P.M. Priyadarshan

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Preface to the Second Edition

The first edition of **Biology of *Hevea* Rubber** was published in 2011. Since then, the science of *Hevea* has made significant progress, especially in genomics that rapidly advances over the last 6 years, which prompted me to prepare a second edition. I am sure if a third edition is to be attempted in 2022, the science of *Hevea* will have been taken over by 'omics'.

Hevea rubber tree is an excellent example of how a soil-tree-atmosphere system can work in tandem. The retrieval of rubber through 'injuring' the tree on alternate days or once in three days or once in seven days is indeed, a universally unique arrangement that ensures income to the planter almost throughout the year. Every molecule of rubber is the end result of meticulous biochemical changes. The biology of *Hevea* rubber tree is a subject that encompasses basic sciences of physics, chemistry and mathematics that finally aggregates technology for the realization of its industrial utility. The science that deals with the origin, upkeep and latex harvest offers immense opportunities for a biologist to encounter and appreciate the intricacies underlying the phenomena that govern the biological processes occurring inside the tree. A book on the biology of *Hevea* rubber integrates such issues in a single volume.

Portions of chapters might have gone 'untouched', because progress in these are minimal, especially in conventional breeding since the improvement of *Hevea* has been largely taken over by genomics. Genomics has conquered every aspect of *Hevea* biology that a comprehensive account of any topic cannot be complete without dealing with it.

Striking a balancing chord between being informative to a beginner and a reference source to a scientist is an arduous job. In my attempt, I hope I have done justice to the hard work of my colleagues.

I am indebted to my wife Bindu and my daughter Sandra for their unflinching support. Finally, I thank Springer for agreeing to publish this revised edition.

Kerala, India P.M. Priyadarshan

Contents

Introduction 1

Natural rubber has been an essential commodity not only for the tyre industry but also for more than 50,000 products that hold elasticity as an attribute. Rubber is obtained from the exudates of certain tropical plants (natural rubber) or derived from petroleum and natural gas (synthetic rubber). Because of its elasticity, resilience and toughness (Table 1.1), rubber is the basic constituent of tyres used in automotive vehicles, aircraft and bicycles. A car has almost 30% of its components made of rubber. The prime source of natural rubber worldwide is *Hevea* rubber or *Hevea brasiliensis*. Some of the alternate sources of rubber are: *Manihot glaziovii* (Ceara rubber), *Manihot dichotoma* (Jeque rubber), *Castilla elastica* (Panama rubber), *Ficus elastica* (India rubber), *Funtimia elastica* (Lagos rubber), *Landolphia kirkii* (Landolphia rubber), *Cryptostegia grandiflora* and *C. madagascariensis* (Madagascar rubber), *Parthenium argentatum* (guayule rubber), *Taraxacum kok-saghys* (Russian dandelion) (Table 1.2).

The molecule of natural rubber is a *cis*-1, 4-polyisoprene (with a molecular mass of 10–10,000 kDa) and is the end product of around 21 biochemical steps. Production of a molecule requires at least 48 h. Shaving of bark of the tree (tapping) yields rubber latex from the turgid *laticifers* and the flow of latex ceases upon plugging of the *laticifers* that happens as a natural phenomenon after 3–4 h of latex flow. Latex is indeed produced by *laticifers* and is a white cytoplasmic colloidal suspension containing mainly rubber particles but also non-rubber particles, organelles, proteins and serum (d'Auzac et al. 1989). *Hevea* latex has a bimodal particle size distribution (Pendle and Swinyard 1991; Gomez and Hamzah 1989), containing spherical particles (Wood and Cornish 2000), smaller than 0.4 μm in diameter (SRP, small rubber particles) or larger than 0.4 μm (LRP, large rubber particles) (Ohya et al. 2000; Singh et al. 2003). Latex particles are negatively charged on their surface (Sansatsadeekul et al. 2011) and latex has a plasticity index (pI) between 3 and 5 (Ho and Ng 1979). This confers a colloidal stability of latex at basic pH, whereas latex coagulates at low pH. A rubber particle has been described as a hydrophobic core of polyisoprene surrounded by a complex lipoprotein layer (Wren 1941; Nawamawat et al. 2011). There are two classes of rubber particles in *Hevea* latex: the large rubber particles (LRPs) with REF (Rubber Elongation Factor) located on their surfaces and the small rubber particles (SRPs) that are coated with SRPP (small rubber particle protein) (Oh et al. 1999; Berthelot et al. 2014). SRPs are far superior in number, accounting for 94% of all rubber particles in the latex, whereas LRPs constitute only the remaining 6% of the rubber particles. However, it is precisely this 6% of rubber particles by number that makes up 93% of the rubber by volume in the latex (Yeang et al. 1995). A striking expansion of the REF/SRPP gene family and its divergence into several laticifer-specific isoforms seem crucial for rubber biosynthesis (Tang et al. 2016). A pivotal point in *Hevea* evolution was the emergence of REF1, which is located on the

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Table 1.1 Properties of NR

Item	Attribute	Properties
Physical properties	Glass transition temperature (°C)	–70
	Melting temperature (°C)	25
	Hardness range (Shore A)	30–100
	Maximum elongation (at 70 °F, %)	750
	Specific gravity	0.915
	Solubility	Insoluble in water, alcohol, acetone, dilute acids and alkalis; soluble in ether, carbon disulphide, carbon tetrachloride, petrol and turpentine
Tensility and vulcanization	Tensile strength Before break	Very strong 2475 psi 13.9 n/mm^2
	Maximum tensile strength (at 70 °F, psi)	4000
	Vulcanizes at	307 °F/ 152 °C
Advantages	Physical resistance	Excellent resilience
		Excellent tear strength
		Excellent abrasion resistance
		Excellent impact strength
		Excellent cut growth resistance
		Good compression set
	Environmental resistance	Excellent water resistance
		Good low temperature flexibility
		Good oxidation resistance
	Chemical resistance	Good resistance to alcohols and oxygenated solvents
		Good resistance to acids
Limits	Environmental resistance	Poor ozone resistance
		Poor sunlight resistance
		Very little flame retardance
	Chemical resistance	Poor oil and gasoline resistance
		Poor resistance to (aliphatic and aromatic) hydrocarbon solvents

surface of large rubber particles that account for 93% of rubber in the latex. The latex available as such is processed further to make sheets and centrifuged latex (*cenex*). The coagulated latex left out over the tapping panel and collection cup are transformed into crepes and block rubber. Such processed rubber is the raw material for making innumerable end products with industrial applications.

Latex works as a defence media against natural enemies and a literature summary shows records in 40 families and more than 20,000 species are estimated to bear laticiferous structures (Lewinsohn 1991). Euphorbiaceae, Apocynaceae, Asclepiadaceae, Asteraceae, Moraceae, Papaveraceae and Sapotaceae are the prominent families that own these species (Archer and Audley 1973; Heywood 1978; Backhaus 1985; John 1992; Cornish et al. 1993) (Table 1.2). But there is another version saying 20 botanical families, 900 genera and 12,500 species produce latex, of which eight families, 300 genera, or 1800 species, produce rubber in their latex (Metcalfe 1967). The history of rubber production shows attempts to identify alternate sources of rubber, both temperate and tropical (Hall and Goodspeed 1919; Buchanan et al. 1978; Carr and Bagby 1987; Bowers 1990), but a few plant species known to produce rubber are capable of producing large amounts of high-molecular-weight rubber (Mooibroek and Cornish 2000). Of those, only *Parthenium argentatum* Gray is currently commercially produced on a small scale under semi-arid regions (Cornish 2001). Russian

Table 1.2 Selected rubber-yielding species (other than *Hevea*)

Scientific name	Common name	Distributional range
Castilla elastica Sessé	Panama rubber tree	*America* (Mexico; Central America; Western South America) widely naturalized in tropics
Ficus vogelii (Miq.) Miq.	West African rubber tree	*Africa* (Micronesia; Northeast Tropical Africa; East Tropical Africa; West-Central Tropical Africa; West Tropical Africa; South Tropical Africa; South Africa; Western Indian Ocean)
Funtumia africana (Benth.) Stapf	Lagos silk rubber tree	*Africa* (East Tropical Africa; West-Central Tropical Africa; West Tropical Africa; South Tropical Africa)
Manihot glaziovii Muell. Arg.	Ceara rubber	Native to Brazil, Colombia; exotic to Gambia, Ghana, Kenya, Malaysia, Nigeria, Senegal, Sierra Leone, Singapore, Sri Lanka, Tanzania, Uganda
Holarrhena floribunda (G. Don) Durand & Schinz	False rubber tree	*Africa* (West-Central Tropical Africa; West Tropical Africa)
Funtumia elastica Stapf	Lagos silk rubber	*Africa* (Northeast Tropical Africa; East Tropical Africa; West-Central Tropical Africa; West Tropical Africa) also cultivated elsewhere
Ficus elastica Roxb.	Indian rubber plant	*Asia-Tropical* (India; China; Malaysia)widely cultivated elsewhere
Parthenium argentatum Gray	Guayule	*Northern America* (South-Central USA; Mexico)
Taraxacum kok-saghyz Rodin	Russian dandelion	*Asia-Temperate* Former Soviet Union; China
Cryptostegia grandiflora R. Br.	Palay rubber	south, central and north America, Asia and Africa

See Chap. 6 'Genetic Resources'

dandelion (*Taraxacum kok-saghyz* Rodin) is yet another rubber-yielding species that is fast coming up for temperate regions (see Box 1.1). At least two fungal species (*Lactarius deceptiva* and *Peziza* spp.) are also known to make natural rubber (Stewart et al. 1955). The Euphorbiaceae family is extremely diverse and considered to be polyphyletic (Webster 1994). It is characterized by typical pentamerous flowers and a tricarpellar ovary.

Hevea brasiliensis (Willd. Ex. A. de. Juss. Müll-Arg.) is the almost exclusive contributor towards natural rubber produced worldwide (Greek 1991). It belongs to Euphorbiaceae, a large family of about 300 genera and 7,500 species. *Hevea* trees descended from seedlings transplanted from Brazil to South and Southeast Asia, that have undergone several cycles of breeding are now the prime source of the modern world's natural rubber. Natural rubber is produced in Southeast Asia (92%), Africa (6%) and Latin America (2%). The main producing countries are (by descending order): Thailand (4.17 million t in 2014, accounting for around 37% of global output), Indonesia, Malaysia, India, China, Vietnam and also Sri Lanka, Brazil, Liberia, Côte d'Ivoire, the Philippines, Cameroon, Nigeria, Cambodia, Guatemala, Myanmar, Ghana, Democratic Republic of Congo, Gabon, Bangladesh, Laos and Papua New Guinea (Fig. 1.1).

The latex found in the inner bark of *H. brasiliensis* is obtained by tapping—shaving the bark with a sharp knife—and collection of latex in cups (Fig. 1.2a–c). Addition of acid, such as formic acid, solidifies rubber. The solidified rubber can then be pressed between twin rollers to remove excess water to form sheets. The sheets are commonly packed in bales for shipping. Rubber is also commonly transported in the form of concentrated latex derived through centrifugation. The strip of latex coagulated on the tapping panel (lace) and the lump left out in the cup (cup lump) that form the 'scrap' of commerce also fetches income to the planter. Despite the competition of synthetic rubber, natural rubber continues to hold an important place; its resistance to heat build-up makes it valuable for tyres used in racing cars, trucks, buses and aircraft. *Hevea* rubber is

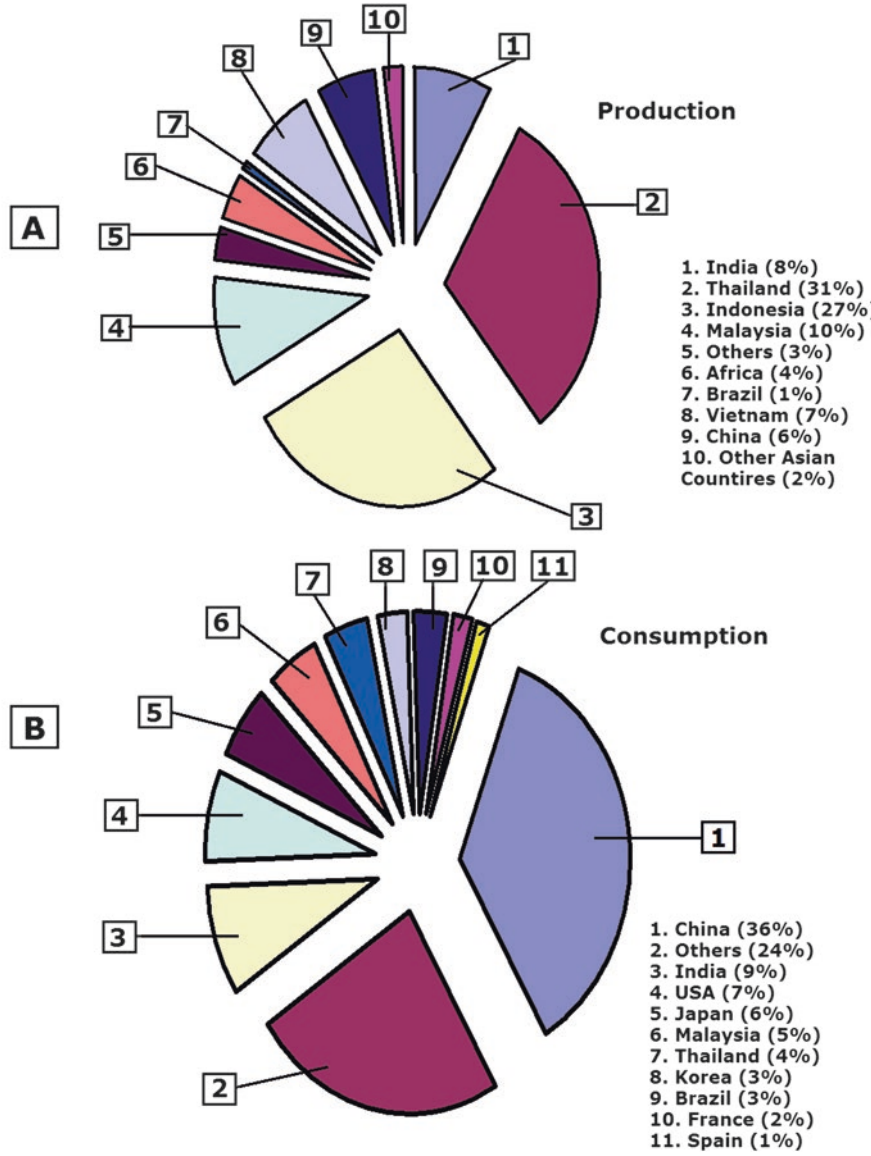

Fig. 1.1 Country-wise production and consumption of natural rubber

depicted in ancient religious documents from Mexico dating back to AD 600 (Serier 1993). Columbus gave the first description of rubber in 1496, and astronomer Charles Marie de la Condamine was the first to send samples of the elastic substance called 'caoutchouc' (the French word meaning 'weeping wood') from Peru to France in 1736 with full details about habit and habitat of the trees and procedures for processing (Dijkman 1951; Baker 1996). Natural rubber was first scientifically described by C.M. de la Condamine and François Fresneau of France following an expedition to South America in 1735. The English chemist Joseph Priestley gave it the name 'rubber' in 1770, when he found it could be used to rub out pencil marks. As a botanist, Fusée Aublet described the genus *Hevea* in 1775. Charles Macintosh in 1818 discovered waterproofing and Thomas Hancock in the 1820s invented mastication by developing a 'prickle' masticator, which gave a homogeneous ball of rubber. But raw rubber did not withstand the

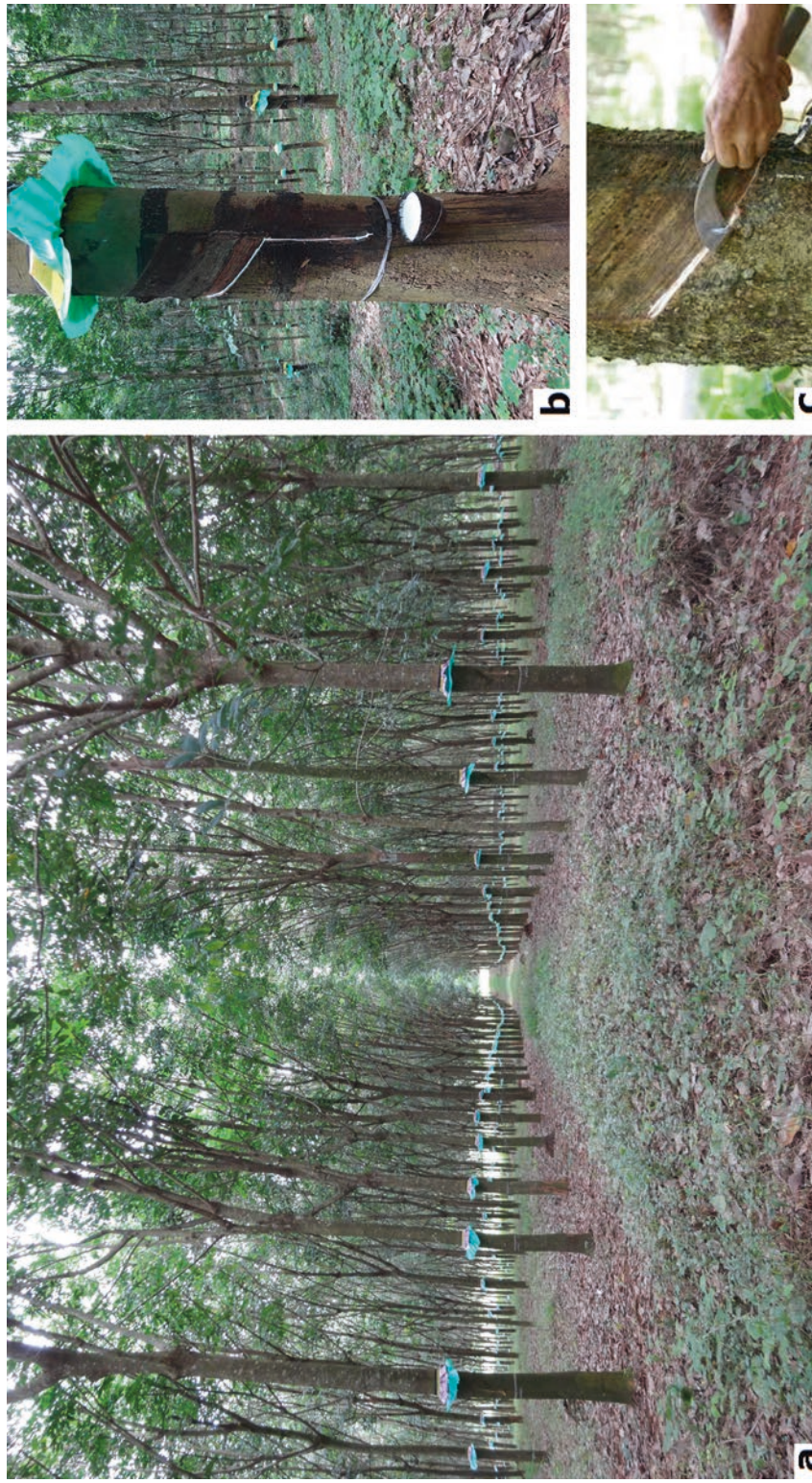

Fig. 1.2 (**a**) Rubber plantation (**b**) Tapped rubber tree (**c**) Tapping of a tree

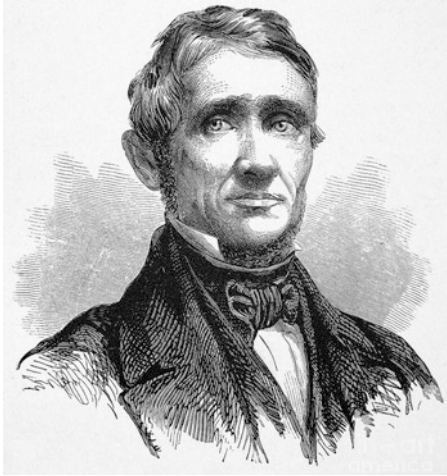

Fig. 1.3 Charles Goodyear (December 29, 1800–July 1, 1860)

extreme changes in temperature and this prompted Charles Goodyear (Fig. 1.3) to discover vulcanization in 1839 (heating rubber with sulphur), which gave explosive advancements in product manufacturing.

Research on the chemistry of natural rubber in the nineteenth century led to the isolation of isoprene, the chemical compound from which natural rubber is polymerized. Polymerization, the process by which long chain-like molecules are built up from smaller molecules, attracted continued research in the early twentieth century. Rubber derived from *H. brasiliensis* is predominantly constituted of *cis*-1,4-polyisoprene $(C_5H_8)_n$ where n may range from 150 to 2,000,000. Carbonyl groups were also detected which significantly help the degree of cross-linking and storage hardening (Pushparajah 2001). The possible roles of latex in plants, though unclear so far, have been suggested as: (i) to provide protection from predation; (ii) to provide a source of stored carbon and moisture; and (iii) to counteract ozone injury (Hunter 1994). One of the earlier arguments says that latex provides *Hevea* with a protective function against boring pests (such as beetles) with its coagulating ability to entrap them in the exuding latex which then self-seals the wound (Sharples 1918). However, further detailed research will only give an insight into the phenomenon of the functions of latex, which is essentially an extensive subject.

The rubber available in the nineteenth century was of varying quality and of uncertain supply when the demand was only for waterproofing of fabric and making of shoes. However, during the second half of the century, circumstances leaned in favour of extension of rubber culture. The widespread adoption and improvement of vulcanization since 1850, coupled with growing demand for mechanical rubber devices, resulted in the expansion of rubber industry both in Europe and North America. The increase of population and the rising standards of living created vast new markets for rubber footwear. The discovery of the pneumatic tyre by John Boyd Dunlop in 1888, the ensuing cycling craze of the 1890s and development of the motor car resulted in greater demand for rubber, compelling the sources of supply to be widened. In the USA, great efforts were made to tap scrap rubber as a supply source, and indeed the US consumption of reclaimed rubber equalled to that of the natural product. The British with a global empire tried to manage the short supplies through imports from Africa and by transplanting rubber seeds from the Amazon valley to their colonies in the East. At the centre of this shift of rubber culture from West to East, as Professor Woodruff reports, was a group of British botanists working with Kew Botanic Gardens (see Chap. 2, 'Genesis and Development'). During World War I, German scientists produced a crude synthetic rubber, and during the 1920s and 1930s several polymerizing processes were developed in Germany, the Soviet Union, Britain and the USA. World War II threatened to shift the rubber wealth. Japan occupied prime rubber-producing areas in Southeast Asia, and the USA feared it would run out of the vital material since every tyre, hose, seal, valve and inch of wiring required rubber. Hence, the USA sought out other sources including establishing a rubber programme that saw explorers going to the Amazon with the ultimate goal of establishing rubber plantations close to home. During the war, the US Congress passed the Emergency Rubber Project Act to solve the rubber shortage problem. With this, the government used lands in the Western states for the production of rubber from another rubber-producing plant, the shrubby guayule (*Parthenium argentatum)*. Much rubber was produced from guayule

during the war. Guayule is still preferred as an alternate source of natural rubber (Mooibroek and Cornish 2000). Also, US chemists had developed (in 1944) synthetic rubber by polymerizing butadiene and styrene that could replace natural rubber. By 1964, synthetic rubber made up 75% of the market. The situation changed drastically with the Oil Producing and Exporting Countries' (OPEC) oil embargo of 1973, which doubled the price of synthetic rubber and made oil consumers more conscious of their petrol mileage, prompting them to own radial tyres. Radial tyres replaced the simple bias tyres (which had made up 90% of the market only five years earlier). Within a few years, virtually all cars were fitted with radials. Synthetic rubber did not have the strength for radials; only natural rubber could provide the required sturdiness. By 1993, natural rubber had recaptured 39% of the US market. Today, nearly 50% of every auto tyre and 100% of all aircraft tyres in the USA are made of natural rubber. In general, car tyres have 12.5–28% natural rubber (higher in radial tyres), truck and bus tyres 50–75% and aircraft tyres 90–100%. Of this rubber, 85% is imported from Southeast Asia. The world consumes about 12.3 million tonnes of natural rubber every year.

Hevea nowadays is cultivated as far north as 25° North (Yunnan province, China) and as far as 21° South in Brazil. The main production zone is confined between 15°N and S. Strong international demand for natural rubber is driving expansion of industrial-scale and small-holder monoculture plantations, with >2 million ha established during the last decade. Mainland Southeast Asia and Southwest China represent the epicentre of rapid rubber expansion (Warren-Thomas et al. 2015). They estimated that 4.3–8.5 million ha of additional rubber plantations are required to meet projected demand by 2024. This demand can threaten significant areas of Asian forests, including many protected areas. The production and consumption of natural rubber worldwide is almost equal—around 12 million metric tonnes.

Box 1.1 Alternate Sources of Rubber

Apart from *Hevea* rubber tree, only two species are known to produce large amounts of rubber with high molecular weight (van Beilen and Poirier 2008): a shrub named guayule (*Parthenium argentatum* Gray) and the Russian dandelion (*Taraxacum koksaghyz*).

Guayule

Guayule *(Parthenium argentatum Gray)*, a shrub grown in semi-arid regions of Mexico and the Southern USA (Fig. 1.4a–c), is the only non-tropical plant that was being used in the early twentieth century as a commercial alternative source of natural rubber and is again being developed as a source of hypoallergenic latex by Yulex Corporation (van Beilen and Poirier 2008). It appears to be a viable alternative to *H. brasiliensis* because it produces relatively high amounts of high-quality rubber with essentially the same molecular weight as that of *Hevea* rubber tree. Guayule rubber has much lower protein content. While *Hevea* rubber production is extremely labour intensive, guayule rubber production can be mechanized fully, but capital and operating costs are significant.

Guayule is a flowering shrub of the aster family, Asteraceae, and is native to the southwestern United States and northern Mexico. It can be found in the US states of New Mexico and Texas and the Mexican states of Zacatecas, Coahuila, Chihuahua, San Luis Potosí, Nuevo Leon and Tamaulipas. Because hypoallergenic rubber and latex products are in high demand, guayule has an extra advantage over *Hevea* rubber. Yield comes to ~900 kg/ha/year.

Russian dandelion

Russian dandelion (*Taraxacum kok-saghyz* Rodin) was identified in Kazakhstan during 1931–1932 to develop a native source of natural rubber in the USSR (Fig. 1.4d–f). Its root is the source of high-quality rubber and was used for rubber production during World War II (Polhamus 1962; Whaley and Bowen 1947).

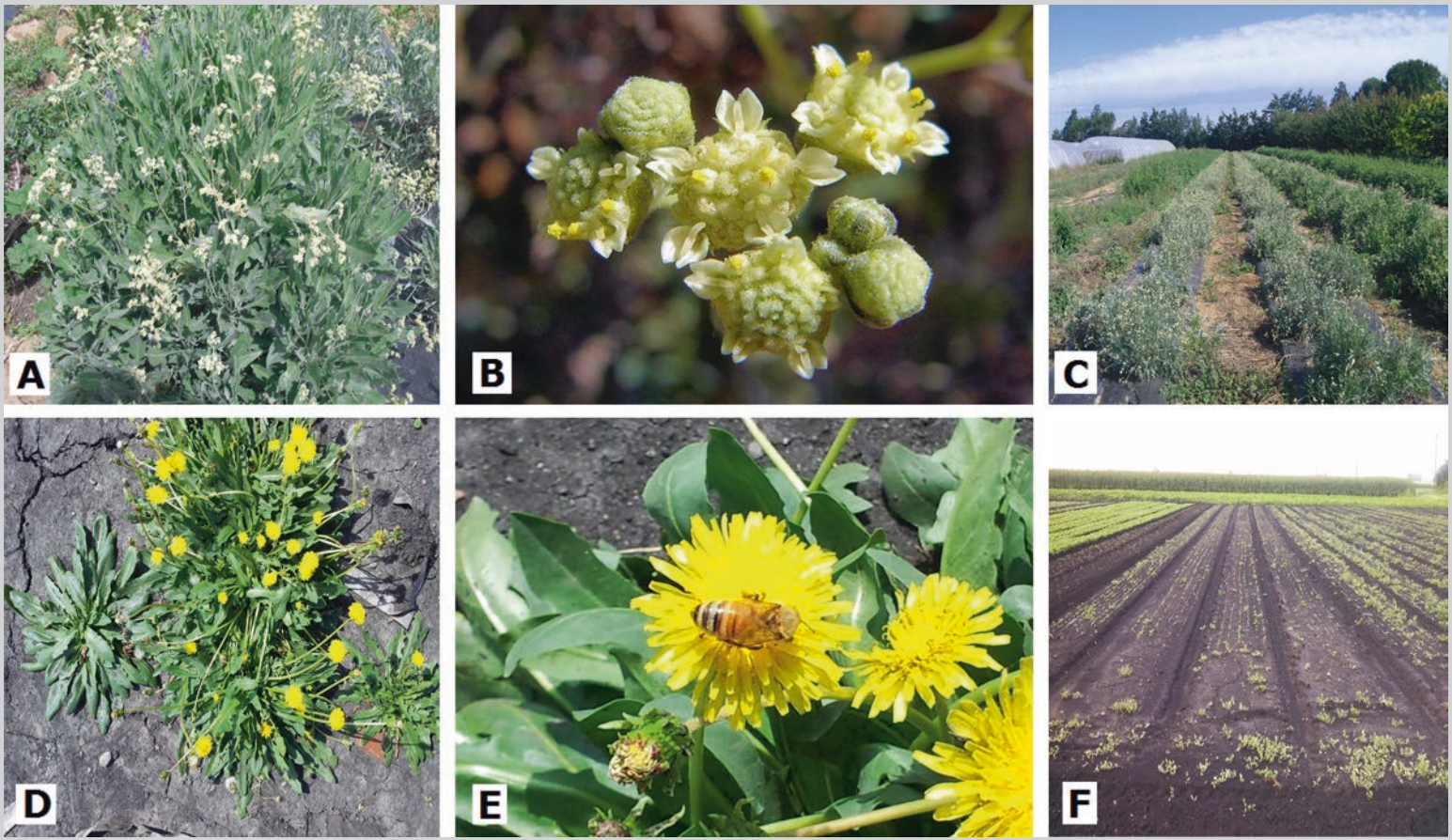

Fig. 1.4 (**a**–**c**) Guayule (waɪˈuːleɪ as in Spanish; why-YOU-lee in English) (*Parthenium argentatum* A. Gray) (Photo courtesy : Serge Palu, CIRAD, France); (**d**–**f**) Russian Dandelion (*Taraxacum koksaghyz* Rodin) (Photo courtesy: Katrina Cornish, Ohio State University, Wooster, USA)

Tyres made from Russian dandelion rubber were as resilient as those produced from *H. brasiliensis* and, furthermore, were better than guayule-rubber-derived tyres (Whaley and Bowen 1947), probably owing to the extraordinarily high molecular weight of the dandelion rubber (Hallahan and Keiper-Hrynko 2004). An attractive feature of the Russian dandelion is that it could be developed as an annual rubber crop for the temperate regions because it can be grown in a similar way to chicory. Although Russian dandelion has laticifers like the rubber tree, the rubber cannot be harvested by tapping; instead, plant roots must be homogenized and the rubber pressed out or extracted.

Russian dandelion is often abbreviated as TKS. Soviet literature on *T. kok-saghyz* records yield up to 10 tonnes/ha and is reported to occupy a small area of 8000 sq. km in Kazakhstan. A perennial species of 4–30 cm height, it grows in several valleys of Alatau mountain range at an altitude of 1800–2100 m. It can withstand −12–18 ^{0}C.

Whatever species identified, as such, there is no commercial alternative to *Hevea* rubber.

Genesis and Development

2

Since the early twentieth century, the chief source of latex has been *Hevea brasiliensis* (Greek 1991), though there are several other tropical and subtropical species that yield rubber from their laticifers (latex vessels)—small tubes found in the inner bark. As its botanical name suggests, *H. brasiliensis* is native to tropical regions of South America, especially Amazonia and adjoining areas.

2.1 The Amazon River Basin

During latter half of the nineteenth century, the Amazon River and its major tributaries were inhabited by relatively dense, sedentary populations of indigenous peoples who practised intensive root-crop farming, supplemented by fishing and hunting of aquatic mammals and reptiles. The higher areas away from the rivers and their flood plains were (and still are) inhabited by small, widely dispersed, semi-nomadic tribes of Indians living on hunting animals and on wild fruits, berries and nuts with some small-patch agriculture of low yield. Amazon Rainforest is as much as 100 million years old. Rainforest covers the largest part of the Amazon region, most of the Guyanas, southern and eastern Venezuela, the Atlantic slopes of the Brazilian Highlands and the Pacific coast of Colombia and northern Ecuador (Fig. 2.1). The huge Amazon region is the largest and probably the oldest forest area in the world; it also ascends to the slopes of the Andes until it merges with subtropical and temperate rainforest. On its southern border it merges with the woodlands of the Brazilian state of Mato Grosso, with galleries of trees extending along the rivers.

The Amazon basin consists of enormous trees, some exceeding a height of 100 m, with an incredible number of species growing side by side in the greatest profusion arranged in different strata. For example, in Manaus (Brazil), 1652 plants belonging to 107 species in 37 different families were found in about 630 m^2. There are about 2500 species of Amazonian trees (Ducke 1941) and as many as 100 arboreal species have been counted on a single acre of forest with hardly any one of them occurring more than once. Papers of Schultes (1945) and Seibert (1947) further confirm this enormous diversity. The Amazon forest has a strikingly layered structure. The canopy of sun-loving giants, soar to as much as 40 m above the ground and a few, known as emergents, rise beyond such canopies, frequently attaining heights of 70 m. Their straight, whitish trunks are covered with lichens and fungus. A characteristic of these giant trees is the buttresses, or basal enlargements of their trunks, which presumably help stabilize the top-heavy trees during infrequent heavy winds. Further characteristics of these trees are their narrow, downward-pointing 'drip-tip' leaves that easily shed water. Flowers are inconspicuous. Among the canopy species, prominent members include the rubber tree (*H. brasiliensis*), the silk cotton (*Ceiba pentandra*), the Brazil nut (*Bertholletia*

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Fig. 2.1 Vegetation map of Brazil

excelsa), the Sapucaia (*Lecythis*) and the Sucupira (*Bowdichia*). Many creatures, including monkeys and sloths, spend their entire lives in this sunlit canopy.

The Amazon River basin is the largest river basin in the world. It is important not only to the seven countries it spreads (Brazil, Peru, Ecuador, Bolivia, Colombia, Venezuela and Guyana), but

to the entire world as it affects the global climate. The name Amazon was given by Spanish explorer Francisco de Orellana in 1541. It slices through the rainforest from the Andes to the Atlantic; it extends beyond the rainforests and reach elevations in the Andes of more than 16,400 ft (5000 msl) at its westernmost watershed (Goulding et al. 2003). The Andes draining into the Amazon span a 2800 mile (4500 km) arc, stretching from Bolivia to Colombia. The Amazon is anywhere between 3903 miles (6259 km) and 4195 miles (6712 km) long. The drainage basin covers an area of over 6,915,000 km^2 (2,722,000 square miles), or roughly 40% of South America (Schroth et al. 2003). The Amazon basin covers a surface area of 4,100,000 km2 (1,583,000 square miles), of which around 3.4 million km^2 (1.3 million square miles) are presently forested (Schroth et al. 2004). The discharge from Amazon River is about 220,800 m^3/s. Or, it drains over 7,381,000 cubic foot of water into the Atlantic Ocean each second. During the monsoons, the width of the Amazon River can reach over 30 miles (50 km). The second longest river in the world (Nile is believed to be the first), the Amazon is by far the largest river in the world, accounting for approximately 20% of the water flowing from the world's rivers into the oceans. It produces about 20% of the Earth's oxygen.

Accounting for parts of the Amazon outside Brazil, the total extent of the Amazon is estimated at 8,235,430 km^2 (3,179,715 square miles). By comparison, the land area of the USA (including Alaska and Hawaii) is 9,629,091 km^2 (3,717,811 square miles). Amazonian evergreen forests account for about 10% of the world's terrestrial primary productivity and 10% of the carbon stores in ecosystems (Melillo et al. 1993)—of the order of 1.1×10^{11} t of carbon (Tian et al. 2000). Amazonian forests are estimated to have accumulated 0.62 ± 0.37 t of carbon ha^{-1} $year^{-1}$ between 1975 and 1996 (Tian et al. 2000). Fires related to Amazonian deforestation have made Brazil one of the top greenhouse gas producers. Brazil produces about 502 million t of CO_2 a year from fossil fuel consumptions. If one includes land use component, this figure would be much higher. CO_2_CAP shall be around 2.2 for Brazil as per World Bank data (see http://data.worldbank.org/indicator/EN.ATM.CO2E.PC). Brazil is listed as one of the lowest per capita (ranked 123; 0.67 CO_2_CAP) in CO_2 emissions according to the US Department of Energy's Carbon Dioxide Information Analysis Center (CDIAC) (Table 2.1). No accurate data on deforestation exists for the Amazon basin as a whole, although annual losses of 8920 square miles to 9420 square miles (more than the size of New Jersey) are frequently cited (Butler 2004) (Fig. 2.2).

Table 2.1 Ranking of the world's countries by 2013 per capita fossil fuel CO_2 emission rates. National per capita estimates (CO_2_CAP) are expressed in metric tons of carbon (not CO_2)

Rank	Nation	CO_2_CAP
1	Qatar	11.03
9	United Arab Emirates	5.10
12	Australia	4.43
13	USA	4.40
21	Canada	3.68
23	Russian Federation	3.40
33	Japan	2.67
35	Germany	2.56
37	Singapore	2.55
39	Israel	2.48
40	South Africa	2.41
50	China (Mainland)	2.05
53	UK	1.95
123	Brazil	0.67
133	Indonesia	0.52
143	Vietnam	0.46
147	India	0.43
159	Philippines	0.27
167	Sri Lanka	0.21

Source: Tom Boden and Bob Andres, Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory; Gregg Marland, Research Institute for Environment, Energy and Economics, Appalachian State University

Currently, *Hevea* rubber is planted in compact areas as rubber plantations that cover vast tracts of Indonesia, Malaysia, Thailand, India, Vietnam, China, Sri Lanka (erstwhile Ceylon) and Nigeria. Cambodia and Laos are the upcoming rubber producers. How a wild plant of the Amazon jungles was domesticated and trained to be the producer of a pre-eminent industrial raw material is

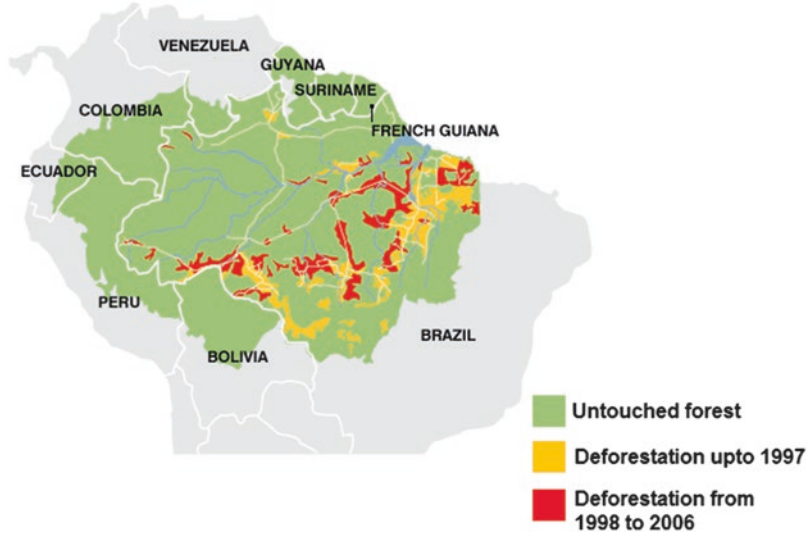

Fig. 2.2 Deforestation in Brazil

the central saga in the history of the so-called indispensable rubber industry. A crucial episode in that narrative is the transport of *Hevea* seeds from Brazil to England and from there to South and Southeast Asia as described in the 14th edition of *Encyclopedia Britannica* by William Woodruff, professor of economic history and author of *The Rise of the British Rubber Industry During the Nineteenth Century* (1958), and later by many authors (Tan 1987; Simmonds 1989; Clément-Demange et al. 2000; Thomas 2001, 2002; Priyadarshan 2003a, 2007; Priyadarshan and Clément-Demange 2004). A brief account of the history of *Hevea* domestication is given here.

2.2 History of Domestication

History of *Hevea* recapitulates the names of five distinguished men: (i) Clement Markham (of the British India Office); (ii) Joseph Hooker (Director of Kew Botanic Gardens); (iii) Henry Wickham (naturalist); (iv) Henry Ridley (Scientific Director of Singapore Botanic Gardens) and (v) R.M. Cross (Kew gardener), with Kew Botanic Gardens playing the nucleus for rubber procurements and distribution. As per directions of Markham, Wickham (Fig. 2.3) collected 70,000 seeds from the Rio Tapajoz region of the Upper Amazon (Boim district) and transported the collection to Kew Botanic Gardens during June 1876 (Wycherley 1968; Schultes 1977b; Baulkwill 1989) (Fig. 2.4). Of the 2899 seeds germinated, 1911 were sent to the Botanic Gardens, Ceylon (now Sri Lanka), during 1876, and 90% of them survived at the Henarathgoda botanical garden. During September 1877, 100 *Hevea* plants specified as 'Cross material' were

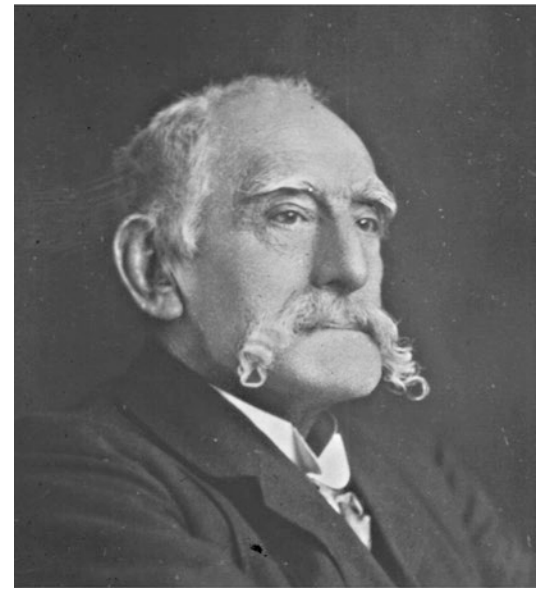

Fig. 2.3 Sir Henry Alexander Wickham (29 May 1846–27 September 1928)

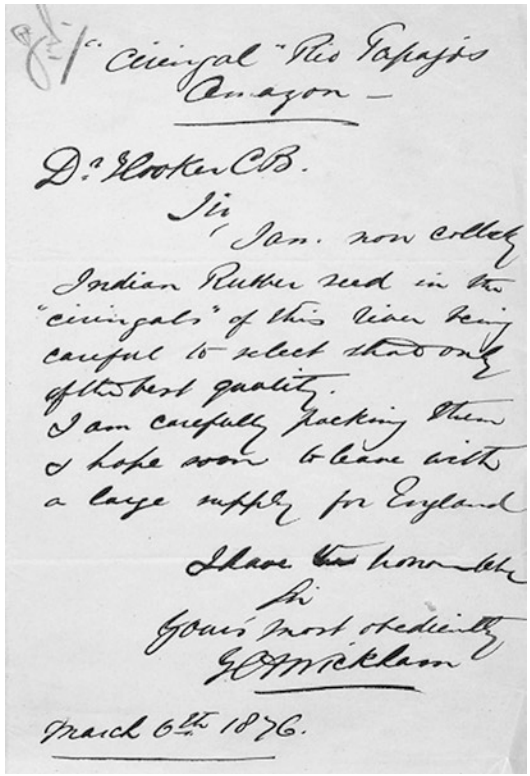

Fig. 2.4 Dispatch notification by Wickham of rubber seeds to from Rio Tapajós region, Upper Amazon to Kew Botanic Gardens, UK (from Kew Botanic Gardens with permission)

also sent to Ceylon. In June 1877, 22 seedlings not specified either as Wickham or Cross were sent from Kew to Singapore, which were distributed in Malaya and formed the prime source of 1000 seedling tappable trees found by Ridley during 1888. An admixture of Cross and Wickham materials might have occurred, as the 22 seedlings were unspecified (Baulkwill 1989). One such parent tree planted during 1877 was available in Malaysia even after 100 years (Schultes 1987). Seedlings from the Wickham collection of Ceylon were also distributed worldwide. Rubber trees covering millions of hectares in Southeast Asia are believed to be derived from very few plants of Wickham's original stock from the banks of the Tapajoz (Imle 1978). After reviewing the history of rubber tree domestication in East Asia, Thomas (2001) drew the conclusion that the modern clones have invariably originated from the 1911 seedlings sent to Ceylon during 1876. Also, Charles Farris could transport some seedlings to Kolkata in India (erstwhile Calcutta) during 1873 (Fig. 2.5). Hence, the contention that the modern clones were derived from '22 seedlings' is debatable. Moreover, if the modern clones were derived from 1911 seedlings, then the argument that they originated from a 'narrow genetic base', as believed even now, needs to be reviewed (Thomas 2002). A chronology of events is given in Table 2.2.

