRPC, 2018). Breeding of *H. brasiliensis* was studied extensively and were mainly focused on finding superior and elite clones which can produce higher latex yield associated with good secondary characteristic such as disease tolerance. Many clones were produced and introduced by Rubber Research Institute of Malaysia (RRIM), Rubber Research Institute of India (RRII) and Rubber Research Institute of Ceylon (RRIC) with the yield gradually increasing throughout decades of research. With the advances of knowledge in agricultural technology, the focus of *Hevea* research was no longer restricted to only finding clones with higher yield. Study has since shifted towards producing *Hevea* clones with better secondary characteristics such as disease and draught resistance and higher wood volume rather than merely high yielding (Ramli *et al.*, 1994; Mignon and Werbrouck, 2018).

The unprecedented climate change due to global warming and the declining of agricultural land area for rubber cultivation have driven the scientific focus of *H.*

brasiliensis research to engineering commercially available elite clones with desirable traits of agronomical interest to accommodate the need for sustaining *H. brasiliensis* tree in a wider planting zone further from the traditional planting area surrounding the equator (Rekha, 2013). Transgenic plants of *Hevea* integrated with osmotin gene and superoxide dismutase gene for abiotic stress tolerance were successfully developed and regenerated by RRII researchers (Jayashree *et al*., 2003; Rekha *et al*., 2014).

Hybridization coupled with vegetative propagation and clonal selection is the most important conventional method of genetic improvement in *Hevea* breeding programs. This, however is an extremely time consuming process and is difficult to achieve with recalcitrant woody species like *H. brasiliensis*. This crop tree is also well known for its recalcitrant nature in rooting. Hence, in commercial nurseries, the conventional propagation of commercially elite clones were carried out by grafting of clonal axillary buds (scion) onto unselected seedlings (rootstock) producing a 2-part tree (Priyadarshan, 2017a). Propagation of a 2-part tree however does not produce true-to-type plants. The interaction between the scion and rootstock were found to affect the genetic potential of the clone (Montoro *et al*., 2012). A true-to-type 1-part tree will be able to avoid the scion-rootstock interaction by producing a more uniform farming material. Hence, clonal propagation of *H. brasiliensis* by tissue culture techniques serve as a powerful tool to bring the rubber industry to a greater extent with a reliable regeneration protocol developed for selected genotype (Mignon and Werbrouck, 2018).

Research on tissue culture of *H. brasiliensis* started as early as 1953 by Bouychou at Institut Francais Caoutchouc. After more than a decade, the Rubber Research Institute of Malaysia only has taken up the research by Chua (1966) (Arokiaraj *et al*., 1994; Nayanakantha & Seneviratne, 2007). The ability to obtain and to express specific foreign or native genes in *H. brasiliensis* (Venkatachalam *et al.*,

2007) brighten the possibility of improving *H. brasiliensis* by genetic manipulation. Agronomic traits of interest can be introduced without compromising the genetic background of the elite clones provided that a reliable plant regeneration method is available. *H. brasiliensis* tissue culture were usually established from immature anther, inner integument and immature inflorescence using modified Murashige and Skoog medium (Ighere *et al*., 2011; Sunderasan *et al*., 2012; Wang *et al*., 2013). With the recent development of suitable regeneration protocols, somatic embryogenic calli derived from immature anthers and inflorescences of *H. brasiliensis* are emerging as suitable target tissues for genetic transformation experiments (Jayasree *et al*., 1999). *H. brasiliensis* tissue culture can be used to produce transgenic *Hevea* plants with desirable agronomic traits quickly and more efficiently as well as to introduce genes that can encode high-value recombinant proteins such as human atrial natriuretic factor, a peptide hormone, human serum albumin and osmotin gene where all these were achieved by Arokiaraj *et al*.(2002), Sunderasan *et al*. (2012) and Rekha *et al*. (2014).

H. brasiliensis has demonstrated strong genotypic response towards culture media and often reacted differently with different regeneration protocol. Specific regeneration protocols are required for different clones of *H. brasiliensis* if they are to be propagated *in vitro* by tissue culture (Mignon and Werbrouck, 2018). With many genotypes of *H. brasiliensis* having been introduced by different rubber research board worldwide, micropropagation of *H. brasiliensis* from mature plants of commercial clones are still limited. Only limited clones have been reported to be successfully propagated *in vitro* by tissue culture (Nayanakantha & Seneviratne, 2007). Many limitations and challenges are yet to be resolved in the microprogation of *H. brasiliensis*. With the emergence of newer clones, the potential of new *H. brasiliensis*

clone to be developed and propagated in tissue culture with desirable agronomic traits is possible.