The first introduction of rubber to India was during 1873 from Ceylon (now Sri Lanka) when 28 *Hevea* plants were planted in the Nilambur Valley of Kerala state in South India (Haridasan and Nair 1980). During the period 1880–1882, plantations on an experimental scale were raised in different parts of South India and the Andaman islands. *Hevea* was first introduced to Vietnam in 1897 by the French, but was rejuvenated only after 1975 because of the long-lasting war (Priyadarshan 2003a).

Developments in domestication of rubber after 1880 commenced in Singapore Botanic Gardens, one of the world's finest in terms of both its aesthetic appeal and the quality of its botanical collection. Approximately 3000 species of tropical and subtropical plants and a herbarium of about 500,000 preserved specimens are the hallmark of this garden. Under the direction of Henry Nicholas Ridley (Fig. 2.6), who took over as Director of Singapore Botanic Gardens in 1888, the garden became a centre for research on *H. brasiliensis*. Ridley developed an improved method of tapping rubber trees that resulted in a better yield of latex. His innovation revolutionized the region's economy. His persistence resulted in the first rubber estate in 1896 using his seeds and thereon the rubber industry grew into one of the economic mainstays of the Malay states. It is also known that 100 plants went to Sri Lanka in the summer of 1877 and a further 50 to India. In all, by the end of 1877, Kew had distributed over 3000 seedlings; vastly more than their primary stock, so there must have been considerable propagation from cuttings. Sri Lanka then forwarded 22 seedlings from that delivery of 100 to Singapore. All of these survived and Henry Ridley, Director of the Singapore Botanic

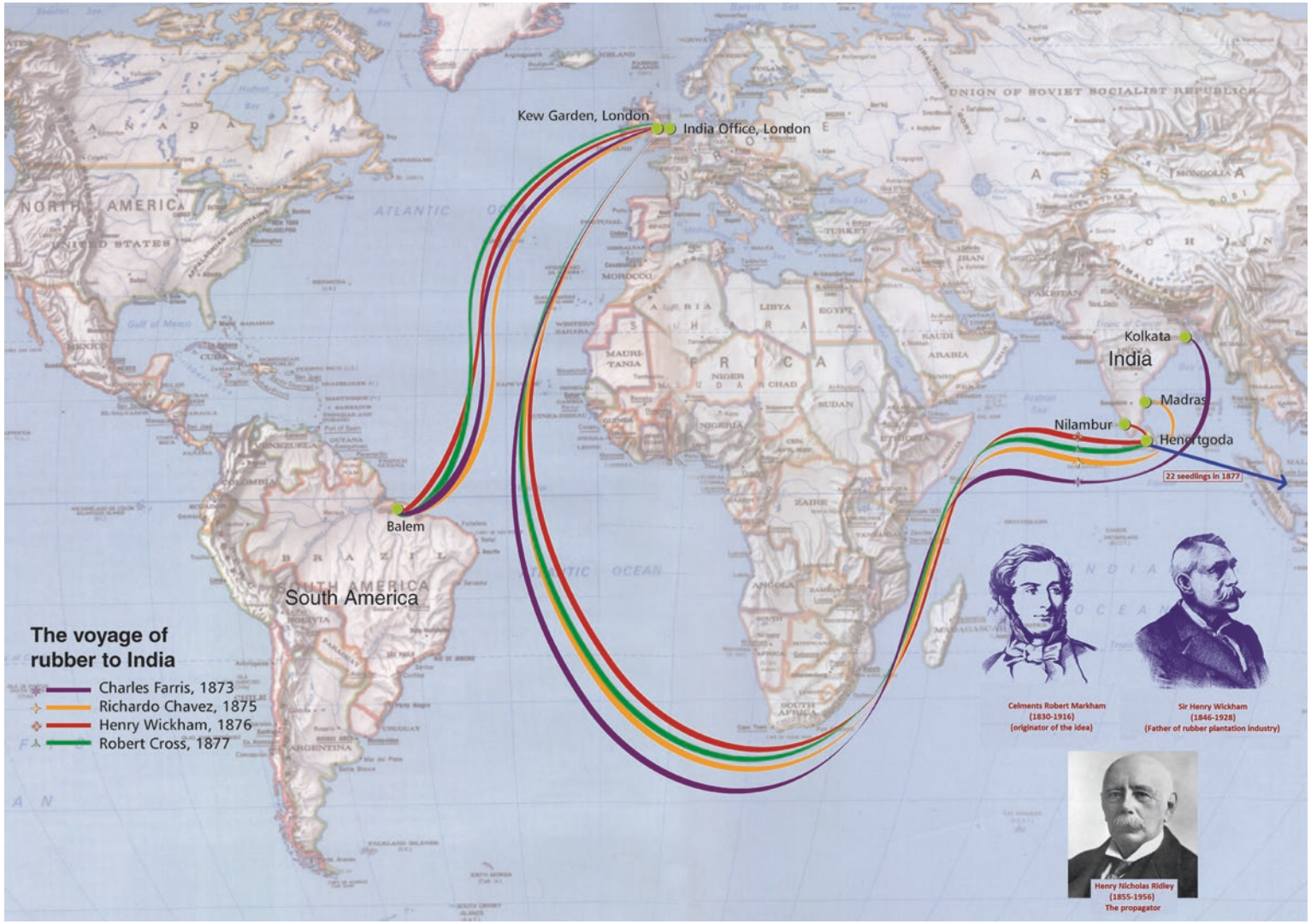

Fig. 2.5 The voyage of rubber to India

Table 2.2 Events in the history of rubber

1.	1735—Rubber samples sent to Europe by Charles-Marie de La Condamine
2.	1763—French (François Fresneau) found caoutchouc could be dissolved in naphtha; suggested use in waterproofing clothing but it became tacky when warm
3.	1770—Joseph Priestly discovered that the material would rub out paper marks, hence the name India rubber, and now simply 'rubber'
4.	1803—The first rubber factory was established near Paris
5.	1823—Mackintosh manufactures waterproof raincoats by coating fabric with rubber dissolved in naphtha
6.	Early 1820s—Hancock invented the masticator, a machine that shredded rubber scraps, allowing rubber to be recycled after being formed into blocks or rolled into sheets
7.	1824—Hancock suggested plantation growing of rubber
8.	1839—Goodyear and/or Hancock discovered vulcanization; when rubber was heated with sulphur, rubber retained physical properties from 0° to 100 °C. This led to rubber boom
9.	Interest in rubber with vulcanization process led to increased demand and exploitation of wild *Hevea* trees (*Hevea* was the native word)
10.	1845—The first patent for a pneumatic tyre was issued to Robert William Thomson in England
11.	1858—The first patent on an integral pencil and eraser was issued in the USA to Joseph Rechendorfer of New York City
12.	1870—Sir Clements Markham of India Office suggested that rubber along with cinchona (source of quinine) be obtained from tropical America and grown in Asia
13.	1872—James Collins reviewed rubber producing plants, published monograph entitled *Caoutchouc of Commerce*
14.	1873—Seeds from Brazil sent to Kew Botanical Gardens; 12 plants raised and sent to Calcutta, but failed
15.	1875—Second consignment of seed failed to germinate
16.	1876—Makham sends Robert Cross to Para, Brazil, where he obtained 1000 plants of *Hevea*, but no plants reach the East. At this time H.A. Wickham, an Englishman residing at Manaus (centre of the rubber boom in Brazil), sent 70,000 seeds from Central Amazon basin (he received £10/100 seeds). This provided the basis for the world's rubber industry. The seeds were sent to Kew. Seeds had short viability but produced 2899 plants. Seedlings were sent to Ceylon and 50 plants to Singapore and a few to Java
17.	1888—In Singapore, there were 9 trees of the original introduction, 21 five-year-old trees and 1000 seedlings. Ceylon had 20,000 seeds
18.	1888 to 1911—H.N. Ridley, scientific director of the Botanical gardens at Singapore demonstrated that *Hevea* was the superior rubber bearing plant; discovered excision method of extracting latex and devised method for coagulating latex
19.	1888—John Boyd Dunlop, a veterinary surgeon of Belfast, obtained patents on a pneumatic tyre for bicycles
20.	1898—Dunlop rediscovers pneumatic tyres (motor cars invented in 1885). Today, 70% of rubber involves transportation, 6% for footwear, 4% for wire and cable
21.	1898—First planting in Malaysia by a Chinese grower named Tan Chan Yoy. At this time, coffee prices slumped and there was interest in establishing a new industry
22.	1910—Rubber boom; rubber reaches $3 a pound
23.	1956—Ridley dies at the age of 101
24.	After 1956 till date—*Hevea* rubber popularized as a cash crop all through east Asia and many countries of Africa. Agencies like ANRPC, IRSG and IRRDB constituted. Development extended to suboptimal climates of various countries

Gardens, was later to remark that it was from these 22 seedlings in the Gardens that more than 75% of the cultivated plants in Malaysia were derived.

The arrival of 22 seedlings in Singapore did not create the Malaysian plantations overnight. *Hevea* seedlings were planted in the Residency gardens at Kuala Kangsar where they were nurtured by the Resident Hugh Low while investigations of both *Hevea* and indigenous rubber producing plants were carried out by H.J. Murton, the Superintendent of the Singapore Botanic Gardens, and by his successor, N Cantly. In 1885, Cantly claimed that the latter offered

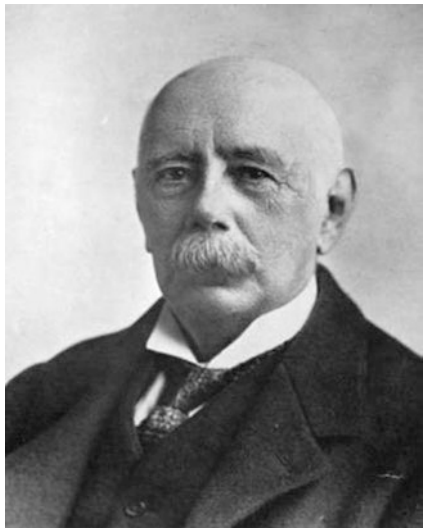

Fig. 2.6 Henry Nicholas Ridley (10 December 1855–24 October 1956)

better commercial potential. Meanwhile, in 1884, Frank Swettenham, later to be the High Commissioner of the Federated Malay States, planted 400 *Hevea* seeds from the Kuala Kangsar trees in Perak. More were planted in Selangor between 1883 and 1885 by T H Hill although these were possibly ornamental rather than commercial plantings.

Henry Ridley suggested that the government should consider large-scale plantings, as there was little private interest in planting crops which would take 5 years or more to start paying their way. He was able to use his additional position as Supervisor of the Straits Forest Department to carry out plantings in both Singapore and around Malacca and to investigate ways of cultivating and tapping the trees for optimum yield. He published his recommendations in 1897 and, following his ideas, Curtis in Penang and Derry in Kuala Kangsar obtained yields of latex from which they were able to calculate that rubber production could be profitable. It was also noted from samples sent to England that there would be a ready market for plantation rubber as it was much cleaner and more consistent in quality than the wild rubbers of either Africa or Amazonia.

It is perhaps ironic that another Brazilian commodity pushed Malaysia into rubber. Various government inducements had encouraged planters to create and expand plantations and many of these chose coffee as their main crop. The price of coffee had been high due to production problems in Brazil but, by the mid-1890s, these problems had been overcome while fungal disease was attacking the Malaysian plants. In 1895, Tan Chay Yan planted 43 acres of *Hevea* on his estate at Bukit Lintang in Malacca and the Kindersley planted a further 5 acres in Selangor. These were the first commercial rubber estates in Malaysia and, as the coffee market collapsed, more and more planters turned to rubber. Initially the plantings were interspersed with cash crops such as coffee but by 1898 Stephens, in Perak, was planting dedicated rubber plantations. At about this time Ridley noted that he had received requests for one million seeds in a single day!

Significant development on the propagation of *Hevea* rubber occurred after 1910. The contribution to propagation and breeding of *Hevea* made by P.J.S. Cramer (Bogor, Indonesia) during 1910–1918 is noteworthy. He made a trip to the Amazon and succeeded in getting seeds of *Hevea spruceana* and *Hevea guianensis*. Cramer also conducted experiments on variations observed in 33 seedlings imported from Malaysia in 1883 from which the first clones of the East Indies were derived (Dijkman 1951). Along with van Helten, a horticulturist, he could standardize vegetative propagation by 1915. The first commercial planting with bud-grafted plants was undertaken during 1918 in Sumatra's east coast. Ct3, Ct9 and Ct38 were the first clones identified by Cramer (Dijkman 1951; Tan et al. 1996). Commercial ventures gradually spread with the introduction of bud grafting; 'generative' and 'vegetative' selection methodologies were simultaneously used that resulted in seedlings and grafted clones (Dijkman 1951). Around 1950, the advantages of grafted clones proved to be overwhelming for yield potential compared to genetically improved seedlings, and the focus shifted to derivation of clones for latex productivity. With all these cultural developments, *H. brasiliensis* soon ousted many other rubber-producing species including *Castilla*, *Manihot glaziovii* (ceara or manicoba rubber tree), *Ficus elastica*,

Landolphia and *Clitandra* vines (African rubber).

Once the *Hevea* tree had been successfully transplanted to Southeast Asia, the development of rubber plantation industry was rapid and considerable quantities of the commodity were in the market by 1910. Factors such as availability of labour and favourable soil and climate contributed to this development. With the growth in world demand increasing, the total area of plantation in the East in 1900 amounted to 5000 acres. In 1910, it was 1 million acres and in 1920, 4 million acres. After the end of World War II in 1945, the total acreage exceeded 9 million, and by the mid-1960s it was 11.5 million. Rubber produced from *Hevea* in Asian countries, ranging from the Philippines to Sri Lanka, accounted for almost 95% of the world's natural rubber supply (Table 2.3) (IRSG 2015). Worldwide, there is almost 15% increase in yield from 2008 to 2012 (IRSG 2015). Currently, the production and consumption of natural rubber is equalized (little more than 12 million metric tons) (Fig. 2.7).

There has been a constant correlation in the prices of oil and natural rubber. World economic recessions also have always experienced a downfall in the prices of natural rubber. An extensive survey of the history and development of natural rubber is beyond the scope of this book. Readers interested in such details may refer to Baulkwill (1989) for an extensive account on the history and various bulletins by IRSG for rubber statistics.

Table 2.3 Natural rubber production- Asian countries against world (in million metric tons)

Country	2012	2013	% of change
Bangladesh	0.18	0.19	2.6
Cambodia	0.64	0.85	32.1
China	0.8	0.86	7.9
India	0.91	0.7	–13.4
Indonesia	3.0	3.2	7.5
Laos	0.07	0.08	17.6
Malaysia	0.92	0.82	–10.4
Myanmar	0.013	0.14	7.9
Papua New Guinea	0.075	0.075	0.0
Philippines	0.11	0.11	0.0
Sri Lanka	0.15	0.13	–14.2
Thailand	3.8	4.17	10.4
Vietnam	0.8	0.9	8.2
Asia total	*10.85*	*12.15*	*11.9*
World total	*11.6*	*12.22*	*5.2*

Hevea rubber invites sharp criticism from environmentalists. For instance, new millennium saw a boom in rubber prices. This led to rapid and widespread land conversion to monoculture rubber plantations in Southeast Asia, where natural rubber production has increased >50% since 2000. Rubber was planted into increasingly suboptimal or marginal environments (nearly 72% of plantation area) where one can anticipate reduced yields. Of this, an estimated 57% of the area is susceptible to insufficient water availability, erosion, frost or wind damage. In 2013, typhoons destroyed plantations worth US$ >250 million in Vietnam alone (Ahrends et al. 2015). Such vagaries in nature can still reduce the potentiality of new areas. Expansion into marginal areas creates potential for loss-loss scenarios: clearing of high-biodiversity value land for economically unsustainable plantations that are poorly adapted to local conditions and alter landscape functions (e.g. hydrology, erosion)—ultimately compromising livelihoods, particularly when rubber prices fall. For example, between 2005 and 2010, >2500 km^2 of natural tree cover and 610 km^2 of protected areas were converted to plantations (Ahrends et al. 2015). More than 500,000 ha may have been converted already in the uplands of China, Laos, Thailand, Vietnam, Cambodia and Myanmar (Ziegler et al. 2009). By 2050, the area of land dedicated to rubber and other diversified farming systems could more than double or triple, largely by replacing lands now occupied by evergreen broadleaf trees and swidden-related (a plot of land cleared for farming by burning away vegetation) secondary vegetation. Ziegler et al. (2012) conducted meta-analysis of over 250 studies reporting above- and below-ground carbon estimates for different land use types that indicated great uncertainty in the net total ecosystem carbon changes that can be expected from many transitions, including the replacement of various types of swidden agriculture with oil palm, rubber or some other types of agroforestry systems. These transitions are underway throughout

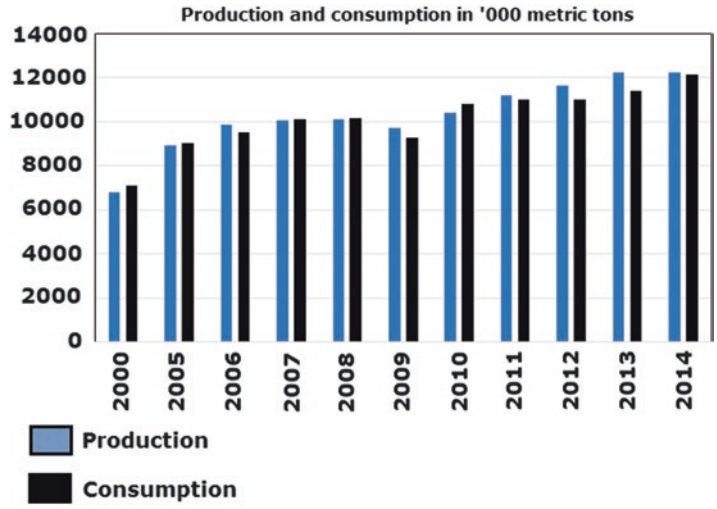

Fig. 2.7 Production and consumption of rubber over years

Southeast Asia and are at the heart of REDD+ (Reduce Emissions from Deforestation and forest Degradation) debates. As some transitions may negatively impact other ecosystem services, food security and local livelihoods, the entire carbon and non-carbon benefit stream should also be taken into account before prescribing transitions with ambiguous carbon benefits. A deeper and more systematic analysis of the multiple consequences of these policies is consequently necessary for the design of successful REDD+ policies in MMSEA (Montane Mainland Southeast Asia) and other areas of the developing world (Fox et al. 2014a, b).

Further, Kumagai et al. (2015) conducted eddy flux measurements over 3 years in two plantation sites of north-eastern Thailand and central Cambodia having distinct dry seasons. They used a combination of actual evapotranspiration (*ET*) flux measurements and an inverted version of a simple 2-layer E_T model for estimating the mean canopy stomatal conductance (g_s). There was less sufficient stomatal regulation at the Thailand site, where there might be little risk of water stress-induced hydraulic failure because of its higher annual rainfall amount. In comparison, at Cambodian site where annual potential water balance (precipitation – potential evaporation: $P - E_{T\text{-}POT}$) was negative and there was stricter stomatal regulation, preventing excessive xylem cavitation. This demonstrates *Hevea* behaves differently under water stress conditions (see Chap. 3 for details). High water use by rubber raises concerns about potential effects of continued expansion of tree plantations on water and food security in MMSEA (Giambelluca et al. 2016). However, this issue demands thorough debate by experts.

Plant Structure and Ecophysiology 3

Rubber is synthesised in over seven families: Euphorbiaceae, Apocynaceae, Asclepiadaceae, Asteraceae, Moraceae, Papaveraceae and Sapotaceae (Backhaus 1985; Lewinsohn 1991; Cornish et al. 1993) (see Chap. 1 for other sources of rubber). Of late, scientists have started exploring the possibility of generating microorganisms that can produce rubber (Steinbüchel 2003). Euphorbiaceae family is extremely diverse and considered to be polyphyletic (Webster 1994). *Hevea brasiliensis,* the prime source of rubber, can survive for more than 100 years, and one such tree borne from the original nine seedlings brought over to Malaya in 1877 is still available (Fig. 3.1).

3.1 Plant Structure

Hevea is easily recognizable from its characteristic trifoliated leaves. The genus is basically composed of ten species: *H. brasiliensis, H. guianensis, H. benthamiana, H. pauciflora, H. spruceana, H. microphylla, H. rigidifolia, H. nitida, H. camporum* and *H. camargoana* (Webster and Paardekooper 1989; Wycherley 1992; Schultes 1990). Seven species are found in the upper Rio Negro region, considered to be the centre of origin of the genus. *Hevea brasiliensis* is found in southern areas outside of this centre, in the upper Rio Madeira, where five other species are represented. It has generally been assumed that the species are freely intercompatible (Seibert 1947; Baldwin 1947). Pires (1981) observed natural hybrids of *H. camargoana* × *H. brasiliensis*, and Gonçalves et al. (1982) analysed progenies issued from hand pollination from this type of crossing. Consequently, *Hevea* species might be considered as a species complex, due to the absence of a strict barrier to recombination between species. Many efforts led to the identification of certain types which were formerly presented as other possible species. *H. paludosa* was identified in Brazil by Ule in 1905 and is often considered as an 11th species (Gonçalves et al. 1990; Priyadarshan and Gonçalves 2003). An elaborate description of taxonomical and botanical aspects of *Hevea* has been reviewed by Schultes (1977a, b, 1987, 1990a, b) and Wycherley (1992) (see Chap. 6 for details).

All *Hevea* species have $2n = 36$ chromosomes, with the exception of one triploid clone of *H. guianensis* ($2n = 54$) and the existence of one genotype of *H. pauciflora* with $2n = 18$ (Baldwin 1947; Majumder 1964). Although *Hevea* behaves as a diploid, it is believed to be an amphidiploid ($2n = 36$; $x = 9$) that stabilized during the course of evolution. This contention is supported by the observance of tetravalents during meiosis (Raemer 1935; Ong 1975; Wycherley 1976). In situ hybridization studies revealed two distinct 18S–25S rDNA loci and one 5S rDNA locus, suggesting a possible allotetraploid origin with the loss of 5S rDNA during the course of evolution (Leitch et al. 1998).

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Fig. 3.1 Para Rubber at Henarathgoda Botanic Garden, Gampaha, near Colombo, Sri Lanka where the first plantation outside of Brazil was established in 1876. The gardens were founded for the purpose of cultivating Para rubber. Fig. 3.1(**a**) Dr. Nigel Taylor, Director of the Singapore Botanic Gardens with his wife Dr Daniela Zappi, colleague Lahiru Wijedasa and the Curator of the Henarathgoda Botanic Garden; Fig. 3.1(**b**) Dr Daniela Zappi, then botanist at Gardens by the Bay, Singapore

But locus duplications are infrequent in *Hevea* genome, and they could have occurred due to chromosomal modifications posterior to the polyploidization event (Seguin et al. 2003); consequently, the two unknown ancestral genomes of *Hevea* would have strongly diverged.

3.1.1 Flowers

Similar to other tropical trees, *Hevea* normally takes 4–5 years to attain the reproductive stage—a phase called ripeness to flower (Kramer and Kozlowski 1979). Though the capacity to flower is retained thereafter, the periodicity and the quantitative importance of flowering vary from clone to clone, as in other tropical trees (Owens 1991). Precocious flowering is rarely observed in rubber seedlings (Sasikumar 2000). Rubber tree is monoecious, with lateral inflorescences (branched panicles) bearing both staminate and pistillate flowers (Fig. 3.2a) that appear in the last phase of the defoliation–refoliation process during wintering (the dry season: March–April in the northern hemisphere and September–October in the southern hemisphere) (Priyadarshan et al. 2001). For all practical reasons, rubber reaches maturity when it attains 50 cm trunk girth (tappable girth) at 125 cm height, so as to harvest latex (real physiological maturity of a tree is achieved when it reaches flowering stage). So, the immature phase of rubber is around six to seven years.

Hevea shows seasonal flowering in response to alternation of seasons. In the northern hemisphere, March–April is the main flowering season, and a short spell of secondary flowering prevails in August–September in many areas. Over much of Malaysia, the main flowering season occurs in February–April, following wintering in January–February, and there is a lesser flowering season during September–October

Fig. 3.2 (**a**) inflorescence, (**b**) fruits, (**c**) extra-floral nectary and (**d**) seeds of *Hevea*

(Yeang 2007). It seems reasonable to presume that geographic location has a bearing on whether the trees flower during the secondary season. While it flowers and sets seeds during both the seasons in Malaysia, the southern parts of India experience flowering in March and April only. In north-east India, Tripura state experiences flowering and seed set during both seasons, but seeds are less viable during the secondary season. This prompts hand-pollination experiments to be centred in March and April when a substantial number of clones undergo flowering for a short span of 10–15 days. The shift in flowering coincides with the latitudinal changes. Flower emergence occurs towards mid-February in all traditional areas north of the equator. However, towards mid-March, the emerging flowers remain apparently dormant until the onset of favourable environmental conditions. The appearance of female flowers takes 10–12 days more than male flowers (dichogamy), and, due to incomplete protandry, some of the male flowers emerge after the appearance of female flowers (Webster and Paardekooper 1989). In Manaus and São Paulo (Brazil), which are located south of the equator, *Hevea* flowers only during September–October (Priyadarshan et al. 2001; Yeang 2007) (see Chap. 9 for details).

Hermaphroditism and the occurrence of bisexual flowers have also been reported in the clones PR 107, AVROS 1328, GT 1 and Tjir 16 (Cuco and Bandel 1994, 1995). However, this could not be confirmed in the north-eastern states of India, perhaps due to altered environmental conditions. GT 1 is a male-sterile clone, and the impediment to the normal process of gametogenesis has been characterized at the histological level (Leconte 1983). Some other clones having GT 1 as the female parent, such as IRCA 41 and IRCA 319, are also proven to be male sterile, indicating those cytoplasmic genes might be responsible for male sterility (Nouy B. and Leconte A 1985, unpublished observations). BPM 24 is also reported to be male sterile in Thailand. Male sterility can be visually observed by the fact that stamens remain small and flat and produce no pollen.

Inflorescences are borne in the axils of the basal leaves of the new shoots that grow out after wintering. The inflorescence is a many-branched, shortly pubescent panicle bearing flowers of both sexes. The larger female flowers are borne at the end of the central axis and main branches, while the smaller and more numerous male flowers appear on other parts of the panicle. Flowers are greenish yellow, with a bell-shaped calyx having five triangular lobes but no petals. Such pentamerous flowers and a tricarpellary ovary are typical of the Euphorbiaceae, and the alternation of vegetative and reproductive phases with the formation of inflorescences at the end of the dry season implies a tight control of flowering time (Dornelas and Rodriguez 2005). Staminate flowers have ten anthers arranged over a staminal column in rows of five each. The pistillate flower consists of a three-celled ovary with three short sessile stigmas. For each pistillate flower, about 70 staminate flowers are found. One floral-meristem-identity gene (*HbLFY*) has been isolated from the rubber tree and the highest level of *HbLFY* expression occurs during the time of flower meristem formation and declines as the organs expand. *HbLFY* works as a functional orthologue of *FLORICAULA/LEAFY* found in other dicot species such as *Arabidopsis*, grape vine and kiwi fruit (Dornelas and Rodriguez 2005). *HbLFY* seems to be responsible for male/female floral inductions. Non-synchronous flowering is a restriction to genetic recombination between available genitors. Interestingly, high solar radiation induces synchronous anthesis and blooming in *Hevea* around the time of spring and autumn equinoxes on the equator (Yeang 2007). This opens the possibility of conducting hand-pollination experiments at the equator which would ensure the seed-set success in desired combinations. The possibility of pollen storage used to counter non-synchronous flowering (Hamzah et al. 1999) cannot be easily mastered for practical use.

Hevea appears to be obligatorily insect pollinated (Rao 1961) and predominantly cross-fertilized (Simmonds 1982). The strongly scented flowers of mature inflorescence attract insects (pollinators) that are mostly midges and ants of the families Heleidae and Ceratopogonidae. Wind appears to play little or no part since no pollen was collected in spore traps placed within 15 m of heavily flowering trees, and inflorescences enclosed in insect-proof bags did not set seeds.

In Malaysia, over 30 species of insects have been seen to visit the flowers, but it is likely that pollination is almost entirely effected by midges and, to a minor extent, by thrips. In Puerto Rico, Brazil and Malaysia, Ceratopogonoidae midges, which are very small and hairy with a capacity for sustained flight, were found to be the most important pollinators, while several species of thrips, which are not very active fliers, probably play a minor role (Warmke 1952; Rao 1961). As per a logarithmic model, pollen grains can travel 0.3–1.1 km (Yeang and Chevallier 1999). Pollen grains are triangular, measuring about 35–40 μm on each side, and their surface is sticky. The viability of pollen grains can be as high as 90%, but on average is only about 50% (Gandhimathi and Yeang 1984; Sowmyalatha et al. 1997). In tests on artificial media, Majumder (1964) found no differences between a large number of clones in percentage germination of healthy, well-filled pollen grains. He observed that pollen taken from male flowers during or soon after rain gave a smaller germination percentage than pollen from dry flowers.

3.1.2 Fruit Set

Fertilization occurs within 24 h after pollination and unfertilized female flowers quickly wither (Majumder 1964). Clones vary greatly in flowering, fertility and fruit set. This ranges from near sterility to prolific fertility. There is no evidence of self-incompatibility (Webster and Paardekooper 1989). The mature fruit is a large three-lobed capsule, 3–5 cm in diameter, having a woody endocarp and a thin, leathery mesocarp, and contains three seeds (Fig. 3.2b). The fruit reaches its maximum size in about 80–90 days and the endocarp becomes woody in about 110 days. The endosperm matures in about 130 days and the cotyledons get pressed to the endosperm. Thereafter, the moisture content of the capsule wall declines when the fruit is about 140 days so that the dry capsules dehisce explosively into six pieces with dispersal of seeds up to 15 m from the tree (Husin et al. 1981).

Low fruit set and its variation among clones, notably in the case of self- pollination, may be regarded as a general characteristic of the reproductive biology of *H. brasiliensis* and are not confined to specific incompatible crosses (Hamzah et al. 2002). This is a major limitation to genetic recombination in rubber breeding. It affects the number of full-sib families that can be evaluated, the size and the balance in sizes of these families, the cost of hand-pollination campaigns, and the quality of mating designs that can be established for genetic analysis. Pollen fertility, which varies from 50 to 98%, does not seem to be a limitation. The development of flowers to fruits is estimated to be very low, around 5% (Husin 1990), and non-fertilized flowers soon wither. In contrast, most of the young fruits that are able to initiate their growth will produce viable seeds. At the time of maturation of fruits, a secondary shedding may occur as a result of infection by *Phytophthora* and *Oidium*. Low fruit set is not due to natural pollination deficiency (Warmke 1952). While in Puerto Rico 5% or less of the female flowers bear fruit (Warmke 1952), in Malaysia it happened to be only 0.3–1.6% (Rao 1961), one of the pertinent reasons being the non-occurrence of pollination in all female flowers. In fact, fruit-set success rate assessed by controlled pollination varies widely, depending on the pollinated clones, from no success at all to a maximum of 5–10% for the more fertile clones such as PB 5/51 or PB 260. This is because the pollinator ensures the pollination of the highest number of female flowers (Maas 1919). The success rate varies from year to year with a coefficient of variation of 45% (Clément-Demange et al. 1995). When all the female flowers of an inflorescence are hand pollinated, the fruit set is 3–8% (Gandhimathi and Yeang 1984; Sowmyalatha et al. 1997).

Paiva et al. (1994) indicated cross-pollination to be 64% through isozyme studies. A clear example is PB 5/51, which is heterozygous for a recessive yellow gene, where open pollination led to an estimation of 16–28% self-pollination (Simmonds 1989). Since many allozymes are produced at different development stages (Adams and Joly 1990), it is always reliable to use DNA analysis as a means to spell out the proportion of cross-pollination. Using five pairs of polymorphic microsatellite primers, Pawsoi et al. (2013) estimated 79% cross-pollination. They used 288

seedlings derived from the seed orchard with five rubber clones (AVROS 2037, BPM 1, IAN 873, PB 260 and RRII 118) that were systematically grown in a random design. The five microsatellite loci chosen for this study were highly polymorphic. Individual female parents varied in their estimated outcrossing rate from 58.62 to 98.36%, while the overall outcrossing rate in the seed orchard was 79% and selfing rate was 21%. Pollen contamination was not observed in this seed orchard. The high outcrossing level and the lack of pollen contamination may be useful for the establishment of a seed production facility and for the management of hybrid production. Dijkman (1951) argued that a clone like LCB 510 (PR 107) is practically self-sterile, and that over 3000 self-pollinations yielded only one seed, indicating clonal variation towards self- incompatibility. As a matter of fact, the self-pollination rate in open pollination is strongly assumed to be much influenced by the specific context of the female trees and of the possible neighbouring cross-pollinators. By comparing eight hand-pollinated full-sib families, including two self-pollinated crosses, Leconte (1983) found no difference in the share of pollinated flowers with pollen tubes growing from stigmas to ovules (an average of 77%). Leconte (1984) and Sedgley and Attanayake (1988) confirm that there is no difference in pollen-tube growth between different clones and between self- or cross-pollinated flowers, so indicating that low fruit set and poor success in self-pollination are not due to incompatibility between the pollen and the stigma. A prezygotic or postzygotic control exerted by the same incompatibility alleles and/or the inbreeding effects due to accumulation of homozygous loci in the embryo appear to be the reasons for low fruit set in self-pollination (Priyadarshan and Clément-Demange 2004).

All three ovules of a fruit need to be fertilized for fruit setting (Gandhimathi and Yeang 1984; Sedgley and Attanayake 1988). Rubber fruits mostly have three carpels (sometimes four, and very rarely five), and fruits with only one or two seeds are almost never observed. Hamzah et al. (2002) confirmed that fruit-set success was clone characteristic of the seed parent and those pistillate flowers of PB 5/51 have a greater propensity for successful fruit set, while PR 107 is a poor seeder. Another study showed that the abortion of ovules never precludes fruit-wall formation in the early stages (Sowmyalatha et al. 1997). A fruit-load compensation phenomenon can be assumed, where fruit set ceases when an optimum number of fruits are formed. Only 25% of total pistillate flowers form fruits, and of these only about 25% attain maturity. The maternal parent might selectively abort genetically inferior progeny. Abortion of fruits is seen even 80 days after pollination. When seed set of PB 5/51, RRIM 600 and PR 107 were studied, flowers with no ovules penetrated were greatly over-represented (non-random distribution), and one explanation for this is the existence of 'receptive' flowers that favour successful fertilization (Hamzah et al. 2002). Hence, understanding the genetics of the female flower is vital in increasing seed set.

Though meagre, studies on the effect of environmental attributes over the fruit set during hand pollination demonstrated that fruit-set success could be negatively correlated with evaporation (Yeang et al. 1986). Maximum temperature and relative humidity (RH) of post-pollinated days are also found to influence fruit set. The distribution of fruits on the floral shoots was found to conform to a negative binomial distribution that supports aggregated distribution, indicating that some of the shoots are favoured for fruit setting (Yeang and Ong 1988). Leconte (1983) found that most of the fruits were borne by flowers from the buds located at the base of assimilatory leaves rather than from the buds of the scale leaves; consequently, he suggested focusing hand pollination on this type of flower.

3.1.3 Post-fertilization Events

Ovule abortion decreases seed production and is a crucial factor for the survival of remaining ovules. On the other hand, the abortion of ovules never precludes fruit-wall formation in the early stages, indicating that the control mechanisms of these two processes are not interdependent (Sowmyalatha et al. 1997). However, due to fruit-load compensation, when the optimum number

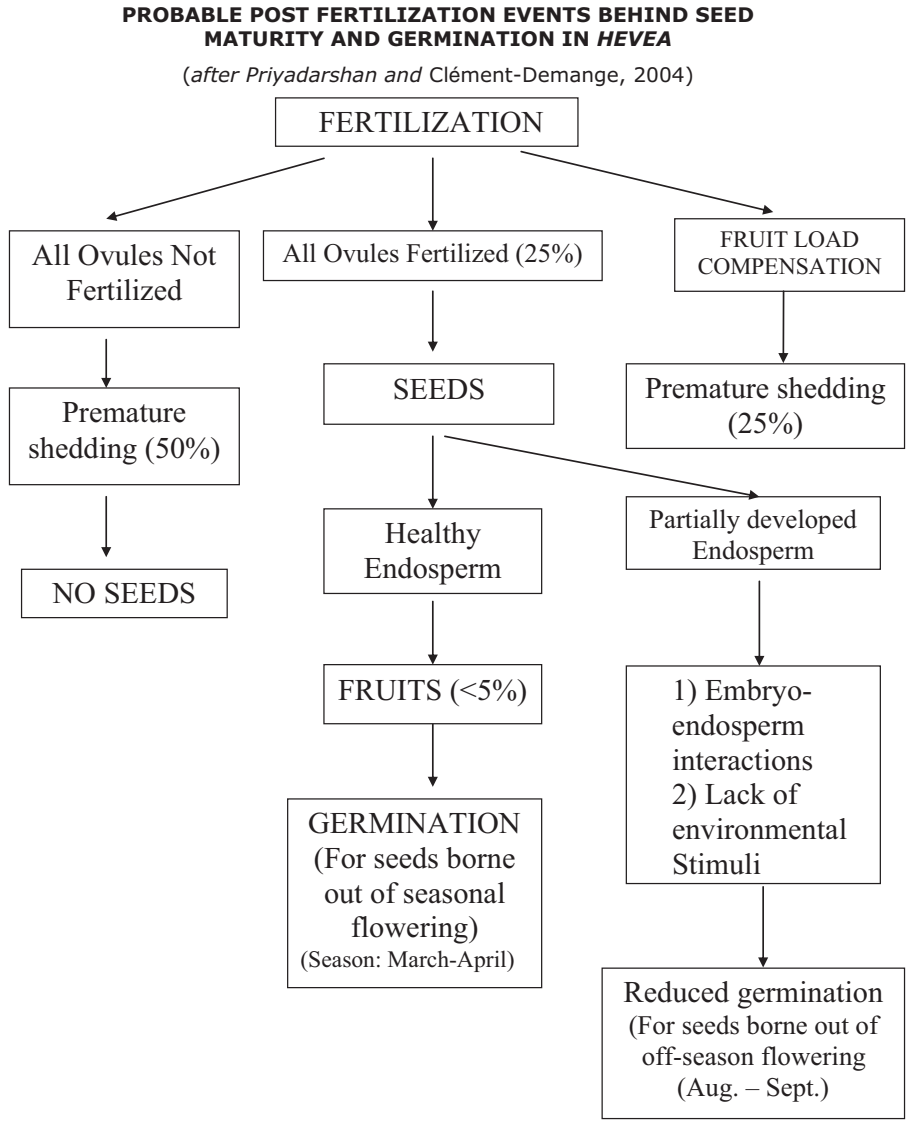

Fig. 3.3 Post-fertilization events behind seed maturity and germination in *Hevea* (After Priyadarshan and Clément-Demange 2004)

of fruits is set, the pistillate flowers arising later may not fulfil their reproductive role. Only 25% of total pistillate flowers form fruits, and of these only about 25% attain maturity. The total number of fruits set will only be < 5% (Fig. 3.3). Since *Hevea* is an outbreeding taxon, reproductive success is expected to be low, as demonstrated in other outbreeding species (Weins et al. 1987). Selfing, or the crossing of related parents, of an outbreeding taxon could result in inbreeding depression, and, since there are no conspicuous incompatibility barriers, it can very well be presumed that an inbred progeny would bring together deleterious recessive alleles largely contributing to reduced seed set. Here, the maternal parent can selectively abort genetically inferior progeny. Such preferential abortion is prevalent in many tree species (Stephenson 1981). The abortion of fruits is seen even 80 days after pollination (see Box 3.1).

Box 3.1
Due to incomplete protandry, *Hevea* clones may undergo assortative mating. Clones that have early anthesis of female flowers preferentially get pollinated by clones wherein female flowers are yet to emerge. So, in a multi-clone population, possibility of random mating can always occur. However, due to incompatibility barriers among clones and issues relating to fruit set, seed set may not occur in the expected rate.

3.1.4 Seed

Seeds are large (3.5–6.0 g) and ovoid with the ventral surface slightly flattened. The seed coat, or testa, is hard and shiny, brown or grey-brown with numerous darker mottles or streaks on the dorsal surface, but few or none on the ventral side (Fig. 3.2c). The female parent of a seed could be identified by its markings and shape since the testa is a maternal tissue and the shape of the seed is determined by the pressure exerted by the fruit capsule during its development. The hilum can be seen as a shallow, approximately circular depression on the ventral surface and the micropyle is adjacent to it. A papery integument lines the inner testa and encloses the endosperm, which fills the seed. The embryo is situated in the middle of the endosperm with the radicle pointing towards the micropyle. The two white, veined cotyledons are pressed against the endosperm and enclose the plumular end of the axis of the embryo. The endosperm, which forms 50–60% of the weight of the seed, contains semi-drying oil which can be used as a rather poor substitute for linseed oil. If seeds are not sown in 10–15 days, they lose viability on storage as a result of the production of hydrocyanic acid (HCN). Seed filling with ultimate growth of the endosperm ensures the germination of the seed.

Hevea seeds are recalcitrant (Roberts 1973). For storage, seeds can be mixed with sawdust and kept at 7 °C to have vigour for up to 4 months (Ang 1976). Treatment with polyethylene glycol 1500 improved storage up to 6 months with 25% germination (Normah and Chin 1995). As mentioned above, *Hevea* seeds lose viability due to production of HCN during storage. But over 90% of the cyanogenic material is consumed to form non-cyanogenic compounds during seedling development. The cyanogenic glucosides are believed to be transported and metabolized in the young growing tissues (Lieberei et al. 1985). Protein profiling of dry and germinated seeds revealed that in both cases, though common proteins are profiled, unique protein profiles are also seen (Wong and Abubakar 2005). Seed germination is hypogeal and commences within 3–5 days after sowing. The radicle breaks through the testa at the hilar depression and very soon produces a ring of primordia which rapidly grow out as lateral roots. The radicle grows rapidly to form the primary taproot. The emerging plumule is bent in a ‘u’-shape, but it soon withdraws its tip from within the seed, straightens up and grows vigorously. The endosperm remains inside the testa. The plumule produces the first pair of leaves in about 10 days after commencement of germination. Subsequently, an internode grows and the first flush of three leaves is produced above it. At the same time, further lateral roots grow out on the primary taproot, which will continue rapid growth and will be well provided with root hairs near its tip (Gomez 1982).

3.1.5 Vegetative Growth

Growth in the length of stem is discontinuous, with rapid elongation of an internode towards the end of which a cluster of leaves is produced. This will be followed by a rest period for the scale leaves to develop around the terminal bud. This sequence is repeated and leaves are produced in whorls separated by bare stem. Young scions of bud grafts elongate internodes for 2–3 weeks followed by a rest period. Although the elongation of stems is intermittent, their girth increases continuously. New flushes in the mature tree appear at any time of the year. The spirally arranged trifoliate leaves hang downwards approximately parallel to the petioles and are reddish or bronze in colour and gradually become green. The angle with the petioles will now be increased to 180°,

in which position they remain until they senesce. The mature laminae are shiny dark green on their upper surfaces and a paler, glaucous green below. Leaves are trifoliate and glabrous, arising on petiolules with long petioles (about 15 cm) bearing extra-floral nectaries at the point where petiolules merge (Fig. 3.2d). Nectar is secreted only on the new flush of leaves during flowering. The leaflets are elliptic or obovate with the base acute and the apex acuminate; they have entire margins and pinnate venation. Clones can be identified through a closer examination of the architecture of leaves (Mercykutty et al. 2002).

The upper epidermis and palisade parenchyma of the leaflets are single layers of cells below which there are several layers of spongy parenchyma and the single layer of the lower epidermis. The latter has many ridge-like appendages and a reticulate cuticle (Rao 1963). Stomata are only present in the lower epidermis. Senanayake and Samaranayake (1970) examined 25 clones and found that these showed a wide variation in stomatal density from 22,000 to 38,000 cm^{-2}, but they found no significant relationship between stomatal density and latex yield. Gomez and Hamzah (1980) investigated variation in leaf morphology and anatomy in 11 clones. They found significant differences between clones in: (i) stomatal density (which ranged from 28,000 to 37,000 cm^{-2}), (ii) cell number in the upper epidermis and (iii) thickness of palisade and spongy layers.