In this study, *H. brasiliensis* clone RRIM 929, a latex timber clone (LTC) was chosen for the establishment of *in vitro* plantlets via somatic embryogenesis and micropropagation for further genetic transformation studies. This clone, does not only produce sufficient yield of NR but also produce wood suitable for furniture production. The potential for clone RRIM 929 for propagation via tissue culture techniques has never been reported, hence this will be the first attempt on developing the tissue culture methods of this LTC. This study will also be useful in further transformation attempts in developing of genetic manipulated rubber clone.

1.1 Objectives

The objectives of the present study are:

i. To establish tissue culture of *H. brasiliensis* clone RRIM 929 from seedling material and to attempt acclimatization of the regenerated plantlets.
ii. To establish tissue cultures of *H. brasiliensis* clone RRIM 929 with juvenile and mature bud-grafted clonal material via somatic embryogenesis and microcutting.
iii. To study the effect of immature anther's age, as determined by the day after flower bud emerge (DAFe) and cytokinin on callus induction of *H. brasiliensis* clone RRIM 929.
iv. To determine the effect of basal medium, glutamic acid and commercial fertilizer incorporated mediums towards tissue culture of *H. brasiliensis* clone RRIM 929.

CHAPTER 2

LITERATURE REVIEW

2.1 *Hevea brasiliensis (Willd. ex A. Juss.)* Müll Arg

2.1.1 History

Hevea brasiliensis (Willd. ex A. Juss.) Müll Arg is a perennial tropical tree originated from the Amazonian basin of South America (Imle, 1978; Wycherley, 1992; MRB, 2009). It is indigenous to the rainforest in countries such as Brazil, Bolivia, Colombia, Ecuador, French Guiana, Guyana, Peru, Suriname and Venezuela (Wycherley, 1992, Priyadarshan, 2007, Lim, 2012).

The initial name of the plant was pará rubber tree which was derived from the name of the province where most latex was extracted and exported from in Brazil when latex was first exploited. The Spanish call it *caucho* to indicate the ecological origin of the majority of rubber-bearing plants. British scientist Joseph Priestly coined the word 'rubber' because of the ability of rubber to rub out pencil marks (Nair, 2010). A complete list of the vernacular name of this plant according to various countries is listed in Lim (2012). In Malaysia, it is commonly known as *Pokok Getah*. French botanist, Jean Baptiste Fusée Aublet published the first taxonomic description of the genus *Hevea* in 1775 whereby the word *Hevea* is a Latinized version of the Ecuadorian Indian name, *Hheve* (Nair, 2010). The taxonomy of the genus has since undergone considerable changes throughout the years and *H. brasiliensis* was finally brought under the genus *Hevea* by Jean Mueller Argoviensis in 1865 (Wycherley, 1992). An elaborate description of the taxonomical aspects of *Hevea* has been reviewed by Schultes (1949, 1970) and Wycherley (1992).

The existence and use of the crude products of *Hevea* was found as early as the 15th century whereby tree gum used by the natives to make bouncing balls caught the

attention of the first European visitors to the New World when Columbus discovered America (Imle, 1978). Since then, raw rubber was exported to Europe from time to time. The discovery of vulcanization by Goodyear in 1839 and the rapid growth of the automotive industry in the late 19th century propelled high demand for NR, hence setting off the widespread planting of rubber trees around the world (MRB, 2009).

H. brasiliensis was introduced into the East and its exploitation was developed through the agency of various British botanical institutions mainly the Kew Garden in England and in the Southeast Asia by the Singapore Botanic Gardens (Wycherley, 1959). Efforts to cultivate the tree commercially on a wide scale back in its native country in South America were unsatisfactory because of the fungal disease known as South American leaf blight (SALB) (Priyardarshan, 2017). In 1876, Sir Henry Wickham collected 70,000 seeds of *H. brasiliensis* from Brazil, near Boim on the Rio Tapajoz where excellent wild rubber was produced. About 2700 of these seedlings were raised in Kew Garden, England. Soon after that, a total of 1919 of the seedlings were dispatched to mainly Sri Lanka and a few went to Malaysia, Singapore, and Indonesia (Wycherley, 1959, Nair, 2010). Most of the commercially planted Malaysian rubber trees and those covering millions of hectares of plantation across Southeast Asia are believed to have originated from the twenty-two surviving seedlings introduced by Wickham in 1876 (Imle, 1978; Ramli *et al.*, 1994; Priyadarshan, 2007). *H. brasiliensis* has since been developed remarkably from a wild Amazonian jungle tree to a worldwide major domesticated crop within a span of about four decades.