3.1.6 Wintering

Trees of more than 4 years exhibit defoliation or 'wintering', a term used to describe the annual shedding of senescent leaves which renders the trees wholly or partly leafless for about 15–20 days. Defoliation is followed by terminal bud bursting in 15 days, and in a week the expansion of new leaves occurs. There used to be a yield depression during defoliation and more markedly during refoliation. In areas experiencing a dry period, the duration of wintering tends to be short and refoliation is completed fast, thus minimizing yield reduction. Most of the non-traditional rubber-growing areas above 15°N fall under this category. For instance, trees of north-east India tend to be completely leafless for 10–15 days. There are marked differences between clones in wintering behaviour. A few tend to shed and replace part of their foliage simultaneously over a relatively long period and may thus show no very obvious signs of wintering, while at the other extreme some become completely leafless for a time. The majority are intermediate between these extremes. Clones also vary considerably in the extent to which they suffer yield depression during refoliation.

3.1.7 Root System

Trees grown as seedlings, or as bud grafts on seedling rootstocks, develop a strong taproot and extensive lateral roots, the whole root system forming about 15% of the total dry weight of a mature tree. In a study of rooting habit on a range of soils, which revealed no marked differences between trees of nine clonal seedling families, it was found that, on deep soils without impediments to root growth, 3-year-old trees had taproots about 1.5 m long and laterals 6–9 m long, while at 7–8 years the taproots were about 2.4 m and the laterals over 9 m. The laterals normally extend well beyond the spread of the branches so that in plantations at the usual spacing they commonly grow through the adjacent planting rows. The roots of neighbouring trees intermingle and some may become grafted together. The major lateral roots almost invariably arise from the taproot in a whorl within 30 cm of the soil surface and grow horizontally, or only slightly downwards. Further laterals are commonly produced at a depth of 40–80 cm, but do not extend horizontally as far as those nearer the surface. All the laterals ultimately give rise to unsuberized, yellow-brown roots of about 1 mm diameter, possessing root hairs, and these are known as feeder roots since they are mainly responsible for absorption of nutrients and water. While the feeder roots are mostly in the top 30 cm of the soil, a proportion arises from the deeper laterals and there is no reason to believe that these are less efficient absorbers than those nearer the surface.

An investigation of the distribution of the feeder roots of clonal seedling trees aged

1–22 years, planted in rows 6.1 m apart on a range of soils, showed that up to 3 years after planting the roots were concentrated near the trunks; at 4 years the feeder roots of trees in adjacent rows met; and at 5–7 years the feeder root density in the centre of the interrows was significantly greater than close to the trees. After the roots of neighbouring trees had met, ramification occurred nearer the trunk with the result that in the mature plantation there was little variation in the concentration of feeder roots across the interrows, except where the roots branched prolifically on entering a patch of particularly well-aerated, moist or nutrient-rich soil.

While the development of the root system always follows the general pattern described above, considerable variations occur due to: (i) soil type, (ii) soil aeration and moisture content, (iii) cultivation, (iv) nature of the ground vegetation and (v) mode of fertilizer application. Soong (1976) investigated the influence of several factors on the density and distribution of feeder roots to a depth of 45 cm by augur sampling the roots of mature trees of four clones, all bud grafted on Tjir1 clonal seedlings and growing on seven different soil series. He found that feeder root development was markedly influenced by the scion clone: for example, the vigorous clone RRIM 605 had about 80% more feeder roots by weight than the slower-growing RRIM 513. Soil texture had a marked effect. On sandy soils the weight of feeder roots was significantly greater than on clayey soils, probably due to the plant's reaction to the lower moisture retention, or better aeration, of the former soils. Over a range of soil types, feeder root densities were positively and significantly correlated with fine sand content and negatively correlated with clay content. However, this did not apply on some clayey soils which possessed a good structure due to their high sesquioxide content. On most soils about 50% of the feeder roots in the 0–45 cm layer were in the top 7.5 cm, and the proportion decreased rapidly with depth, only about 10% occurring between 30 and 45 cm below the surface. Exceptions to this were found on soils with a compact or poorly structured subsurface layer, where 70% or more of the feeder roots could occur in the top 7.5 cm, and also on uncompact soils with little or no profile differentiation, where distribution was fairly uniform throughout the 0–45 cm layer. In the 7.5–45 cm soil layer, the lowest feeder root density occurred at the same time as in the surface soil, but peak root development was about 3 months later than it was in the upper layer.

The presence of mycorrhizae on the roots of rubber trees was recorded by Park in Ceylon in 1928 (Dijkman 1951). In Malaysia, the occurrence of endotrophic, vesicular arbuscular mycorrhizae of the endogone type on the roots was found to be general on rubber trees of all ages and on a variety of soils (Wastie 1965). Spores of several species of endomycorrhizal fungi have been identified in soil samples from rubber plantations examined by Jayaratne (1982) in Sri Lanka and by Ikram and Mahmud (1984) in Malaysia. It is not known whether the mycorrhizae are of significance in the nutrition of the tree. Young rubber trees grow well from sterilized seed in sterile sand if supplied with the requisite nutrients, but mycorrhizae may be of value on soils high in organic matter or of low phosphate content.

3.1.8 Juvenile and Mature Characteristics

When grown from seed, the rubber tree passes through a juvenile stage to a mature stage, the latter normally beginning with the formation of the branches. Throughout its life a seedling tree exhibits certain juvenile characteristics, in that its bark is somewhat rough, and with increasing height the trunk tapers, the thickness of the bark decreases and the number of latex vessel rings declines. The 'mature-type' bud grafting used commercially is formed by bud grafting seedling rootstocks with buds which are mature-stage tissue. The scion does not pass through a juvenile phase and therefore grows into a trunk which lacks juvenile characteristics; it does not taper but is almost cylindrical, the bark is smoother than that of a seedling and both its thickness and the number of latex vessel rings within it remain virtually constant with increasing height. Virtually a primary branch is being tapped in bud-grafted trees.

3.1.9 Growth Studies

Templeton (1968, 1969) studied the growth before and during tapping of two clones, RRIM 501 and RRIM 513, bud grafted in the field on seedling stocks planted at the normal spacing of 9.15 × 2.44 m (30 × 8 ft), giving 444 trees ha^{-1}. The determination of the dry weight of the different parts of the plants, including the roots, was done by destructive sampling of complete trees at 9, 15, 21, 27, 39, 55, 63 and 81 months after bud grafting. The total dry weight per tree increased approximately exponentially up to 39 months from bud grafting, after which the rate was slower. Trees of RRIM 501 exceeded 300 kg within 81 months, a figure agreeing well with that obtained by Shorrocks (1965) for the same clone. The rate of girth increment rose to a maximum of 1.0 cm $month^{-1}$ between 27 and 39 months and then declined. The relative growth rate (RGR; the rate of increase in dry weight per unit of dry matter present per unit of time) declined steadily from 0.04 g $week^{-1}$ at 9 months to 0.005 g $week^{-1}$ at 81 months. The leaf area ratio (LAR; leaf area per unit of dry weight) was naturally low at first with a small scion on a 1-year-old rootstock, but rose to 12 cm^2 g^{-1} by 9 months from bud grafting and remained at about this level until 39 months, after which it declined steadily, reflecting the increasing proportion of plant weight in the non-photosynthetic tissues of trunk and branches. The leaf area index (LAI; the area of leaf laminae per unit area of ground) increased rapidly to reach a maximum of 5.8 m^2 m^{-2} between 50 and 60 months, when a complete canopy over the ground was achieved, and continued at this level up to 81 months. The net assimilation rate (NAR; the rate of increase in dry weight per unit area of leaf) declined slowly from 0.0032 g cm^{-2} $week^{-1}$ at 9 months to 0.0013 g cm^{-2} $week^{-1}$ at 81 months due to increased self-shading of the foliage as the LAI increased, resulting in a lower photosynthetic rate per unit leaf area. The crop growth rate (CGR; the rate of dry matter production per unit area of ground per year, which is proportional to LAI × NAR) increased to a peak of 35.5 t ha^{-1} $year^{-1}$ by 55 months, after which it declined. The cumulative dry weight for clone RRIM 501 at 81 months was 135 t ha^{-1}, which is close to the figure for the same clone quoted by Shorrocks (1965). Templeton (1968) considered that the RGR, LAR, NAR and CGR would all decline slowly after canopy closure, but that the LAI, which had levelled off at 5.8 m^2 m^{-2} after 63 months, would remain near this value for many years. However, Shorrocks (1965), who reported a similar LAI value (6.3 m^2 m^{-2} at 6 years) for RRIM 501 to that of Templeton, found that the LAI rose to 14 m^2 m^{-2} at 10 years and was still about 9 m^2 m^{-2} at 24 years. Premature senescence and fall of lower leaves is frequently observed in plantations with dense canopies. It is also common for some of the lower branches of mature stands to die back and be shed. Both these features suggest that the LAI is high enough for self-shading to reduce the light intensity to below the compensation point low down in the canopy. Templeton's data show that the CGR of RRIM 501 fell off after 4–5 years, while the LAI rose to 5.8 m^2 m^{-2} at 5 years and remained at this level. Similarly, Shorrocks's figures indicate that the CGR fell from the sixth to the tenth year, while the LAI rose over the same period. Thus, there is some evidence to suggest that the LAI may be above the optimum for dry matter production (but not necessarily for latex production) over much of the life of the plantation. Templeton (1969) estimated the efficiency of the rubber tree from the maximum recorded CGR of 35.5 t ha^{-1} $year^{-1}$ of dry matter which, at 4800 cal g^{-1}, is equivalent to 1704×10^8 cal ha^{-1}. The average solar radiation in Malaysia is 420 cal cm^{-2} day^{-1} and, assuming 40% utilization of this energy for photosynthesis (including 10% loss of radiation energy to non-photosynthetic pigments), the available energy amounts to about 61,320 × 108 cal ha^{-1} $year^{-1}$, so that the efficiency of utilization of solar radiation by the closed canopy of the rubber plantation is about 2.8%.

3.1.10 Root Heterogeneity and Stock-Scion Interactions

A major part of rubber breeding efficiency can be attributed to the grafting technique which enables the multiplication of elite genotypes at the level

of the bud-grafted part (aerial part of the tree) and which determines the use of clones as the almost exclusive varietal type in rubber cropping. Unfortunately, cloning the whole tree (aerial part and roots) for the development of single-component clonal trees by the cutting technique (self-rooted marcots and mist-propagated cuttings) generates a high ratio of uprooting due to lack of taproot and inadequate anchorage. Notwithstanding, a bud-grafted population has a high level of homogeneity and should exhibit intraclonal variation in yield to a minimum, barring factors such as: (i) soil heterogeneity, (ii) difference in juvenility of buds and (iii) variable seedling rootstocks. However, experience with RRII 105 monoclonal population spelled a difference of 10–310 ml in the total volume of latex per tap and a range of 28.1–43.9% in dry rubber content (drc) during the peak yielding period (October–January). An estimate showed 20% of the trees yielded more than 150 ml per tree per tap and 20% showed higher drc (more than 38%). In another experiment with RRII 105, the total volume of latex and dry rubber yield were 5.0–325.0 ml and 1.8–144.0 g, respectively (Chandrashekar et al. 1997). Such variation was reported from countries such as Malaysia (Hardon 1969), Indonesia (La Rue 1921) and Sri Lanka (Philpott 1946). The differences exhibited are significant and refutable for a homogeneous population. Two factors may affect these results. The first factor is soil heterogeneity, a key attribute manoeuvring the overall yield of a stand and one that can be geared at will through observance of appropriate agronomic practices. Soil can be tested for any deficiencies, and a fertilizer dosage can be followed in cognizance with the soil test data. The second factor is the difference of juvenility in bud-grafted plants. This factor has actually not been assessed so far. However, this should not make a significant difference, since bud-grafted plants of a given population would normally arise from the same bud-grafting generation (see Box 3.2).

The effect of rootstock has been most intriguing. Studies conducted in the past have proved there are reliable and marked effects of rootstock on the yield of scion when bud grafting was made on to illegitimate seedlings, where variation in terms of yield and girth was significant (Buttery 1961; Abbas and Ginting 1981). Further, it has been demonstrated that monoclonal or selfed seedlings from monoclonal blocks of bud-grafted plants showed marked differences in yield Ng et al. (1982). From these results, it was suggested that vigorous hybrid seedlings issued from polycross seed gardens would provide better rootstocks (Simmonds 1985). Also, monoclonal seedlings of PB 5/51 and RRIM 623 were found to be significantly superior to other stocks (Ng 1983). In an unpublished experiment (Centre National de Recherche Agronomique (CNRA)–Centre International de Recherche pour l'Agriculture et le Développement (CIRAD), Côte d'Ivoire), monoclonal seedlings of GT 1 used as the rootstock were found to be significantly better than three other monoclonal seedling rootstocks (issued from clones RRIM 600, PB 5/51 and LCB 1320). This confirms the good performance of GT 1 seedling rootstock already published earlier (Combe and Gener 1977). This performance of GT 1 seedling rootstock is assumed to be linked with the male-sterile behaviour of clone GT 1 which displays exclusive unselfed seedling progenies, with no inbreeding effect and a better growth.

Another experiment confirms that the most important part of yield variation is due to differences between the bud-grafted clones rather than between the seedling rootstocks, so mitigating the importance of the choice of seedling rootstock which is heavily dependent on the availability of monoclonal seedlings in the planting areas. It was

Box 3.2

The bud wood nursery needs to be replanted every 10–12 years. Otherwise, the juvenility of the bud wood becomes very minimal. The stocks are juvenile since they are arising from fresh seeds. If the bud wood is also juvenile (within 5 years of age), the population so arisen is expected to have more or less uniform growth and yield. No data and evidences are available in favour of such an argument, but the logic behind juvenility prompts one to think so.

also shown that the rootstock can affect the thickness of scion bark and the number of latex vessel rings. Babilioff (1923) made a notable observation that during the formation of new bark, when the scion cambium made 12 latex vessels, stock could make nine latex vessels, so making the superiority of the scion cambium more prominent. Some logical inferences are also worthy here. Many clones especially in Kerala of South India perform significantly different under varied soil and climatic conditions. While RRII 105 and RRIM 600 yield almost uniformly, RRII 400 series clones yield differently even under nearest fields. It is highly reasonable to presume that these clones have a high preference towards a right stock that needs to be researched intensively, because recommended clones must yield near uniformly in planters' fields.

The rationale enunciated here indicates that, though the soil heterogeneity and juvenility of buds may not necessarily influence the yielding pattern of a bud-grafted population, the variable rootstock should exert effects over yield that are largely uncontrollable. Although poorly addressed by breeding in the lack of an efficient cloning technique, the root system directly affects: (i) soil–plant relationships, (ii) water and mineral uptake, (iii) water stress resistance (Ahmad 2001) and (iv) resistance to uprooting by wind. Moreover, efficient breeding for growth of bud-grafted clones and the increasing use of fast-growing clones may have generated an imbalance between stock and scion, so emphasizing uprooting hazard (Clément-Demange et al. 1995). Consequently, cloning the root system is a major challenge for rubber tree breeding, as it would greatly facilitate growth and yield improvement as well as adaptation to various environments. A comparative study of shoot tip cultured and bud-grafted plants of RRII 105 with DDRT-PCR (differential display reverse transcription polymerase chain reactions) to track down the transcript level changes (mRNA differential display) in latex showed a similarity index of 57% in bud-grafted plants and 80% in plants raised through shoot tip culture (Geetha et al. 2010). The increased similarity index in shoot tip culture-derived plants could be due to lack of stock-scion interactions.

3.2 Ecophysiology

Rubber planters are under permanent pressure to raise land productivity while protecting the environment as a result of: (i) decrease in the amount of cultivable land, (ii) the change in human lifestyles increasing the demand for rubber and (iii) environmental issues generating a demand for natural rubber over synthetic rubber. During the past decade, this increasing demand has prompted rubber cultivation to be rapidly expanded into non-traditional rubber-growing areas. The principal way of achieving high productivity has been the development of high-yielding clones with desirable characteristics through rigorous breeding and selection. Supplementary approaches to achieve greater productivity have been the determination of an optimum tapping schedule and soil management to maintain fertility. All these parameters affect the overall growth and physiology of trees. In this section, aspects of ecophysiology are considered in some detail.

Before the rubber tree can produce latex, it needs to attain maturation of the leaves, the organs of photosynthesis. Leaves attain maturity in around 35–40 days after emergence. In addition to phytohormonal equilibrium, leaf maturity can be regarded in terms of CO_2 balance (Samsuddin and Impens 1979), and the maturity leads to a series of characteristics such as leaf expansion, chlorophyll accumulation and formation of photosynthetic apparatus (photosystems I and II and carboxylative enzymes), stomata, cell wall and supporting structures. Knowledge about phenological behaviour is especially relevant when one intends to determine the required time to start leaf net photosynthesis (Senevirathna et al. 2003). Studies on leaf ontogeny, relative to CO_2 balance, even done under greenhouse conditions (Bergonci 1981; Pita 1984; Schwob et al. 1998) are scarce under field conditions. The dry matter increase in rubber tree leaves is directly related to the balance between CO_2 assimilation from photosynthesis and its release by respiration. During the juvenile phase, the CO_2 balance in the rubber tree is especially affected by significantly higher respiration. The higher respiration rates that occur in the juvenile phase seems to indicate a higher metabolic activity (growth

respiration), during which energy released is required to synthesize structural compounds and chlorophyll. To reach net photosynthesis, the young leaves need to increase the concentration of CO_2 (Miguel et al. 2007).

The dynamic aspects of the competition between latex and wood production and the spatial distribution of radial growth rates around the tapping cut have been studied by Silpi et al. (2006). In untapped control trees, radial growth started with the onset of the rainy season and lasted until the onset of the dry season, with no growth during the driest and winter periods. Also, on reaching the flowering stage, the annual girth increment is seen to be significantly greater, and after the trees are tapped, the girth increment is drastically reduced (Priyadarshan and Clément-Demange 2004). While the former is due to attainment of physiological maturity, the latter is due to source–sink adjustments and the cumulative growth was about half that of untapped trees. When the latex production increased over the years, establishment of a latex sink becomes a long-term process probably involving many aspects of metabolism.

3.2.1 Photosynthetic Efficiency

As *Hevea* rubber is a perennial crop requiring over 6 years' growth before latex can be harvested and another 7 years to assess yield, breeding programmes take more than 20 years to produce a suitable clone. Based on the principle that plants produce basic compounds for growth and yield through photosynthesis, photosynthetic efficiency, together with associated factors such as water vapour diffusion, was coined as the early determinant of high-yielding genotypes (Nugawela and Aluthhewage 1985; Nugawela et al. 1995a). Knowing the high variability of the instantaneous rate of photosynthesis due to its dependency on the incident radiation levels, key parameters of the light response curve (LRC) of photosynthesis (i.e. light-saturated level of photosynthesis (A_{max}, the photosynthetic rate at which the LRC tends to plateau) and apparent quantum yield (φ_{app}, initial slope of the LRC) could be used in such studies (Rodrigo 2007). The rate of A_{max} denotes the maximum expected rate of photosynthesis under the ambient temperature and CO_2 levels (light saturation stage), while φ_{app} demonstrates the photosynthetic performance under low light levels (light-limiting stage). In general, an equation of either a rectangular hyperbola or quadratic function is used to construct the LRC, and the latter provides a more accurate measure of A_{max} and φ_{app} and also provides an additional parameter on the convexity (θ) of the LRC which represents the transition stage between light limitation and saturation (Hay and Walker 1989; Thornley and Johnson 1990). Radiant energy (P_{sat}), carboxylation efficiency (CE), stomatal conductance (g_s) and CO_2 compensation concentration (Γ) are the attributes that influence the biomass production and water use efficiency (WUE). A clone with high P_{sat}, high CE, low g_s and low Γ will be more dependent on mesophyll factors than stomata, producing relatively more biomass and maintaining high WUE (Nataraja and Jacob 1999). Attempts to use photosynthetic parameters to judge yield potential for early screening for newly bred *Hevea* genotypes were made in Sri Lanka. The daily photosynthetic integral was estimated using the Charles-Edwards equation (Charles-Edwards 1982), where A_{max} and φ_{app} are the key components. Photosynthetic rates and WUE were greater in high-yielding clones and vice versa for dark respiration rates (Nugawela et al. 1995b). Even though the approach is unique, genotype separation is only into three broad categories of high-, medium- and low-yielding groups. While seedlings behave as discrete units exposing most of the leaves to incoming solar radiation, there is a high level of light attenuation within the closed canopies of mature rubber trees. Photosynthesis in mature trees is highly dependent on canopy architecture, which is generally represented by the light extinction coefficient (k) and the Leaf Area Index (LAI). Hence, it would be unrealistic to predict the values of k and LAI of a mature crop from the assessment in seedlings (see Chap. 7 for parameters used for early selection). Attributing a fixed value for k across all genotypes and estimation of LAI of different genotypes with known differences in LAI and

leaf area per whorl of seedlings were also attempted (Nugawela et al. 1995b). This exercise was too simplistic, offering large variation in the seedling population. Essentially, developing a simple model to predict k and LAI of mature crops would also require consideration of canopy architecture.

3.2.2 Dry Matter Production and Water Use Efficiency (WUE)

As said earlier, rubber cultivation has been extended to sub-optimal non-traditional rubber-growing areas. These areas experience a multitude of stresses including soil moisture and cold stresses. Trees avoid the effects of water stress by judicious maintenance of water uptake, reducing transpirational loss or by osmotic adjustment. The mechanism and the magnitude of the response depend on the genotype and its sustainability and productivity. Stomata of *Hevea* leaves are highly reactive to environmental changes (Rodrigo et al. 2005b). Diffusive resistance to water vapour and CO_2 of stomata depends mainly on atmospheric vapour pressure deficit, irradiance and temperature. Trees will be responsive to internal water status as measured by leaf water potential (ψ_μ), which is highly dependent on soil water status. Therefore, clonal responses to dry conditions are generally assessed with measurements of stomatal conductance (g_s) or resistance (r_s) (each being the reciprocal of the other), ψ_μ and relative water content.

Trees grown in wet regions of Sri Lanka (annual rainfall is ~ 5000 mm) have shown a wide range of r_s, varying from 1 to 8 s cm^{-1} (Rodrigo et al. 2005b). Stomatal resistance is distinct with diurnal variation commencing with high values around midday, particularly after dry spells. Under no major soil moisture deficit, the maximum r_s is ~ 4 s cm^{-1} which could increase over twofold under dry conditions. An increase in r_s can adversely affect the photosynthetic rate by limiting CO_2 transfer (Rodrigo 1997). Similarly, trees grown under two distinct agroclimatic regions, namely, Dapchari of Western India (with high temperature summer stress) and Agartala of north-east India (with cold winter stress), experienced high photosynthetic photon flux density (PPFD) and severe inhibition of photosynthesis. The upper canopy leaves exposed to high PPFD fixed little car- bon through the day. Photosynthetic rates were higher in the shaded leaves with low PPFD. Inhibition of photosynthesis due to high PPFD was also evident in the decreased quantum yield of CO_2 assimilation and in vivo photosystem II (PSII) activity in the stressed leaves (Devakumar et al. 2002). Photosynthesis is one of the foremost processes that is inhibited when plants are exposed to drought and cold (Baker 1996). High solar radiation can cause an imbalance between light and dark reactions of photosynthesis leading to increased diversion of photosynthetic electrons for the production of oxygen species that will further lead to senescence of stressed leaves (Jacob et al. 1999). Under an ideal climate, leaf photosynthesis saturated at a photosynthetically active radiation (PAR) of 1000 μmol m^{-2} s^{-2} (Nataraja and Jacob 1999). On the other hand, a higher PAR will induce imbalance between the photochemical and the biochemical reactions resulting in the overenergization of thylakoid membranes, diverting photosynthetic electrons for the production of active oxygen species (AOS) like superoxide, hydrogen peroxide and singlet oxygen (Jacob et al. 1999). Photosynthetic response to higher temperatures was similar in different clones. Subjecting RRIM 600 and PB 260 to a wide range of temperatures between 10 and 45 °C, Kositsup et al. (2007) concluded that the rate of photosynthesis stayed constant between 23 and 37 °C and decreased above or below this range.

The WUE that gives the measure of how much dry matter is produced per unit of water consumed under moisture stress situations is most important. Only a limited amount of work has been conducted in this line since growth and water use assessments on *Hevea* rubber are tedious. Other studies have been dependent mainly on the instantaneous rate of CO_2 assimilation and predicted transpiration rates based on stomatal conductance without properly taking into account the boundary layer conductance at different canopy levels. Values of WUE were within the range of 1.5–3.5 g mm^{-1} m^{-2} (Rodrigo et al. 2005a), while that

calculated using CO_2 assimilation and stomatal conductance was below 1 μ mol CO_2 $mmol^{-1}$ H_2O for water stress conditions (Dey and Vijayakumar 2005) and 3 μmol CO_2 $mmol^{-1}$ H_2O without stress (Nugawela et al. 1995b). Since *Hevea* rubber originated in the wet tropics, moisture stress situations do not favour dry matter production owing to low values of WUE. The extreme moisture stress conditions in Dapchari resulted in low plant moisture status and high plugging indices coupled with inhibition of stomatal conductance and transpiration rates. A comparative study with two clones (GT 1 and RRIM 600) revealed that attributes influencing latex flow and production, namely, pre- and post-tapping turgor (P_{LV}), latex solute potential (ψ_π), leaf water potential (ψ_μ) and stomatal conductance (g_s), significantly decline in the dry season (February–May) compared with the wet season (June–December). Water deficit during the non-rainy season can be as high as 1070 mm as against 350 mm in the traditional rubber-growing areas (Jacob et al. 1999). Adequate irrigation reduced the immature period to six years (Devakumar et al. 1998). Only a few rain-fed trees attained tappable girth even after nine years (Devakumar et al. 1998). The first-year yield for these two clones was 550 kg and 622 kg ha^{-1}, respectively (Chandrashekar et al. 1990). These clones gave 672 and 681 kg ha^{-1} in the traditional rubber-growing areas of India, whereas in the cold-stressed environment of Tripura (north-east India), it was 577 kg and 1085 kg ha^{-1}, respectively (Priyadarshan et al. 1998b).

Generally, the Penman–Monteith equation is used to estimate the amount of water loss through transpiration (Monteith 1965; Monteith and Unsworth 1990). Though data can be retrieved through sophisticated instruments, the assessment of boundary layer conductance (g_b) is time-consuming and requires continuous weighing of leaf samples in the crop canopy and calculations to find g_b values. A study with *Picea sitchensis* gives a classic example to demonstrate the tediousness of assessment of g_b where a huge tree had to be suspended in the air and provided with artificial rain (Teklehaimanot and Jarvis 1991). Therefore, suitable ecophysiological models are required to estimate g_b. A model has been developed to estimate g_b of tree crops (Rodrigo et al. 2005a), and this has been used for rubber ($g_b = 0.048 \times U \times LAI0.381$ where U refers to wind speed at 2 m above the canopy, in m s^{-1}). Nevertheless, only the LAI represented the canopy architecture in this model (Rodrigo et al. 2005a), and therefore future investigations should incorporate other parameters of canopy architecture such as leaf distribution. The thermocouple-based heat pulse/sap flow system provides direct measurements of transpiration water loss with no difficulty in assessing either g_b or g_s. This system has not been used effectively in rubber. The adaptability of trees in dry regions to conserve water on overall canopy photosynthesis and latex productivity also needs attention. Rubber is grown in cooler climates in China and India (in areas above 21°N). *Hevea* rubber is tropical and is predominantly grown in areas where conditions are above 20 °C (ideally 28 ± 2 °C with a diurnal variation of about 7 °C; Barry and Chorley 1976) and at altitudes below 200 m mean sea level (MSL) (see Chap. 8). High temperatures result in high evapotranspiration and water stress, while low temperatures lead to low growth rates and cold damage. Low temperatures can cause permanent damage to the photosynthetic apparatus. Photoinhibition is the final result under such circumstances even under moderate light levels, which otherwise are beneficial under optimum temperatures. A few clones have proved to be tolerant to low temperature. For instance, GT 1 has performed reasonably well at 5 °C in China, and SCATC 93/114 (now named REYAN 93/114) can tolerate temperatures even below 0 °C for a short period (Zongdao and Xuequin 1983; Priyadarshan 2003a). Though such clones survive the cold stress, overall productivity appears to be rather lower (Priyadarshan et al. 1998a). However, photosynthetic efficiency can be a measure for selecting such clones. Although A_{max} provides a useful measure, assessments of Φ_{app} and chlorophyll *a* fluorescence emission in terms of the ratio of variable to maximum emission (F_v/F_m) indicate the level of any damage to the photosynthetic apparatus (Ireland et al. 1989). Photoinhibition of the leaf photosynthetic apparatus results in a major decrease in the F_v/F_m ratio and can be measured with a commonly available portable plant efficiency analyser. Being a rapid

measurement, fluorescence emission can be assessed in a large number of plants within a short period and hence it would appear to be a suitable measurement for shortlisting suitable clones. Thereafter, analysis of the light response of photosynthesis would be appropriate in early screening. The same procedure could be adopted in genotypic evaluation to some extent under dry conditions; however, in rubber plants, no permanent damage of the photosynthetic apparatus has been recorded in such conditions. Instead, any short-term drop in the F_v/F_m ratio and Φ_{app} could be attributed to downregulation of photosynthesis. In both wet and dry climatic conditions in Sri Lanka, the F_v/F_m ratio was in the range of 0.75–0.85 (Senevirathna et al. 2003; Iqbal and Rodrigo 2006). Only under sunny conditions has some level of decrease in the Fv/Fm ratio been recorded around midday (Senevirathna et al. 2003). Such downregulation of photosynthesis limits the maximum capacity of photosynthesis, affecting the A_{max}, and, in addition, is more prominent in the early stages of field establishment when rubber trees are small (Iqbal and Rodrigo 2006).

Physiological responses and expressions of genes involved in energy biosynthesis and reactive oxygen species (ROS) scavenging in plants under drought conditions are noteworthy. Relative water content (RWC) in leaves was continuously decreased with the severity of drought stress in plant of GT 1. Wilting of leaves were observed at 7 day without water (dww). The contents of malondialdehyde (MDA) and proline were significantly increased under drought stress. Peroxidase (POD) and superoxide dismutase (SOD) activities were markedly enhanced at 1 and 3 dww. Meanwhile, the soluble sugar content was constant under drought stress. These indicated that photosynthetic activity and membrane lipid integrity were quickly attenuated by drought stress in rubber tree, and osmoregulation participated in drought tolerance mechanism in rubber tree. Expressions of energy biosynthesis and ROS scavenging systems related genes, including *HbCuZnSOD, HbMnSOD, HbAPX, HbCAT, HbCOA, HbATP* and *HbACAT*, demonstrated that these genes were significantly up-regulated by drought stress, and reached a maximum at 3 dww, then followed by a decrease from 5 dww (Wang 2014a). That the drought stress adaption in rubber tree was governed by energy biosynthesis, antioxidative enzymes and osmoregulation was evident from the studies of Wang (2014a).

Light being the pivotal factor for photosynthesis, Wang (2014b) conducted gene expression analyses under different light intensities in the seedlings of GT 1. When light intensity increased from 20 to 1000 μ mol m^{-2} s^{-1}, there was no effect on the maximal quantum yield of photosystem II (PSII) photochemistry (Fv/Fm), indicating that high light intensity did not damage the structure and function of PSII reaction centre. However, the effective photochemical quantum yield of PSII (Y(II)), photochemical quenching coefficient (qP), electron transfer rate (ETR) and coefficient of photochemical fluorescence quenching assuming interconnected PSII antennae (qL) were increased significantly as the light intensity increased, reached a maximum at 200 μ mol m^{-2} s^{-1}, but decreased from 400 μ mol m^{-2} s^{-1}. These results suggested that the PSII photochemistry showed an optimum performance at 200 μ mol m^{-2} s^{-1} light intensity. The chlorophyll content was increased along with the increase of light intensity when it was no more than 400 μ mol m^{-2} s^{-1}. Since increasing light intensity caused significant increase in H_2O_2 content and decreases in the per unit activity of antioxidant enzymes SOD and POD, the malondialdehyde (MDA) content was preserved at a low level even under high light intensity of 1000 μ mol m^{-2} s^{-1}, suggesting that high light irradiation did not induce membrane lipid peroxidation in rubber tree. Moreover, expressions of antioxidant-related genes were significantly up-regulated with the increase of light intensity. They reached the maximum expression at 400 μ mol m^{-2} s^{-1}, but decreased at 1000 μ mol m^{-2} s^{-1}. In conclusion, rubber tree could endure strong light irradiation via a specific mechanism. Adaptation to high light intensity is a complex process by regulating antioxidant enzyme activities, chloroplast formation and related gene expressions in rubber tree (see Chap. 9 for physiology under suboptimal environments).

Propagation Systems

4

As in any other tree species, *Hevea* is also multiplied by both seeds and vegetative means. There are different kinds of rubber seeds such as (i) legitimate, (ii) illegitimate, (iii) ordinary, (iv) monoclonal and (v) polyclonal seeds. Among these, monoclonal and polyclonal seeds are produced in specially raised plantations. The other types of seeds are collected from commercially established plantations. Monoclonal seeds, especially those of Tjir 1, were once commonly used for propagation. For this, plantations consisting of this clone alone were established. Special care was taken not to have any tree of any other clone in the garden to prevent contamination of pollen grains. This ensured that all the seeds produced were selfed seeds of Tjir 1. However, monoclonal seeds are not recommended now for raising plantations due to their inferiority compared with the modern clones.

4.1 Polyclonal Seed Generation

Polyclonal (polycross) seeds, which are hybrid seeds, are produced in plantations called polyclonal seed gardens. In these gardens, several clones are planted intermixed so as to maximize cross-pollination. Clones planted in these gardens should possess desirable characters such as (i) high yield, (ii) disease resistance, (iii) vigour, (iv) ability to produce good seedling families and (v) profuse production of seeds. All the clones should flower simultaneously. Genetic factors like genetic divergence and inbreeding depression also have to be taken into account while selecting clones (Mydin et al. 1990). Some of the clones usually planted in polyclonal seed gardens are RRIM 600, RRIM 605, RRIM 623, PB 5/51, PB 28/59, Tjir 1 and PR 107. Other clones having the required desirable attributes mentioned above could also be included. The number of clones in the seed gardens usually varies from three to seven (Simmonds 1986). To maximize cross-pollination, special designs are adopted while planting. Selection of at least four clones enables better randomization so that trees of the same clone are not planted adjacent to each other (see Chap. 7). A wider spacing is adopted in seed gardens for proper development of the crown, essential for profuse flowering and fruit set. A spacing of 9.1 × 3.0 m (358 trees ha^{-1}) is considered suitable for this purpose. The stand is reduced to 247 by the sixth year by progressive thinning out.

Pollinating insects may carry pollen from other nearby rubber trees. Such contamination of pollen grains can result in production of undesirable seeds with different genetic constitutions and their mixing up with the desirable ones. To prevent this, an isolation belt, about 100 m wide, is provided around the garden. This isolation belt is planted with a non-rubber crop. If rubber has to be planted, one of the clones included in the garden has to be used. Production of seeds in a garden depends to a great extent on clones, climate and diseases. On average, a tree produces 150 seeds in well-maintained gardens.

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4.2 Vegetative Methods

Propagation through asexual (vegetative) parts such as buds, leaves and stem cuttings is termed vegetative propagation. Vegetative propagation of rubber is carried out mainly by bud grafting (budding is the colloquial term). Propagation through rooted cuttings is possible in rubber but is not generally practised due to unsatisfactory development of the root system, especially the taproot. The principle involved in bud grafting is the replacement of the shoot system of a genotype with that of another more desirable genotype. The method of bud grafting adopted is a modified form of the Forket method of patch bud grafting. In this process, a patch of the bark of the seedling plant (stock) is replaced by a bud patch taken from the clone to be multiplied (scion). A thin film of polythene is wound over the bud patch for waterproofing. After 21 days, the polythene is taken off. The bud patch gets attached to the stock permanently and becomes a part of it. The stock is then cut off above the bud-grafted portion and the grafted bud develops into a new shoot (scion) and then into a two-part tree.

Depending on the colour and age of the buds, three main types of bud grafting are recognized. These are (i) brown (conventional), (ii) green and (iii) young bud grafts. In the first method, older buds having a brown colour are used while, in the other two, tender green buds are utilized (Marattukalam and Saraswathyamma 1992). Depending on the part of the stock where bud grafting is carried out, the classification would be (i) base bud grafting, (ii) crown bud grafting, (iii) over bud grafting and (iv) high bud grafting.

4.2.1 Brown Bud Grafting

This was developed in 1916 in Indonesia by van Helten, a horticulturist, in collaboration with two planters, Bodde and Tass. The first handbook on this subject was published by Bodde in 1918 (Dijkman 1951). Brown bud grafting is generally carried out by grafting brown-coloured buds taken from budwood of about 1 year growth on to stock plants of 10 months old. Vigorously growing healthy stocks having a girth of 7.5 cm are ideal for grafting. Stocks should be grafted when the bark peels off very easily. Test peeling of a small patch of bark 15 cm above the base is the surest method of assessing the peeling quality of the bark. Brown buds are usually obtained from brown budwood produced by bud-grafted plants raised as source bush nurseries (SBNs). Buds found in the axils of fallen leaves are generally utilized for bud grafting. Budwood should be collected when the top whorl of leaves has fully expanded but not hardened to ensure proper peeling of the bark and high bud-grafting success. Budwood should, as far as possible, be collected in the morning or evening, and should preferably be utilized for bud grafting as soon as collected. If bud grafting is delayed, special measures should be adopted for preventing moisture loss. Budwood is harvested as per the requirement and cut into pieces of convenient length, usually 1 m.

Bud grafting is usually carried out with a specially designed knife with two blades called a bud-grafting knife. Taking the stock plant, two parallel vertical cuts that reach the wood are made, starting from about 2.5 cm above the collar. The cuts should be a little more than 5 cm in length and 1.5 cm apart. Then a horizontal cut joining the bottom ends of the vertical cuts is made. Latex oozes out for a few minutes through the cuts and this can be wiped off. The flap of bark separated by the three cuts is then gently lifted with the aid of the knife and peeled upwards. The practice of removing the flap completely is also adopted. The exposed region is called the bud-grafting panel.

The bud patch used for brown bud grafting has a length of about 5 cm and a width of about 1.5 cm. For preparing the bud patch, two parallel vertical cuts having a length of 5 cm are made on two sides of a bud, 1.5 cm apart. Then, two horizontal cuts are made connecting the lower and upper ends of these cuts. Latex is allowed to ooze out and meanwhile incisions are made around neighbouring buds of the same budwood. When the oozing of latex stops, it is wiped off and the bud patch marked out by the four cuts is stripped off. The inner side of the bud patch is examined for the presence of the core of the bud, which

appears as a slight projection. If that is not present, the bud patch should be discarded. The bud patch is then gently placed in the bud-grafting panel after lifting the flap. Due care must be taken not to injure the cambium. The panel is bandaged using a polythene strip. Bandaging should commence at the bottom and move upwards in a close tight spiral that can end with a knot. It requires 15–20 days for the bud patch to heal and form part of the stock. The presence of green colour on the bud when the bud is scratched indicates initial success of bud grafting.

4.2.2 Green Bud Grafting

Developed in Indonesia in 1960 by H.R. Hurov, this process involves a young stock plant and budwood. Stock seedlings would be 2–8 months old and the budwood 6–8 weeks old. Buds found above the scale leaves of the shoots alone are used for grafting. For proper peeling of the bud patch, harvesting should be done when the leaves are copper brown to dark green. After cleaning the basal portion of the stock, two vertical incisions, a little more than 5 cm long and 1 cm apart, are made, starting from a point about 2.5 cm above the collar region. The lower ends of these cuts are joined by a horizontal cut and a few minutes allowed for the cessation of latex flow. The flap of bark thus separated out is then gently lifted upwards exposing the bud-grafting panel. The flap is then cut off, leaving a short 'tongue' of about 1.5 cm at the top.

The bud patch can be stripped from the budwood in the same way as in the case of brown bud grafting. The upper end of the bud patch is gently inserted under the 'tongue' and placed in the bud-grafting panel (Tinley 1962). Then the bud patch is secured firmly by bandaging with a transparent polythene strip as in the case of brown bud grafting. This strip can be about 25 cm long and 2 cm wide. Transparent tape allows light to fall on the green bud patch, which in turn enhances the grafting success. Buds can be examined after 3 weeks. Retention of green colour is the indication of success (see Fig. 4.1). The final observation to check on the success of grafting is made after 10 or more days. Bud grafting can be carried out throughout the year, but predominantly during the rainy season (de Silva 1957). However, days with heavy rainfall are not suitable for bud grafting (Marattukalam and Premakumari 1982).

4.2.3 Young Bud Grafting

Young bud grafting is carried out on plants of less than 2 months old (Ooi et al. 1976). Stocks are raised in small bags of 33 × 15 cm size. The plants are given intensive nursing such as foliar application of fertilizers and fungicides twice weekly (Leong et al. 1986) and soil application of an N,P,K,Mg mixture weekly. Four weeks after bud grafting, plants are cut back to a height of 20–25 cm (Yoon et al. 1987). When the scion develops two or three whorls of leaves, it is transplanted in the field just like other bag plants. Using this technique, bag plants could be produced within 7 months after the planting of germinated seeds in the bags during August–September. These plants will also have a well-developed root system (Seneviratne 1995).

4.2.4 Crown Grafting

Susceptibility to diseases and wind are the undesirable characters of modern clones. An undesirable crown can be replaced by a desirable one through crown bud grafting. Crown bud grafting was first adopted in Indonesia (Java) in 1928 and in South America in the 1930s to prevent the damage caused by South American leaf blight (SALB). The tree produced by crown bud grafting is a three-part tree comprising (i) the root system of the stock plant, (ii) the trunk of one clone and (iii) the crown of another clone (Yoon 1973).

Crown grafting is ideally carried out when the scion has attained a height of 2.4–3.0 m; 1–2 years are usually required for the plants to attain such a growth. The height of the plant is more important than the age. Grafting is carried out at a height of 2.1–2.4 m on the inter-whorl region below the top whorl of leaves. Grafting

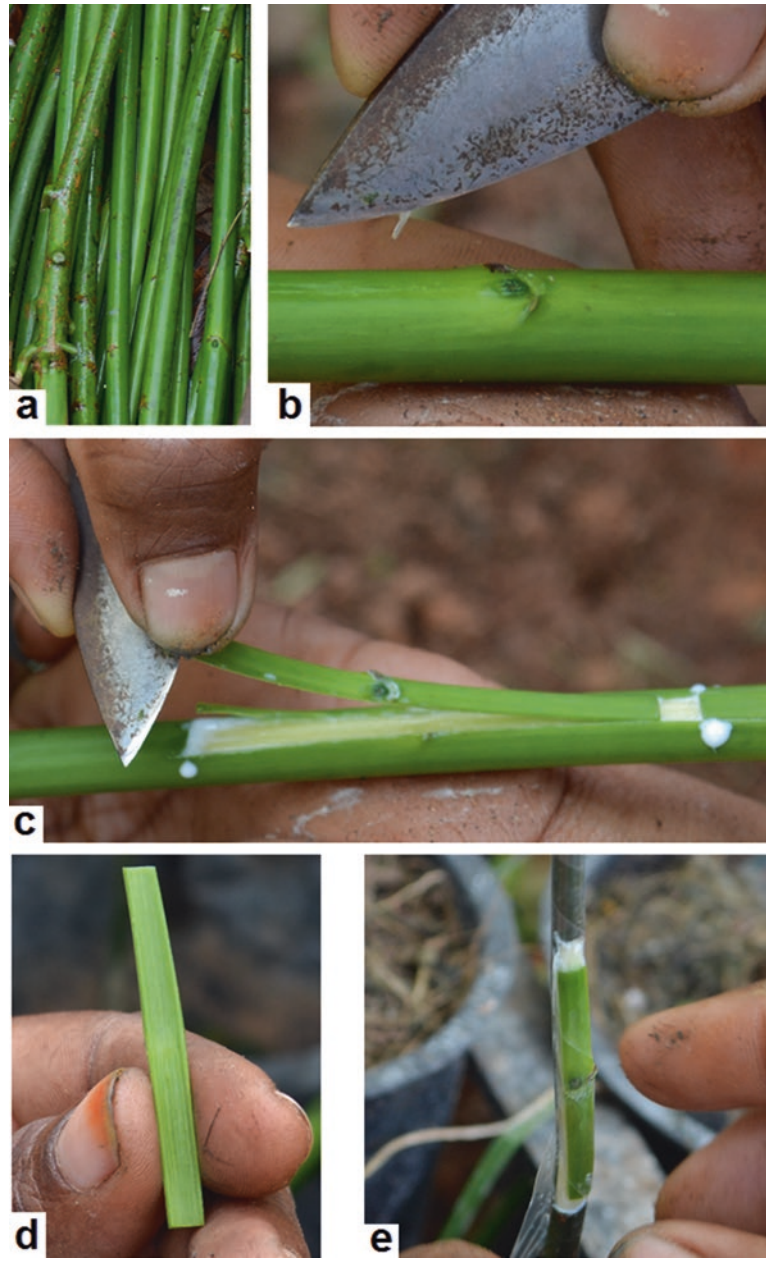

Fig. 4.1 (**a**) Set of budwood, (**b**) selected budwood, (**c**) peeling of budwood, (**d**) peeled budwood and (**e**) grafted budwood being sealed with polyethylene strip

should be done only when the top flush of leaves is fully expanded and hardened and the stem tissue should be green or dark green. Plants having a height up to 4.5 m can also be used for crown grafting. For crown grafting, the green grafting technique is followed. If grafting fails, re-grafting is done on the opposite side of the stem, 5 cm above or below the first attempt. Successfully grafted plants are cut back, leaving a snag of about 5 cm. Treating the cut ends of the stem with some wound-dressing compound is desirable. About 9 months after cutting back, when the crown–trunk union is firmly established, the trunk shoots are pruned. The crown shoot later on fully establishes itself and in due course develops to be the crown of the three-part tree

(Yoon 1973). Similarly, crown grafting can be done on to plants grown in polythene (poly) bags, which can grow into a full three-part tree.