2.1.2 Botany

H. brasiliensis (Willd. ex A. Juss.) Müll Arg is the main cultivated species for obtaining natural rubber among nine others in the genus *Hevea* and belongs to the family of Euphorbiaceae (Chen, 1984, Lim, 2012). Wild *Hevea* is defined as a megaphanerophyte back in its native habitat in the Amazonian forest with an average stem girth of 250 cm and over 30 m in height but the cultivated *H. brasiliensis* are usually much smaller in size (Chen, 1984). The crown of *H. brasiliensis* are found in a conical shape with a broad base made up of spirally-arranged trifoliate compound leaves attached to a long stalk or petioles. Plate 2.1 shows a typical *H. brasiliensis* tree in a Malaysian rubber plantation. Young leaflets are bronze and slowly turn into dark green upon maturation (Lim, 2012) as shown in Plate 2.2. In Malaysia, the rubber tree defoliates twice a year during the short spell of dry weather usually at the beginning of the year and around August/September. The trunk is cylindrical in shape with a swollen, bottle-shaped base. It consists of a central pith surrounded by layers of bark where the latex vessels-laticifers are found in the outer most layer. This is where it oozes latex upon tapping which make this plant economically important (Petch, 1911). Plate 2.3 shows white latex oozing out from a newly tapped bark of a young *H. brasiliensis* tree. *H. brasiliensis* is a monoecious flower-bearing tree with both the male and female flowers found on the same panicle (Yeang, 2007). The inflorescences are of creamy-yellow colour and without petals. Male flowers are slightly smaller and numerous while female flowers are few, larger, and occupy the terminal end of the panicle as shown in Plate 2.4. A detailed description of the floral structure has been given by Yeang and Ong (1988), Nair (2010) and Lim (2012). It has a main flowering season annually and a minor secondary flowering which depending on the location and cultivated clone (Yeang, 2007).

Plate 2.1 *H. brasiliensis* in a typical rubber plantation in Malaysia. Bar represents 50 cm.

Plate 2.2 Bronze young leaflets and mature green leaves of *H. brasiliensis*. Bar represents 5 cm.

Plate 2.3: White colour latex oozing out from a freshly tapped bark of a young *H.brasiliensis* tree. Bar represents 2 cm.

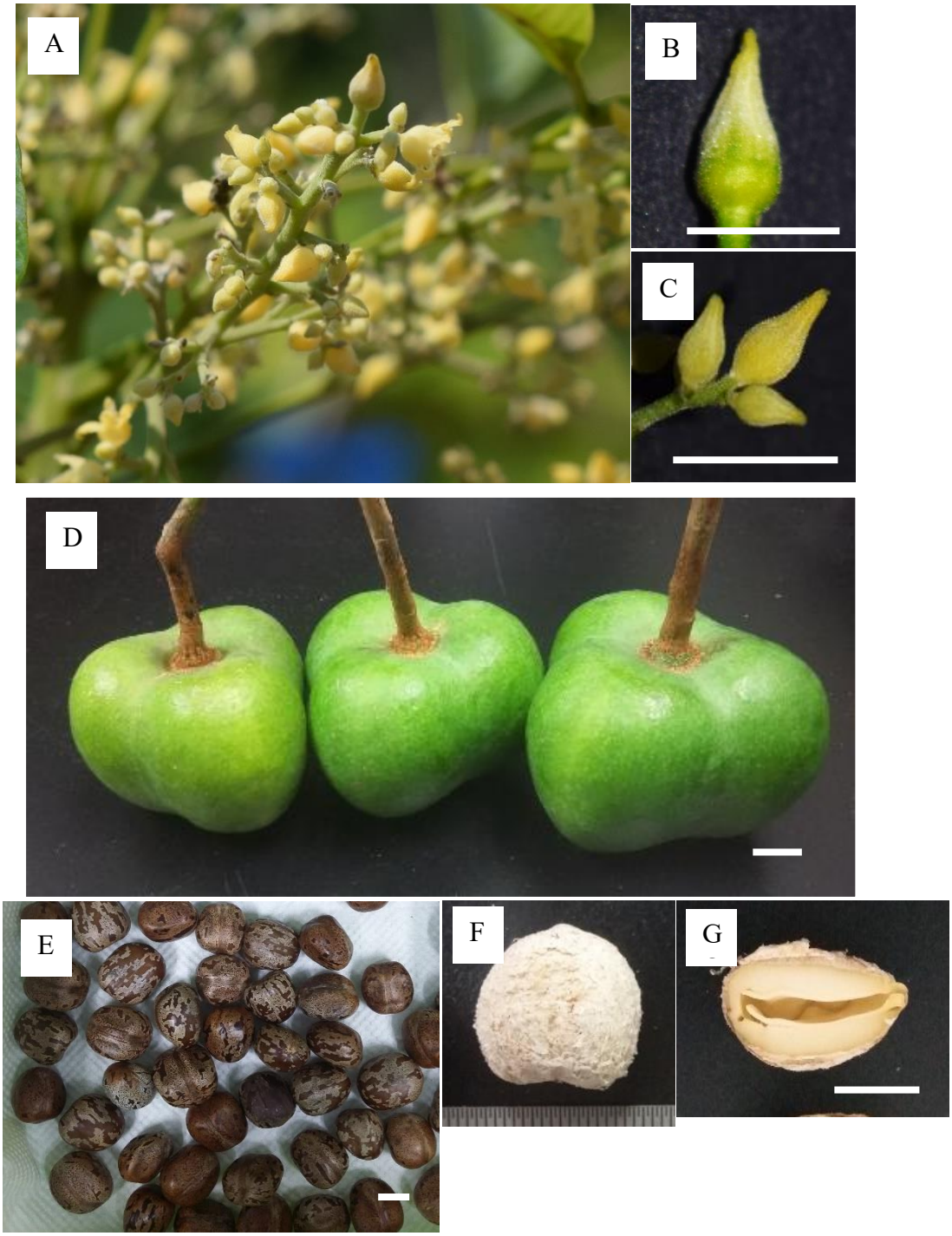

Plate 2.4: Explants of *Hevea* tissue culture. (A) Inflorescence (panicles) of *H. brasiliensis*; (B) Hairy female flower; (C) Hairy male flower; (D) Young green 3-lobed capsular fruits of *H. brasiliensis*; (E) *H. brasiliensis* seeds with distinct seed coat pattern (F) De-coated seed with the exposed kernel covered with tegmen and (G) Cross section of the kernel exposing the endosperm and cotyledon. (Bars represent 0.5 cm in B and C, 1 cm in D, E and G).

In Malaysia, the flowering season followed soon after the tree defoliates during March/April and August/September (MRB, 2009). Flowering is restricted to only a few months per year and depending on the clone and the surrounding weather conditions. Non-synchronization of flowering among different clones limits possible cross clonal pollination (Sedgley and Attanayake, 1988). Pollination is by insects and the fruits ripen in 5–6 months after fertilization (Nair, 2010). The tree produces woody capsular fruits usually having 3 lobes that contain a shiny seed in each lobe. Seeds are ejected from the capsules via explosion of the capsule upon ripening to disperse the seeds as far as 100 feet away from the mother tree (Gomez, 1982). Seeds of a single clone exhibit distinct visual characteristics in terms of seed coat pattern, size and shape which enable accurate visual identification (Gomez, 1982, MRB, 2009). Gomez (1982) has described the seed morphological characteristics in detail based on the seed of the clone Tjir 1. Plate 2.4 shows the inflorescence, fruits and seeds of *H. brasiliensis*.

H. brasiliensis is an interspecific hybrid; although *it* behaves as a diploid, it is proposed that *Hevea* has an amphidiploid origin (Ong, 1975; Lespinasse *et al.*, 2000). Detailed cytological investigations have confirmed the chromosome complement of *H. brasiliensis* in the somatic cells as $2n = 2x = 36$ (Ramaer, 1935; Nair, 2010).

Originated from regions with the mean annual temperature around 26-27 °C along with relative humidity of over 90 % and about 2500 mm annual rainfall (Chen, 1984), *H. brasiliensis* is a tropical crop which can survive within 1000 km north and south of the equator except for arid regions and can be planted to a maximum elevation of 500 m above sea level (MRB, 2009). It is currently being cultivated and planted in major natural rubber producing countries mainly in the tropical regions such as: Malaysia, Thailand, Indonesia, Sri Lanka, India, China, Vietnam, Cambodia, Myanmar, Bangladesh, Philippines, Papua New Guinea, Brazil, Mexico, Nigeria,

Ghana, The Ivory Coast and Central Africa (Chen, 1984, MRB, 2009, Nair, 2010, Lim, 2012, Anis and Ahmad, 2016). In Malaysia, a total of 1,315,000 ha area were used for the cultivation of *H. brasiliensis* in the late 1900's (Nair, 2010).