4.2.5 Rooting of Cuttings

Terminal cuttings with one whorl of mature leaves are used for rooting. Rooting is done in a mist-propagation chamber, in raised beds filled with rooting media under artificial mist generating systems. Proper shade and coverage are provided to protect the cuttings from intense sunlight as well as to prevent mist drift (RRIM 1959). Shoots of about 30 cm length are planted in the beds and mist is applied continuously during the daytime. After 5–9 weeks, the cuttings produce roots. Subsequently, they are transferred to poly bags and kept in hardening beds (RRIM 1962). In these beds they are initially provided with continuous mist for 1 week, and thereafter the duration of misting is gradually reduced. Shade also is progressively removed. Within 3–6 weeks the process of hardening is over and the plants are ready for field planting. Corpuz (2013) could use alpha Naphthalene Acetic Acid for inducing rooting in brown stem cutting, but feasibility of such technique for large scale propagation is to be proved in order to implement it commercially.

4.2.6 Layering

Development of roots on a stem while it is still attached to the parent plant is known as layering. The type of layering adopted in the case of rubber is air layering. Young branches are used for this purpose (RRIM 1960). The stem is first girdled by removing the bark around it to a width of about 2–5 cm. The cambium of the exposed wood is completely removed by scraping and growth hormones are applied to this region to enhance rooting. Then this portion is completely covered with a ball of any moist rooting medium (such as sphagnum moss, soil containing plenty of organic matter, coconut husk–soil mixture) and finally covered with polythene sheet to prevent loss of moisture (Yoon and Ooi 1976). Within a few weeks, roots develop from the upper edge of the girdle and grow into the rooting medium. When the roots are properly developed, the layer is separated from the plant by cutting the branch below the ball. The layer is then planted after careful removal of the polythene cover without damaging the rooting medium. Studies conducted by the Rubber Research Institute of India (RRII) have proved that sphagnum moss is far superior to other rooting media (Sobhana et al. 1995).

4.2.7 Root Trainers

Planting materials of *Hevea* have traditionally been raised in poly bags. However, a drawback of poly-bag plants is that coiling of the taproot and lateral roots lead to slow growth and poor wind endurance (Sharma 1987; Josiah and Jones 1992; Ginwal et al. 2001). An alternative to raising plants in poly bags is using root trainer containers. These are made of polypropylene and have an inner diameter of 7.5 cm at the top, tapering and ending with a drainage hole at the bottom, are 30 cm in depth and have a holding capacity of 800 cm^3. The growth medium used in root trainer containers is sieved coir pith that has been previously dipped in water for 2 weeks to remove tannin. The dried coir pith can be mixed with powdered charcoal (in a ratio of 9:1) and rock phosphate (200 g) and neem cake (100 g) can be added. Appropriate quantities of fungicides and pesticides can also be added. One-third of the container is filled with this mixture and green bud-grafted stumps are planted into the container and the container is topped up with growth medium. Plants raised in root trainers showed better sturdiness (height:diameter ratio) than poly-bag plants (Soman et al. 2002). The lateral roots were also found to be significantly higher in root trainer plants compared with poly-bag plants (Soman and Saraswathy Amma 2005). In poly bags, the taproot reached the bottom of the bag in 6–7 weeks after planting the budded stumps and it started coiling thereafter leading to root strangling and distortion (Soman and Saraswathy Amma 1999). Compared with poly-bag plants, root trainer plants showed uniform distribution of roots (Fig. 4.2).

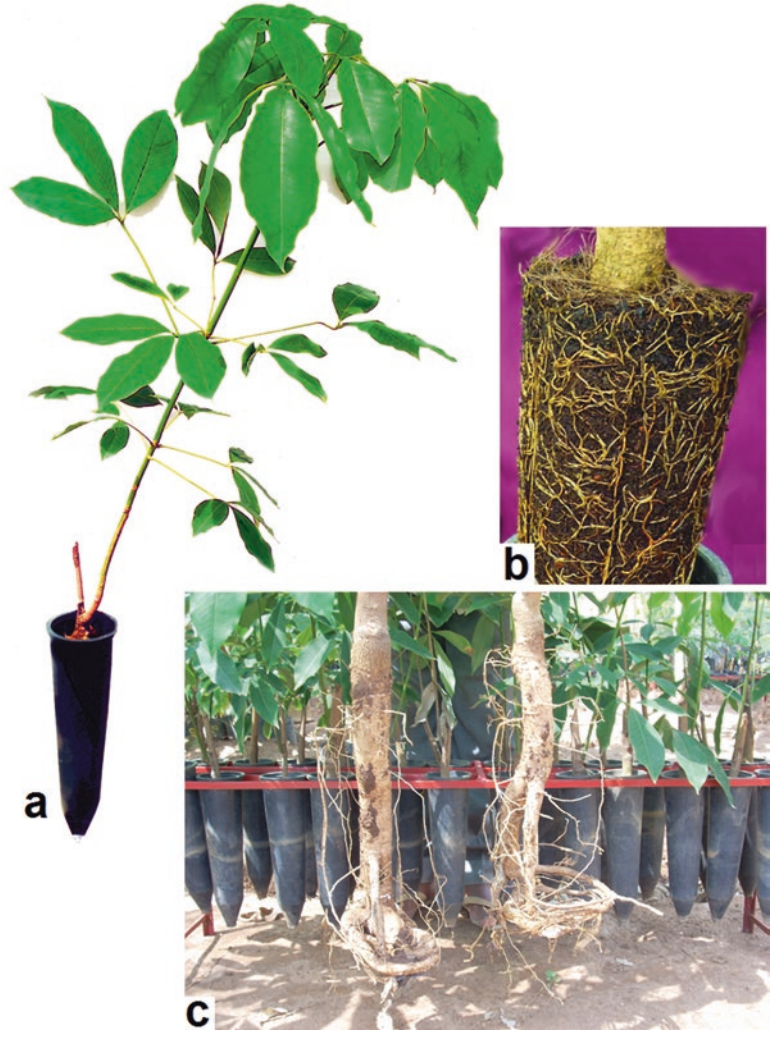

Fig. 4.2 (**a**) Root trainer plant, (**b**) root core of root trainer plant and (**c**) entangled roots of plants raised in poly bags

4.3 Preparation and Packing of Propagation Materials

The propagation materials handled by rubber growers are (i) fresh seeds, (ii) germinated seeds, (iii) seedling stumps, (iv) brown budwood, (v) green bud shoot, (vi) brown bud-grafted stumps, (vii) green bud-grafted stumps, (viii) poly-bag plants, (ix) stumped bud grafts and (x) three-part stumps. During storage and transit, they are likely to get damaged by loss of moisture.

Fresh and healthy seeds collected from the field can be kept under shade without much loss of viability for about 7 days and storing fresh seeds in water at ambient temperature increases their water content, which in turn prolongs viability (Mercykutty et al. 1996). By packing seeds loosely in well-aerated containers with powdered charcoal that has 40% moisture, 70% viability could be retained for up to 30 days (Eikema 1941). The viability of seeds can be prolonged to 2 months by mixing them with an equal volume of sawdust (10% initial moisture content) in perforated poly bags (RRIM 1966). Storage of seeds at 4 °C in sealed poly bags is also considered to be a reliable method for retaining viability for up to 4 months (Wycherley 1971). Germinated seeds are collected from germination beds when the radicle just comes out. To prevent root damage,

beds are inspected every day and germinated seeds are picked up. For transporting short distances and immediate use, germinated seeds are carried in water. For transporting long distances, they are packed in boxes between layers (2.5 cm thick) of aged sawdust, charcoal powder or damp coconut fibre (Subramaniam 1980). They should be laid in such a manner that the radicle points downwards. Brown budwood is cut into pieces of 1 m length. For use on the same day and transporting over short distances, brown budwood is kept wrapped in wet sacking. For longer storage and transport, their cut ends are sealed with molten wax and each piece covered with a banana sheath, wet sacking, coconut fibre or grass leaves. Viability can be retained up to 3 days. While collecting green bud shoots, the leaf-bearing top portion is cut off. The leafless lower part with scale leaves alone is used for taking buds. Since green bud shoots are tender it is better to use them for bud grafting immediately. In the seedling nursery, they are carried in trays or buckets containing water. Storage is possible up to 6 days with their cut ends sealed with wax (Subramaniam 1980). Seedlings prepared to a convenient size by pruning the stem and roots are called seedling stumps. They should have a minimum girth of about 7.5 cm at the base and brown colour up to a height of 45 cm or more. For stumping, the seedlings are cut back at some point between 45 and 60 cm, where the brown colour ends. Pruning is always done with a slanting cut, preferably above a whorl of buds. While cutting back, green or partially brown stem should not be retained on the stump as transpiration can take place through such regions and the resulting loss of water may lead to the drying of the stumps after planting. The plants are left in the nursery for 7–10 days. During this period, a few buds below the cut end become activated and swell. At this stage the decapitated plants are pulled out without causing much damage to the roots and bark of the stem. If it is difficult to pull out the plants due to drying of soil or extensive development of the root system, the lateral roots can be loosened by digging the soil on one side of the taproot or all around the plant to make it easier. Once it is pulled out, the taproot is pruned to the maximum possible length, but not more than 60 cm and not less than 45 cm. After preparing the seedling stumps by proper pruning of roots and stem, the cut end is sealed with molten paraffin wax. For storing overnight, they should be kept in fresh water. For longer storage, the procedure followed for brown budwood can be replicated (RRIM 1968).

Brown bud-grafted plants with pruned stem and roots are known as brown bud-grafted stumps. To prepare a brown bud-grafted stump, the plant is cut at a height of 7.5 cm above the upper end of the bud patch. The cut should have a downward slant of around 45° from the side of the bud to the opposite side. The cutting back is done about 10 days before pulling out. During this period the bud becomes activated, which in turn will speed up the establishment of the bud-grafted stump after planting. The plants are then pulled out and the taproot is pruned to a length of 45–60 cm and the laterals to a length of 10–15 cm. Where it is difficult to pull out the plant, it can be lifted before cutting back and then pruned. If the bud-grafted stumps are intended for planting in poly bags, the taproot should be pruned to a length about 15 cm less than the depth of the soil core and laterals to around 5 cm length. The cut end of the stem is sealed with wax and the bud patch is protected by covering with a small piece of banana sheath. The viability of brown bud-grafted stumps can be retained for up to 30 days by packing them in boxes with wet sawdust (Premakumari and Nair 1974).

Poly-bag plants are raised from green bud grafts or brown bud grafts. The poly-bag plants are field planted usually at the two- to three-whorl stage. While transporting poly-bag plants, utmost care should be given to prevent any damage to the soil core. If the soil core is damaged, casualties may arise. Three-part stumps are produced by proper pruning of the stem and roots of crown bud-grafted plants raised in the nursery. To produce a three-part stump, a bud-grafted plant in the nursery is first cut back above the bud patch as in the case of stumped bud grafts. When the scion grows to a height of 240 cm, crown bud grafting is done below the top whorl of leaves. Then the scion (trunk shoot) is cut back at about 7.5 cm above the crown bud. The crown bud grows and produces the new crown shoot.

4.4 Somatic Embryogenesis and Meristem Culture

Somatic embryogenesis research has been reviewed by Carron et al. (1995a, b, 2001). *Hevea* somatic embryogenesis was first developed in China and Malaysia, using the anther wall as the initial mother-tissue explant (Carron et al. 1989). Paranjothy (1974) obtained the first *Hevea* somatic embryos. Successful plantlet formation and acclimatization were achieved in Haiken 1, Haiken 2 and SCATC 88/13 (Wang et al. 1980). In 1985, the first plantlets from clones RRIM 600 and GL 1 were transferred to the soil in Malaysia. Research on somatic embryogenesis from immature inflorescences was also reported by Sushamakumari et al. (2000a). Chen et al. (1979) reported the production of somatic plants through embryogenesis issued from anther explants, and their use as explants for microcuttings, to generate a new type of 'self-rooting juvenile clone'.

At the RRII, high-frequency somatic embryogenesis and plant regeneration were achieved from immature anthers of Indian *Hevea* clones by Kumari Jayasree et al. (1999). Optimum callus induction was observed on modified Murashige and Skoog (MS) medium supplemented with 2.0 mg l^{-1} 2,4-D and 0.5 mg l^{-1} kinetin. The maximum number of somatic embryos was produced on a medium supplemented with 0.7 mg l^{-1} kinetin and 0.2 mg l^{-1} NAA. Further development of the embryos into plantlets was achieved on a hormone-free medium and these were subsequently established in the field. Cytological analysis revealed that all the plantlets tested were diploid. Sushamakumari et al. (2000a) evaluated the independent effect of kinetin and benzylaminopurine (BA) in combination with NAA and GA3 on normal *Hevea* somatic embryo induction. The promotive effect of GA3 on normal *Hevea* somatic embryogenesis was assessed by Kumari Jayashree and Thulaseedharan (2001). Embryo germination in *Hevea* was affected at higher concentrations of GA3 (Kumari Jayasree et al. 2001). The protocols developed for *Hevea* regeneration were published by various research groups.

At CIRAD, the inner integument of immature seeds was chosen as the maternal tissue explant (Carron and Enjalric 1982) for developing somatic embryogenesis through four successive phases: (i) callogenesis, (ii) differentiation of embryos, (iii) multiplication of embryos and (iv) germination of embryos and development into plantlets. The first development of embryos was achieved with RRIM 623, PB 260 and some others. Procedures were developed for reducing callus browning due to culture stresses and optimizing each component of the process. This involved histology and biochemistry, water balance between the explants and the culture medium, the exchanges of minerals, CO_2, ethylene and polyamine synthesis, growth regulator types and concentration, type of medium support and oxidative stress. The simultaneous presence of embryos from unicellular and multicellular origins was demonstrated. Following the primary somatic embryos issued from explants, subculturing of the primary calli was investigated in order to achieve friable and embryogenic secondary calli to increase the multiplication process (proliferation). The reliable induction of friable calli with a high calcium supply has been analysed by Montoro et al. (1995). Suspension cultures issued from the disaggregation of friable calli were sustained for more than 1 year for clones PB 260, PR 107 and GT 1, with embryogenic rates from 6% to 12%, but plantlets were obtained only from PB 260 and PR 107. The dynamics of somatic embryo development was compared with that of zygotic embryos for development and maturation stages and for their efficiency to germinate into plantlets (Etienne et al. 1993). Two protocols were established for somatic embryogenesis from the inner integument of immature seed: (i) a short method, which could not be used for mass propagation, using the rapid but ephemeral formation of embryos on compact calli (150 days), with six phases (initiation of callogenesis, embryogenesis expression, pro-embryo development, maturation of somatic embryos, germination of somatic embryos and plantlet development); and (ii) a longer method which involves inducing and proliferating friable embryogenic calli, and then isolating microcalli

Fig. 4.3 Micropropagated plantlets

from the suspension for regeneration through the 'short' process (Carron et al. 1995b). Carron et al. (1995a) presented a qualitative and quantitative study comparing somatic embryogenesis in four clones (PB 260, PR 107, RRIM 600 and PB 235), starting from 600 explants for each clone, following the primary embryogenesis protocol (the 'short method'). The following percentages of plantlets were obtained: 77%, 31%, 17% and 2% for the four clones, respectively. PB 260 exhibited a high embryogenic efficiency (77%) but a corresponding low rate of conversion into plantlets. Eventually in 1992, 18 plantlets obtained from PB 260, 66 from PR 107 and 2 from RRIM 600 were acclimatized and transferred to a field trial at CNRA (Côte d'Ivoire) for comparison with the corresponding bud-grafted clones. In the absence of an embryo maturation phase, survival during acclimatization was very low (see Fig. 4.3 for micropropagated plantlets). Carron et al. (1995a) compared the micropropagation capacities of clones with explants issued either from mature plants or from theoretically rejuvenated somaplants (plants from somatic embryos). The micropropagation capacity of explants from somaplants was much greater than that of explants from mature trees. This suggests that plants from somaplants are completely rejuvenated and behave as seedlings. Etienne et al. (1997b) standardized a pulsed-air temporary immersion system (the RITA® system) for enhancing embryo production, through culturing embryogenic calli under immersion in an autoclavable filtration unit. A high concentration of calcium in the culture medium stimulated the regeneration potential of embryogenic calli (Etienne et al. 1997a). Somatic embryo production was three to four times greater than that on a semi-solid medium, producing 400 embryos g^{-1} fresh weight with a smaller number of abnormal embryos (Fig. 4.4). Lardet et al. (1999) demonstrated that protein accumulation in zygotic embryos commenced from week 13, and starch accumulation from week 15, leading to development and maturity. The smaller size of somatic embryos accumulating less starch and protein reserves leads to lower vigour and lower conversion rates and lower acclimatization success.

CIRAD is now conducting field trials in cooperation with various partners (Rubber Research Institute of Thailand, RRIT; CNRA, Côte d'Ivoire; and the Michelin Company, Nigeria) with the planting of 13,000 somaclonal plantlets, predominantly from clone PB 260 (Carron et al. 2001). Research is now focusing on the limiting factors of mass propagation, which include (i) developing new friable embryogenic callus lines, (ii) selecting among the lines for improved quality, (iii) maintaining those callus lines through proliferation or cryopreservation, (iv) improving

Fig. 4.4 (**a**) RITA system for rapid multiplication, (**b**) development of somatic embryo, (**c**) *in vitro* plantlet and (**d**) plantlet with taproot

the regeneration of embryos into plantlets and (v) mastering the acclimatization conditions. The quality of embryogenic lines is assessed with regard to the embryogenic capacity (production of somatic embryos), the ability for regeneration and the vigour of young somaplants at field level. An attempt based on the comparison of mRNAs from different lines (differential display) was carried out for identifying markers specific for embryogenic capacity (Charbit et al. 2001). The final acclimatization of *in vitro* plantlets is still a bottleneck for field evaluation. Apart from their use in mass propagation, somaplants are being considered as juvenile mother trees for developing juvenile bud-grafted clones on classical rootstocks and for developing clonal rootstocks by micropropagation.

Meristem culture follows three phases: (i) primary culture, (ii) multiplication and rooting and (iii) acclimatization. Selection of explants is crucial. Indeed, very juvenile stem pieces have exhibited a higher rate of successful aseptic cultures than older material. Culture treatments are rather rigorous due to higher anticipated infection by fungi and bacteria. A mixture of gentamycin, kanamycin, chlortetracycline,

chloramphenicol, rifampicin and the fungicide benomyl is found to be ideal for disinfecting the explants. Primary culture involves soaking explants in a solution of growth regulators—indole butyric acid (IBA) and benzylaminopurine (BAP)—for 2–3 h. Bud grafting is initiated in MB medium (Carron et al. 1989) without growth regulators. Isolated buds are cultured in half-strength Lepoivre medium with IBA and BAP. These buds are subcultured to form microshoots that will in turn be cultured as explants in the multiplication phase. Soaking the base of the root in an IBA-NAA mixture for 3–4 days induces roots. Rooted microcuttings can be transferred to soil in 4–5 weeks. A number of clones, such as RRII 105, PB 5/51, PB 235, IRCA 438, IRCA 440, IRCA 442, PR 107 and GT 1, have been multiplied through micropropagation (Carron et al. 1995a). However, the acclimatization of plants is crucial, with a balance between RH and temperature governing the establishment of plants in the soil (Leconte and Carron 1988). The commercial value of the aforesaid procedures needs to be debated. Although gross experimentation was conducted in the past for standardizing *in vitro* technologies, there had been many setbacks in commercializing these procedures (Carron et al. 1992; Thulaseedharan et al. 2000). A number of aspects inherent in the explant tissue are responsible for the delay in commercialization. These include (i) the release of phenols, (ii) contamination by bacteria and fungi, (iii) their recalcitrant status, (iv) reduced axillary branches, (v) lack of sufficient juvenility and above all (vi) increased sensitivity of *in vitro* raised plantlets towards environmental attributes. There are, however, remedial measures for these setbacks. Since the contamination of microorganisms is location specific, newer chemicals are to be tried to raise aseptic cultures. Instead of treating the explants with antioxidants, the incorporation of them in the media decreased browning (Seneviratne and Wijesekara 1996). The use of support systems like cellulose plugs in liquid media reduced synthesis of polyphenols, and embryogenesis activity could be maintained for more than 200 days (Housti et al. 1992). On the other hand, the growth regulators used to induce axillary branches and somatic embryogenesis are more or less the same throughout. Judicious combination of new growth regulators that have shown positive results in other tree species can be tried in rubber. Also, metabolism of ethylene and polyamines during callus development must be controlled by appropriate adjustment of growth regulators (Carron et al. 1992). More prominently, the water status of the embryogenic callus is a governing factor to enhance embryogenesis (Etienne et al. 1991). Further, Lardet et al. (1999) demonstrated that protein and starch accumulation commenced from the 13th and 15th weeks, respectively, and the development and maturity of zygotic embryos happen accordingly. Somatic embryos also display the same accumulation abilities. However, the smaller size of somatic embryos can accomplish a relatively small mass of starch and protein reserves. This is related to lower vigour and conversion rates. The vigour is directly related to acclimatization success. Hence, increasing the size of somatic embryos through nutrient supplies must deserve priority. Techniques like air layering to achieve rooted cuttings and progression of this conventional multiplication for three to four generations can produce juvenile plants. They can be used as source plants for explants with increased juvenility *in vitro.* Increased turgidity and increased absorption of N, P and K are prerequisites for embryo induction. Juvenility is yet another crucial factor, wherein the successful micropropagation of mature stem apices micrografted on to 3-week-old seedlings grown *in vitro* increased the success of micropropagation (Perrin et al. 1994). Such basic studies must be conducted in meristem culture also, in order to implement these technologies in a commercial way. If commercialized in the strict sense, these technologies can assist breeding programmes and enhance productivity significantly. For this, researchers must know what chemicals and growth regulators are needed to improve the *in vitro* propagation system and how meticulously they be used in commercializing the system.

4.4.1 Stock-Scion Interaction

A grafted plant comprises a root system contributed by the stock plant and the shoot system by the scion. Stock-scion interaction is obvious since both these coexist as they exert mutual influence. Vigorous stocks can increase the vigour and yield of the scion (Dijkman 1951; Ooi et al. 1980). Stocks raised from monoclonal seeds of clones like PB 5/51 are found to favourably influence the growth and yield of several scion clones, while some other stocks like RRIM 600 affect the performance of the scion negatively. Polyclonal seeds are generally more vigorous and impart better growth to the scions (Ng et al. 1981). Stocks produced from vigorous clones like GT 1 also enhance growth of the scion, resulting in reduction of the immaturity period (Combe and Gener 1977). Similarly, vigorous scions induce more growth in the root system (Dijkman 1951). However, of the two parts of a bud-grafted tree, namely, the stock and scion, it is primarily the scion that determines the performance of the plant. Though a sizeable number of publications appeared in the literature, none of them gave a clear and comprehensive account of the intricacies of stock-scion interaction in *Hevea* rubber.

The stock-scion interaction in *Hevea* rubber has been a puzzle for the scientists all over. It will remain as a puzzle as long as the stock is an open pollinated seedling. What could be done is to bud-graft a given clone (say RRII 105) onto micropropogated (tissue cultured; true to the type) plants. Trees borne from bud-grafting RRII 105 onto open pollinated seedlings must also be raised. Also, a third population with trees borne out of bud-grafting RRII 105 onto the seedlings of RRII 105 must also be raised. Comparison of all these three populations may yield some insights into the intricacies of stock-scion interactions (see also sec. 13.8).

Latex Production, Diagnosis and Harvest

5

Latex is a colloidal suspension. Wikipedia explains that latex is a stable dispersion (emulsion) of polymer microparticles in an aqueous medium. Biochemically, latex is true cytoplasm. Generally speaking, it's a complex emulsion consisting of proteins, alkaloids, starches, sugars, oils, tannins, resins and gums that coagulate on exposure to air. Latex contains most of the subcellular elements that include, besides rubber particles, lutoids—an important vacuo-lysosomal compartment—(Pujarniscle 1968; Ribaillier et al. 1971; D'Auzac et al. 1982) (see Box 5.1), plastids, the Frey-Wyssling particles whose role is not clearly understood (Gomez and Moir 1979; Hebant 1981) and ribosomes (Coupe et al. 1976). However, neither the nuclei nor the mitochondria are expelled during tapping, probably because of their parietal position (Dickenson 1965) which makes investigations on nuclear and energy metabolism difficult.

The cytoplasmic origin of *Hevea* latex, as expressed for the first time by Berthold (1886), was partly confirmed by Milanez (1951) using optical microscopy. Later, with the advent of electron microscopy, the same could be established with certainty (Andrews and Dickenson 1961). While the electron microscopy enabled in situ observation of the main organelles present in laticiferous tissue, the development of ultracentrifugation and biochemical analyses have contributed to extensive knowledge of the various structured elements of latex in vitro. Huret (1948) was the first to carry out ultracentrifugation of fresh latex using a compressed air ultracentrifuge. The Dutch school that worked in Bogor (Indonesia, 1925–1950) obtained the first fundamental knowledge of latex organelles. This school which notably included Frey-Wyssling used optical microscopy and centrifugation. Homans and Van Gils (1948) of the Dutch school used low-speed centrifugation (2000 rpm × 20 min) to separate latex into a white supernatant fraction and a heavier yellow fraction accounting for 15–35% of the initial volume of latex (Resing 1955). The white fraction is colloidal, made of rubber particles, whereas the yellow fraction contains carotenoids as pointed by Frey-Wissling (1929) and thus named Frey-Wyssling particles. However, the yellow fraction consists essentially of organelles discovered by Homans and Van Gils (1948) and was named as lutoids by Ruinen (1950). The aqueous phase of latex plays the role of dispersal phase for these two fractions.

The use of refrigerated ultracentrifuge (50,000 g × 60 min) separated latex into four clearly distinct zones (Cook and Sekhar 1953). However, the investigations of Moir (1959) using vital stains characterized 11 distinct fractions in centrifuged latex that was maintained at temperature less than 5 °C.

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Box 5.1: Lutoids

Lutoids are specific vacuole-based organelles within the latex-producing laticifers. Lutoids, which comprise nearly 20% of the latex volume, are unit-membrane organelles from 1 to 5 μm in diameter. They constitute a dispersed lysosomal vacuum in the latex. Most of the proteins in lutoids are involved in pathogen defense, chitin catabolism, and proton transport. Lutoids accumulate Pi, citrate and Mg^{2+} that are about 10 times more concentrated. Acid hydrolases are present in the lutoids which also contain peroxidase, lysozyme and ∞-mannosidase. The lutoid membrane has an Mg-dependent ATPase which ensures an influx of protons and, therefore, vacuolar acidification. A membranous NADH-cytochrome c-reductase may ensure a proton efflux from the lutoids; it could evolve into a NADH-O_2 reductase, generator of superoxide ions.

5.1 Rubber Particles

Latex usually contains 25–50% dry matter, 90% of which is made up of rubber. Among the 11 distinct zones obtained (Moir 1959), by means of ultracentrifugation of fresh latex (Fig. 5.1), zones 1, 2 and 3 contain the rubber particles, and the biggest particles are found in zone 1, which is by far the largest (Southorn 1961). The size of the particles in zone 2 varies from 0.05 to 0.25 μm, and the particles are frequently elliptical and sometimes connected by fragments of membrane which might be endoplasmic reticulum. The particles in zone 3 are of lower average size (0.035–0.2 μm) and often appear to be linked to each other. Molecular weight and protein content are believed to be responsible for the differences in location of particles in zones 1, 2 and 3 (Hamzah and Gomez 1982). Rubber particles are 0.1 μm in diameter and contain several hundred *cis*-polyisoprene molecules. Electron microscopy reveals that rubber particles have a fully homogeneous internal structure (d'Auzac and Jacob 1989).

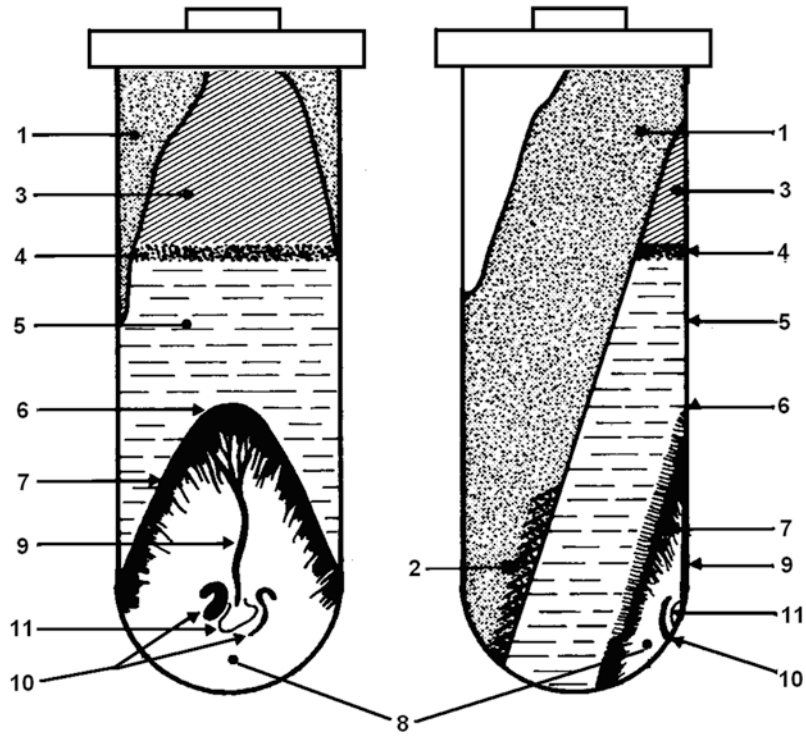

Fig. 5.1 Ultracentrifugation of *Hevea* latex. Fractions 1–3 are white rubber phase. Fraction 4 is the Frey-Wyssling particles. Fraction 5 is the clear serum (C-serum) corresponding to latex cytosol. Fractions 6–11 constitute the 'bottom fraction' of which the fraction 8 is the lutoid fraction

The existence of a protein film surrounding rubber particles that contributes to their stability has been accepted for a very long time. Weber in 1903 gave the isoelectric pH of latex as 3.0–5.0; this is the characteristic value of many proteins (Verhaar 1959). As early as 1906, Henri showed that rubber particles of *Hevea* latex in an electrical field move towards the anode and therefore have a negative charge (Verhaar 1959). One of the most important proteins in *Hevea* latex from the quantitative point of view has been characterized by Archer et al. (1963a, b) as being an ∞-globulin with a low sulphur content (0.06%), with the same isoelectric pH as latex (4.5), and easily absorbed at the surface of the rubber particles to ensure its colloidal stability. The de novo formation of rubber molecules occurs, at least in the very last stages, at the surface of the rubber particles. *Rubber transferase* responsible for this is normally distributed between the cytosol and the rubber particles (McMullen and McSweeny 1966). This enzyme has been isolated and purified from cytosolic serum. It remains inactive as long as it has not been absorbed on particles of rubber, even when the latter have been purified by centrifugation and repeated washing. The reaction catalysed by this enzyme appears to be essentially a chain extension process (Archer and Cockbain 1969).

Some 20% of the dry weight of the bottom fraction, i.e. of the lutoids, consists of soluble proteins. *Hevein* forms 70% of the total bottom fraction. Lutoids contain an acid serum, divalent cations such as Mg^{2+} and Ca^{2+} and positively charged proteins that are effectively capable of provoking the formation of microflocculates and the creaming of a dilute suspension of rubber (Gomez and Tata 1977). According to Sherief and Sethuraj (1978), a high ratio of cationic and anionic proteins in B-serum may also increase plugging. Lutoids sometimes contain springlike proteinic *microhelices* (Dickenson 1969). These microhelices appear to occur more frequently in the latex of trees whose production has been stimulated by treatment with Ethrel®. Clusters of proteinic microfibrils with a double helical structure are also seen in lutoids filling the intravacuolar space (Gomez 1976). Purified microfibrils are thought to contain up to 4% carbohydrates (Audley 1966). Their presence in young lutoids and their disappearance during the ontogeny of laticiferous vessels led to suggest that they may form nitrogen reserves which can be degraded by lutoid proteases (Dickenson 1969).

Frey-Wissling (1929) revealed another kind of globules 'lipoids', with carotenoids for yellow colouring which later became known as Frey-Wyssling particles (Dickenson 1965). It consists of complex system of branching single-membrane tubules associated with several concentric double-membrane lamellae. The Frey-Wyssling complex thus described is 4–6 nm in diameter and enclosed in a typical double membrane (Dickenson 1965, 1969). Their high carotenoid content suggested that they contain the enzymes of the isoprenic synthesis pathway (d'Auzac and Jacob 1989). Their double membrane resembles plastids whose physiological role remains mysterious.

5.2 Organic Non-rubber Constituents

Proteins are prominent among the non-rubber constituents of latex (Tata 1975, 1980b). The earliest report of the presence of proteins in latex was by Spencer (1908) who detected peroxidase and catalase activities in dialysed aqueous extracts of rubber sheets, and subsequently, in dialysed latex. The total protein content in latex has been estimated to be about 1% (Archer and McMullen 1961; Archer et al. 1963b; Tata 1980a). While 27.2% of the total proteins were absorbed on the rubber surface, 47.5% was seen in the C-serum and 25.3% in the bottom fraction (Tata 1980b).

Proteins over the rubber particles are responsible for their colloidal stability. The existence of protein-phospholipid layer imparting a negative charge on the surface of rubber particles contributed to the colloidal stability of these particles (Bowler 1953). Isopentenyl pyrophosphate polymerase (Lynen 1967; Archer et al. 1963a) and rubber transferase are the two enzymes attached with the rubber particle surface (Lynen 1967; Archer et al. 1963a, b; Archer and Cockbain 1969;

McMullen and McSweeney 1966; Archer et al. 1966). They are involved in rubber biosynthesis, and the fact that only two are associated with rubber particles needs to be explained further since several enzymes are expected to be involved in rubber biosynthesis. Proteins located in the C-serum include enzymes for the glycolytic pathway and rubber biosynthesis (Bealing 1969; d'Auzac and Jacob 1969; Archer and Audley 1967). Recently, 27 enzymes were separated by electrophoresis by Jacob and co-workers, of which 17 were shown to exist in multiple forms (Jacob et al. 1978). Studies of Wititsuwaannakul et al. (2004) showed osmotically sensitive bottom fraction membrane was found to be highly active for rubber biosynthesis, in contrast to previous reports that describe rubber biosynthesis only occurring on the rubber particle surface. It was clearly shown that washed bottom fraction membrane was much more active than fresh rubber particles for rubber biosynthesis. Serial acetone extraction of washed bottom fraction proteins showed a distinct profile of the fractions with different rubber biosynthesis activity, indicating that washed bottom fraction has both an enzyme system and a factor for regulation of rubber biosynthesis.

The first protein to be isolated from latex was from C-serum. It was named ∞-globulin, the major component of C-serum (Archer and Cockbain 1955). It is readily adsorbed at a water-air or oil-water interface with a resulting fall in the interfacial tension. This led to the suggestion that ∞-globulin was one of the proteins on the surface of rubber particles and that it contributed to the colloidal stability of fresh latex (Archer and Cockbain 1955). However, ∞-globulin was later found not to be present on the surface of the rubber particles (RRIM 1982). With the advent of sensitive techniques like starch gel electrophoresis, Tata and Moir (1964) reported the presence of 22 protein bands in C-serum. Seventeen of these were anionic at pH 8.2, while five were cationic and existed in much lower concentrations. A comparative study on the proteins in the C-serum from four clones, viz. RRIM 501, GT 1, Tjir 1 and Pil A44, revealed very little differences between their general electrophoretic patterns (RRIM 1963). Later, the list of proteins in C-serum was enlarged to 24 (Tata and Edwin 1970), using the same starch gel electrophoretic technique. Using polyacrylamide gel electrophoresis, Yeang et al. (1977) reported 26 protein bands from C-serum at alkaline pH and 15 bands at acid pH. These workers also did not observe significant differences in the protein patterns of C-sera between clones (Tjir 1, PR 107, GT 1 and PB 86).

Proteins in the bottom fraction are essentially studied as the soluble proteins in B-serum. The use of paper electrophoresis (Moir and Tata 1960), starch gel electrophoresis (Tata 1975; Tata and Edwin 1969) and polyacrylamide gel electrophoresis (Yeang et al. 1977) all lead to the conclusion that the proteins of B-serum were found to be markedly different from those of C-serum. Upon electrophoresis, the B-serum proteins were usually separated into two major protein bands at the extreme anionic and cationic ends, with several minor bands in between. The major protein in B-serum is *hevein*, which accounts for about 70% *of* the water soluble proteins in the bottom fraction (Archer et al. 1969). *Hevein* is a low molecular weight anionic protein (Approx. 5000 daltons) with higher (5%) sulphur content (Tata 1975; Archer 1960; Tata 1976). All the sulphur in *hevein* exist as eight disulphide bridges (S-S) of cystine (Archer 1960; Tata 1976). Because of its low molecular weight and the large number of S-S bridges, *hevein* is heat stable (Tata 1975, 1976).

Subsequent analysis showed that earlier preparations of *hevein* were mixtures containing *hevein*, traces *of* esterase and a protein with slightly less anionic mobility termed *pseudo-hevein* (Archer 1960; Karunakaran et al. 1961; Tata 1975, 1976). When pure *hevein* (free of *pseudo-hevein*) was isolated and characterized, it was found to be a single peptide chain with glutamic acid as the N-terminus and a molecular weight of approximately 5000 daltons (Tata 1975, 1976). The molecular weight of *pseudo-hevein* was also 5000 daltons. Later, an almost complete amino acid sequence of *hevein* was reported (Walujono et al. 1976) that contained 43 amino acid residues in a single polypeptide chain and an estimated molecular weight of 4729 daltons.

Dickenson (1965, 1969, 1963) in his ultrastructural studies and electron microscopic investigations of lutoids, described some fibrillar components having a tightly coiled helical structure, which he named microfibrils. These structures were observed within lutoids of young latex vessels but were absent from mature vessels. These microfibrils were later shown to be proteins containing up to 4% carbohydrate and having an isoelectric pH of about 4 (Audley 1965, 1966). At ambient temperature (20 °C), the microfibrils break up into smaller segments which reassemble on freezing (Audley 1965, 1966). Later, Southorn and Yip (1968) and Gomez and Yip (1974, 1975, 1976) carried out detailed investigations and reported that these zig-zag structures differed from microfibrils in that they were larger in dimensions and were open helices (not lightly coiled helices of the microfibrils). They were called *microhelices* by Gomez and Yip (1975). Lowering of the ionic concentration of B-serum by dialysis against water or by dilution with water resulted in the formation of microhelices (Tata 1975; Gomez and Yip 1974, 1975, 1976).

The presence of basic proteins in B-serum was first demonstrated when B-serum or an aqueous extract of freeze-dried bottom fraction was electrophoresed (Tata and Edwin 1970; Moir and Tata 1960; Karunakaran et al. 1961). A major and a minor basic protein, which account for about 4% of the total, were found to have lysozyme and chitinase activities (Tata 1980; Tata et al. 1983). The major basic protein has been crystallized and its molecular weight determined (approx. 26,000).

The biomembrane surrounding rubber particles from the *hevea* latex is well known for its content of numerous allergen proteins. HbREF (Hevb1) and HbSRPP (Hevb3) are major components, linked on rubber particles, and they have been shown to be involved in rubber synthesis or quality (mass regulation), but their exact function is still to be determined. Berthelot et al. (2014) highlighted the different modes of interactions of both recombinant proteins with various membrane models (lipid monolayers, liposomes or supported bilayers, and multilamellar vesicles) to mimic the latex particle membrane. They combined various biophysical methods (polarization-modulation-infrared reflection-adsorption spectroscopy (PM-IRRAS)/ellipsometry, attenuated-total reflectance Fourier-transform infrared (ATR-FTIR), solid-state nuclear magnetic resonance (NMR), plasmon waveguide resonance (PWR), fluorescence spectroscopy) to elucidate their interactions. Small rubber particle protein (SRPP) shows less affinity than rubber elongation factor (REF) for the membranes but displays a kind of 'covering' effect on the lipid head groups. Its structure is conserved in the presence of lipids. In contrary, REF demonstrates higher membrane affinity with changes in its aggregation properties. REF binds and inserts into membranes. The membrane integrity is highly perturbed, and that REF is even able to remove lipids from the membrane leading to the formation of mixed micelles. These two homologous proteins show affinity to all membrane models tested but neatly differ in their interacting features. This could imply differential roles on the surface of rubber particles.

The major soluble carbohydrates in the latex are the cyclitols, sucrose and glucose (Low 1978). Though the latex was believed to have mainly sucrose and a smaller amount of raffinose (Tupy and Resing 1969), glucose and fructose are also present in significant quantity (Bealing 1969). Low fructose concentration in latex sera is believed to be due to its rapid metabolism in preference to glucose (Bealing 1969; d'Auzac and Jacob 1967). The distribution and concentration of the major soluble carbohydrates in latex have been described (Low 1978). The concentration of total cyclitols i.e. quebrachitol and R- and m-inositols, appears to vary with clones (13.0–32.0 mg/ml of C-serum). Like total cyclitols, the concentration of sucrose in C-serum also varies with clones (4.0–10.5 mg/ml). While cyclitols and sucrose are confined largely to C-serum, glucose is located mainly in the lutoids (Bealing 1969; d'Auzac and Jacob 1969). Whether the carbohydrates used for latex regeneration in tapped trees are coming from recent photosynthates or from stored carbohydrates was quiet unknown. Recently, Kanpanon et al. (2015) studied temporal variations of carbon isotope composition

of trunk latex (δ^{13}C-L) leaf soluble compounds (δ^{13}C-S) and bulk leaf material (δ^{13}C-B) collected from tapped and untapped 20-year-old trees. A lack of correlation between δ^{13}C-L and δ^{13}C-S suggested that recent photosynthates are mixed in the large pool of stored carbohydrates that are involved in latex regeneration after tapping.

Lipids and phospholipids associated with the rubber and non-rubber particles in latex play a vital role in the stability and colloidal behaviour of latex. Earlier studies (Cockbain and Philpott 1963; Blackley 1966) demonstrated that the rubber particles are strongly protected by a complex film of protein and lipid material. It is believed that some of the lipids are present within the rubber particles. The concentration and distribution of lipids between the rubber cream and the bottom fraction had been studied (Ho et al. 1976). These lipids were isolated and divided into neutral lipids and phospholipids for further analysis. There appeared to be distinct clonal variation in the total amount of neutral lipids extractable from rubber cream and from bottom fraction. Colloidal stability of latex was found related to the natural lipid content of rubber particles (Sherief and Sethuraj 1978). Lipids from different clones, however, were qualitatively similar. Triglycerides and sterols were the main components of the neutral lipids of rubber particles. While sterols and long-chain free fatty acids are mainly made up of the neutral lipids of the bottom fraction. A furanoid fatty acid containing a methylfuran group was found mainly in the triglyceride fraction of the neutral lipids (Hasma and Subramaniam 1978). It constituted about 90% of the total esterified acids. It was suggested that the main triglyceride in latex contained three furanoid fatty acids, hence making it a rare triglyceride known in nature. The phospholipid content of the rubber particles (approx. 1% on the dry weight of rubber) was similar between different clones. The total phospholipid content of the bottom fraction was much less (only about 10%) than that in the rubber cream. Ho et al. (1976) suggested that the amount of neutral lipid (especially triglycerides) associated with the rubber particle was inversely related to the plugging index (PI; plugging index was introduced as a measure of the rate of plugging by Milford et al. (1968), as the ratio of the initial flow rate per minute over the first 5 min or 10 min to the total volume of latex obtained, and for convenience multiplied the ratio by 100) of the clone. Lutoid stability, as indicated by bursting index (BI); which measures the ratio of lutoidic free acid phosphatase activities to total acid phosphatase activities determined after bursting of lutoids by a detergent (0.1% Triton® X-100), was found to be negatively correlated with the phospholipid content of the bottom fraction of latex (Sherief and Sethuraj 1978). BI is generally negatively correlated with the yield—the more bursted lutoids, the lower will be the yield and higher will be the plugging index.

5.3 Nucleic Acids and Polysomes

The presence of nucleic acids in latex was discovered by McMullen (McMullen 1962). Latex contains both ribosomal RNA, soluble RNA, DNA and messenger RNA (Tupy 1969). These are all present in the serum fraction of latex. Functional polysomes were also discovered in the serum phase of latex (Coupe and d'Auzac 1972). More recently, ribonucleic acid (Marin and Trouslot 1975) and ribosomes (Marin 1978) have been found to be located in lutoids. The lutoid ribosomes represent 11.9% of the total ribosomal content of the latex. Two high-molecular-weight RNA components have also been identified and their nucleotide base composition determined (Tupy 1969). The presence of these membrane-bound ribosomes in lutoids led to the speculation that these ribosomes were transported from the cytoplasm to the lutoids (which are also lysosomes) where they are rapidly destroyed (Marin 1978).

5.4 Latex Metabolism

Tapping causes loss of cell constituents from the laticifers. When flow stops as a result of the complex phenomena which lead to the coagulation of

rubber particles and plugging of the wound, regeneration of the latex becomes necessary (Southorn 1969). This involves intense metabolic activity. If there is a sufficiently long interval between two tappings, this regeneration can be complete and the intralaticiferous metabolism then slows down (Jacob et al. 1988). Although metabolism plays a major role in the production through the reconstitution of latex in the laticiferous tissue, factors regulating the latex flow also determine the amount of latex loss and the subsequent rate of catabolic activities (Sethuraj and Raghavendra 1987). Also, transport of water and solutes to the laticiferous system requires biochemical energy (Jacob et al. 1988). The biochemical composition of latex (Compagnon 1986) shows very clearly that the 'royal metabolic pathway' of the laticiferous system is the synthesis of rubber, which forms 35–45% of fresh weight and over 90% of dry weight of latex. All the enzymatic processes are thus coordinated and arranged to result in the biosynthesis of rubber.

Hevea rubber is a macromolecule formed by chains of 5-carbon isoprenic units (Bouchardat 1875)—the *cis* 1,4 polyisoprene $(C_5H_8)_n$—where *n* may range from 150 to 2,000,000 (Pushparajah 2001). This high-molecular-weight polymer is formed from sequential condensation of isopentenyl diphsophate (IDP) units. IDP is a common intermediate for the production of numerous classes of isoprenoids produced in plant kingdom. These units are the precursor of numerous other natural isoprenic substances (sterols, carotenoids, etc.). A close study of its structure has shown that the isoprenic bonds are mainly of the *cis* form; less than 0.2% is in the *trans* form and these make the first 'geranylgeranyl' links in the polyisoprene chain (Archer et al. 1982; Audley and Archer 1988). According to Kekwick (1988), the average molecular weight is between 200,000 and 800,000.

Laticifers are the major location of rubber biosynthesis (Gomez and Moir 1979). Numerous classes of isoprenoids are produced through IDP as a common intermediate (Kekwick 1989). The mevalonate (MVA) pathway has been the conventionally studied pathway for isoprenoid biosynthesis since the 1950s (Fig. 5.2). This cytosolic pathway of rubber formation was demonstrated through incorporation of radiolabelled pathway intermediates such as mevalonate and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA) (Hepper and Audley 1969; Skilleter and Kekwick 1971) into rubber. Recently, the plastidic I-deoxy-D-xylulose 5-phosphate/2-C-methyl-D-erythritol 4-phosphate (MEP) pathway is being considered as a possible alternative route for rubber biosynthesis (Lichtenthaler 1999; Rodriguez-Concepcion and Boronat 2002). The expression of l-deoxy- D-xylulose 5-phosphate synthase (DXPS) in *Hevea* latex and leaves suggests that the MEP pathway exists in the laticifer (Ko et al. 2003) and therefore could provide an alternative means of generating IDP for *cis*-polyisoprene synthesis (Rahman et al. 2013) (Fig. 5.3).

The initiator molecules of rubber (short-chain allylic diphosphates) are synthesized from IDP by soluble *trans*-prenyltransferase (Archer et al. 1963a, b; Archer and Audley 1987). A membrane-bound *cis*-prenyltransferase or rubber transferase is thought to facilitate the condensation of new IDP units from *trans* to *cis* configuration (Tanaka 1989). There are numerous reports on the identification of *Hevea* rubber transferase (Archer and Cockbain 1969; Archer and Audley 1987; Light and Dennis 1989; Cornish 1993); the involvement of *Hevea* cis-prenyltransferase in generating high-molecular-weight rubber molecules was more recently reported (Asawatreratanakul et al. 2003). A number of other proteins have also been shown to participate in *cis*-polyisoprene biosynthesis. Initially, most attention was directed to the major membrane proteins of rubber particles, rubber elongation factor (REF) (Dennis and Light 1989) and small rubber particle protein (SRPP) (Oh et al. 1999), which share 72% protein sequence similarity. In addition, the cytosolic proteins identified were the rubber biosynthesis stimulator protein which corresponds to elF-5A (Yusof et al. 2000; Chow et al. 2003) and a patatin-like inhibitor protein (Yusof et al. 1998). The surface of pre-existing rubber particles is the presumed site for the synthesis of allylic diphosphate initiators and cis-polyisoprene (Archer et al. 1963a, b; Archer and Audley 1987). Also, Tangpakdee et al. (1997) and Wititsuwaannakul

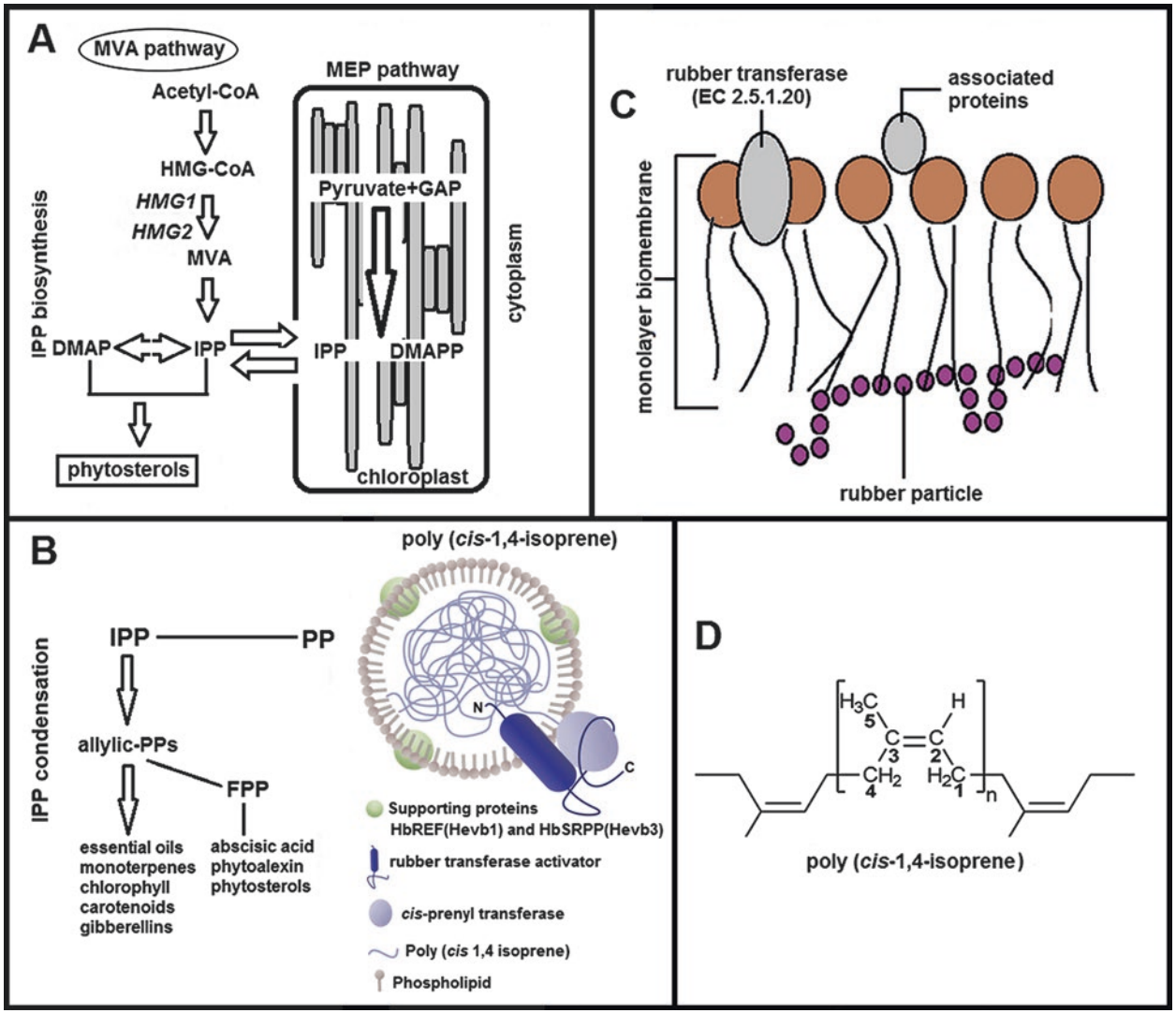

Fig. 5.2 Biosynthesis of rubber particles: (**a**) The monomeric subunit of natural rubber IPP is synthesized by the MVA pathway and the MEP pathway in higher plants from acetyl-CoA or glyceraldehydes-3-phosphate and pyruvate, respectively; (**b**) IPP is used for the synthesis of isoprenoids such as allylic diphosphates, as side chains of chlorophylls, and natural rubber. (**c**) Natural rubber is synthesized and regulated by the activity of rubber particle-associated proteins such as a rubber transferase and other proteinaceous factors at the monolayer biomembrane surface of rubber particles. (**d**) Chemical structure of the natural rubber polymer—poly (*cis*-1,4-isoprene)

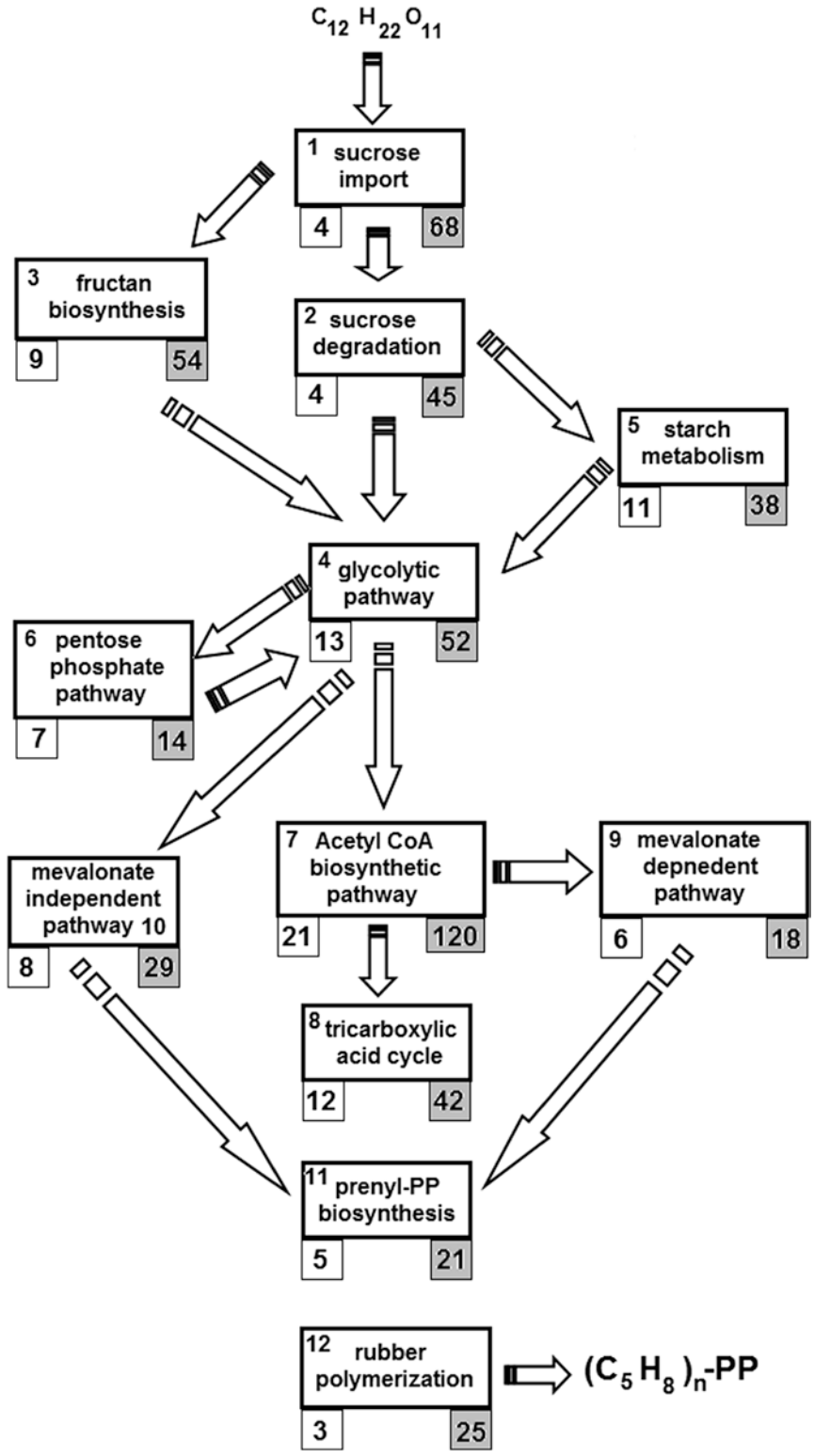

Fig. 5.3 Schematic representation of the metabolic pathway leading to natural rubber biosynthesis. The number of enzymes and associated proteins in each individual pathway is shown in *plain boxes* and the number of orthologs are in *shaded boxes* (after Rahman et al. 2013). http://www.biomedcnetral.com/1471-2164/14/75

et al. (2004) suggested that non-rubber particles may be the site for rubber initiation. Recently, the expressed sequence tags (ESTs) sequencing has enabled insights into laticifer gene expression more precisely. Chow et al. (2007) did a genomic analysis of the latex transcriptome based on a collection of 10,040 latex ESTs with emphasis on genes known to be related to rubber biosynthesis. Majority of ESTs encoded proteins related to rubber biosynthesis and stress or defence responses. Both ESTs and quantitative reverse transcription PCR (QRT-PCR) analyses revealed rubber elongation factor (REF) and small rubber particle protein (SRPP) are the most abundant transcripts in the latex. Numerous proteins with varying regulatory control with mutual interactions are involved in whole rubber biosynthesis machinery (Chow et al. 2007).

Quantitative real-time PCR (qRT-PCR) expression profiles of genes from both MVA and MEP pathways in latex showed that subcellular compartmentalization of IPP for cis-polyisoprene synthesis is related to the degree of plastidic carotenoid synthesis (Chow et al. 2012). From this, the occurrence of two schemes of IPP partitioning and utilization within one species is proposed whereby the supply of IPP for *cis-polyisoprene* from the MEP pathway is related to carotenoid production in latex. Subsequently, Chow et al. (2012) sequenced a set of latex unique gene transcripts, and they were then mapped to IPP-requiring pathways. Up to eight such pathways, including *cis-polyisoprene* biosynthesis, were identified. This insight into rubber biosynthesis can enlighten the pre- and post-IPP metabolic routes that can form a knowledge-driven approach to enhancing *cis-polyisoprene* biosynthesis.

5.5 Factors Regulating Metabolism of Latex

Availability of sugar in the laticifers is the main attribute regulating the metabolism that depends on the carbohydrate loading to laticiferous tissue and its use at cell level (Tupy 1988). Indeed sucrose catabolism supplies the acetate molecules which initiate the isoprene chain and provide the energy necessary for the functioning of the laticifers (Jacob et al. 1988). Positive, highly significant correlations have been established between sugar concentration and latex production (Jacob et al. 1986). Sucrose loading of the laticifers is thus an extremely important phenomenon. Yield in Hevea rubber with high sucrose content during summer is used to be low compared to other months. This high sucrose concentration is demonstrated to be due to high sucrose synthase and thiols which is an activator of sucrose synthase (Yeang et al. 1984; Sreelatha et al. 2007). High sucrose might indicate less utilization of the same through glycolysis, thus low rubber yield. Lacrotte showed the existence of an intermembrane transport process which requires energy. It operates at laticifer plasmalemma level and involves a cotransport H^+-sucrose energized by an ATPase proton pump (Lacrotte et al. 1985, 1988a, b). Contents of ions such as Mg^{2+}, PO^- or other products like citrate and thiols influence the activity of certain key enzymes as activators or inhibitors.

The process of active lutoid loading regulates the cytosolic concentration of certain ions (Mg^{2+}, Pi, Ca^{2+}), organic acids (citrate) and basic amino acids (Ribaillier et al. 1971) and thus detoxifies the cytosol of certain ions which could be powerful enzymatic inhibitors like citrate. These transport phenomena are linked with the functioning of the lutoid membrane ATPase proton pump (d'Auzac 1975) which, by inducing an electrochemical proton gradient, enables the cotransport of molecules with H^+ symport or antiport (Marin et al. 1981). A lutoid membrane pyrophosphatase, which is also a proton pump (Prevot et al. 1988), probably plays a similar role to that of the ATPase.

pH is yet another essential factor in the regulation of the laticiferous metabolism which has an effect on glycolysis. Indeed, invertase and phosphoenolpyruvate carboxylase (PEPcase), the two key enzymes involved in sugar catabolism, are extremely sensitive to physiological variations in pH (Tupy 1973; Jacob et al. 1983). These organic acids are not connected with isoprenic synthesis but may be connected with the energy-producing oxidation reactions. The pH of cytosol and lutoid compartment is different. While the cytosol pH

corresponds to that of whole latex, (neutral and varies between 6.5 and 7.4), the lutoid pH is much more acidic that ranges from 5.2 to 5.8 (Brzozowska-Hanower et al. 1979). Numerous factors regulate the pH of the cytosol connected with the functioning of specific ATPases, which are expected to have a role in carbohydrate supply to the laticifers (Lacrotte et al. 1985, 1988a, b). Highly significant correlations have been found in intraclonal experiments between cytosol pH and production. Highly significant negative correlations were also seen between lutoid pH and production (Brzozowska-Hanower et al. 1979). The difference between cytosol and lutoid pH values (ΔpH) has also been positively and significantly correlated with production (Marin and Chrestin 1984).

Efficient water inflow into laticifers is crucial for latex flow and production since it is the determinant of the total solid content of latex and its fluidity after tapping. As the mature laticifer vessel rings are devoid of plasmodesmata, water exchange between laticifers and surrounding cells is believed to be governed by plasma membrane intrinsic proteins (PIPs). To identify the most important PIP aquaporin in the water balance of laticifers, the transcriptional profiles of ten-latex-expressed PIPs were analysed by An et al. (2015). One of the most abundant transcripts, designated HbPIP2;3, was characterized. When tested in *Xenopus laevis* oocytes, HbPIP2;3 showed a high efficiency in increasing plasmalemma water conductance. Expression analysis indicated that the HbPIP2;3 gene was preferentially expressed in latex, and the transcripts were up-regulated by both wounding and exogenously applied Ethrel (a commonly used ethylene releaser). Although regular tapping up-regulated the expression of HbPIP2;3 during the first few tappings of the virginal rubber trees, the transcriptional kinetics of HbPIP2;3 to Ethrel stimulation in the regularly tapped tree exhibited a similar pattern to that of the previously reported HbPIP2;1 in the virginal rubber trees. Furthermore, the mRNA level of HbPIP2;3 was associated with clonal yield potential and the Ethrel stimulation response. These results have revealed the central regulatory role of HbPIP2;3 in laticifer water balance and ethylene stimulation of latex production in *Hevea*.

5.6 Latex Vessels and Turgour Pressure

On tapping, initial latex flow is fast but recedes rapidly and ceases after a period that lasts from few minutes to several hours. Subsequent tappings at regular intervals result in increased yield due to longer duration of flow and more dilute latex until it attains equilibrium. The increase in yield before reaching a state of equilibrium was termed by early workers as wound response (Pakianathan 1967; Pakianathan and Milford 1977). Regular and controlled tapping not only increases the time of flow but also enhances the biosynthesis of rubber in the drained vessels below the tapping cut. Longer latex flow is equated to higher yield, provided the other circumstances and attributes remain unaffected. Ethephon (chloroethylphosphonic acid) is a popular stimulant to extend the latex flow (Abraham et al. 1971).

The physiological mechanisms of latex exudation and cessation of flow had been a subject of much research in the past (Southorn 1969; Gomez 1983; d'Auzac et al. 1989; Yeang 2005). Latex accumulates in the latex vessels with the turgour pressure of 7.9 to 15 atmospheres (Arisz 1920; Buttery and Boatman 1964, 1966). Pakianathan and Milford (1977), using a vapour pressure osmometer, obtained values of 10–12 atmospheres on drop samples of latex. Diurnal turgor and osmotic pressure measurements taken at various intervals from 0530 to 1900 h showed maximum turgor values at 0530 h, whereas maximum osmotic pressure values were recorded between 1300 and 1600 h (Buttery and Boatman 1966). The extent of dilutions, 5 min after the tapping had commenced, were 24.7, 18.8 and 12.1%, for trees tapped at 0400 h, 0800 h and 1230 h, respectively. The diffusion pressure deficit was highest in trees tapped at 1230 h. Trees tapped at 0400 h yielded more latex than those tapped at 0830 h or 1230 h (Buttery and Boatman 1966). Thus, it appeared that latex production was largely influenced by the internal water relations of the tapping panel. These observations showed that latex vessels behaved as a relatively simple osmotic system. Turgor pressure falls during the day as a result of withdrawal of water under transpirational stress (Pakianathan 1967; Buttery and Boatman 1964).

Upon tapping, the high turgour pressure expels latex from the vessels, and over the period, the loss of turgour pressure tends to cease the flow with the mechanism of latex vessel plugging (Boatman 1966). Almost all hypotheses implicate the vacuole-like organelles called lutoids found in the bottom fraction of the centrifuged latex to be responsible for latex vessel plugging (Pakianathan et al. 1966; Kongsawadworakul and Chrestin 2003). The lutoidic serum (B-serum) contains latex de-stabilizing factors, and its release from damaged lutoids leads to the formation of plugs of flocculated or coagulated rubber at the cut ends of the latex vessels that lead to plugging of latex vessels. The most commonly used measure of latex vessel plugging rate is the 'plugging index' which estimates the average plugging rate over the entire flow. Higher the plugging index, lower will be the latex yield. Total solids content and dry rubber content (drc) are two other measures to give an idea of yield. Low total solid content depends very much on tapping intensity and is reflected in the dry rubber content. A low drc (<30%) is indicative of the tree getting overexploited.

Gomez (1983) studied extensively the events leading from opening of latex vessel to plugging. Through measuring flow rates at definite intervals, the exact shape of flow curve can be determined; the total yield depends on initial flow rate and duration of flow. Trees exhibiting different flow patterns can have more or less same yield. The logarithmic transformation of flow rates show linear trends except for initial 30 min. Incorporating all these, the model of Paardekooper and Samosorn (1969) is

$$Y = b.e^{-at}$$

where Y is the flow rate at time t after tapping; b is the initial flow rate; e is the base of natural logarithms; and a is a constant mainly depending on clone.

When successive tappings were conducted at 10 min intervals, flow rate recovered markedly after each reopening so that stepped flow curves could be obtained (Boatman 1966; Buttery and Boatman 1966, 1967), indicating that some impediment develop at the cut ends of the latex vessels. Southorn (1968) could undertake optical and EM studies on longitudinal section of latex vessels near the tapping cut. Rubber particles and lutoids from internal plugs in some of the vessels and other latex vessels could get plugged successfully. With all these information in hand, Milford et al. (1969) studied clonal variations in plugging and suggested plugging phenomena could be explained through 'plugging index' derived from initial flow rate and total volume of latex:

$$\text{PI} = \frac{\text{Mean initial flow rate (ml / min)}}{\text{Total yield volume (ml)}} \times 100$$

It is a clonal characteristic that varies with season and the tapping system and stimulation schedule adopted (Paardekooper 1989).

The major cause of latex vessel plugging is damage of lutoids. Changes in osmotic concentration of the latex during latex flow damaged lutoids which form aggregates with rubber particles that are found in large numbers at the bottom of the tapping cut (Pakianathan et al. 1966; Pakianathan and Milford 1973). The intensity of plugging was found to be proportional to the square root of time in trees tapped half spiral (medium flow) and quarter spiral (short duration) and trees that were stimulated (long duration) (Yeang 2005). Hence,

$$y = a + b\sqrt{x}$$

y = the percent cumulative latex vessel plugging; a and b are constants.

If all latex vessel plugs are removed on tapping, then the plugging would be 0 immediately after tapping and y would be 1000%. At flow cessation, $100 = b\sqrt{t}$ or $b = 100/\sqrt{t}$, where t is the total flow duration. Since $y = b\sqrt{x}$.

$$y = (100/\sqrt{t}.\sqrt{x} = 100\sqrt{x/t}$$

To estimate the proportion of latex vessels at the midpoint of duration, $x = t/2$ is substituted and that would give y = 70.7%; 71% of the latex vessels are plugged at the midpoint of latex flow. Yeang (2005) suggested tapping panel turgour

pressure and cumulative latex vessel plugging are major determinants regulating latex flow rate. Multiple regression models were examined. Since cumulative latex vessel plugging is proportional to $\sqrt{(x/t)}$ where t is the total duration, turgour pressure (TP) and cumulative plugging data can be fitted in linear regression model:

$$y = a + b_1 (TP) + b_2 \sqrt{x / t}$$

where a, b_1 *and* b_2 are constants. Since t is constant for a tapping, cumulative plugging index is a function of time (x) and the model can be

$$y = a + b_1(TP) + b_2 \sqrt{x}$$

Since cumulative latex vessel plugging is a function of time, latex flow can be expressed as a function of the laticifer turgour pressure and time.

Another measure of plugging, the 'intensity of plugging', calculates the cumulative plugging from the time of plugging to a given point of time of latex flow (Southorn and Gomez 1970).

5.7 Anatomy and Latex Flow

Latex flow rate and the changes in tapping panel turgour pressure have a direct relation with the anatomical aspects of the laticifer system. The latex vessels (laticifers) are arranged in concentric cylinders among the phloem tissue (Riches and Gooding 1952). Elongated laticifer cells are laid down in each cylinder end to end with their end walls dissolved, thus forming sets of continuous articulated tubes (Fig. 5.4). These cylinders appear as rings in a cross section, known as 'latex vessel rings'. Lateral connections between adjacent latex vessels within the same ring occur, and the laticiferous system is thus made up of a complex network of interconnected vessels gaining the name 'anastomosing latex vessels'. There are no connections between adjacent latex vessel rings. Hence, when the tree is tapped, latex thus exuding originates not only from the latex vessels of the trunk that are cut, but also from connected latex vessels of the same latex vessel ring that are uncut, but that lie within the proximity of the 'drainage area' of the tapping cut (Frey-Wyssling 1932). Similarly, tapping panel turgour pressure has a bearing on the changes in the drainage area as a whole.

On tapping, release of pressure occurs to a greater extent in the latex vessels than in the surrounding tissues. This results in a rapid elastic expulsion of latex flow through the vessels along the pressure gradient. The gradient is highest near the cut and becomes smaller with increasing distance away from the tapping cut. Frey-Wyssling (1952) and Riches and Gooding (1952) made extensive studies on the mechanism of latex flow and cessation of flow. Further work by Boatman (1966) and Buttery and Boatman (1967) demonstrated that flow is rapidly restricted by plugging of the vessels at or near the cut surface, and this was usually the major factor causing a decline in the flow rate.

The collapse of latex vessel elasticity in relation to turgour pressure of the tapping panel was studied in the past in some detail. Latex vessels could contract by up to one fifth of their diameter when cut (Frey-Wyssling 1932). Frey-Wyssling (1952) also observed that latex was forcibly expelled when the turgid latex vessels collapsed at tapping. Pyke (1941), and Gooding (1952), who measured the minute contraction of the rubber tree trunk using a dendrometer later, confirmed these observations. However, the dendrometer measurements were made against a background of diurnal contraction and expansion of the trunk that was 4–6 times the magnitude of change due to tapping itself. This was not surprising since the dendrometer measured changes in the dimension of the entire tree trunk, whereas latex vessels constituted only 2% of the bark (Yeang 2005). The measurements should be restricted to the laticifer system if a better understanding is needed on the presumed latex vessel collapse and consequent loss of turgour. Buttery and Boatman (1964, 1966, 1967) who measured the laticifer turgour pressure using a manometer that allowed latex flow into its capillary tubing duly meet this requirement. Since latex vessels are the only articulated cellular elements in the tapped bark, primarily only latex enters the glass capillary of the manometer that is visually verified (Yeang 2005; Buttery and Boatman 1967). Panel turgour pressure and the corresponding latex vessel wall

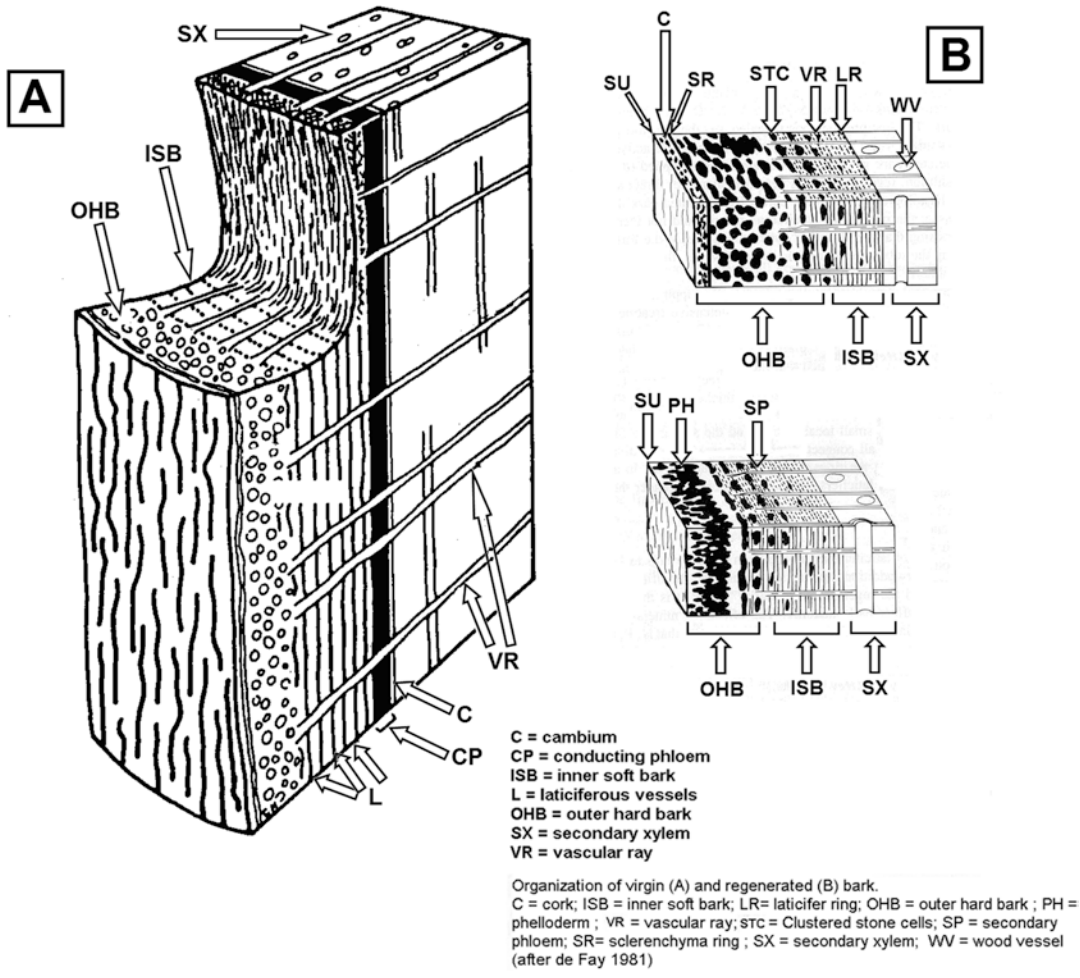

Fig. 5.4 Organization of virgin and regenerated bark. (**a**) Cross section of virgin bark (*C* cambium, *CP* conducting phloem, *ISB* inner soft bark, *L* laticiferous vessels, *OHB* outer hard bark, *SX* secondary xylem, *VR* vascular ray). (**b**) Cross section of regenerated bark (*C* cork, *ISB* inner soft bark, *LR* laticifer ring, *OHB* outer soft bark, *PH* phelloderm, *VR* vascular ray, *STC* clustered stone cells, *SP* secondary phloem, *SR* sclerenchyma ring, *SX* secondary xylem, *WV* wood vessel) (After de Fay 1981)

pressure close to the tapping cut drops immediately after tapping. This is consistent with the collapse of latex vessels after tapping envisioned by Pyke (1941) and Gooding (1952). The proportional changes in latex flow rate and the change in turgour pressure envisage a direct relationship between these attributes.

As an alternative to estimate laticifer turgour pressure, the trees were re-tapped and the manometric readings taken (Yeang 2005). Cumulative latex vessel plugging at any point during latex flow can be eliminated by re-tapping the tree to remove all latex vessel plugs. Hence, the rate of latex flow immediately after re-tapping should reflect the laticifer turgour pressure. As shown in Fig. 5.5, the change in latex flow rate upon re-tapping the tree is indeed proportional to the change in the manometer reading. Especially, turgour pressure readings in the early flow (within 15 min of tapping) are lower than expected as compared with the corresponding latex flow rate. This indicates that though turgour pressure is primarily responsible for expelling latex from the tree when it is tapped, the manometric readings are,

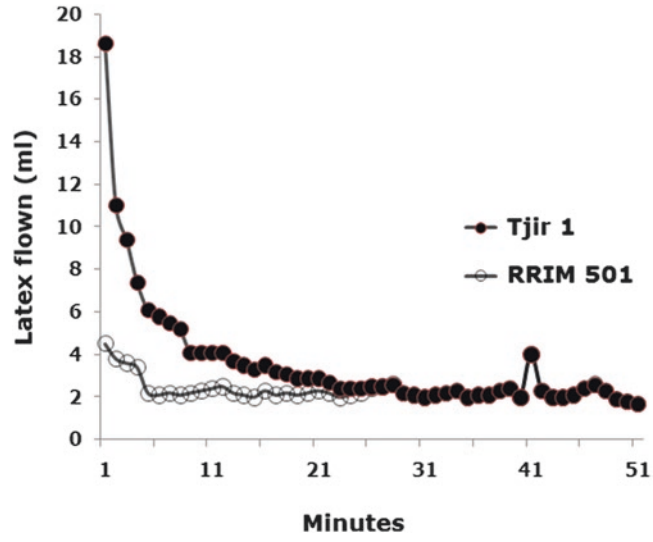

Fig. 5.5 Progression of latex flow of RRIM 501 and Tjir 1

perhaps, under-estimated during the early flow. Following the initial drop immediately after tapping, panel turgor recovers to a considerable extent before flow cessation (Buttery and Boatman 1967). Hence, the latex flow cessation cannot be attributed entirely to turgour loss. Instead, there appears to be barriers that seal latex vessels progressively until flow ceases eventually.

It is clear that latex contains destabilising factors normally located in the lutoid particles. Consequently, any physiological or biochemical factor which affects the stability of the lutoids would undoubtedly affect the latex flow and plugging of the vessels. By repeated reopening of the tapping cut, Boatman (1966) demonstrated that flow was restricted rather rapidly by some process occurring at or near the surface of the cut. Pakianathan et al. (1966) observed flocs of damaged lutoids in tapped latex and suggested that dilution of latex during flow might damage the osmotically sensitive lutoids and provide a possible mechanism of latex vessel plugging. Electron microscopical observation of the ends of the tapping cut revealed both a cap of coagulum on the surface of the cut and internal plugs within the latex vessels (Southorn 1968). Lutoid counts taken before tapping and at various intervals during flow showed a rapid loss during the initial 30 min of flow indicating that lutoids were trapped on the cut surface and initial cap formation during the early stages of flow. Shear may play an important part in lutoid damage. Internal plugging occurs mainly during the fast initial flow, whereas coagulation on the surface of the cut is effective when the flow is slow. It seems that there is no substantial reason to suppose that the two types of sealing processes are separated in time (Southern 1968).

5.8 Lutoids and Coagulation of Latex

Lutoids can destabilize the negatively charged colloidal suspension of rubber particles (Southorn 1969). The negative charges of rubber particles can be neutralized with the attributes like acidic pH, divalent cations (Mg^{2+} and Ca^{2+}) and entrapped positively charged proteins that are available in lutoids. In addition, some of the acid hydrolases trapped in lutoids can attack the protective coating of rubber particles. The coagulant role of intralutoid serum (B-serum) has been demonstrated globally in a dilute suspension (2.7%) of rubber particles. In a few seconds, the serum stops the Brownian movement that causes flocculation.

The breakdown of lutoids during or after tapping may liberate some hydrolytic enzymes that are able to attack the phospholipoprotein films

which protect the stability of rubber particles. Among the lutoid enzymes discovered by Pujarniscle (1968, 1969), only a protease (cathepsin) displaying a very acid optimum pH (~3.5) was believed to be involved in this process, but no experimental proof has been proposed. Lysozyme, a quantitatively important hydrolytic lutoid enzyme, is not suited to attack the protective film of rubber particles, and in fact an exogenous lysozyme is unable to coagulate a suspension of rubber particles. Thus, proof of involvement of lutoid enzymes in latex coagulation was yet to be found. However, an exception must be made of the case of an NADH-quinone reductase originating from lutoid membrane and which plays a role at least in bark dryness induced by overexploitation. The mechanism of action of the latter enzyme is quite different; the forms of toxic oxygen produced attacked the double bonds of the ethylenic fatty acid in the organelles and cell membranes. Leakage of the organelle components (lutoids and Frey-Wyssling particles) follows and then the destabilization of the colloidal suspension occurs. The efficiency of the NADH-quinone reductase depends on the equilibrium between the oxidizing and reducing molecules of the latex. Such equilibrium is itself related to the concentration of certain reducing molecules such as glutathione or ascorbic acid and to the activity of various protective enzymes like catalase and superoxide dismutase or oxidizing enzymes like peroxidases and phenol oxidases. Studies by Hao et al. (2004) demonstrated that during latex flow, the activities of chitinase and β-1, 3-glucanase, the well-known defense proteins of lutoids, are responsible for making a protein network with rubber particle that protects wounded laticifers. They also argued that lack of protein network is the factor that leads to tapping panel dryness. Wititsuwannakul et al. (2008) demonstrated that a *Hevea* latex lectin-like protein (HLL) present on the lutoid membrane is demonstrated to be responsible for the rubber particle aggregation (RPA). A binding protein (BP) ligand counterpart for HLL was also identified. Based on protein identification by peptide mass fingerprinting, the RP-HLLBP was confirmed to be the small rubber particle protein (SRPP). Hence, an intrinsic RP glycoprotein (RP-HLLBP or SRPP) is the key component in the formation of rubber latex coagulum and hence latex vessel plugging. Wang et al. (2013) argued that primary and secondary lutoids are available in the primary and secondary laticifers. Though both perform similar roles in rubber particle aggregation (RPA) and latex coagulation, they vary greatly at the morphological and proteomic levels. Wang et al. (2013) did a comparative proteomic analysis of lutoid membranes revealing 169 proteins that were functionally classified into 14 families pointing that most of the proteins are involved in pathogen defense, chitin catabolism and proton transport. Chitinase and glucanase appeared to play crucial synergistic roles in RPA.

5.8.1 Lutoid Breakdown Mechanisms

Natural coagulation, both in situ and in vitro, begins by the appearance of microflocs of degraded lutoids and rubber particles, and lutoids are the main elements for the stopping of flow sooner or later. The question now is to know how tapping the lutoids can release their contents into the latex, thus leading to the appearance of microflocs, which accumulate to form caps which block the tubes and stop the flow.

The duration of latex flow depends on the quantity of lutoids when they flow out of the laticiferous tubes. That in *Hevea* with a high PI, the removal of a layer of bark about 1 mm thick from the tapping cut reactivates flow show that the plugging of laticiferous vessels is limited to the immediate proximity of the cut. This observation led Lim et al. (1969) to put forward the hypothesis according to which the wound may cause an action potential at the wound itself which might lead to depolarization of cellular or intracellular membranes which had been polarized following active phenomena. Such depolarization might act as a trigger for the release or leakage of solutes across membranes. Insofar as lutoid membranes are concerned, it is conceivable that the formation of microflocs may be induced near the wound site.

The yellow fraction, which is essentially lutoidic and usually viscous, may be observed

under the microscopy to stiffen and flocculate after the addition of water (Homans et al. 1948). The dilution of fresh latex in vitro, with increasing quantities of water, causes progressive damage to the bottom, essentially lutoidic fraction, which disappears progressively (Pakianathan et al. 1966). With increased dilution, the damaged lutoid particles tend to aggregate with rubber particles and formed clusters which are lighter than lutoids. These clusters floated in the clear C-serum and finally, with the highest percentages of dilution, collected just beneath the rubber particles after ultracentrifugation, and the bottom fraction disappeared completely. The mechanism of lutoid degradation by dilution with water is related to the absorption of water through the semipermeable lutoid membrane, the osmotic potential (ψ_s) that is clearly negative. Further, it was demonstrated that the dilution of latex by mannitol 0.3 *M* buffered to pH 7 prevented the rapid degradation of lutoids (Pakianathan et al. 1966). Osmolarity determination of whole latex performed either using the freezing point technique or with a vapour pressure osmometer (Pakianathan 1967) gave values of ψ_s ranging from −330 to −450 mosmol. An increase of +143 mosmol between the beginning and the end of flow was frequently observed. These increases were correlated with the well-known dilution of latex which occurs during tapping (Ferrand 1941). All the processes described above involve the degradation of lutoid membrane. The main reason is probably the shear stress caused at the open extremity of the latex tubes early on in the tapping operation when the turgor pressure drops by nearly 10 atmospheres (Southorn 1969). In addition, dilution of latex with water during tapping can increase its osmotic pressure to a certain extent, but it is not clear whether the hypotonic conditions which appear in this way are strong enough to cause much bursting of lutoids (Pakianathan et al. 1966).

5.9 Harvest

One of the main reasons for the successful establishment of *Hevea rubber* on plantation scale in the Far East was the discovery of excision method of *tapping* for harvesting rubber latex from the tree (see Fig. 1.2b, c). In this method, the same cut is regularly reopened by the removal at each tapping of a thin shaving of bark from a sloping cut, a principle which is in general use today (Ridley 1890–1891). On each occasion, a tree is tapped by means of a suitable knife, so that a channel is prepared along which the latex can flow. This method avoids wounding of the trees as the tissues of the tree can be recognized.

Hevea does not accumulate more than a certain amount of rubber in the latex vessels, and draining out the latex by tapping stimulates the production of latex to replace it. Thus, a tree can be trained by regular tapping to a continuous process of rubber regeneration leading to high cumulative yields. Regeneration of rubber and maintenance of yield is directly in response to regular tapping, requiring the continual application of manual labour (Wycherley 1964). Different tapping systems modify the amount of rubber produced per unit labour or per unit capital invested in the planting and abandoning tapping means permanent loss of crop. The importance of excision tapping lay in the fact that the method was based on the specific characteristics of bark.