The plantations of *H. brasiliensis* in the peninsular Malaysia is mainly distributed in the northern region and states along the west coast such as Perlis, Kedah, Perak, Selangor, Negeri Sembilan and the two Eastern Malaysian states in Borneo - Sabah and Sarawak. A wild *H. brasiliensis* can live up to 100 years. However, in plantation, *H. brasiliensis* will only be cultivated for a lifespan of 30 years based on the feasibility of managing the tapping panel and the decline in latex production (Nair, 2010, Munasinghe and Rodrigo, 2018). Systematic classification and nomenclature of *H. brasiliensis* according to MoEF&CC (n.d.) is as follow:

Kingdom : Plantae
Division : Magnoliophyta
Class : Magnoliopsida
Order : Euphorbiales
Family : Euphorbiaceae
Subfamily : Crotonoideae
Tribe : Micrandreae
Sub-tribe : Heveinae
Genus : *Hevea*
Species : *brasiliensis* (Müll Arg)

2.1.3 *H. brasiliensis* clone RRIM 929

Clone RRIM 929 was introduced as the outcome of the Phase V (1966-1973) breeding program by Rubber Research Institue Malaysia (RRIM). A total of 43 cultivars produced under Phase V (1966-1973) breeding program have been selected and given numbers ranging from RRIM 901 to RRIM 943. Clone RRIM 929 is a cultivar derived from the cross between clone RRIM 605 × RRIM 725 (Ramli *et al.*, 1994). Clone RRIM605 is a high yielder while clone RRIM725 is a cultivar that exhibited high resistance against South American leaf blight disease (Silva *et al.*, 2014; Das *et al*., 2010). As the outcome from the cross, RRIM 929 is a clone with resistance to wind damage, moderate resistance against *Oidium* and *Colletotrichum*, and high resistance against *Corynespora* leaf disease (MRB, 2009). This clone is a latex timber clone (LTC) which is recommended for both latex and timber production in plantation (MRB, 2009). It was included in the MRB Planting Recommendations in year 2003 as one of the recommended planting materials, which implied that this clone is capable of producing high latex of up to 3,143 kg per hectare per year, and could produce about 1.20 m^3 of wood volume per tree after 21 years of planting (MRB, 2009).

Characteristic of each *H. brasiliensis* clone in the RRIM series are determined by intense and continuous observations of dominant features of seeds, leaves (shape, venation, orientation), branching patterns, trunk posture and latex color. (Ramli *et al.*, 1994). The specific characteristics of a clone, be it morphological features as mentioned or other secondary characteristics such as disease and wind resistance make it recognizably different from other clones. The key vegetative and morphological characteristics for RRIM 929 clonal identification are stated in Table 2.1. Specific morphological features of the leaves and seeds are shown in Plate 2.5 and 2.6 respectively.

Table 2.1: Key vegetative and morphological characteristics for clonal identification of clone RRIM 929 (UPOV, 2009)

Organ	Characteristics	Description
Leaf	Leaflet positions	Separated
	Intensity of green colouration on adaxial surface	Dark
	Glossiness of adaxial surface	Medium
	Adaxial surface texture	Smooth
	Leaflet blade attitude relative to petiole	Semi drooping
	Orientation of broadest part in leaf blade relative to leaf length	Towards apex
	Axis in longitudinal section of leaflet blade	Convex
	Margin undulation	Medium
	Shape at base	Cuneate
	Shape of apex	Acuminate
Petiole	Attitude	Semi erect
	Length	Small
	Width	Medium
Seed	Thickness	Thin
	Shape from dorsal view	Obovate
	Colouration	Faded, light brown
Trunk	Main colour of bark	Light colour
	Axis	Long straight
	Texture of bark	Smooth
	Colour of latex	White

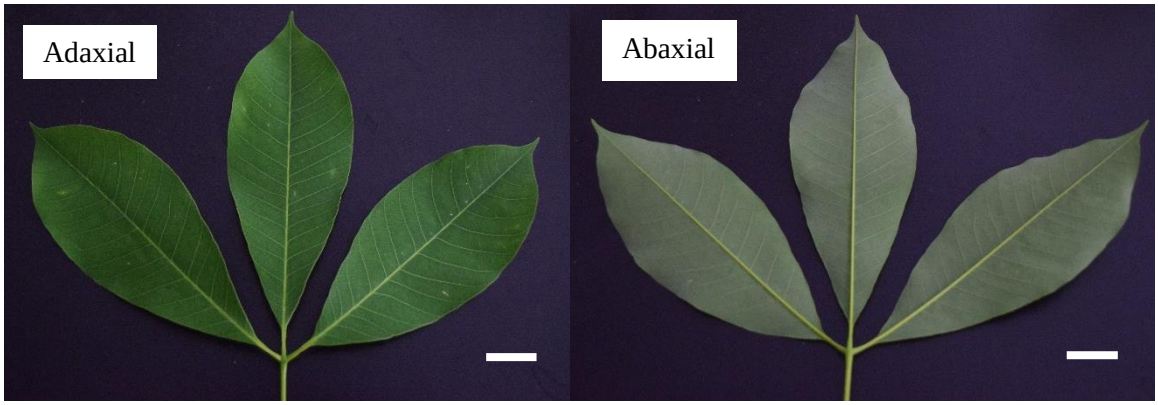

Plate 2.5: Trifoliate, mature and separated leaflets of *H. brasiliensis* clone RRIM 929 with adaxial and abaxial surface view showing medium undulated leaf margin. Bars represent 1 cm.