When the demand for rubber increased in the beginning of the twentieth century, planters became daring and began increasing the length of the tapping cut and practised intensive tapping systems to obtain greater yields. However, experience soon taught them that with lengthening the cut, the yield per unit length of the tapping cut became less and they learned that yield was not proportional to the amount of bark incised. They also noticed that though they obtained good yield responses with intensive tapping at the beginning, the yield declined after some time. Bark renewal too became poor and the planters returned to less intensive tapping systems (Dijkman 1951). Since on those days nothing was known of the physiology of latex flow, it was a matter of finding a tapping system by empirical means. This situation prevailed until 1920. The results of Dutch scientists (De Jong 1916; Maas 1925; Bobilioff 1923) who carried out many tapping experiments and studies on anatomy and physiology of *Hevea* helped to formulate tapping systems on a scientific and rational basis. Thus, the early tapping

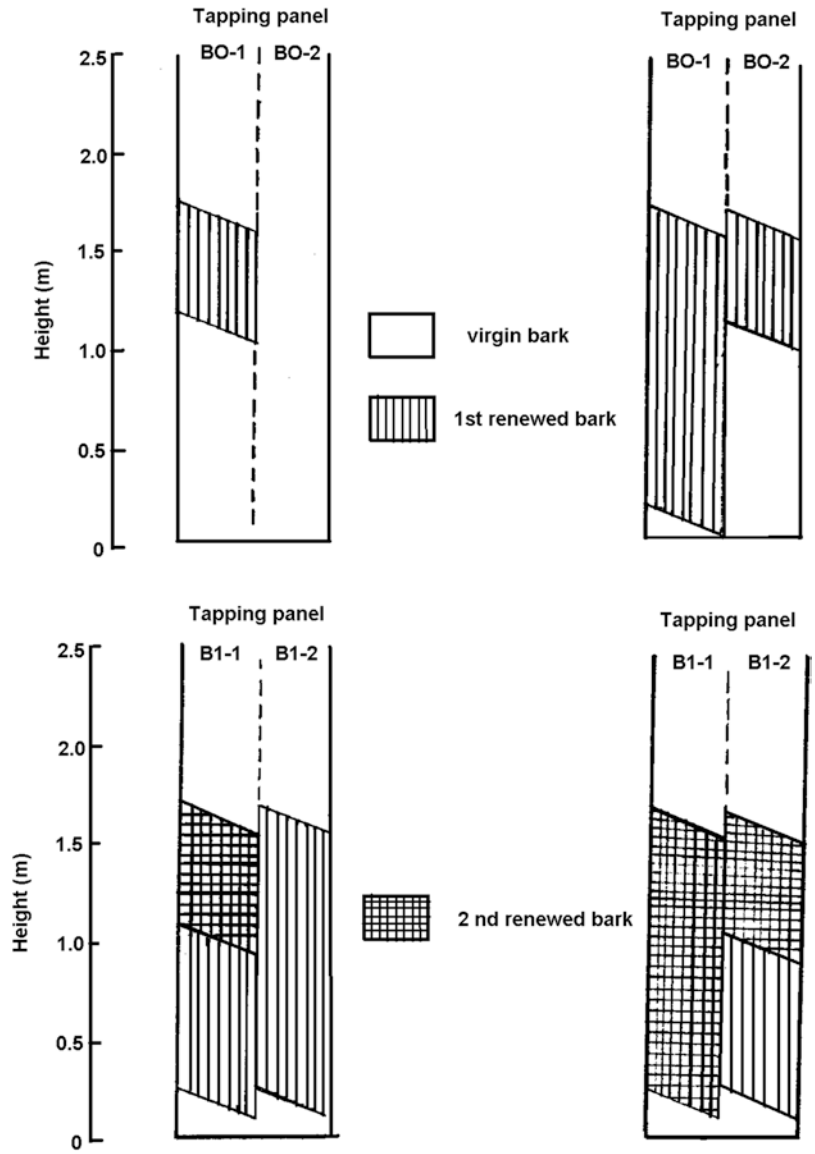

Fig. 5.6 Representation of tapping panels

systems slowly evolved into the modern systems largely by reducing the number of cuts and the frequency of tapping with BO-1, BO-2, BI-1 and BI-2 panels (Fig. 5.6).

5.9.1 Tapping Notations

Many tapping systems were evolved through the years which included complicated techniques such as the herringbone system. Local names were given for each system, and this led to a lot of confusion and difficulty. Thus, the various rubber research institutes formulated a uniform method of expressing various tapping systems commonly used. At the initiative of the International Rubber Research and Development Board (IRRDB), a standard international tapping notation for tapping systems was revised (Lukman 1983). The notation consists of a set of symbols which should be used in regular sequence.

The first symbol describes the number and nature of cuts. The symbols representing the cuts are:

1. S—a spiral cut.
2. V—a V cut.
3. C—a circumferential cut; this could be a V cut or a spiral extending around the entire circumference of the tree.
4. Mc—minicut (5 cm or less in length).

Length of the cut is represented by a fraction preceding the symbol of cut. For minicut, the actual length is denoted in cm:

S = One full spiral cut
V = One full V cut
C = One full circumference
½ S = One half spiral cut
¼ S = One fourth spiral cut
1/3 V = One third V cut
3/4 S = Three fourth spiral cut
½ C = One half circumference cut
Mc5 = Minicut the length being 5 cm

While undertaking an upward tapping, an upward arrow (↑) is given immediately after the tapping notation. Bidirectional tapping is denoted by both upward and downward arrows. Hence:

½ S = One half spiral cut downward
½ S ↑ = One half spiral cut tapped upward
2 × ½ ↑↓ = Two half spiral cuts; one upward and another downward
¼ S↑ + ½ S = One one-fourth spiral cut upward and another half spiral downward

The unit is a day (d) for frequency of tapping and the denominator will be the actual interval between tapping. Hence, the actual frequency will be:

d/1—daily tapping
d/2—alternate day tapping
d/3—third day tapping
d/4—fourth day tapping

If the practical frequency is broken by a day of rest or a regular day (say a holiday), the numerator will be the total days tapped and the denominator will be the total period. For example, alternate days tapping followed by 1 day rest will be denoted as: d/2 6d/7. Similarly, if the tapping is every third day followed by 1 day rest, then the notation will be: d/3 6d/7. Periodicity consists of details of week (w), months (m) and years (y). For example, every third day tapping followed by 1 day rest done for 4 weeks, followed by 1 week rest undertaken for 9 months followed by 3 months rest will be d/3 6d/7 4w/5 9 m/12. If the length of tapping cut is shortened or lengthened, a horizontal arrow will separate old and new notations. For example, one fourth tapping downward changed to half spiral tapping downward will be denoted as: ¼ S→½ S.

Panel notations were described as A, B, C, D, E and F. The latest panel notations are BO-1 (first virgin bark), BO-2 (second virgin bark), BI-1 (first renewed bark of BO-1), BI-2 (first renewed bark of BO-2), BII-1 (second renewed bark of BO-1) and BII-2 (second renewed bark of BO-2).

While the tree is stimulated for yield, the notations are different but not separated from the tapping notations. If the tree is stimulated with 1.0% Ethephon (ET) applied to the panel (Pa) with 1 g of stimulant per application on 1 cm band with 16 applications per year at weekly intervals, then the notation will be ET1.0%Pa1(1).16/y(1w).

As a modification of the above, Vijayakumar et al. (2009) gave the following tapping notations with the approval of IRRDB:

S/2 d3 6d/7. ET 2.5,% Pa2(2) 8/y(m).
that is, half spiral cut without rainguard tapped downward, at third daily frequency, 6 days in tapping followed by 1 day of tapping rest, stimulated with ethephon of 2.5% active ingredient with 2 g of stimulant applied on panel on 2 cm band, eight applications per year at monthly intervals.

S/2(RG) d3 6d/7 95/104. ET 2.5% Pa2(2) 8/y(m) 6/8
that is, half spiral rainguarded cut tapped downward at third daily frequency, 6 days in tapping followed by 1 day rest, with 95 tapping achieved against 104 scheduled tapping days per year. Stimulated with 2.5% ethephon with 2 g of the stimulant applied on panel on 2 cm band, eight scheduled applications per year at monthly

intervals. Six stimulations could be done against the scheduled eight per year.

S/2(RG)·d3 6d/7 6 m(JUN-NOV)/12. ET 2.5% Pa2(2) 4/6 m(6 w); S/4 U d3 6d/7 6 m(DEC-MAY)/12. ET5.0% Lal(−) 9/6 m(3 w) (6 m,6 m)

that is, half rainguarded cut tapped downward at third daily frequency, 6 days in tapping followed by 1 day tapping rest, 6 months of tapping from June to November, stimulation with 2.5% ethephon with 2 g of the stimulant applied on panel on 2 cm band, 4 applications in 6 months at interval of 6 weeks between applications, changed to one fourth spiral cut tapped upward for the next 6 months from December to May, stimulation with 5.0% ethephon with 1.0 g of stimulant applied on lace, 9 applications in 6 months at interval of 3 weeks between applications. The cycle is repeated.

S/4 d4 6d/7 9 m (MAR-NOV)/12. ET 2.5% Pa 1 (2) 18/9 m (2 w) + S/4 U d4 6d/79 m (MAR-NOV) /12. ET 5% La1(−) 18/9 m (2 w).

that is, two quarter spiral cuts, one tapped downward and the other tapped upward, once in 4 days on the same tapping day, 6 days in tapping followed by 1 day of tapping rest, 9 months of tapping from March to November followed by 3 months of rest, both cuts stimulated, the lower cut with 2.5% ethephon, 1.0 g of stimulant applied on the panel on 2 cm band, 18 applications in 9 months at fortnightly interval, while upward tapped cut is stimulated with 5.0% ethephon, 1.0 g of stimulant applied on the lace, 18 applications in 9 months at fortnightly interval. While expressing data, number of tappings realized may be shown as fraction of maximum I number of tapping days possible.

The aforesaid tapping notations are the latest and approved by the IRRDB. Though little complicated, these notations are to be followed at least while presenting scientific data. Planters, however, follow to use the age old A, B, C and D panels for their convenience (see Table 5.1).

5.9.2 Tapping Techniques

There is a need to open trees for tapping as soon as the required minimum girth has been obtained. While the budded trees have cylindrical trunks and can be opened at a height which tappers can reach without any aid, seedling trees are conical with a bigger girth at the base of the tree and hence, a lower height of opening is recommended. With conventional tapping, the recommendation is to open bud-grafted trees for tapping with a girth of 46 cm and above (when 70% of the trees have attained) attained at a height of 1.5 m from the ground. For seedling trees, the convention is to open when a similar girth is reached at a height of 75 cm from the ground. Immature field can be brought into opening for tapping and above at the recommended height.

The latex vessels in the bark traverse from bottom left to top right at an angle of 30° in an anti-clockwise direction. Hence, a cut from the high left to low right will severe a greater number of latex vessels which lead to the current practice of sloping cut from high left to low right on all spiral cuts. Similarly, 25° slope is preferred for seedling because it results in lesser bark consumption and a smaller area of bark that will be lost when the cuts reach ground level without much loss of yield. Further, the presence of a thick corky layer in bark provides a channel for the flow of latex (Fig. 5.7). Since the bark thickness is less in bud-grafted trees, the latex may overflow the sides of the tapping cut with a 25° slope, which is an additional reason for having 30° slope in bud-grafted trees. When the tapping cut approaches the base, a new cut on the opposite panel can be similarly opened.

The yield obtained from the tree is greatly influenced by the skill of the tapper. A skilled tapper will tap to optimum depth to within 1 mm of the cambium without wounding the cambium. Greatest number of latex vessels is situated near the cambium to realize better yield (Fig. 5.8). This is where the skill of the tapper is critical in that he is able to tap deep without wounding the trees. Low intensity tapping systems benefit more from deep tapping than high intensity systems

Table 5.1 Some tapping notations and descriptions

S/2 d4 6d/7 12 m/12. ET2.5% Pa1(1) 0/y	Half spiral cut tapped downward at four daily frequency, 6 days in tapping followed by 1 day rest, 12 months in 12; stimulated with Ethephon of 2.5% active ingredient with 1 g of stimulant applied on 1 cm band on scraped bark below the tapping cut, 0 application per year
S/2 d4 6d/7 12 m/12. ET2.5% Pa1(1) 2/y	Half spiral cut tapped downward at four daily frequency, 6 days in tapping followed by 1 day rest, 12 months in 12; stimulated with Ethephon of 2.5% active ingredient with 1 g of stimulant applied on 1 cm band on scraped bark below the tapping cut, 2 applications per year at irregular interval between applications
S/2 d4 6d/7 12 m/12. ET2.5% Pa1(1) 4/y	Half spiral cut tapped downward at four daily frequency, 6 days in tapping followed by 1 day rest, 12 months in 12; stimulated with Ethephon of 2.5% active ingredient with 1 g of stimulant applied on 1 cm band on scraped bark below the tapping cut, 4 applications per year at irregular between applications
S/2 d4 6d/7 12 m/12. ET2.5% Pa1(1) 8/y (m)	Half spiral cut tapped downward at four daily frequency, 6 days in tapping followed by 1 day rest, 12 months in 12; stimulated with Ethephon of 2.5% active ingredient with 1 g of stimulant applied on 1 cm band on scraped bark below the tapping cut, 8 applications per year at interval of 1 month between applications
S/2 d4 6d/7 12 m/12. ET2.5% Pa1(1) 13/y(3w)	Half spiral cut tapped downward at four daily frequency, 6 days in tapping followed by 1 day rest, 12 months in 12; stimulated with Ethephon of 2.5% active ingredient with 1 g of stimulant applied on 1 cm band on scraped bark below the tapping cut, 13 applications per year at interval of 3 weeks between applications
S/2 d4 6d/7 12 m/12. ET2.5% Pa1(1) 26/y(2w)	Half spiral cut tapped downward at four daily frequency, 6 days in tapping followed by 1 day rest, 12 months in 12; stimulated with Ethephon of 2.5% active ingredient with 1 g of stimulant applied on 1 cm band on scraped bark below the tapping cut, 26 applications per year at interval of 2 weeks between applications.
S/2 d4 6d/7 12 m/12. ET2.5% Pa1(1) 39/y(w)	Half spiral cut tapped downward at four daily frequency, 6 days in tapping followed by 1 day rest, 12 months in 12; stimulated with Ethephon of 2.5% active ingredient with 1 g of stimulant applied on 1 cm band on scraped bark below the tapping cut, 39 applications per year at interval of 1 week between applications
S/2 d4 6d/7 12 m/12. ET2.5% Pa1(1) 78/y(4d)	Half spiral cut tapped downward at four daily frequency, 6 days in tapping followed by 1 day rest, 12 months in 12; stimulated with Ethephon of 2.5% active ingredient with 1 g of stimulant applied on 1 cm band on scraped bark below the tapping cut, 0 application per year at interval of 4 days between applications.

(Abraham and Tayler 1967). Experiments have shown that beyond a minimum bark consumption, yield is not enhanced with increasing thickness of bark shaving (De Jonge and Warriar 1965). Low frequency tapping systems caused more drying of the bark tissue between tappings. A thicker bark shaving per tapping is required which experienced and skilled tappers adjust automatically. Annual bark consumption from different frequencies of tapping on a half spiral cut will be 20–23 cm for alternate daily, 16–18 cm for third daily and 14–16 cm for fourth daily. Higher yield can be obtained at dawn. This is believed to have a direct bearing on the turgidity of the tree with its transpiration to the minimum under a high atmosphere relative humidity. The tapping task for a tapper will depend on the tapping system, stand per hectare and topography of land. In Malaysia, normal task size with ½ S d/2 tapping is between 550–600 trees. Tapping is controlled wounding, and it retards the growth of all trees especially budded trees. This is clone dependent and such trees are likely to have unbalanced development and will become prone to wind damage. Hence, tapping systems must be tailored to the growth habit of cultivars after tapping. Longer cuts than half spiral tend to reduce the rate of girth increment and hence, long cut systems are not preferred on young rubber trees (Ng et al. 1969). Two micro-tapping systems have been used in the planting industry, viz. puncture tapping and micro-X tapping systems. Both systems only work with stimulation and hence have their limitations. Puncture tapping has some attractions for bringing trees into early tapping (Abraham 1981). Micro-X combines the use of puncture tapping and excision tapping (Ismail Hashim et al. 1979).

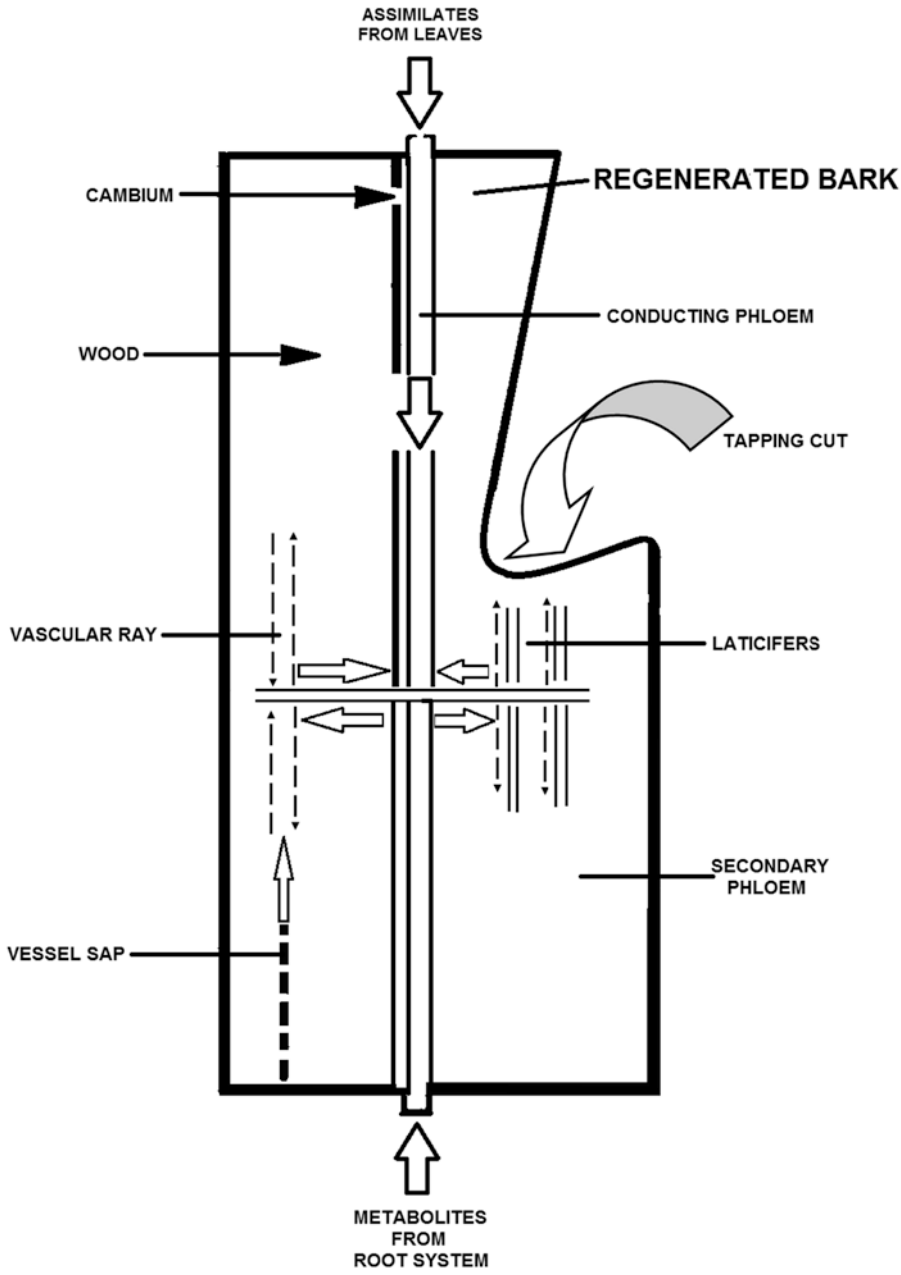

Fig. 5.7 Diagrammatic representation of tapping panel and its functions

5.9.3 Factors Affecting Tapping Efficiency

Tapping is a skilled operation and hence quality of tapping varies from person to person. Usually the best tappers are given young trees to tap where tapping should be carried out with minimum wounding. Field supervision is a must to ensure that latex never spills over the panel and tapping is carried out to proper depth to reduce yield loss. On a flat land, a tapper may be able to tap 600 trees on Panel B0-1 and on a steep hill slope only

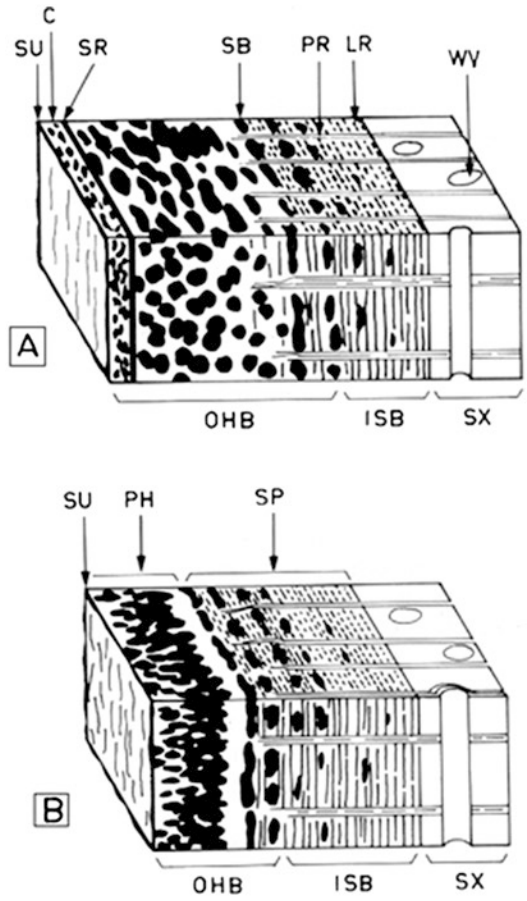

Fig. 5.8 (**a**) Virgin bark (**b**) regenerated bark. *C* cork, *ISB* inner soft bark, *LR* laticifer ring, *OHB* outer hard bark, *PH* phelloderm, *PR* vascular ray, *SB* clustered stone cells, *SP* secondary phloem, *SR* sclerenchyma ring, *SX* secondary xylem, *WV* wood vessel

500 trees. Tapping at dawn is most preferred since hydrostatic pressure of the tree used to be high (1.0–1.5 MPa), and diurnal variation in latex flow follows vapour pressure deficit of air. This implies the role of transpiration on turgour pressure (Rao et al. 1990).

The girth of older trees is usually larger, and hence the tapping cuts are longer. Consequently, it requires more time to tap the older trees and a tapper who taps 600 trees on Panel BO-1 may only be required to tap 575 trees on Panel BO-2, 530 trees on Panel BI-1 and so on. The length of the tapping cut is also determined by the tapping system. It goes with the wisdom of the owner of the estate to give task for a tapper. For example, where tapping involves the use of a ladder, the tapper should be given only 65% of the appropriate numbers of trees that are given for low-level tapping. In controlled upward tapping (CUT), a tapper is given a slightly bigger task size than ladder tapping (Ismail Hashim et al. 1981).

Two tapping knives are commonly used in the industry, i.e. *Michie-Golledge* and *Jebong*. The third type is *Gouge* meant mainly for controlled upward tapping (CUT). While Jebong is suitable for shaving off a thin layer of bark, Gouge is used to push along the tapping cut to shave off the bark instead of being pulled along as with Jebong. A modified Gouge with a long handle is widely used for CUT (Figs. 5.9 and 5.10). Bidirectional knives are also available and are used for upward

Fig. 5.9 Various tapping knives

Fig. 5.10 Controlled upward tapping (CUT)

and downward tapping systems (Abraham 1981). Spouts made of galvanized iron are to be fixed (without injuring the cambium) to the trees at the end of the tapping cut to enable the latex to flow from the tapping cut into the cup.

5.9.4 Yield Stimulation

Tolerance to recurrent mechanical wounding and exogenous ethylene is a feature of the rubber tree. Latex harvesting involves tapping of the tree bark, and ethephon is applied to increase latex flow. Ethylene is an essential element in controlling latex production. Stimulation of latex flow is principally an exogenous process to increase the yield above that is normally obtained by tapping a rubber tree. The first known report on yield stimulation is that periodic scraping of bark led to an increase in yield (Kamerun 1912). Stimulation is now an integral part of most exploitation methods. The early history of stimulation has been reviewed tracing the development to the commercial use of synthetic growth substances such as 2,4-D and 2,4,5-T (Abraham and Tayler 1967). A wide range of substituted phenoxyacetic acids and substituted benzoic acids were screened for stimulant activity during the period 1956–1968. A number of experiments led to the conclusion that only 2, 4, dichloro-5-fluoro-phenoxyacetic acid gave comparable or better response than 2, 4, 5-T, but was not considered because of economic reasons (Blackman 1961; Abraham et al. 1968). The results supported the continued use of 2,4-D and 2,4,5-T as yield stimulant of *Hevea.*

The first use of a gas (ethylene oxide, which is toxic) was reported to increase latex flow (Taysum 1961). Subsequently, acetylene was found to be a yield stimulant (Banchi 1968; Banchi and Poliniere 1969). The yield stimulant action of acetylene and ethylene was later confirmed (D'Auzac and Ribaillier 1969a, b), and all effective non-gaseous stimulants generate ethylene in common (Abraham et al. 1968). Ethylene gas is believed to act more or less directly by inhibiting the plugging reaction of the trees (Abraham et al. 1972). The success of (2-chloroethyl) phosphonic acid (ethephon) as a yield stimulant generated an intensive search for alternative ethylene-based stimulants. Though numerous chemicals were screened covering a

wide spectrum, no alternative to ethephon could be named (Pakianathan et al. 1971). However, derivatives of ethephon, especially poly-silylene phosphate derivatives, could constantly prolong the production of ethylene over time (Derouet et al. 2003).

There are several methods of applying ethephon that are not labour demanding but consequently cheap, simple and very practical. These methods are described here briefly with variations. A common method is applying over the scraped bark below the tapping cut. This method entails demarcating a narrow band below the tapping cut, scraping off the outer corky tissue and then applying a thin layer of stimulant mixture over the scraped area. The applied portion is consumed before the next application (Abraham et al. 1975). In the second method, application to the regenerating bark immediately above the cut with no scraping is done where width of application varies with the frequency (Puddy and Warriar 1961). In the third method, the stimulant is applied to the groove of the tapping cut by means of a paint brush after removal of tree lace, on a non-tapping day at monthly intervals that is as effective as the scraped bark method. Fourth is the lace application, in which stimulant is applied on the groove of tapping cut without removal of tree lace.

Gaseous stimulations are available using ethylene popularly known as HLE and RRIMFLOW (Guha et al. 1992). While in HLE, a hypodermic puncture of 1 mm is made in the bark and latex extracted in a container having 8–10% ammonia solution, RRIMFLOW is practiced through taking a minicut (2.5 mm) with d/4 frequency. Both the cases, a small portion of the bark is exposed to ethylene gas with the help of applicators. A reduced frequency of d/4 or d/6 will maximize yield with a large task of 900–1000 trees that will improve labour productivity. Tapping at low intensity and frequency along with low dosage of stimulation using ethephon has been suggested as an effective approach to increase productivity per tapper and thus reducing cost of production (Zarin et al. 1991; Thanh et al. 1996).

The incidence of dryness in stimulated tree is generally higher than that of unstimulated trees and increases markedly in stimulated trees when the cut approaches the union (Sivakumaran et al. 1981). Generally, trees on which stimulation was first introduced on virgin panels have higher incidence than trees which first had stimulation on renewed panels. A previously tapped and stimulated panel will have greater chances of going into dryness.

However, extent of depression in girth increment is largely influenced by age of trees and intensity of tapping in stimulated trees. Thus, stimulation on virgin panels generally has a greater adverse effect on girth increment than stimulation on renewed panel when growth rate is lower and competition for assimilates is less. Generally, the depression in girth increases in proportion to the increase in length of cut with the most depression obtained in trees that are intensively tapped.

The effect of stimulation on bark thickness and number of latex rings varied according to cultivars. Thus, in a study of eight clones, bark thickness of renewed bark was significantly increased in ethephon-stimulated trees of five clones relative to respective controls, while in three others the increase was not significant (Sivakumaran et al. 1981). Similarly, for latex vessel number, there was an increase in six clones, while in two there was a marginal decrease (Ping 1982). In trees stimulated for 3 years with ethephon, both initial flow rate and turgor pressure got reduced in comparison to controls (Pakianathan 1977). Also, a marked reduction in initial flow rates in long-term ethephon-treated trees was observed relative to unstimulated trees (Pakianathan et al. 1982). Anatomical examination of bark taken from such low-pressure areas has shown increase in stone cells in the soft bark and partial emptiness of latex vessels along with a reduction in sucrose levels (Tupy and Primot 1976).

Ethylene stimulation of latex production results in high sugar flow from the surrounding cells of inner bark towards the latex cells. Dusotoit-Coucaud et al. (2010) studied the role of seven sucrose transporters (HbSUTs) and one hexose transporter (HbHXT1) sucrose flow. In PB217 and PB260, the expression pattern of these sugar transporters (HbSUTs and HbHXT1) was monitored under different physiological conditions and

found to be maximal in latex cells. HbSUT1, one of the most abundant isoforms, displayed the greatest response to ethylene treatment. In PB217, ethylene treatment led to a higher accumulation of HbSUT1B in latex cells than in the inner bark tissues. Conversely, stronger expression of HbSUT1B was observed in inner bark tissues than in latex cells of PB260. A positive correlation with HbSUT1B transcript accumulation and increased latex production was further supported by its lower expression in latex cells of the virgin clone PB217. A diagrammatic representation of the probable steps involved in Ethrel stimulation is available in Fig. 5.11.

The ethylene signalling pathway leads to the activation of Ethylene Response Factor (ERF) transcription factors. This family has been identified in *Hevea brasiliensis.* This study set out to understand the regulation of ERF genes during latex harvesting in relation to abiotic stress and hormonal treatments. Analyses of the relative transcript abundance were carried out for 35 HbERF genes in latex, in bark from mature trees and in leaves from juvenile plants under multiple abiotic stresses. Twenty-one HbERF genes were regulated by harvesting stress in laticifers, revealing an over-representation of genes in group IX. Transcripts of three HbERF-IX genes from HbERF-IXc4, HbERF-IXc5 and HbERF-IXc6 were dramatically accumulated by combining wounding, methyl jasmonate and ethylene treatments. When an ethylene inhibitor was used, the transcript accumulation for these three genes was halted, showing ethylene-dependent induction. Subcellular localization and transactivation experiments confirmed that several members of HbERF-IX are activator-type transcription factors. This study suggested that latex harvesting induces mechanisms developed for the response to abiotic stress. These mechanisms probably depend on various hormonal signalling pathways. Several members of HbERF-IX could be essential integrators of complex hormonal signalling pathways in *Hevea* (Putranto et al. 2015).

There are special protein families called the AP2/ERF proteins. This family contains transcription factors that play a crucial role in plant growth and development and in response to biotic and abiotic stress conditions in plants (AP for APETALA and ERF for Ethylene Response Factor). The AP2/ERF super family is one of the largest groups of transcription factors in plants. It includes all genes coding for at least one APETALA2 (AP2) domain and can be further separated into the Ethylene Response Factor (ERF), the AP2 and the RAV families (Piyatrakul et al. 2014). The RAV family encodes proteins possessing a single AP2 domain plus an additional B3 domain, which is also present in other, non-ERF transcription factors. The AP2/ERF super family encodes transcription factors that play a key role in plant development and responses to abiotic and biotic stress. In *Hevea brasiliensis*, ERF genes have been identified by RNA sequencing. This study set out to validate the number of HbERF genes and identify ERF genes involved in the regulation of latex cell metabolism. A comprehensive *Hevea* transcriptome was improved using additional RNA reads from reproductive tissues. Newly assembled contigs were annotated in the Gene Ontology database and were assigned to three main categories. The AP2/ERF super family is the third most represented compared with other transcription factor families. A comparison with genomic scaffolds led to an estimation of 114 AP2/ERF genes and one soloist in *Hevea brasiliensis*. Based on a phylogenetic analysis, functions were predicted for 26 HbERF genes. A relative transcript abundance analysis was performed by real-time RT-PCR in various tissues. Transcripts of ERFs from group I and VIII were very abundant in all tissues, while those of group VII were highly accumulated in latex cells. Seven of the 35 ERF expression marker genes were highly expressed in latex. Subcellular localization and transactivation analyses suggested that HbERF-VII candidate genes encoded functional transcription factors (Piyatrakul et al. 2014).

The identification of aquaporin genes has extended new insights into the water use efficiency and latex production. Zou et al. (2015) identified 51 full-length aquaporin genes (AQP) from the rubber tree genome. The phylogenetic analysis assigned these AQPs to five subfamilies, including 15 plasma membrane intrinsic proteins

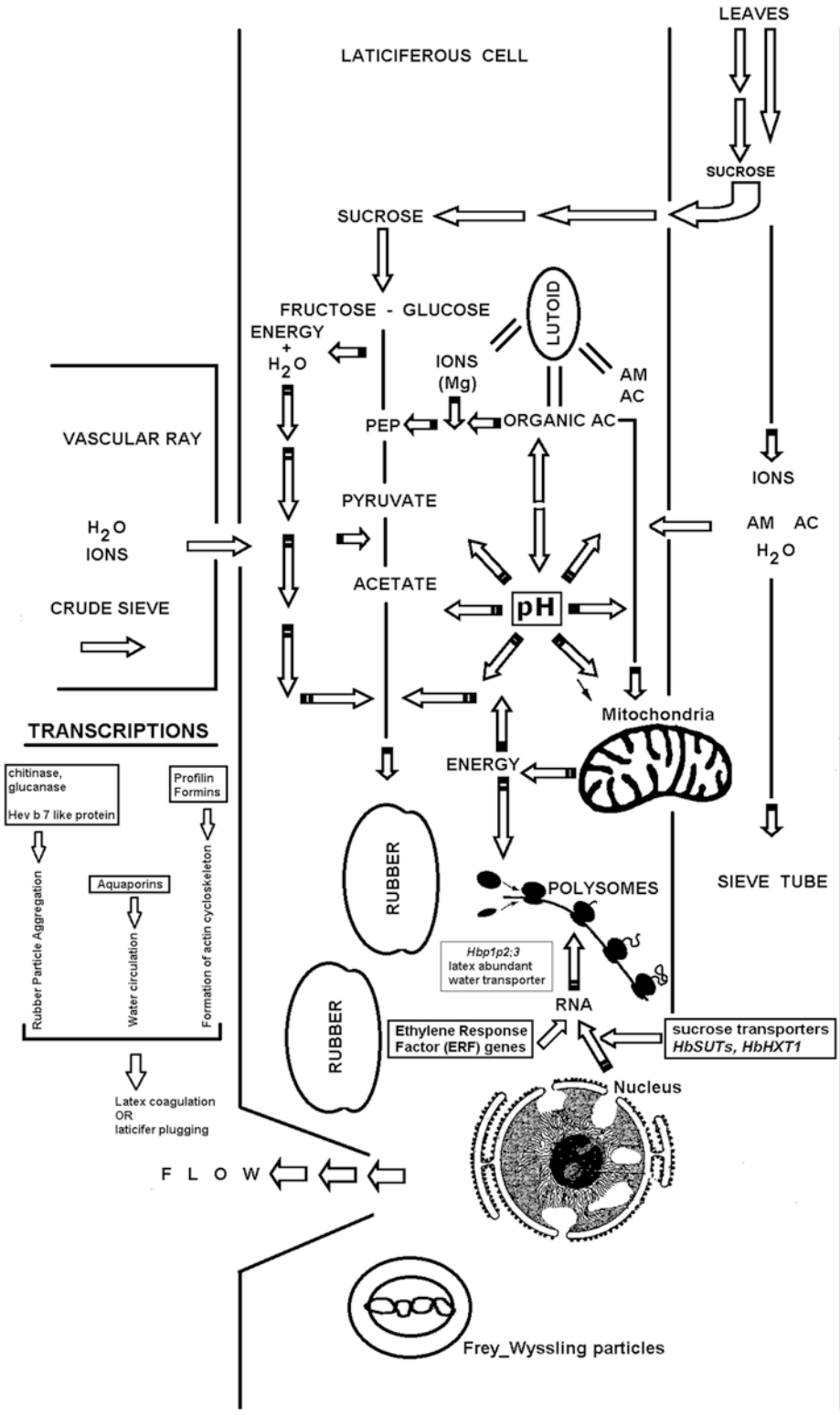

Fig. 5.11 Diagrammatic representation of the probable events after ethylene stimulation

(PIPs), 17 tonoplast intrinsic proteins (TIPs), 9 NOD26-like intrinsic proteins (NIPs), 4 small basic intrinsic proteins (SIPs) and 6 X intrinsic proteins (XIPs). qRT-PCR analysis showed diverse expression patterns of laticifer-expressed HbAQP genes upon ethephon treatment, a widely used practice for the stimulation of latex yield. This study of Zou et al. (2015) provides an important genetic resource of HbAQP genes, which will be useful to improve the water use efficiency and latex yield of *Hevea.* On the other hand, sucrose transporters are having a direct bearing on laticifers and ethylene application. Dusotoit-Coucaud et al. (2009) cloned seven putative full-length cDNAs of sucrose transporters from a latex-specific cDNA library. These transporters belong to all SUT (sucrose transporter) groups and differ by their basal gene expression in latex and inner soft bark, with a predominance of HbSUT1A and HbSUT1B. Of these sucrose transporters, only HbSUT1A and HbSUT2A were distinctly increased by ethylene. Moreover, this increase was shown to be specific to laticifers and to ethylene application. The data and all previous information on sucrose transport show that HbSUT1A and HbSUT2A are related to the increase in sucrose import into laticifers, required for the stimulation of latex yield by ethylene in virgin trees (see Fig. 5.12 for a scheme of ethylene-induced biochemical pathways in latex cells).

Transcriptome sequencing of genes in the bark is yet another emerging area that can extend ideas on gene action relating to stimulation. Liu et al. (2016) performed de novo sequencing and assembly of the bark transcriptomes of *Hevea brasiliensis* induced with ethephon for 8 h (E8) and 24 h (E24). Compared with control, 10,216 and 9374 differentially expressed genes (DEGs) in E8 and E24 were respectively detected. The expression of several enzymes in crucial points of regulation in glycolysis was up-regulated, and DEGs were not significantly enriched in isopentenyl diphosphate (IPP) biosynthesis pathway. In addition, up-regulated genes of great regulatory importance in carbon fixation (Calvin cycle) were identified. The rapid acceleration of glycolytic pathway supplying precursors for the biosynthesis of IPP and natural rubber, instead of rubber biosynthesis per se, may be responsible for ethylene stimulation of latex yield in rubber tree.

5.10 Tapping Panel Dryness and Necrosis

Tapping Panel Dryness (TPD) is a syndrome encountered in rubber trees, characterized by spontaneous drying up of the tapping cut resulting in abnormally low yield or stoppage of latex production (Fig. 5.13). The disease was reported first in Brazil in 1887 in the Amazon forest and at the beginning of the century in plantations in Asia (Rutgers and Dammerman 1914). The symptoms range from partial dryness with no browning of the tapping cut, browning and thickening of the bark and cracking and deformation of the bark in some instances. The syndrome is characterized by the appearance of tylosoids and the coagulation of latex (de Fay 1981; de Fay and Hebant 1980; Paranjothy et al. 1976), abnormal behaviour of the parenchyma cells adjoining the laticifers and general increase in synthesis of polyphenols (Rands 1921). A detailed review of the histological, histochemical and cytological study of the diseased bark was presented by de Fay and Jacob (1989).

Nandris et al. (2004) made a bifurcation between Tapping Panel Dryness and Trunk Phloem Necrosis (TPN). While TPN almost invariably results in irreversible panel dryness, TPD can be either reversible or irreversible. There are several reasons by which TPD or TPN can occur. Some are (a) reduced water availability due to compaction of soils combined with disturbed sap flow (Nandris et al. 2004), (b) involvement of impaired cyanogenesis in the necrotic process of bark tissue (Chrestin et al. 2004; Kongsawadworakul et al. 2006), (c) occurrence of oxidative stress within the latex cells (Sookmark et al. 2006) and overstimulation (Sookmark et al. 2006). Further, involvement of a causative organism (Keuchenius 1924; Rands 1921; Sharples 1922), existence of cortical necrosis (Peries and Brohier 1965; Guanbiao et al.

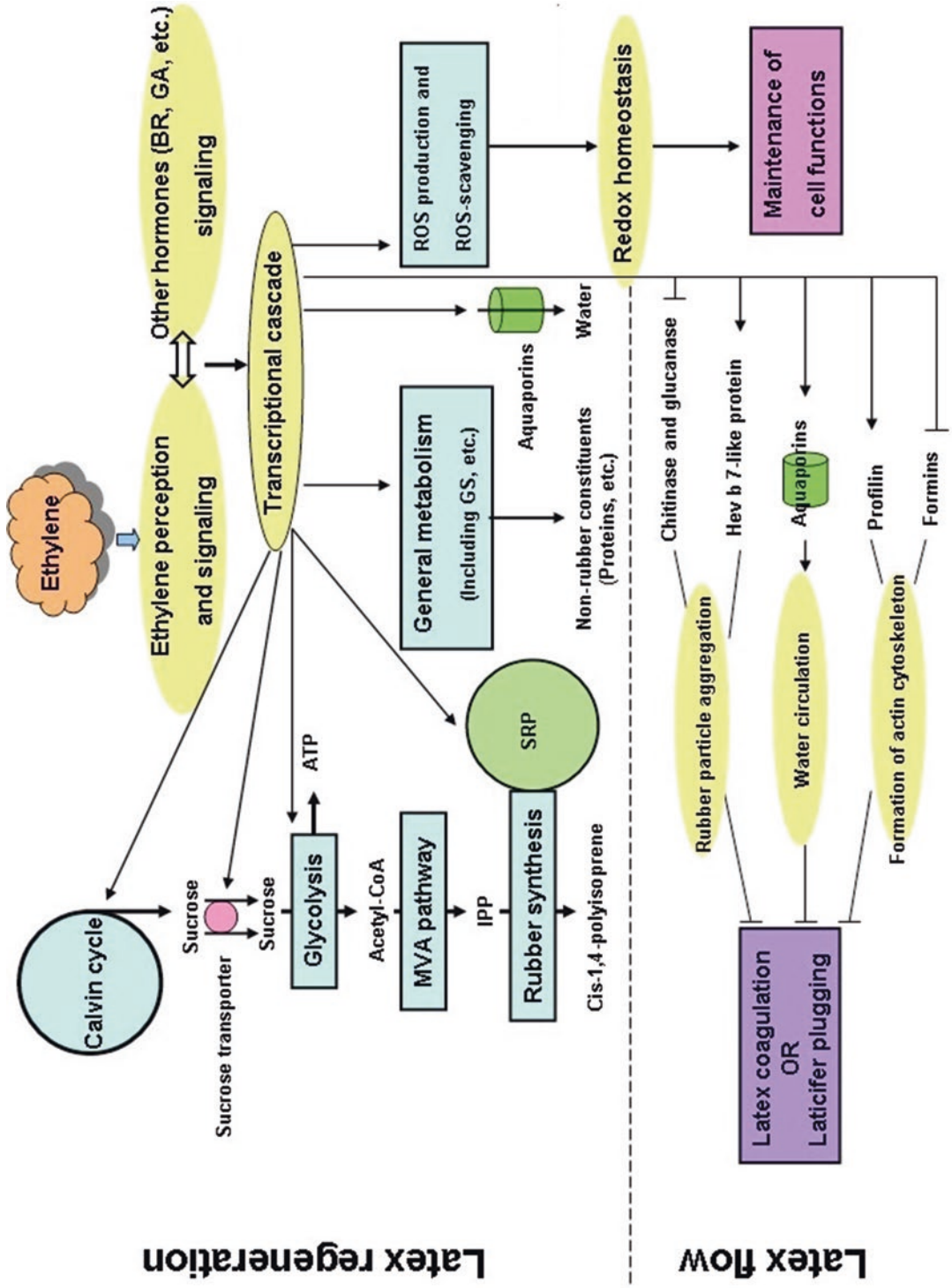

Fig. 5.12 General scheme of ethylene-induced biochemical pathways in latex cells. Factors in shaded boxes are activated and inhibited by ethephon or ethylene, respectively. Factors are: *Chi* (chitinase), *Glu* (glucanase), *GS* (glutamine synthetase), *HEV* (hevein), *HMG* (3-hydroxy-3-methylglutaryl-coenzyme A reductase), *HXT* (hexose transporter), *PIP* (plasma membrane intrinsic protein), *RBOH* (NADPH oxidase), *REF* (rubber elongating factor), *SUS* (sucrose synthase), *SUT* (sucrose transporter) (After Piyatrakul et al. 2014). http://dx.doi.org/10.1371/journal.pone.0099367.g001

Fig. 5.13 (**a**–**c**) Various kinds of tapping panel dryness

1982) and rickettsia-like organisms (RLO) (Guanbiao et al. 1988) were suspected to be responsible for tapping panel dryness. No confirmatory evidence could so far been made available for any of these contentions. High intensity of exploitation is known to promote incidence of tapping panel dryness in plantations; the proportion of dry trees increases with tapping intensity and particularly with tapping frequency (Bealing and Chua 1972; Chua 1967). The intensive exploitation is reported to result in excessive outflow of latex and consequent nutritional stress (Chua 1967; Schweizer 1949; Taylor 1926), inadequate organic resources (Chua 1966; Tupy 1984) and Cu and K deficiency (Compagnon et al. 1953).

Influence of climate and growth period was also believed to be the reasons for dryness (Harmsen 1919; Vollema 1949; Compagnon et al. 1953; Bealing and Chua 1972). Unbalanced nutrition favouring the incidence of disease was reported by Pushpadas et al. (1975). Clonal sensitivity was also observed by many workers as a reason (Dijkman 1951; Omokhafe 2004). Though evidences are many, the real reason and cause of dryness is yet to be confirmed that is accepted by all.

The most common symptom of TPD/TPN is a phase of excessive and/or late dripping of latex and a simultaneous fall in the drc, followed by a sharp decline in the volume per tapping. The colloidal stability of the latex will also be reduced resulting in particle damage, flocculation of rubber particles and early plugging of latex vessels (Chrestin et al. 1985). A reduction in turgor pressure (Sethuraj et al. 1977), change in latex flow pattern (Sethuraj 1968) and a sharp increase in bursting index (Eschbach et al. 1983) can also occur. The starch reserve is not depleted (Chua 1967) and the vascular rays function normally (de Fay 1981).

Certain forms of bark dryness are transitory and do not display the characteristic symptoms of the formation of tylosoids or activation of the phenolic metabolism (de Fay and Jacob 1989). Numerous traumatism (mechanical such as tapping, chemical or pathological infection) cause the formation of ethylene (Yang and Pratt 1978), and its influence in biochemical, anatomical and histological phenomena is proved (Liebermann 1973). Overstimulation (dose and frequency) or overtapping can lead to excessive endogenous ethylene production and deleterious effect on cellular systems (Chrestin 1984a, b, 1985). Deliberate overstimulation with Ethrel can also result in imbalanced peroxidase activity and consequently the disorganization of membrane structures thus leading to bark dryness. A reduction in sucrose, thiol and Mg contents and increase in

redox potential (RP) are connected with a higher rate of bark dryness (Eschbach et al. 1986). Though tapping rest for varying periods can revive certain trees, in many cases reoccurrence is not uncommon. Thiols, which delay latex coagulation, are composed of glutathione (GSH), cysteine and methionine. The rate-limiting enzyme, γ-ECS, plays an important role in regulating the biosynthesis of glutathione. Fang et al. (2016) cloned and derived the full length of one γ-ECS from rubber tree latex (*Hbγ-ECS1*). According to quantitative PCR analysis, the expression levels of *Hbγ-ECS1* were induced by tapping and Ethrel stimulation, and the expression was related to thiols content in the latex. The expression of *HbγECS1* increased with routine tapping and tapping with Ethrel stimulation indicating that *HbγECS1* has bearing on thiol content.

Ecophysiological studies showed that TPN-affected trees were experiencing significant water deficit with higher stomatal resistance, and cytologically an abnormal vascular connection between rootstock and scion was also seen. At the ultrastructural level, signs of degeneration were observed in mitochondria—a typical feature of stress and ageing (Nandris et al. 2004). Construction of inner phloem cDNA Suppression Subtractive Hybridization (SSH) libraries and bioinformatic analysis of more than 2000 ESTs sequenced from the SSH libraries (healthy vs TPN and TPN vs healthy) suggest differential gene expression (Kongsawadworakul et al. 2006). While investigating the biochemical and/or molecular markers related to stress response and the role of oxidative stress in the onset of bark disorders, Sookmark et al. (2006) cloned and characterized three full-length cDNAs encoding Cu/Zn-superoxide dismutase (Cu/Zn-SOD), ascorbate peroxidase (APX) and glutathione peroxidase (GPX). While healthy trees showed a positive relationship between rubber yield and latex cytosolic GPX activity and gene expression, TPD trees were exactly vice versa. On the contrary, trees exhibiting TPN were with both higher GPX activity and gene expression. So, though both TPD and TPN trees end up in dry panel, they differ in their origin (Sookmark et al. 2006). Much emphasis has been laid for the study of TPD/TPN, but a comprehensive picture on this syndrome is yet to emerge.

Protein markers, yield potential and susceptibility to tapping panel dryness (TPD) are interrelated (Eliathe et al. 2012). They compared yield and susceptibility to TPD in 11 clones (stimulated and non-stimulated). Lutoid fraction polypeptides were analysed using one and two-dimensional electrophoresis. Susceptibility to TPD appeared as a clonal trait which is not related to yield potential. TPD can occur either in stimulated or non-stimulated clones, but overstimulation increase TPD symptoms. While PB 235, PB 260 and IRCA 130 were seen highly susceptible to TPD, IRCA 41, PB 217, AF 261, AVROS 2037 and GT 1 were less susceptible. Eliathe et al. (2012) analysed 32 KDa and 35 KDa lutoidic proteins. High yielding with less TPD were characterized by abundant quantity of 35 KDa lutoidic polypeptide. On the contrary, clones susceptible to TPD were characterized by abundant quantity of 32 KDa polypeptide. In low-yielding clones (RO 38, Tjir 1), 32 KDa protein was more abundant than 35 KDa. Overstimulation induces a decrease of 35 KDa protein intensity. This is a lead to say that 32 and 35 KDa polypeptides are having a direct bearing on yielding potential and susceptibility to TPD.

Forward and reverse cDNA libraries from the latex of healthy and TPD trees using suppression subtractive hybridization (SSH) method were constructed to identify the genes related to TPD (Li et al. 2010). Of the 1106 clones obtained from the two cDNA libraries, 822 clones showed differential expression in two libraries by reverse Northern blot analyses. Sequence analyses indicated that the 822 clones represented 237 unique genes; and most of them have not been reported to be associated with TPD in rubber tree. The expression patterns of 20 differentially expressed genes were further investigated to validate the SSH data by reverse transcription PCR (RT-PCR) and real-time PCR analysis. According to the Gene Ontology convention, 237 unique genes were classified into ten functional groups, such as stress/defense response, protein metabolism, transcription and post-transcription, rubber biosynthesis, etc. Among the genes with known function, the genes preferentially expressed were

associated with stress/defence response in the reverse library, whereas metabolism and energy in the forward one. Li et al. (2010) concluded that the production and scavenging of reactive oxygen species (ROS), ubiquitin proteasome pathway, programmed cell death might play important roles in TPD. This is a new insight into understanding of TPD process. Transcriptome sequencing of putative genes in healthy and TPD-affected trees revealed that in TPD-affected trees, the expression of most genes related to the latex biosynthesis and jasmonate synthesis was severely inhibited (Liu et al. 2015). This could be the direct cause of TPD.

Studying the regulation of micro-RNA genes (*MIR*) under harvesting stress (tapping, ethephon stimulation) and reactive oxygen species (ROS)-induced TPD in mature rubber trees remains difficult. One *MIR* gene (*MIR159b)* is differentially regulated upon TPD which was shown upregulated upon TPD occurrence (Griffiths-Jones et al. 2006). The expression of this gene was increased in response to cold in leaves and bark. In order to get a full understanding of mechanisms involved in latex production and TPD syndrome, a complete validation of miRNA/target messenger pairs is first needed using high throughput 'degradome' analysis (German et al. 2009). Combination of analyses on juvenile and mature plant materials will help developing model of *MIR* gene regulations under abiotic stress and further characterization of the TPD-regulated miRNAs and their targets. Regulation of *MIR* genes differs depending on the tissue and abiotic stress applied (Gébelin et al. 2013a). Deep sequencing of small RNAs could be carried out on latex from trees affected by TPD using Solexa technology (Gébelin et al. 2013a, b). The most abundant small RNA class size was 21 nucleotides for TPD trees compared with 24 nucleotides in healthy trees. By combining the LeARN pipeline, data from the Plant MicroRNA database and *Hevea* EST sequences, Gébelin et al. (2013a, b) identified 19 additional conserved and four putative species-specific miRNA families not found in previous studies on rubber. The relative transcript abundance of the *Hbpre-MIR159b* gene increased with TPD thus indicating small RNA-specific signature of TPD-affected trees. Such studies at molecular level can only elucidate the intricacies of TPD.

Trunk phloem necrosis (TPN) is known as a physiological disorder since 1980s. Distinguished from rubber tree tapping panel dryness (TPD), by its macroscopic symptoms and presumed origin, little attention has been paid to its microscopic features. de Faÿ (2011) has come out with some evidence that both syndromes could be linked to an impaired cyanide metabolism. In order to characterize TPN and compare it with TPD microscopically, the inner phloem of tapping panels was investigated by light and transmission electronmicroscopy in healthy trees and TPN-affected trees. TPN-affected phloem presented numerous and varied structural and ultrastructural features. Signs of cellular deterioration could be seen in a great number of specialized cells, i.e. laticifers and sieve tubes, but not in very specialized cells, i.e. parenchyma cells and companion cells. There were also signs of cellular dedifferentiation in other parenchymatous cells, e.g. in tylosoids and hyperplasic cells. These cells were derived from parenchyma cells that ensheath laticifers in which the latex coagulated. Numerous structural features of TPN are common to TPD, notably tylosoids associated with in situ coagulated latex, which are also known to be early structural markers of TPD and cyanide-induced. de Faÿ (2011) therefore concluded that TPN is identical to or a variant of TPD and is a degenerative disorder of rubber tree trunk phloem resembling plant stress response, programmed cell death and plant tumourigenesis in some aspects.

Genetic Resources

6

Genetic diversity plays a crucial role in the stability of our ecological system. Every species fulfils a role in the earth's biosphere and assures ecological survival. By this, biodiversity keeps the soil fertile, recycles all nutrients and cleans the air and water. The richer the genetic baggage, the higher shall be the capacity to fight different fungi, virus or bacteria. It is the diversity of genetic baggage that makes natural extinction so rare. Basically, biodiversity provides everything humans need to survive, like food, fresh air, clean water, clothing, medicine, wood and various raw materials for industrial uses. A rich ecological environment is indeed very complex and is impossible for humans to recreate. Genetic erosion is the loss of genetic diversity—often magnified or accelerated by human activities. Quite often, cultivation of a limited number of high-yielding genotypes can also lead to genetic erosion. Much of the diversity of the centre of origin of *Hevea* (Amazon basin, Brazil) is being lost due to extensive deforestation (see Chap. 2).

6.1 *Hevea* as a Species Complex

The genus *Hevea* includes ten species, which are inter-crossable (Clément-Demange et al. 2000). Schultes (1977a, b) and Wycherley (1992) refer readers to excellent reviews on the subject. The taxonomic considerations from 1874 to 1970 delineated the genus with several species on different occasions. Although the genus was considered to include 24 species in 1906, the species concept crystallized with nine species in 1970 (Schultes 1977a, b). The tenth species, *Hevea camargoana*, was added in 1971 (Schultes 1987). *Hevea paludosa* has been identified in Brazil and is often considered as the 11th species (Pires 1973; Goncalves et al. 1990). Three botanists are considered to be the principal workers on species delineation—Baldwin, Seibert and Schultes—who during their classical exploratory studies contributed significantly towards the botany of *Hevea*. A *Harvard University Gazette* (from the archive) says 'Schultes' field work, conducted mostly in the Colombian Amazon beginning in 1941, made him a leading voice in the field and one of the first in the 1960s to warn about destruction of the rainforests and disappearance of their native people' (Dijkman 1951).

The ten species recognized today as belonging to the genus *Hevea* are: *H. brasiliensis*, *H. guianensis*, *H. benthamiana*, *H. pauciflora*, *H. spruceana*, *H. microphylla*, *H. rigidifolia*, *H. nitida*, *H. camporum* and *H. camargoana* (Webster and Paardekooper 1989; Wycherley 1992; Schultes 1990a, b) (see Figs. 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7 and 6.8). Seven species are found in the upper Rio Negro region, considered to be the centre of origin of the genus. *H. brasiliensis* is found in southern areas outside this centre, in the upper Rio Madeira, where five other species are represented. It has generally been assumed that the species are freely inter-compatible (Baldwin 1947; Seibert 1947). Pires (1981)

P.M. Priyadarshan, *Biology of* Hevea *Rubber*, DOI 10.1007/978-3-319-54506-6_6

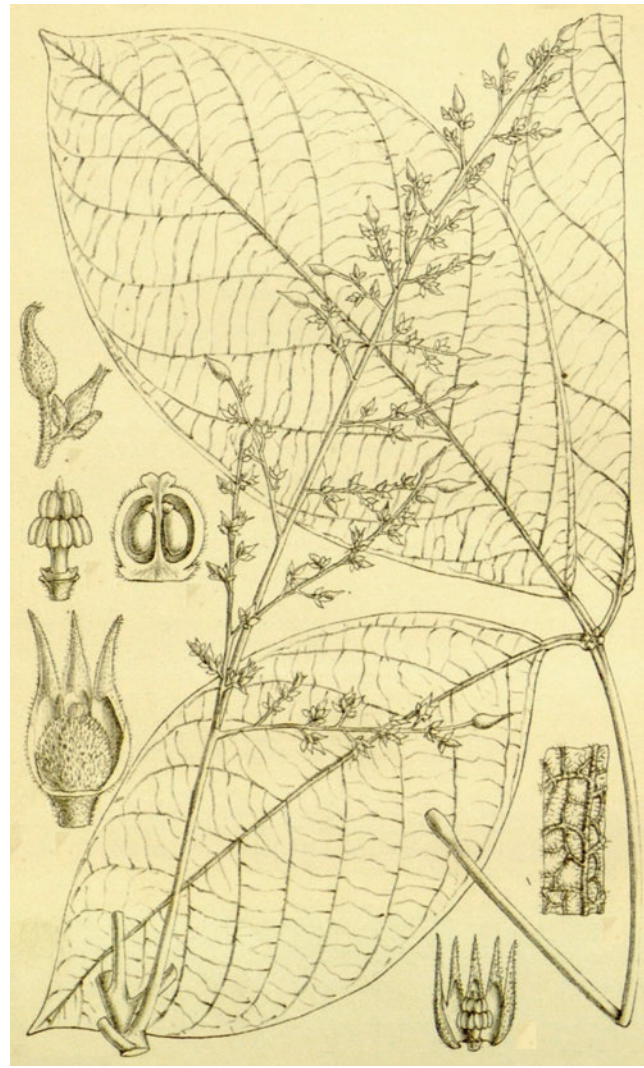

Fig. 6.1 *Hevea benthamiana* Müll. Arg

observed natural hybrids of *H. camargoana* × *H. brasiliensis*, and Gonçalves et al. (1982) analysed the progeny derived from hand pollination from this type of crossing. Consequently, *Hevea* species might be considered as a species complex due to the absence of a strict barrier to recombination between species. Many efforts have led to the identification of certain types which were formerly presented as other possible species. *H. paludosa* was identified in Brazil by Ule in 1905 and is often considered as an 11th species (Goncalves et al. 1990; Priyadarshan and Gonçalves 2003). An elaborate description of taxonomical and botanical aspects of *Hevea* has been reviewed by Schultes (1977a, b, 1987, 1990a, b) and Wycherley (1992). A summary of the salient features of different species of *Hevea* is presented in Table 6.1.

The species are inter-crossable (Clément-Demange et al. 2000). Schultes (1977a, b) and Wycherley (1992) refer the readers to excellent reviews on this subject. The taxonomic considerations from 1874 to 1970 delineated the genus with several species on different occasions. Even though 24 species were considered during 1906, the species concept crystallized with nine species in 1970 (Schultes 1977a, b). A tenth species, *H. camargoana*, was added during 1971 (Schultes

Fig. 6.2 *Hevea camporum* Ducke

1987). Brazil considers 11 species including *H. paludosa* (Pires 1973; Goncalves et al. 1990). Three botanists shall be considered principal workers on species delineation—Baldwin, Seibert and Schultes—who during their classical exploratory studies contributed significantly towards the botany of *Hevea*. The *Harvard University Gazette* (from the archives) says 'Schultes' field work, conducted mostly in the Colombian Amazon beginning in 1941, made him a leading voice in the field and one of the first in the 1960s to warn about destruction of the rainforests and disappearance of their native people' (see www.harvard.edu).

6.1.1 Distribution of Allied Species

The distribution of allied species of *Hevea* is wide among the countries of South America. *Hevea* species are indigenous to Bolivia, Brazil, Colombia, French Guiana, Guyana, Peru, Suriname and Venezuela. All species except *H. microphylla* occur in Brazil, the centre of origin (Goncalves et al. 1990). Four species have been found in Colombia and three occur in Venezuela. Two occur in Bolivia, French Guiana and Guyana (Fig. 6.9 a and b). *H. guianensis* is the most widely adapted species (Pushparajah 2001). Temperate type rubber thrives up to 2500–3000 m in the Andes Mountains (Senyuan 1990). These species of *Hevea* evolved in the Amazonian forests over 100,000 years ago (Clément-Demange et al. 2000). It is pertinent that species adaptation to a particular area is as per climatic and edaphic requirements. The centre of diversity lies within the constantly humid equatorial zone where the amount of precipitation is at least twice the evaporation losses on a yearly basis (Pushparajah 2001). Species like *H. camporum*, *H. paludosa* and *H. rigidifolia* show only limited adaptation. The specific adaptation needs to be closely studied, with reference to climatic and edaphic factors, when clones are to be developed for new environments, especially for marginal areas. It is worthwhile noting that except for *H. benthamiana*

Fig. 6.3 *Hevea guianensis* Aublet

(clones F 4512, F 4542) none of the other species have been utilized for the improvement of the rubber tree.

All *Hevea* species have $2n = 36$ chromosomes, with the exception of one triploid clone of *H. guianensis* ($2n = 54$) and the existence of one genotype of *H. pauciflora* with $2n = 18$ (Baldwin 1947; Majumder 1964). Although *Hevea* behaves as a diploid, it is believed to be an amphidiploid ($2n = 36$; $x = 9$) that stabilized during the course of evolution. This contention is supported by the observation of tetravalents during meiosis (Raemer 1935; Ong 1976; Wycherley 1976). In situ hybridization studies revealed two distinct 18S–25S rDNA loci and one 5S rDNA locus, suggesting a possible allotetraploid origin with the loss of 5S rDNA during the course of evolution (Leitch et al. 1998). But locus duplications are infrequent in the *Hevea* genome, and they could have occurred due to chromosomal modifications posterior to the polyploidization event (Seguin et al. 2003); consequently, the two unknown ancestral genomes of *Hevea* would have strongly diverged.

Low and Bonner (1985b) characterized the *Hevea* nuclear genome as containing 48% of

Fig. 6.4 *Hevea microphylla* Ule

Fig. 6.5 *Hevea nitida* Muell-Arg

Fig. 6.6 *Hevea pauciflora* (Spr.ex Penth) Muell.-Arg. Var coriacea Ducke

Fig. 6.7 *Hevea rigidifolia* (Spruce ex. Benth)Muell.-Arg

Fig. 6.8 *Hevea spruceana* (Benth) Muell. Arg

slowly annealing DNA (putative single copy) and 32% middle repetitive sequences with the remaining DNA being highly repetitive or palindromic. The size of the whole nuclear genome was first estimated as 6×10^8 base pairs. An estimation of the size, using flux cytometry, demonstrated 1.9×10^9 base pairs for *H. brasiliensis*, *H. benthamiana*, *H. guianensis*, *H. pauciflora* and *H. spruceana* (Seguin et al. 2003). The evolution of the cytoplasmic genome was slower due to the lack of genetic recombination through meiosis. The estimated mean molecular size of chloroplast DNA (cpDNA) is 152 kb (Fong et al. 1994). Differentiation of the genus into species appears to be linked with the evolution of the Amazonian forest over the last 100,000 years. Alternations of humid and semi-arid periods responsible for the forest extension or fragmentation resulted in the formation of forest islets. These are assumed to have become zones of protection and differentiation under local selection pressures.

6.1.2 New Genetic Resources

The 'Wickham' population developed in Asia, issued from the collection of seeds in Brazil by Wickham in 1876, has been the basis for rubber domestication and was reputed to have a narrow genetic base. This justified the organization of other collections and transfers of wild germplasm from Amazonia to the main rubber-producing countries, mainly for *H. brasiliensis* but also for allied species. Moreover, the Ford Company and Firestone (companies owning rubber estates in Latin America) as well as Brazilian research contributed to the creation of a stock of selected 'Amazonian' and 'Wickham × Amazonian' germplasm (F, FX, MDF, FDR, IAN, IAC clones). From Brazil to Asia (Dean 1987; Baulkwill 1989), it is difficult to evaluate how narrow the genetic base initially was for what has now become the 'Wickham' domesticated population. Much importance was conferred to a small

Table 6.1 Allied species of the genus *Hevea* - occurrence and features

Species	Occurrence	Notable features[a]
H. benthamiana Muell.-Arg.	North and West of Amazon forest basin, upper Orinoco basin (Brazil)	Complete defoliation of leaves. Medium-size tree. Habitat: swamp forests
H. brasiliensis (Willd. ex. A. de. Juss.) Muell.-Arg.	South of Amazon River (Brazil, Bolivia, Ecuador, Peru)	Complete defoliation of leaves. From medium to big tree size. Habitat: well-drained soils
H. camargoana Pires	Restricted to Marajo island of Amazon River delta (Brazil)	Possibility of natural hybridization with *H. brasiliensis.* From 2to 25 m tree height. Habitat: seasonally flooded swamps.
H. camporum Ducke	South of Amazon between Marmelos and Manicoré rivers tributaries of Madeira river.	Retain old leaves until new leaves appear. Maximum 2 m tall. Habitat: dry savannahs.
H. guianensis Aublet	Throughout the geographic range of the genus (Brazil, Venezuela, Bolivia, French Guyana, Peru, Colombia, Surinam, Ecuador)	Retain old leaves until new leaves and inflorescences appear. Grows at higher altitudes (1100 m MSL) Medium-size tree. Habitat: well-drained soils.
H. microphylla Ule	Upper reaches of Negro River in Venezuela. It is not found in other region of geographic range of the genus	Complete defoliation of leaves. Small trees. They live on flooded area (igapós). Habitat: sandy or *lateritic* soils
H. nitida Mart. ex Muell.-Arg.	Between the rivers Uaupes and Icana tributaries of the upper Negro River (Brazil, Peru, Colombia).	Inflorescences appear when leaves are mature. Small- to medium-size trees (2 m). Habitat: *quartzitic* soils.
H. pauciflora (Spr.ex Bth.) Muell.-Arg.	North and West of Amazon River (Brazil, Guyana, Peru). Distribution discontinuous due to habitat preferences.	Retain old leaves until new leaves and inflorescences appear. No wintering. Small to big size trees. Habitat: well-drained soils, rocky hill sides.
H. rigidifolia (Spr. ex Bth.) Muell-Arg.	Among Negro River and its *affluents.* Uaupes and Içana Rivers (Brazil, Colombia and Venezuela)	Retain old leaves even after inflorescences appear. Small tree from savannahs. Sometime tall, with small crown on the top. Habitat: well-drained soils.
H. spruceana (Bth.) Muell.- Arg.	Banks of Amazon, Rio Negro and lower Madeira (Brazil)	Retain old leaves until new leaves and inflorescences appear. Flowers reddish purple. Medium-size tree. Habitat: muddy soils of islands
H. paludosa Ule[b]	Marshy areas of Iquitos, Peru	Small leaflets, narrow and thin in the fertile branches; up to 30 m. height. Habitat: marshy areas.

After Wycherley (1992), Schultes (1977a, b) Goncalves et al. (1990), Pires (1973) and Brazil (1971)

[a]Wintering characteristics mentioned here has a bearing on the incidence of fungal diseases especially secondary leaf fall (Oidium) since retention of older leaves may make the tree 'oidium escape'. Dwarf types are desirable of the possible wind fastness. All species are diploid ($2n = 36$) (Majumder 1964), and are crossable among themselves (Clément-Demange et al. 2000)

[b]Pires (1973) considered 11 species including H. paludosa; Brazil (1971) considers 11 species

number of 22 seedlings disseminated from Singapore to Malaysia after 1876, but a significant part of the Wickham seedlings which germinated in Kew Botanic Gardens was then sent to Ceylon (now Sri Lanka), raised and disseminated to different countries, especially India. However, it must be underlined that the original Wickham stock was collected in only one Brazilian site, Boïm, on the western banks of the Tapajós river, not far from Santarem. From then, directional selection applied to this population for more than one century and the limitation of the low fruit set

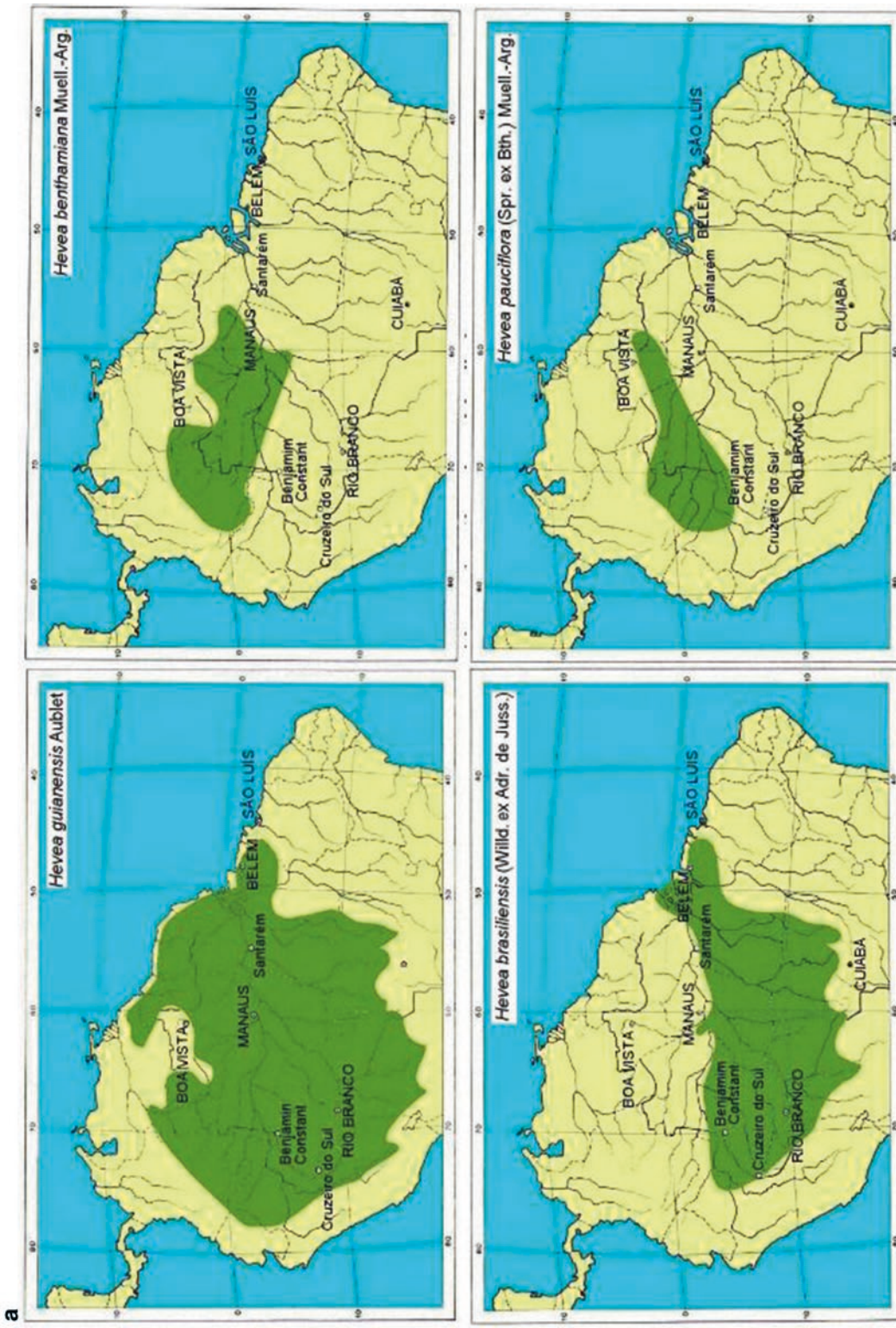

Fig. 6.9 (**a**) and (**b**) Distribution of *Hevea* species in the Amazon (Adopted from Brazil, 1971)

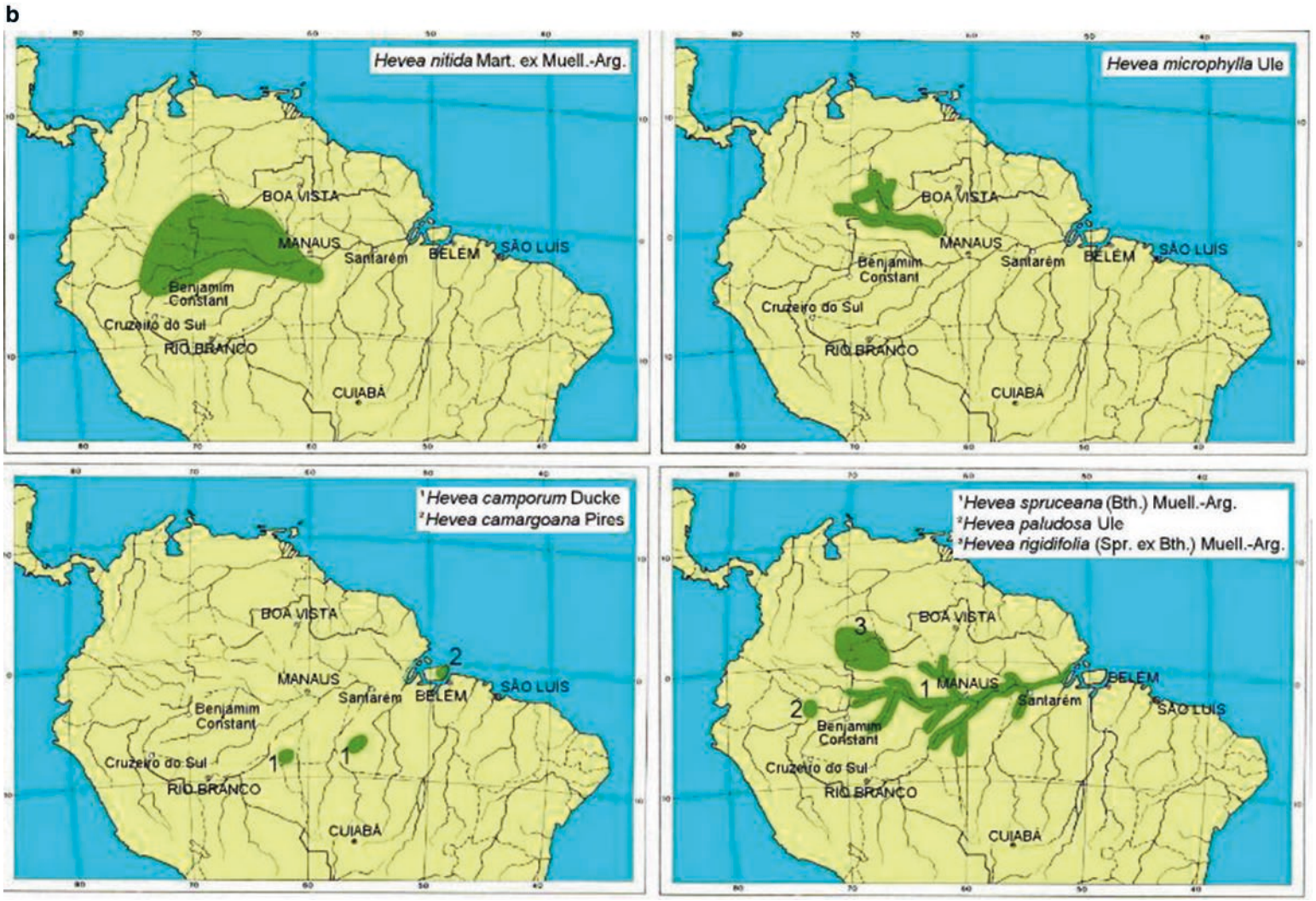

Fig. 6.9 (continued)

in *Hevea* probably further contributed to reducing the extension of this genetic base. Genetic diversity can now be compared with that of the available wild Amazonian populations by use of molecular genetic markers.

Many other introductions from Brazil to Asia and also Africa were carried out between 1896 and 1974, including some species that differed from *H. brasiliensis* (Dijkman 1951; Brookson 1956; Baptist 1961; Wycherley 1968; Hallé and Combe 1975; Nicolas 1976; Ong et al. 1983; Ong and Tan 1987; Tan 1987). All collections were quantitatively rather limited, especially for non-*brasiliensis* species.

In 1981, the IRRDB organized an international collection in Brazil predominantly of seeds, but also of budwood and seedlings (Nicolas 1981; Nouy 1982; Tan 1987; Simmonds 1989). This collection was carried out over three states (Acre, Rondonia and Mato Grosso) from 60 different locations spread over 16 districts. It resulted in the provision of around 10,000 new accessions for breeding. Of this, 37.5% of the seeds were sent to Malaysia and 12.5% to Côte d'Ivoire. Half of the collections were maintained in Brazil. The accessions from the budwood collection were brought to Malaysia and Côte d'Ivoire after a quarantine period of 1 year on the island of Guadalupe (as a protection from SALB disease). After the establishment of two IRRDB germplasm centres in Malaysia and Côte d'Ivoire, other IRRDB member countries were supplied with budwood from this material according to their request.

The field evaluation of this wild Amazonian germplasm showed that the latex yield was as low as about 10% of GT 1, one of the most cultivated clones. Attempts to improve it through Wickham × Amazonian crosses resulted in recombinants that still had a low yield, ranging between 30% and 50% of the level of GT 1, probably due to the important genetic gap lying between the two populations. Conversely, a wide variability was found within these crosses for growth, enabling the selection of very vigorous Wickham × Amazonian clones. A clear difference in branching habit could be observed between accessions from Acre and Rondonia, which more often have tall trunks with poor branching located at a high height, and those from Mato Grosso, which display abundant branching at a low height. Obviously, this wild Amazonian germplasm is bearing an important genetic burden in terms of unfavourable alleles. From the evaluation of IRRDB 1981 germplasm in Côte d'Ivoire, a working population of 287 accessions was selected, taking into account genetic diversity but mainly based on yield; the average yield level of this population is estimated at 36% of the level of GT 1 (Nicolas et al. 1988; Clément-Demange et al. 1998). Four genetic groups of this population could be the basis of pre-breeding work aimed at improving their yield level before testing them by crossing with the Wickham population. In 1995, an expedition was launched by the Rubber Research Institute of Malaysia (RRIM) to collect rubber seeds from Brazil. From this collection, about 50,231 seedlings were planted in Malaysia, including allied species (RRIM 1997; MRB 1999). In order to enlarge the genetic variability of *Hevea*, some research was carried out on mutation breeding (Ong and Subramaniam 1973; Markose et al. 1977) and on polyploidization of the $2n = 36$ *H. brasiliensis* species (Mendes and Mendes 1963; Shepherd 1969; Zheng et al. 1980, 1981). An artificial triploid has been produced by crossing a diploid and a tetraploid (Saraswathyamma et al. 1988). Naturally occurring triploids have also been reported (Nazeer and Saraswathyamma 1987). The existence of some putative genetically dwarf or semi-dwarf genotypes have been mentioned (Ong et al. 1983); *H. camargoana* would have a dwarf growth habit (Gonçalves et al. 1982). Some molecular genetic markers were associated with the dwarfing trait (Venkatachalam et al. 2004).

6.2 Molecular Diversity

The association between DNA sequence variation and heritable attributes has helped to define variations in plants at the molecular level. However, identification and utilization of recombinants with desirable traits is time con-

suming and laborious in rubber due to long generation time and larger size of the crop. With the advent of DNA markers, localization of desirable traits has become routine. The molecular marker systems can be broadly classified into three, viz., first-generation (restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD) and modifications); second-generation (simple sequence repeats (SSRs), amplified fragment length polymorphisms (AFLPs)) and third-generation markers (expressed sequence tags (ESTs), single nucleotide polymorphisms (SNPs)) (Gupta et al. 2001). Of these, SNPs are the new-generation markers used for marker-assisted selection (MAS). All marker systems, except SNPs, have been applied in *Hevea* to facilitate identification and characterization of genes (Saha and Priyadarshan 2012). Recently, a saturated linkage map of *H. brasiliensis* has been accomplished (Lespinasse et al. 2000a). Efforts were on for breeding *Hevea* at the molecular level ever since Low and Bonner (1985b) characterized nuclear genomes containing 48% of most slowly annealing DNA (putative single copy) and 32% middle repetitive sequences with remaining highly repetitive or palindromic ones. Also, the whole genome size was calculated as 6×10^8 base pairs.

Low and Bonner (1985b) characterized *Hevea* nuclear genome as containing 48% of slowly annealing DNA (putative single copy) and 32% middle repetitive sequences with remaining highly repetitive or palindromic DNA. The whole nuclear genome size was first estimated as 6 × 108 base pairs. Estimation with flux cytometry demonstrated 1.9 × 109 base pairs for *H. brasiliensis*, *H. benthamiana*, *H. guianensis, H. pauciflora, and H. spruceana* (Seguin et al. 2003). The evolution of cytoplasmic genome was slower, due to the lack of genetic recombination through meiosis. The estimated mean molecular size of chloroplast DNA (ct DNA) is 152 kb (Fong et al. 1994). Differentiation of the genus into species appears to be linked with the evolution of the Amazonian forest over the last one hundred thousand years. Alternations of humid and semi-arid periods responsible for the forest extension or fragmentation resulted in the formation of forest islets. These are assumed to have become zones of protection and differentiation under local selection pressures.

Seguin et al. (2003) proposed a general organization of *H. brasiliensis* germplasm with 6 genetic groups: *group 1* made up with the two districts AC/T (Tarauaca) and AC/F (Feijo) in the western part of Acre, and with the Calima component of the Schultes' collection; *group 2* made up with the three districts AC/B (Brasileia), AC/S (Sena Madureira), and AC/X (Xapuri) in the eastern part of Acre; *group 3* made up with the six following districts of Rondonia: RO/A (Ariquemenes), RO/C (Calama), RO/CM (Costa Marques), RO/J (Jaru), RO/JP (Jiparana), RO/OP (Ouro Preto), the district MT/VB (Vila Bella) of Mato Grosso, and accessions MDF (Madre de Dios Firestone) from the Firestone collection in Peru; *group 4* made up with three districts MT/A (Aracatuba), MT/C (Juruena), and MT/IT (Itauba) of Mato Grosso, and the district RO/PB (Pimenta Bueno) of Rondonia; *group 5* made up with the Palmira component of the Schultes collection and *group 6* made up with the domesticated Wickham population (Fig. 6.10). Even if no prediction can be made about the progenies of crosses between these groups, they can be used as a base for managing the genetic variability in the long term and organizing the recombination process. Methodological researches have been carried out in order to select the genotypes for making up a collection of reduced size of the Amazonian germplasm, representative of the predominant part of the total variability of this germplasm, according to the concept of 'core collection' (Hamon et al. 1998). The germplasm characterization and diversity analysis studies coordinated by Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD) were funded by the European Union from 1985 to 1997. On the contrary, Lekawipat et al. (2003) used twelve microsatellite markers to detect DNA polymorphism among 108 accessions of *H. brasiliensis* including 40 Wickham clones and 68 wild accessions (1981 Amazonian accessions). Genetic similarity values between genotypes calculated from all the

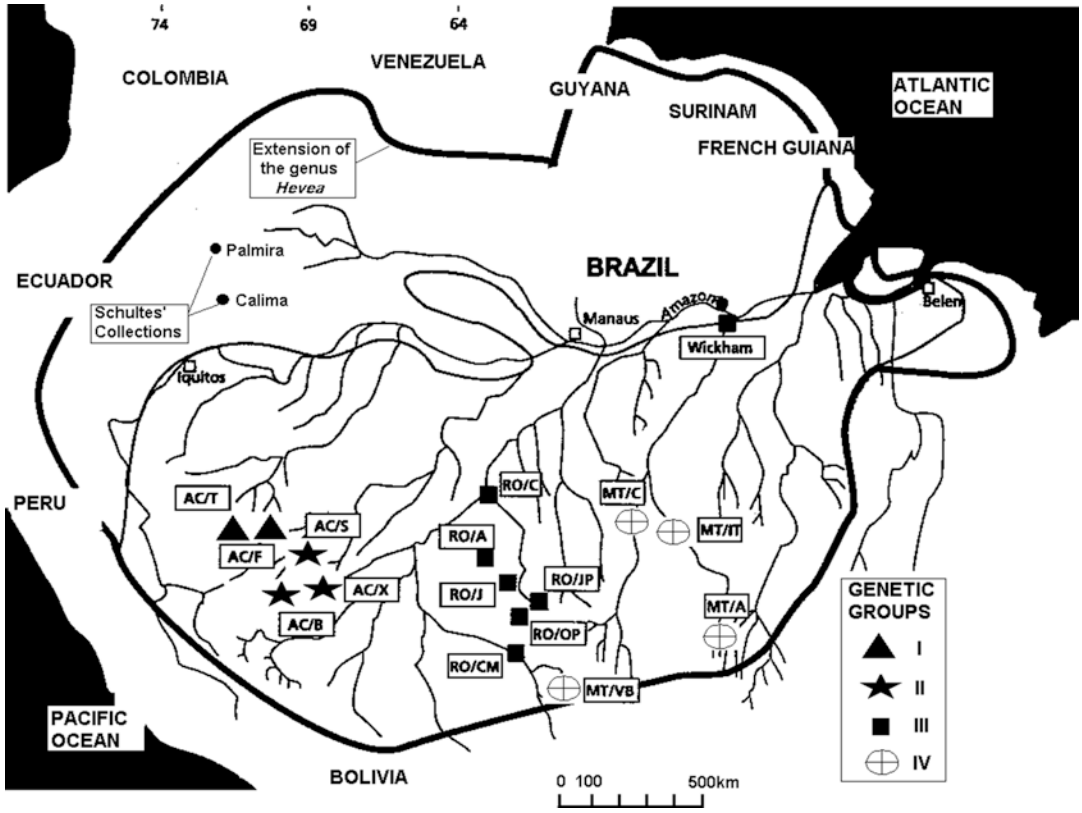

Fig. 6.10 Geographical origin of *Hevea* clone analysed with isozymes or RFLP markers for genetic diversity assessment

microsatellite markers were used to produce a dendrogram of the relationship among accessions, using the unweighted pair-groups method with arithmetic average. A total of 170 alleles were detected. The number of alleles ranged from 5 to 21, with an average of 14 alleles per marker. The results clearly demonstrated that wild accessions are more polymorphic than cultivated Wickham clones and could be divided into three clusters, depending on the geographical origin of collection areas such as Acre, Rondonia and Mato Grosso state. Despite the narrow genetic basis of Wickham clones, their high level of polymorphism could be detected. García et al. (2011) designated primers from sequences reported in GenBank using Primer3, PrimerQuest and OlgoPerfect software for PCR amplification of microsatellites. The primers so obtained were thermodynamically analysed by Oligo Analyzer 3.1 software and experimentally evaluated on 12 *Hevea* clones (GT1, PB 235, PB 260, RRIM 600, IAN 710, IAN 713, IAN 873, FX 3864, FX 3899, GU 198, AVROS 1581 and AVROS 2037). Four microsatellite markers were seen to be sufficient for discriminating 10 of the 12 clones. Clustering analysis showed narrow genetic base of Brazilian clones compared to Asiatic ones.

6.3 Gene Flow and Paternity Identification

Pre-breeding of the Amazonian genetic groups was considered based on recombination through seed gardens. For methodological purposes, one seed garden made up with 50 Amazonian genotypes and GT1 clone, planted at CNRA (Côte d'Ivoire), was subjected to the analysis of gene flux and paternity identification with isozymes and microsatellites (Blanc et al. 2001; Lidah

2005). Paternity identification with microsatellites was carried out with the Cervus software (Marshall et al. 1998). A high level of confidence was found for paternity identification carried out with 8 microsatellite markers. The distribution of the contribution of the different genotypes to pollination was found to be highly unequal, with 4 genotypes accounting for 40%, 14 genotypes accounting for 80% and 25 genotypes accounting for 95% of the total fertilization of the seed garden. The variation of selfing rate was assessed among the genotypes with an average of 5%, and no selfing was found on GT1 as expected for a male sterile clone. The isolation of the seed garden was confirmed since no allele other than those belonging to the parental population was found. The efficiency in paternity identification which is made possible by microsatellites suggests the new possibility to exercise selection on seedlings raised from natural pollination and to identify paternity a posteriori only on the best trees. Here, male sterile clones GT 1 and BPM 24 can be used to ensure cross pollination (see Sect. 7.5.2).