Plate 2.6: Dorsal and ventral view of the seed of *H. brasiliensis* clone RRIM 929. An obovate dorsal view of the seeds is the key feature of this clone. Bar represents 1 cm.

2.2 *H. brasiliensis* breeding and propagation: Objectives, achievements and limitations

The increasing demand for natural rubber mainly for the manufacture of tyres in the automobile industry and the uprising price were the main motivations for the rapid expansion of *H. brasiliensis* cultivation worldwide. Hence, breeders have been worked in tune by the interest and demand from growers as well as the rubber industry to release new cultivars with increasing yield from time to time.

In Malaysia, with the original Wickham gene pool, *Hevea* breeding was initiated 90 years ago since 1928. Breeding objectives were mainly focused on developing clones with higher latex yield and better secondary characteristics like high initial vigour and growth, abundant latex vessel, good bark renewal and resistance to major diseases such as Tapping Panel Dryness (TPD) (Priyadarshan *et al*., 2009). Clones with early attainment of tappable girth and high initial yield were also included as one of the breeding objectives. Specific breeding objectives vary depending on agro climatic and socioeconomic requirements in certain countries (Priyadarshan, 2017b).

The expansion of plantation in marginal and non-traditional areas has demanded priority for the development of clone resistant to prolonged drought, with high and low temperature tolerant during summer and winter season, strong winds resistance and adaptability to higher altitudes. Since rubber planters are predominantly small holders, breeding objectives were streamlined to take care of their specific needs as well (Nair, 2010). For the past few decades, systematic breeding with careful selection and evaluation have since resulted in several outstanding clones with substantial enhancement in latex productivity from about 500 kg/ha/year for unselected seedlings to about 3,000 kg/ha/year in the modern clones by various rubber research institutes around the world (Chen, 1984; Priyadarshan, 2007).

The development of new clones by conventional breeding method has traditionally relied on generating crosses and progeny lines by controlled hybridization between selected parental clones. Parental clone with good characteristics are crossed using hand pollination whereby the pollen grains from male anther are placed onto the stigma of female flower (MRB, 2009). Evaluation of obtained hybrids by screening and selection of promising recombinants will be subjected to further selective breeding and testing schemes (Priyadarshan, 2017b). In *H. brasiliensis*, the juvenile period is relatively long, ranging from 5 to 7 years until a plant can start to produce latex depending on environmental conditions and management practices. Hence, one testing cycle might take about 20 to 30 years before a new clone is released (Pethin *et al.*, 2015; Priyadarshan, 2017b).

In Malaysia, *H. brasiliensis* flowering is restricted to only a few months' time from February to April and July to September (MRB, 2009). Non-synchronous flowering in certain clones limits the possibility of accessing all possible cross combinations of different clones in the breeding program (Nair, 2010). However, study by Hamzah *et al.* (1999) and Kaewbunjong and Tongkaemkaew (2015) on pollen storage and induction of off-season flowering have since been proved successful in overcoming the limitation of non-synchronous flowering despite being only for specific Malaysian and Thai commercial clones. Poor fruit set following controlled pollination which demands further investigation is another setback that limits the hybridization progress (Sedgley and Attanayake, 1988; Yeang and Ong, 1988). Moreover, limitations such as long breeding and selection cycle, as well as insufficiency of land for field experimentation on newly developed genotype are making the breeding process somehow a more difficult task.

Propagation of *H. brasiliensis* can be done either by sexual propagation or by

vegetative propagation (MRB, 2009). Characteristics and performance of offspring produced from sexual propagations are various and inconsistent. Hence, vegetative propagation is preferred. Most of the planting materials available in the majority of plantations are vegetatively propagated by bud-grafting scions of desirable clone onto selected rootstocks originated from either unselected or clonal seedlings (Priyadarshan, 2007). This method of propagation produces almost exactly the same type of plant from which the scion was obtained. Despite the fact that *H. brasiliensis* can be easily propagated in this way, there are restrictions on the use of clonal root stocks, mainly due to the lack of taproot formation by cuttings obtained from mature plants and the recalcitrance in rooting of many *H. brasiliensis* clones (Paranjothy, 1987). Rooting of certain clonal cuttings have been made possible with root inducing chemicals, nevertheless, the success rate still remains low. The root system produced is fibrous or adventitious with the absence of tap root, as a consequence, causing susceptibility to drought and frequent uprooting (Seneviratne, 1996). Therefore, the propagation of rubber by conventional grafting of buds from selected clones on to unselected seedlings is still widely practiced. But this leads to another problem; root stock derived from cross pollinated seeds are heterozygous by nature and hence led to inevitable root stock-scion interaction causing undesirable intraclonal variation (Seneviratne and Flegmann, 1996).

The same drawbacks reappeared when cuttings of *H. brasiliensis* were propagated *in vitro* with microcutting technique (Mignon and Werbrouck, 2018). Cuttings from young seedlings could generally be propagated *in vitro,* but cuttings of shoots initiated from mature elite clones propagated from conventional bud grafting were still very much recalcitrant (Seneviratne, 1996) and rooting attempts had repeatedly failed (Nayanakantha and Seneviratne, 2007). *In vitro* propagation of *H.*

brasiliensis via microcutting was then not taken forward due to bottlenecks in the multiplication and acclimatization phases even though much effort had been made during the 1980s (Enjalric and Carron, 1982, Gunatilleke and Samaranayake, 1988, Mendanha *et al.*, 1998).

Somatic embryogenesis mediated regeneration of *H. brasiliensis* was established more than 3 decades ago (Mignon and Werbrouck, 2018). However, commercial clones have only been systematically regenerated during the last few decades (Carron *et al.*, 1995). Extensive experiments were carried out by many researchers to enhance the frequency of somatic embryo induction and plant regeneration. Studies were conducted to optimize culture conditions as well as nutritional and hormonal requirements during somatic embryogenesis (Michaux-Ferrière and Carron, 1989, El Hadrami *et al.*, 1991, Etienne *et al.*, 1993, Veisseire *et al.*, 1994, Montoro *et al.*, 1995, Etienne *et al.*, 1997, Blanc *et al.*, 2002, Zhou *et al.*, 2010). The intensive efforts invested in research on the somatic embryogenesis turned out to be fascinating as the associated rejuvenation allows vegetative multiplication of elite clones for studies of cryopreservation, genetic modification and genome editing. Undeniably, there are always some drawbacks. Published protocols of *H. brasiliensis* somatic embryogenesis are based on trial and error with limited number of clones only. With the genotypic dependent nature of *H. brasiliensis*, painstaking optimization of the basic protocol has to be performed for each and every new genotype (Mignon and Werbrouck, 2018).

Despite all the major constraints of the conventional breeding and propagation method, great progress had been achieved along the way since the booming of the rubber planting industry. High yielding modern clones were released with significant increment in yield (from 500 kg/ha/year to about 3,000 kg/ha/year) and with better

secondary characteristics (drought and disease resistance) (MRB, 2009; Priyadarshan, 2017b). Propagation methods has since made progress by switching from multiplication by seeds to propagation by budding, and subsequently with the most recent advancement in the development of new techniques, the *in vitro* micropropagation by tissue culture (Priyadarshan *et. al*., 2009). Substantial time and effort has been poured and yet much more is needed to overcome the existing constraints and to improve for the better.

2.3 Plant tissue culture: *In vitro* culture techniques

2.3.1 An overview

Plant tissue cultures refer to culture of any plant parts be it cells, tissues or organs in artificial nutrient culture media with control environments under *in vitro* condition (Loyola-Vargas and Ochoa-Alejo, 2018). It is usually initiated from pieces of isolated parts of a whole plants called the explant, and must be established and maintained under aseptic conditions to avoid microbial organisms in particularly bacteria and fungi from competing adversely for the provided nutrient (George, 1993; Thorpe, 2012). Tissue culture technology relies on the basis of plant cells totipotency, in which when appropriate chemical and physiological environment is provided, it should be capable of inducing any cell to regulate its metabolism, growth and development to regenerate the plant cell into a complete true-to-type plant (Hopkins and Hüner, 2008).

Plant tissue culture generally can be divided into either cultures of unorganized tissues or organized structures. Callus culture, suspension culture, protoplast culture and anther culture are typical unorganized tissue culture, while organ culture such as the culture of meristem, shoot tip, node, embryo and isolated roots are organized tissue culture according to George *et al.* (2007). Each type of culture have been applied to a

range of different purposes with micropropagation as the most extended application of tissue culture in commercial use (Loyola-Vargas and Ochoa-Alejo, 2018).

The success of a tissue culture is greatly influenced by several factors namely the nature of the culture medium, appropriate explant, elimination of microbial contamination and proper controlled environment conditions (George *et al.*, 2007; Loyola-Vargas and Ochoa-Alejo, 2018). The major focus of research during the past few decades in plant tissue culture has been in the manipulation of growth media and growth conditions. Numerous studies have been conducted aiming to optimize culture medium for specific plant species of interest and hundreds of media have since been developed (George, 1993; Kondamudi *et al.*, 2009). Formulating culture media with optimal concentration and ratio of auxin and cytokinin is deemed the most challenging part for a successful plant tissue culture. Optimization of protocol for every new species or cultivars studied has to be performed differently, this is due to different species or even cultivar has different exogenous hormonal requirement depending on the available endogenous phytohormone levels within the plant cell (Bhojwani and Razdan, 1986; Mignon and Werbrouck, 2018).

H. brasiliensis is one of the important commodity crops, tissue culture will come in handy to aid in solving problems and to provide studies on the agronomical traits. Tissue culture is proved to be of immense practical value as a powerful tool for true-to-type propagation, crop improvement, germplasm storage and secondary metabolites production in commercial application (Bhojwani and Razdan, 1986; Naik and Chand, 2011). Plant tissue culture might be the key to solve the global crisis of decreasing arable land due to climate change, increased urbanization, soil degradation and pollution by overcoming the disadvantages of seasonality, geographical and environmental constraints since *in vitro* culture is not limited by seasons, is available

all year round, has reduced possibility of disease, and enables easy distribution of *in vitro* propagated plants.

2.3.2 Development of *in vitro H. brasiliensis* tissue culture

Development of tissue culture for *H. b*rasiliensis is not a new and advance research work. Initial tissue culture of *H. brasiliensis* involved embryo culture to raise seedlings (Muzik and Cruzado, 1956; Chua, 1966; Paranjothy and Ghandimathi, 1975). The i*n vitro* culture work done are mostly directed towards micropropagation through shoot tip culture, nodal cultures, somatic embryogenesis and with the recent trend, genetic transformation. Overall, *in vitro* culture research of *H. brasiliensis* has led to three types of micropropagation techniques, namely, microcutting, short-term somatic embryogenesis and long-term somatic embryogenesis along with genetic transformation (Montoro *et al.*, 2010).

The first known *in vitro* culture of *H. brasiliensis* was carried out by Bouychou of the Institut Français du Caoutchouc in 1953 using initiated calli as convenient material to study the laticiferous system (Carron *et al.*, 1989). This work was then followed by RRIM, with successful callus initiation from the plumule sectioned from juvenile seedlings and noticeable root differentiation after 5 to 6 months were achieved. However, the callus failed to grow upon subculturing (Nayanakantha and Seneviratne, 2007). This was then followed by Wilson and Street (1975) whereby upon culturing stem derived callus in MS liquid medium with the use of PGRs, morphogenesis was obtained. The first successful attempt at continuous subculturing of the initiated callus was then established from anther culture by Satchuthananthavale and Irugalbandara (1972). Differentiation of roots and shoots from cotyledon cultured were reported by Paranjothy and Gandimathi in 1976, shoot apices from aseptic seedlings were cultured

and rooted plantlets were obtained but the plantlets could not be multiplied (Nayanakantha and Seneviratne, 2007). Enjalric and Carron (1982) achieved shooting from the stem nodes of juvenile greenhouse plants but was subsequently reported that the propagation of elite clonal stock material from stem cuttings was a failure due to poor rooting as reviewed by Venkatachalam *et al.* (2007). Later, Chen (1984) of the Chinese research team claimed to have successfully obtained complete plantlets from seedling stem segments with buds as well as from decotylated embryos followed by successful transfer of some of these plants to the field in 1984. However, the results were unpublished (Chen,1984). Carron *et al.* (1989) also obtained complete plantlets from culture of apices and buds of seedlings. Gunatilleke and Samaranayake (1988) used shoot tips from aseptically grown seedlings as explants for micropropagation. Te-chato and Muangkaewngam (1992) and Sirisom and Te-chato (2012) successfully produced multiple shoots from various explants derived from *in vitro* seedlings.

Plant regeneration via somatic embryogenesis was then achieved using various explant, namely, anther, inner integument of immature fruit and root (Carron *et al.*, 1995; Jayasree *et al.*, 1999; Wang *et al.*, 2005; Lardet *et al.*, 2009; Zhou *et al.*, 2010; Srichuay *et al.*, 2014a; Nor Mayati, 2015; Zhao *et al.*, 2015; Nor Mayati and Izilawati, 2017). The effect of various factors such as type and concentration of exogenous hormones, source and timing of explant collection, types of carbohydrates used and calcium concentration on somatic embryogenesis were studied extensively by various group of researchers all with the aim on improvising the success rate of somatic embryogenesis in *H. brasiliensis* (El Hadrami *et al.*, 1989; Auboiron *et al.*, 1990; El Hadrami *et al.*, 1991; Michaux-Ferriere *et al.*, 1992; Etienne *et al.*, 1993; Veisseire *et al.*, 1994; Etienne *et al.*, 1997; Blanc *et al.*, 2002; Srichuay and Te-chato, 2012; Srichuay *et al.*, 2014b).