6.4 Genetic Mapping

The availability of numerous molecular genetic markers (MGMs) led to the development of genetic linkage mapping based on the analysis of the percentage of crossing over between the loci of two markers during meiosis (a genetic and not a physical distance) and the ranking of the different loci on the different chromosomes of one species. Due to the heterozygous nature of rubber clones, the construction of genetic linkage maps in *Hevea* requires specific methodology. Unlike annual crops, a cross between two heterozygous parents in *Hevea* can yield information up to four alleles, which are segregated further. The first comprehensive genetic linkage map of *H. brasiliensis* has been built recently, mainly by use of RFLP markers but also AFLPs, microsatellites and isozymes (Lespinasse et al. 2000a). This was accomplished through a double pseudo-test cross as per the methodology of Grattapaglia and Sederoff (1994), and a map was constituted separately for each parent. Further, homologous markers segregating in both parents were ascertained and consensus map prepared. The parents used were PB260 and RO38. The F_1 synthetic map of 717 markers was distributed in 18 linkage groups corresponding to the 18 chromosomes. This comprised of 301 RFLP, 388 AFLP, 18 microsatellite and 10 isozyme markers. The genetic length of the 18 chromosomes was fairly homogeneous, with an average map length per chromosome of 120 cM. Many AFLP markers were seen in clusters, which were attributed as reduced recombination frequency regions. Though the RFLP markers were well distributed all over the 18 linkage groups, these were insufficient to saturate the map. AFLPs and a few microsatellites together contributed to saturating the map. A partially non-random arrangement of duplicate loci observed in RFLP profiles indicate that they have homology descending from a common ancestor (Lespinasse et al. 2000a). The origin of such duplications is still unknown and *H. brasiliensis* continues to behave as a diploid.

In yet another study, Souza et al. (2011) found 603 microsatellite markers, with 309 of them (51%) showing polymorphism. Chi-square test carried out on the genotyping polymorphic loci showed that 110 loci followed a segregation ratio of 1:1, 28 followed a ratio of 1:2:1 and 87 (38.7%) followed a ratio of 1:1:1:1. The map consists of 225 markers, distributed in 23 linkage groups (LG) and 2471.2 cM in length with an average genetic distance of 11 cM between adjacent markers. The largest group has 215.9 cM (18 markers) and the smallest has 2.71 cM (2 markers). This reflects a real polymorphism in a full-sib cross.

Genetic linkage maps associated with phenotyping studies (field evaluation of the genotypes) can generate phenotypic comparisons between a huge number of classes of alleles and lead to the identification of quantitative trait loci (QTL). The research developed on the cross PB260 × RO38 was targeted to understanding the genetic determinism of the resistance of this cross to SALB, first with manual infection at the laboratory level (Lespinasse et al. 2000b). Eight QTLs, with one predominant on linkage group

g13, were identified for resistance in RO38 map through Kruskal-Wallis marker-by-marker test and interval mapping method (Lander and Botstein 1989; van Oojen et al. 1992). The F_1 consensus map confirmed results obtained in parental maps. It was further rationalized that the resistance alleles of RO 38 have inherited from its wild grandparent (*H. benthamiana*) and no favourable allele came from AVROS 363, the Wickham parent. Eight different QTLs for five strains of fungus were found available in RO38, with specificity of resistance to different strains. Field evaluation against the pool of *Microcyclus* strains available in French Guyana was carried out under the real infestation conditions, and it confirmed the presence of the predominant QTL in g13 previously found under controlled infestation (Le Guen et al. 2003). Then it was shown that this major QTL was no more efficient against two widely virulent and highly aggressive strains; for one of them, another QTL located on the linkage group g12 was able to reduce the aggressiveness. This genetic mapping and QTL approach is currently being continued with other crosses for analysing the genetic determinism to different sources of South American Leaf Blight (SALB) resistance. Research for identifying and cloning the real genes responsible for this QTL in linkage group g13 is undertaken at CIRAD in the framework of the building of a bacterial artificial chromosome (BAC) bank and of a physical map of the rubber tree genome based on the clone RO38 that inherited the resistance trait from F4542. Among other applications, this will make possible the search for the DNA fragments bearing the QTL g13 and the development of the 'chromosome walking' technique towards genes associated with QTL g13 on these fragments. This physical map with a high density of MGMs (fine mapping) will also allow one to assess the stability of linkage between the neighbouring genetic markers.

Studies on microsatellite markers and their transferability allied species is an upcoming area of research. Mantello et al. (2012) constructed di- and trinucleotide-enriched libraries. From these two libraries, 153 primer pairs were designed and initially evaluated using 9 genotypes of *H. brasiliensis.* A total of 119 primer pairs had a good amplification product, 90 of which were polymorphic. A total of 46 polymorphic markers were characterized in 36 genotypes of *H. brasiliensis*. The expected and observed heterozygosities ranged from 0.1387 to 0.8629 and 0.0909 to 0.9167, respectively. The polymorphism information content (PIC) values ranged from 0.097 to 0.8339, and the mean number of alleles was 6.4 (2–17). The microsatellites were also tested in 6 other *Hevea* species. The percentage of transferability ranged from 82% to 87%. Locus duplication was found in *H. brasiliensis* and also in 5 of other species in which transferability was tested. Six other species (*H. guianensis, H. rigidifolia, H. nitida, H. pauciflora, H. benthamiana and H. camargoana*) were used to evaluate the transferability of the markers. All loci were tested under the same PCR conditions used for *H. brasiliensis*. Of the 46 loci tested, 40 (87%) were amplified for *H. guianensis* and *H. pauciflora*, 39 (85%) were amplified for *H. camargoana, H. nitida and H. pauciflora* and 38 (82%) were amplified for *H. benthamiana*. This high percentage of transferability may be useful in the evaluations of genetic variability and to monitor introgression of genetic variability from different *Hevea* species into breeding programmes. Earlier, cross-species amplification of the SSR markers developed for *H. brasiliensis* was successful in the wild *Hevea* species *H. guianensis, H. rigidifolia, H. nitida, H. pauciflora, H. benthamiana and H. camargoana* (Souza et al. 2009). The data indicated a high degree of sequence homology in the microsatellite flanking regions of these species.

The main ex situ collections of South America including Amazonian populations that have never been previously described have been subjected to genetic diversity studies (de Souza et al. 2015) (Fig. 6.11). Genetic data were analysed to determine the genetic structure of the wild populations, quantify the allelic diversity and suggest the composition of a core collection to capture the maximum genetic diversity within a minimal sample size. A total of 1117 accessions were genotyped with 13 microsatellite markers. A total of 408 alleles, 319 of which were shared between

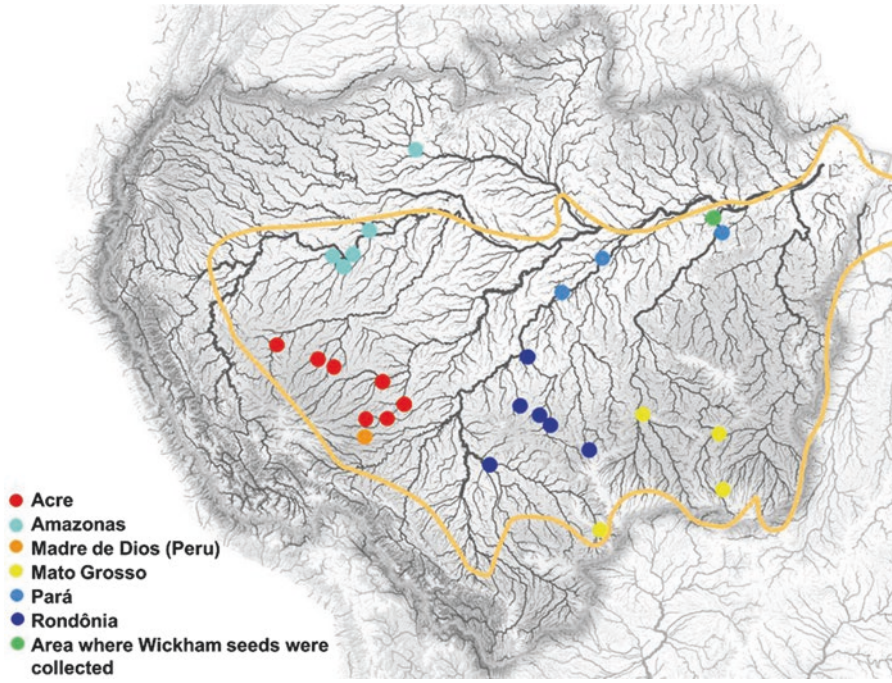

Fig. 6.11 The locations of the sampled sites within Brazil (de Souza et al. 2015)

groups and 89 that were private in different groups of accessions were identified. Principal component analysis revealed primary division into the following two subgroups: cluster 1, which consisted of varieties from the advanced breeding germplasm that originated from the Wickham and Mato Grosso accessions; and cluster 2, which consisted of the wild germplasm from the Acre, Amazonas, Pará and Rondônia populations and *Hevea* spp. These analyses revealed a high frequency of gene flow between the groups, with the genetic differentiation coefficient (GST) estimated to be 0.018. Additionally, no distinct separation among the *H. brasiliensis* accessions and the other species from Amazonas was observed. A core collection of 99 accessions was identified that captured the maximum genetic diversity. Such a core collection could provide resources for forming an association panel to evaluate traits with agronomic and commercial importance and to have genomic breeding (please see Chap. 13 for further details). Phumichai et al. (2015) investigated the genetic diversity and population structure of eight populations of *Hevea* rubber genotypes from Malaysia, India, Sri Lanka, Indonesia, France, Thailand, Brazil and China, in addition to individual primary clones, using 10 nuclear and 11 polymorphic novel chloroplast (cp) microsatellite markers. The Brazilian clones exhibited the greatest genetic diversity. Polymorphisms among different cpSSRs allowed delineation of chlorotypes. Such results provide valuable data for in situ or ex situ conservation and utilization of germplasm collections for breeding programmes.

6.5 Nuclear Vs. Cytoplasmic Diversity

Accessions of Amazonia could be categorized into genetic groups according to their geographic origin (Acre, Rondonia, Mato Grosso). This was revealed with a RFLP analysis of 92 clones of Amazonian and 73 of Wickham origin (Besse et al. 1994). On the other hand, cultivated clones conserved relatively high level of polymorphism, despite narrow genetic base and continuous assor-

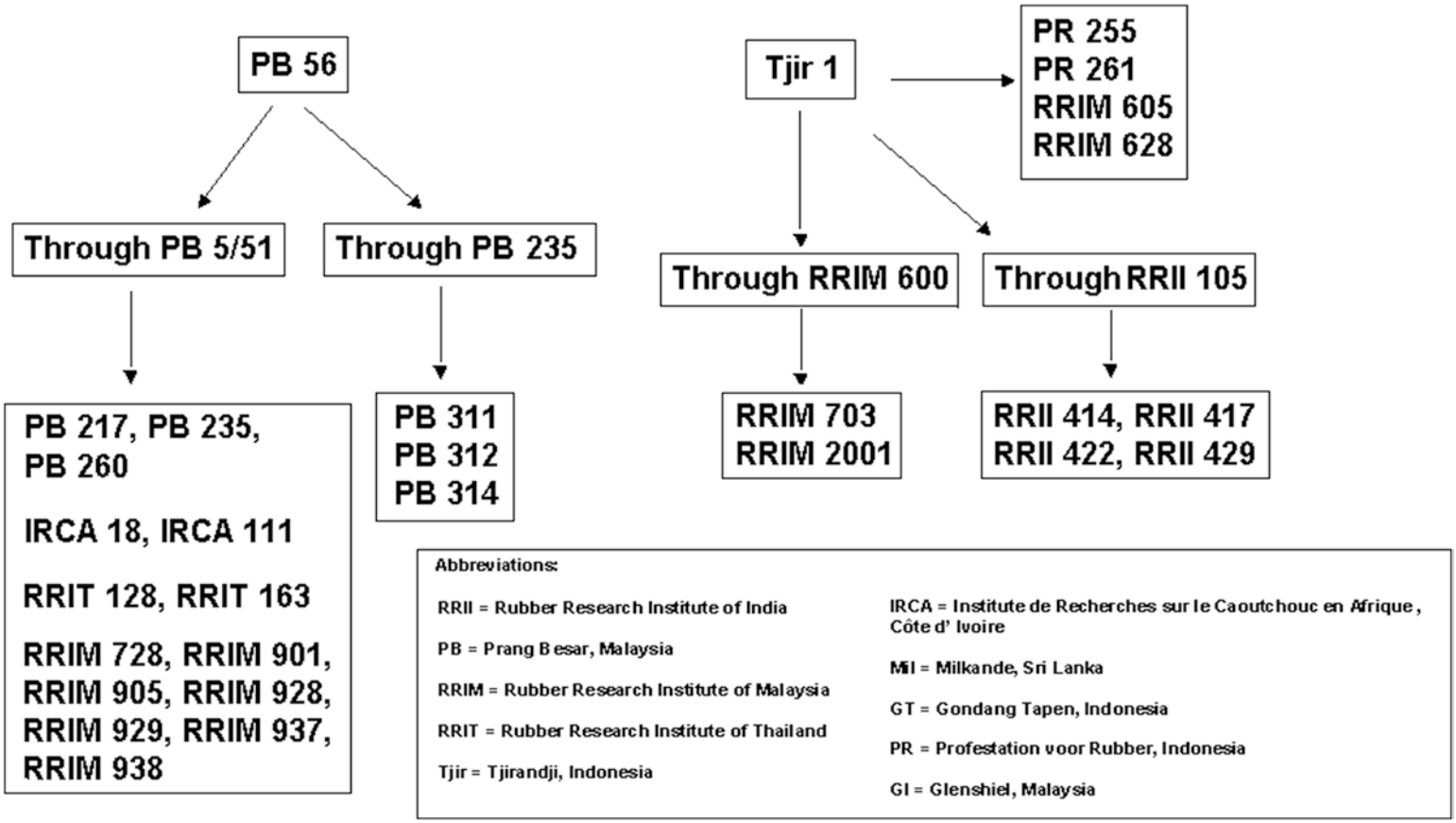

Fig. 6.12 Transmission of cytoplasm of PB 56 and Tjir 1 in the derivation of modern clones

tative mating and selection. As expected, polymorphism is very prudent among allied species of *Hevea*. A comparison of isozyme analysis (Lebrun and Chevallier 1990) with that of DNA markers showed much similarity (Besse et al. 1994). Identification of all Wickham clones could be done with 13 probes associated with restriction enzyme *Eco RI* (Besse et al. 1993a, b). The cultivated clones are genetically close to the Mato Grosso genotypes. Rondonia and Mato Grosso clones are more polymorphic as per RFLP data (Besse et al. 1994). A Rondonia clone (RO/C/8/9) showed eight specific restriction fragments and a unique malate dehydrogenase (MDH) allele, indicating this clone is of inter-specific origin. Such molecular markers are useful in rubber tree breeding since no distinct morphological traits exist. Mitochondrial DNA (mt DNA) polymorphism was analysed in 345 Amazonian accessions, 50 Wickham clones and two allied species (*H. benthamiana, H. pauciflora*) (Luo et al., 1995). While the variation in wild accessions was considerable, the cultivated clones formed only two clusters.

6.5.1 Potentiality of mtDNA

The aforesaid observations amply indicate that the selection was indirectly towards nuclear DNA polymorphism, while evolving modern clones. Luo et al. (1995) argue that the geographic specificity towards nuclear and mtDNA polymorphisms are due to great level of genetic structuring among natural populations in the Amazon forests in relation to hydrographic network. In wild accessions, seed dispersal and selection are as per the environmental conditions. If this is true, we observe that much of the variations produced in the natural habitat are being lost due to selection pressure of environmental factors. This is a matter of concern since the wild accessions have not rendered much contribution in evolving high-yielding clones so far, after introduction to other parts of the globe. On the other hand, Wickham clones exhibited high nuclear DNA polymorphism, perhaps due to breeding under different climates. It is presumable that the nuclear genome has been forced to enhance variation to suit the diverse hydrothermal situations of newly introduced areas, resulting in selection of rightly adapted clones under a given environment. The mtDNA of Wickham clones has lesser variation because their female progenitors are all primary clones, naturally bred under the similar environmental conditions of Malaysia and Indonesia. These clones were introduced later into India and Sri Lanka for further breeding programmes. Moreover, cytoplasmic donors for most of the improved clones are either PB 56 or Tjir 1 (Fig. 6.12). While the cytoplasm of PB 56 is transferred through PB 5/51, the cytoplasm of Tjir 1 was through RRII 105, RRIM 600 and RRIM 605. In conventional breeding systems followed in rubber, the best parents of one generation are used as parents for the next cycle of breeding (Simmonds 1989). Obviously, this is the reason for the mtDNA profile showing only two clusters. A possible explanation for greater polymorphism in mtDNA in wild accessions is that they must have been evolved through inter-specific hybridization. mtDNA polymorphism in wild accessions needs to be exploited fully. A molecular survey of available Amazon accessions and isolation of competent molecular variants in their progeny are the possible exercises that would give meaningful results.

Plant mitochondrial genomes encode tRNAs, rRNAs, proteins and ribosomal proteins and range in size from 200 Kb in *Brassica hirta* (Palmer and Herbon 1987) to 2.74 Mb in *Cucumis melo* (Rodríguez-Moreno et al. 2011). Mitochondrial genome expansion in land plants is primarily due to large intergenic regions, repeated segments, intron expansion and incorporation of foreign DNA such as plastid and nuclear DNA (Turmel et al. 2003; Bullerwell and Gray 2004). Accumulation of repetitive sequences in plant mitochondrial genomes cause frequent recombination events and dynamic genome rearrangements within a species (Chang et al. 2011; Allen et al. 2007). Several mutations by gene rearrangement of the mitochondrial genes were found associated with cytoplasmic male sterility (CMS) such as the T-urf13 gene in maize (Dewey et al. 1986), pcf gene (a fusion of atp9 and cox2 portions) in petunia (Young and Hanson 1987), cox1 in rice

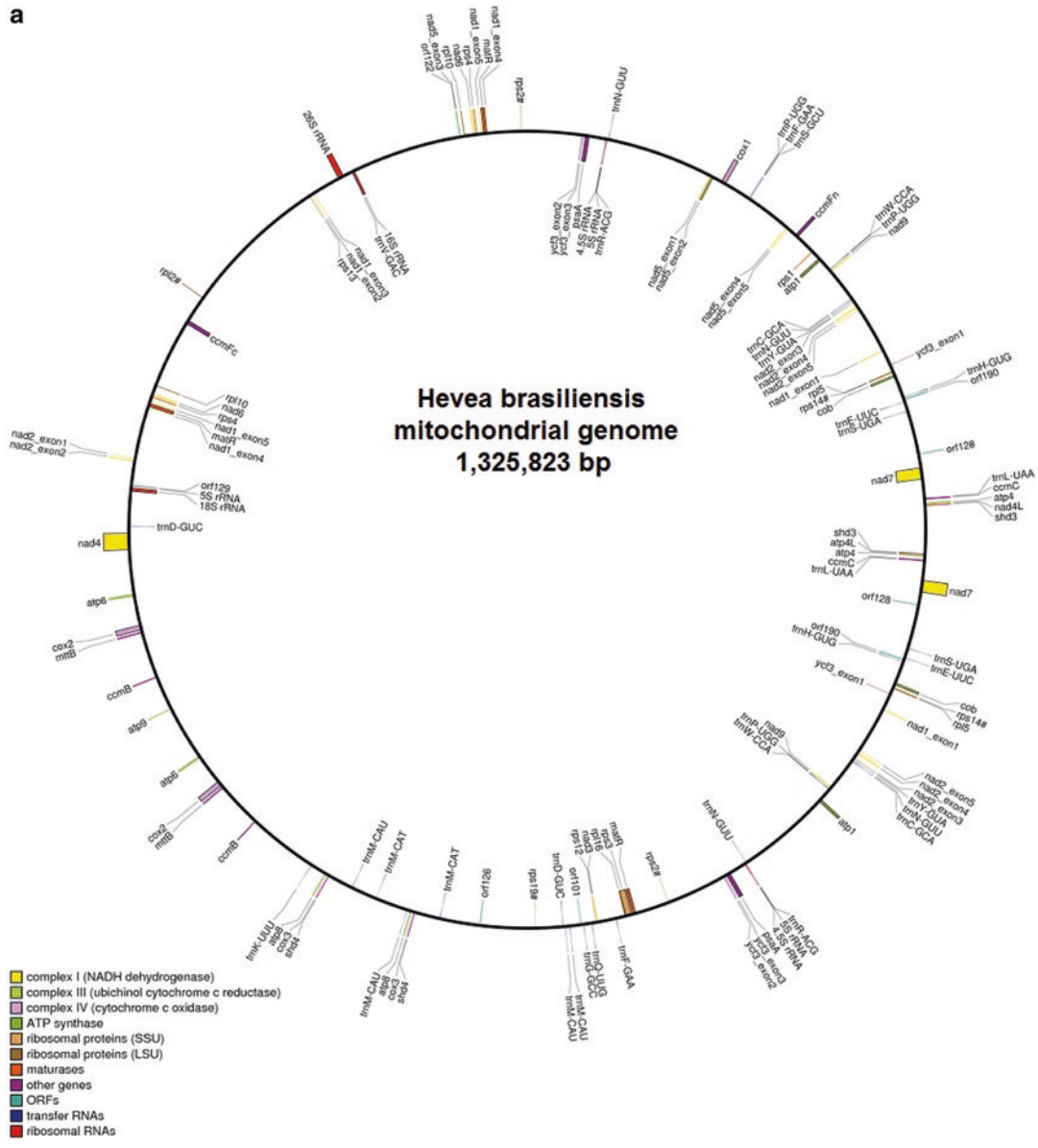

Fig. 6.13 (**a**) Annotated representation of the rubber tree mitochondrial genome. (**b**) *Hevea* mitochondrial genome: *Grey arches* indicate the mapping of each pair of the Illumina paired-end sequence data. Direct repeats are shown as *blue arches* and inverted repeats as *orange arches* (After Shearman et al. 2014)

(Wang et al. 2006) and mutations in ATPase subunits in sunflower (Laver et al. 1991) and Brassica (Landgren et al. 1996). RNA processing also plays an important role in controlling CMS as evidenced in *orf355/orf77* (*atp9*) and *T-urf13* in maize (Gallagher et al. 2002; Dill et al. 1997). With the development of next generation sequencing (NGS) technologies, new strategies have been used to obtain plant mitochondrial genomes. A combination approach of shotgun and paired-end NGS sequencing from non-enriched whole genome DNA libraries have been successfully used to obtain the mitochondrial genomes. Clone BPM 24 exhibits cytoplasmic male sterility, inherited

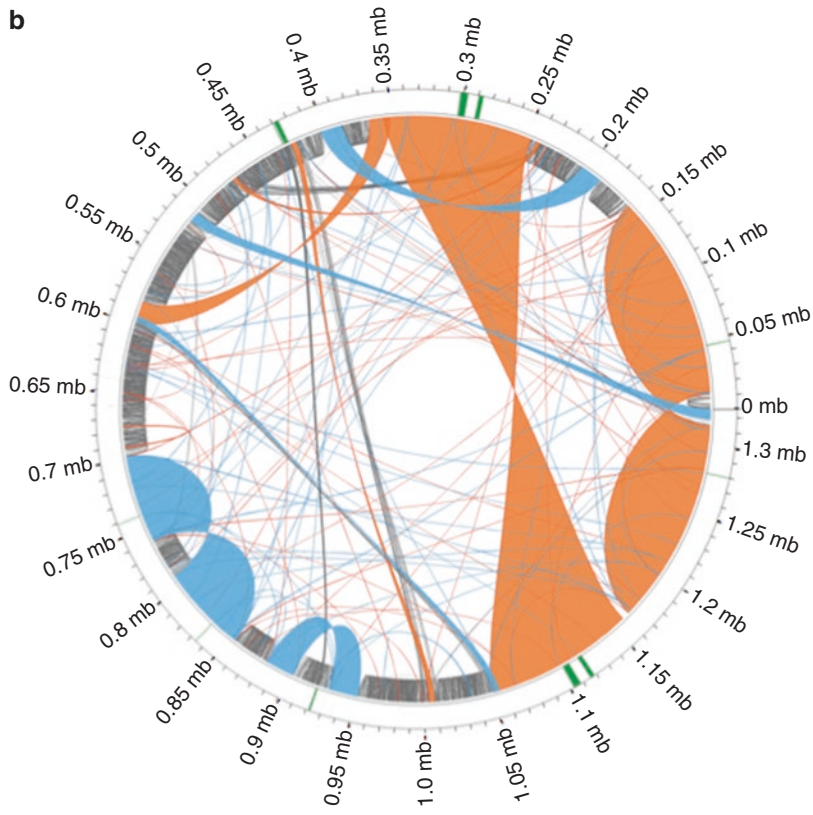

Fig. 6.13 (continued)

from the variety GT 1. Shearman et al. (2014) constructed the rubber tree mitochondrial genome of a cytoplasmic male sterile variety, BPM 24, using 454 sequencing, including 8 kb paired-end libraries, plus Illumina paired-end sequencing. They further annotated this mitochondrial genome with the aid of Illumina RNA-seq data and performed comparative analysis. Shearman et al. (2014) then compared the sequence of BPM 24 to the contigs of the published rubber tree, variety RRIM 600, and identified a rearrangement that is unique to BPM 24, resulting in a novel transcript containing a portion of atp9 (Fig. 6.13 a, b). The novel transcript is consistent with changes that cause cytoplasmic male sterility through a slight reduction to ATP production efficiency. The exhaustive nature of the search rules out alternative causes and supports previous findings of novel transcripts causing cytoplasmic male sterility.

6.5.2 Potentiality of cpDNA

Chloroplast genomes are sufficiently large and complex to include structural and point mutations that are useful for evolutionary studies from intra-specific to inter-specific levels (Neale et al. 1988; McCauley 1992; Graham and Olmstead 2000; Provan et al. 2001). Since the first complete chloroplast (cp) genome sequence of liverwort (*Marchantia polymorpha*) was reported in 1986 (Ohyama et al. 1986), more than 150 chloroplast genomes have been sequenced and characterized thus disclosing an enormous amount of evolutionary and functional information of chloroplasts. In chloroplasts, transcripts undergo a series of RNA processing steps such as intron splicing, polycistronic cleavage and RNA editing. RNA editing is a mechanism to change genetic information at the transcript level by nucleotide insertion, deletion or conversion

(Bock 2000; Knoop 2010). The chemical composition of natural rubber is cis-polyisoprene, a high-molecular weight polymer formed from sequential condensation of isopentenyl diphosphate (IDP) units catalysed by the action of rubber transferase (Cornish 2001). IDP is also an important intermediate for biosynthesis of essential oils, abscisic acid, cytokinin, phytoalexin, sterols, chlorophyll, carotenoids and gibberellins (Chappell 1995a; McGarvey and Croteau 1995; Lichtenthaler et al. 1997; Cornish 2001). There are two IDP biosynthesis pathways: the mevalonate (MVA) pathway which occurs in cytosol (Chappell 1995b); and the 1-deoxy-D-xylulose 5-phosphate/2-C-methyl-Derythritol 4-phosphate (MEP) pathway which occurs in plastids (Lichtenthaler 1999; Ko et al. 2003). One approach to improving rubber production in *H. brasiliensis* would be to engineer chloroplasts and modify metabolic flux to produce more biosynthetic intermediates. The availability of the complete chloroplast genome sequence should also facilitate the chloroplast transformation technique. The improved transformation efficiency and foreign gene expression can be achieved through utilization of endogenous flanking sequences and regulatory elements (Birch-Machin et al. 2004; Maliga 2004; Tangphatsornruang et al. 2010). Transformation of chloroplast genome offers a number of advantages over nuclear transformation including a high level of transgene expression, polycistronic transcription, lack of gene silencing or positional effect and transgene containment (Daniell et al. 2002; Maliga 2002, 2004; Bock 2007). Tangphatsornruang et al. (2011a, b) reported the complete chloroplast genome sequence of rubber tree as being 161,191 bp in length including a pair of inverted repeats of 26,810 bp separated by a small single-copy region of 18,362 bp and a large single-copy region of 89,209 bp (Fig. 6.14). The chloroplast genome contains 112 unique genes, 16 of which are duplicated in the inverted repeat. Of the 112 unique genes, 78 are predicted protein-coding genes, 4 are ribosomal RNA genes and 30 are tRNA genes. Relative to other plant chloroplast genomes, Tangphatsornruang et al. (2011a, b) observed a unique rearrangement in the rubber tree chloroplast genome: a 30-kb inversion between the *trnE(UUC)-trnS(GCU)* and the *trnT(GGU)-trnR(UCU)*. A comparison between the rubber tree chloroplast genes and cDNA sequences revealed 51 RNA editing sites in which most (48 sites) were located in 26 protein-coding genes and the other 3 sites were in introns. Phylogenetic analysis based on chloroplast genes demonstrated a close relationship between *Hevea* and *Manihot* in Euphorbiaceae.

Shotgun genome sequencing of *H. brasiliensis* using pyrosequencing technology revealed the complete chloroplast genome sequence (Tangphatsornruang et al. 2011a, b). Gene content and structural organization of the rubber tree chloroplast genome is similar to that of *M. esculenta*, with an exception of the 30-kb-fragment rearrangement. By comparing the rubber tree chloroplast genes and the cDNA sequences, the distribution and the location of RNA editing sites in the chloroplast genome could be determined (Tangphatsornruang et al. 2011a, b). The phylogenetic relationships among angiosperms, based on chloroplast DNA sequences including those of the rubber tree chloroplast DNA provided a strong support for a monophyletic group of the eurosid I and demonstrated a close relationship between *Hevea, Manihot, Jatropha* and *Populus* in *Malpighiales*.

As a synthesis of these diversity studies on DNAs (nuclear, mt and cp), good relationships were found between the results issued from the different genetic markers. Even if the contribution of isozymes is important by itself, molecular markers provided important clarifications for the distinction of different groups. There would be no barrier to migration of *Hevea* genes within the Amazonian basin. However, the wideness of the area and the limited dispersion of *Hevea* seeds allowed the preservation of the current structure, which is assumed to have initially resulted from the fragmentation of the Amazonian forest during the pleistocene period, according to the refuge theory presented by Haffer (1982). Moreover, the *Hevea* germplasm genetic structure clearly appears as geographically structured in relationship with the hydrographic network of the Amazonian forest, which confirms the role of riv-

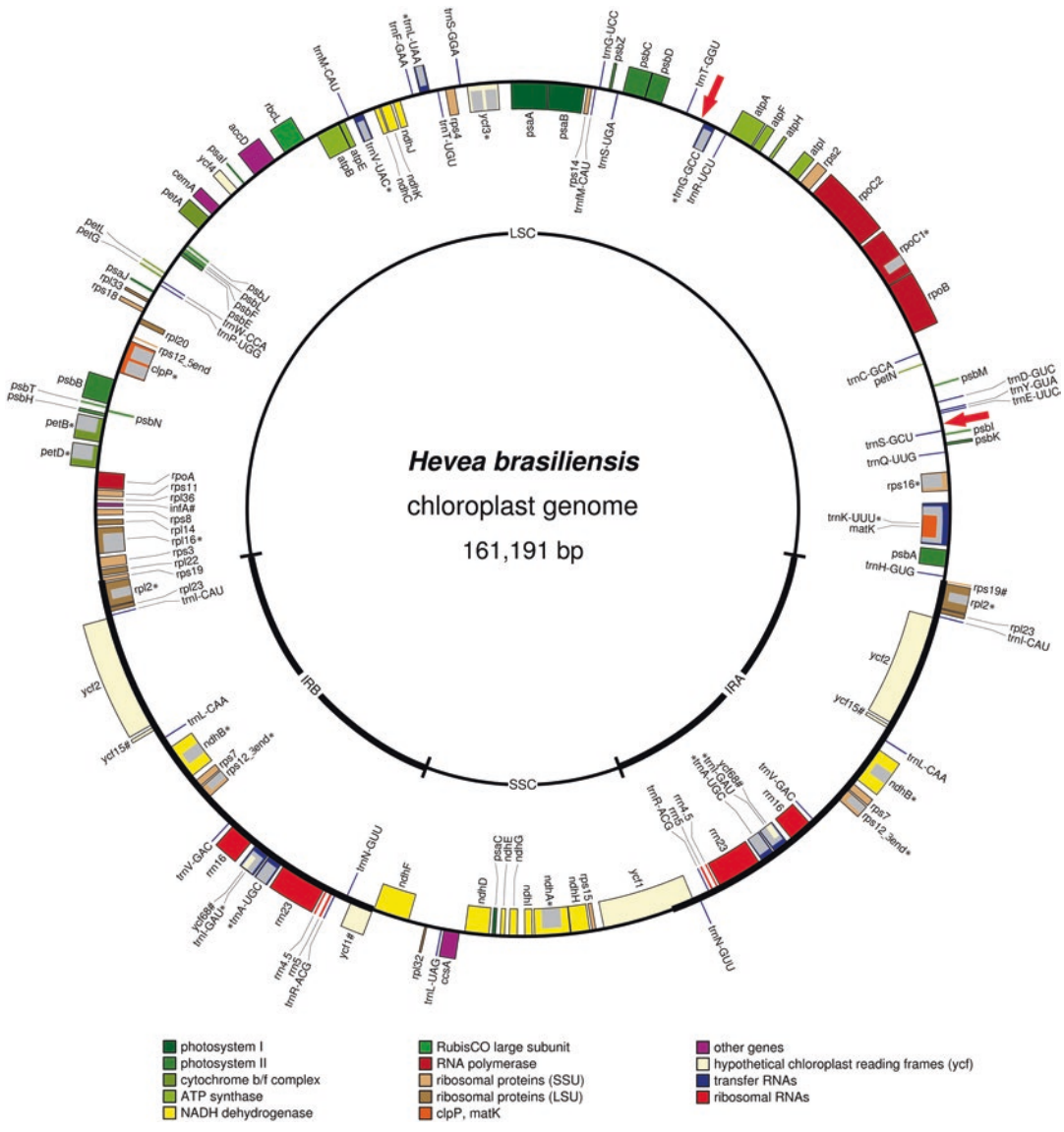

Fig. 6.14 Map of *H. brasiliensis* chloroplast genome. The *thick lines* indicate the extent of the inverted repeats (IRa and IRb) which separate the genome into small and large single-copy regions. Genes on the outside of the map are transcribed clockwise and those on the inside of the map are transcribed counter clockwise. Genes containing introns and pseudogenes are marked with * and #, respectively. *Arrows* indicate the positions of a 30-kb-unique rearrangement in relative to the cassava chloroplast genome (After Tangphatsornruang et al. 2011a, b) (Photo courtesy: Sithichoke Tangphatsornruang, National Center for Genetic Engineering and Biotechnology, Bangkok)

ers and inundated zones in the transport of seeds and dissemination of the species (Besse et al. 1993a; Luo et al. 1995; Seguin et al. 1996). Also, differentiation among populations could be jointly explained by both geographical location within the hydrographical Amazon network and by isolation by distance among populations belonging to distinct catchments (Le Guen et al. 2009). Mato Grosso (Brazil) populations were seen to be genetically more distant from all other populations. The mt DNA of Wickham population has lesser variation since their female progenitors

are restricted to a very small set of primary clones. Cytoplasm donors for most of the improved clones are either PB56 or Tjir1. Obviously, this is the reason for the mt DNA profile showing only two clusters (Priyadarshan and Gonçalves 2003). A possible explanation for greater polymorphism in mtDNA of wild accessions is that many might have been evolved through inter-specific hybridization.

6.6 Impact of Genetic Erosion

As said earlier, genetic erosion can result from a narrow genetic base in the original collections or by practices that reduce genetic diversity. That the original 22 seedlings of Wickham collection, as it is believed till date, is the base population from which the day-to-day *Hevea* clones were evolved had been genetically narrow to enrich the *Hevea* gene pool. In addition, these populations were subjected to several rounds of controlled crossing that further narrowed the diversity. Moreover, the strategy followed by the breeders to select only the desirable genotypes and to reject the unwanted ones (without assessing the utility other than yield) is the main reason that reduces diversity. Concerted efforts to infuse the Amazonian germplasm through controlled crossings never met with enriching the diversity as desired as expected. This is because selection was, and has always been in favour of higher yield only. Preserving other genotypes/entries cannot be accomplished due to space constraints unlike annual species. This drawback needs to be addressed resolutely if the diversity of *Hevea* rubber is to be increased. Genetic diversity not produced or preserved is equivalent to genetic diversity lost. The total number of clones is not more than hundred that are being cultivated worldwide for natural rubber production.

Molecular characterization of *Hevea* has not been done systemically. Only molecular diversity of Amazonian accessions and a few clones had been studied to an extent. A very systematic study of all *Hevea* clones at molecular level is appreciable, since the wisdom of understanding differences in morphological and molecular diversity has accumulated of late. QTL mapping is yet another area that needs to be undertaken with international co-ordination. As mentioned in this article, much work at the molecular level had been carried out like for Tapping Panel Dryness, latex production, defence genes and alike. Only growth related traits have been attempted for QTL mapping (Souza et al. 2013). But a sincere and systematic effort to tie up this variation with QTLs for yield had not been done so far. This exercise is difficult but not impossible. This systematic exercise can only elucidate the intricacies underlying diversity of *Hevea* rubber. Such an exercise can lead to setting up of a molecular library for *Hevea* and scientists working worldwide can contribute to this molecular library. The deposition of microsatellites, SSRs and ESTs is not enough, but a library that includes genes for QTLs is most warranted. The contribution of Rahman et al. (2013) on gene sequencing of *Hevea* is a sincere and systematic step towards this. Nevertheless, the attempts of Salgado et al. (2014) on de novo transcriptome analysis in *Hevea* stems promise. This tempo needs to be accelerated further, should there be a comprehensive gene library for *Hevea* rubber.

One of the early contributors to the science of plant genetic resources, Harlan (1970) remarked: 'The varietal wealth of the plants that feed and clothe the world is slipping away before our eyes, and the human race simply cannot afford to lose it', and he also predicted a 'genetic wipe out of centres of diversity' (Harlan 1975). Genetic wipe out has not really happened but the modern varieties have replaced traditional varieties or land races. One of the primary duties of a Plant Breeder is to evolve, document and manage genetic diversity. As such, there are no land races in *Hevea* rubber, but only modern clones. In this context, how much genetic diversity is getting conserved, catalogued and utilized and how much genetic erosion happens are the options left to one's own wisdom.

Heterozygosis and Breeding

7

The penultimate aim of any breeding programme is to derive a clone with enhanced dry rubber yield (g tree^{-1} tap^{-1}) and, if possible, with improved secondary attributes like resistance to diseases and high/low temperature stresses. The efficient use of the available genetic variability in the form of clones is further augmented through bud grafting. However, the long time needed for genotype evaluation and low seed set added constraints for the production of recombinants. The main source of modern clones is the Wickham population with much lower genetic variability. Of late, concerted efforts to integrate new wild Amazonian population offer new vistas for the production of new clones.

7.1 Early History of Rubber Breeding

Dijkman (1951), in a global presentation of rubber research in the first half of the twentieth century, related how rubber breeding was pioneered in the East Indies (Indonesia). Seeds from the first Wickham trees planted in Asia after 1876 were used for the establishment of new plantations ('random unselected seedling populations'). Cramer analysed the variability of these seedlings for latex yield (Cramer 1914, 1934). Almost at the same period in Malaysia, it was reported that 9.8% of a population of seedlings produced 28% of the total crop (Whitby 1919). Such variation analyses led to the identification of better-yielding trees and to the preferential use of their seeds for new plantings ('mother-tree seedlings') in an extensive process of mass selection. While vegetative multiplication by bud grafting was being developed by van Helten (1918) for multiplying the best seedlings as clones (Dijkman 1951; Cramer 1956), the method for producing recombinants (full-sib (FS) families) by hand pollination was also standardized. During this period, this method was seen as a way to produce elite seeds directly for commercial planting. Cramer and Dijkman were distinguishing the two methods by calling them: (i) vegetative selection and (ii) generative selection or 'breeding'. However, generative selection could produce only limited quantities of seeds, while the quality of 'mother-tree seedlings' gradually improved as a result of the intensive directional selection of the mother trees. In 1916, the bud-grafting method was good enough to be used commercially, but no clone had been selected, and the bud-grafting technique had to overcome the many doubts of the planters. This sceptical attitude was motivated by varied practical and economic reasons. For example, the raffia strips which were used in bud grafting for binding the buds to the rootstock were not rainproof and were poorly effective as compared with the plastic strips that became available more than 30 years later. As a result, bud-grafting success at that time was relatively low. The competition between mother-tree seedlings and bud-grafted clones led to two tree types available to planters until the

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end of World War II when bud-grafted clones began to emerge as the most productive. These two ways were not only competitive but also complementary for state-owned research institutions and large companies (see Dijkman 1951, p. 97: Flow chart of vegetative selection and breeding in *Hevea*). The selection of mother trees and the observations on their seedlings were important sources of information for the selection of new clones, and 'breeding', the generative way, became the basis for clone selection, the vegetative way. Also, the best clones were used for establishing polyclonal seed gardens. All lines of breeding can be streamlined under three main headings: (i) evaluation of established clones for quick recommendations, (ii) recombination breeding and derivation of hybrids and (iii) evaluation of polycross progenies like ortets (mother trees) and polyclonal seedlings (Priyadarshan and Clément-Demange 2004). All these, either individually or in combination, will lead to new clones. Details of these programmes will be considered here. However, while presenting these, a strict separation is not possible since these lines of work are mutually complementary.

7.2 Evaluation of Clones

When considering planting a new area that may be suboptimal for rubber growth, the best plants to use are polyclonal seedlings to ensure maximum stand development, sacrificing yield since these areas are affected by stress conditions. However, evaluation of established clones gives quick information on what clones are suitable for a new area. Many planting recommendations made in several countries are based on such clone evaluations. Detailed accounts of the performance of clones in non-traditional rubber-growing areas of India, Brazil, Vietnam and China are available in Chap. 10. A worldwide account of prominent clones is given in Table 7.1. A comparison of yield and other attributes of polyclonal seedlings and clones is given in Table 7.2. Varied performances were noticed among these clones in all those areas. While the rubber-producing countries have selected vivid clones suitable to their traditional environment, RRIM 600 seems to be the universally accepted clone for all non-traditional areas of the world (see Chaps. 9 and 10 for further details).

7.3 Recombination Breeding

Natural pollination was the basis of 'random seedling populations' and of 'mother-tree seedlings'. Natural pollination was exploited by creating polyclonal seed gardens with the best mixed clones, planted in isolated sites in order to protect them from outside pollen. They produce 'polyclonal seeds', such as the outstanding PBIG/GG seeds (Prang Besar Isolation Gardens/Gough Gardens) which were used for commercial plantings and proved to be tough competitors of the best clones, even after World War II (Simmonds 1996a, b). Natural pollination in seed gardens can lead to a certain amount of selfed seedlings with potential inbreeding. This is why it was advised not to use seeds from monoclone plots ('monoclone seedlings'), where selfing could reach rather high rates. This inbreeding effect, noted by Sharp (1940, 1951) and discussed by Ross and Brookson (1966) and other authors (Tan 1981a, b), is suspected to be mainly due to the highly heterozygous nature of rubber, but its real importance has not actually been estimated. This 'generative' way of breeding still has some supporters. Arguments in its favour would be that: (i) planting seeds is easier for smallholders, (ii) seed gardens can provide seeds for growing improved rootstocks and (iii) they are sources for creating 'rubber forest plantations' for timber (in both cases by the use of vigorous parental clones). However, the limitations are: (i) the economic competitiveness of such synthetic cultivars cannot be guaranteed (unless intensive research is committed to this), (ii) a long time is required between the setting of seed gardens and the availability of seeds and (iii) the low seed productivity of seed gardens. On the other hand, the 'clone revolution' achieved through bud grafting of elite genotypes has been the main factor in the increase in rubber production, first in Malaysia, then in Thailand (with smallholders) and in other countries.

Table 7.1 Profile of prominent clones

Clone	Parentage	Yield (kg/ha)	Girth increment during tapping	*Resistance to*						
				Wind damage	Panel dryness	Pink disease	*Oidium*	*Colletotrichum*	*Corynespora*	*Phytophthora*
RRII 105[I]	Tjir 1 × Gl 1	2210	3	3	5	5	3	5	5	1
RRII 203[I]	PB 86 × Mil 3/2	1618	4	3	2	3	3	NA	3	3
RRII 208[I]	Mil 3/2 × AVROS 255	1587	3	3	3	NA	3	NA	NA	NA
RRIC 100[M]	RRIC 52 × PB 83	1774	3	5	3	3	4	3	5	NA
RRIM 600[M]	Tjir 1 × PB 86	2199	4	4	4	1	3	3	1	1
RRIM 712[M]	RRIM 605 × RRIM 71	2264	2	5	4	3	3	1	3	3
RRIM 936[M]	GT 1 × PR 107	2146	3	4	3	4	3	4	4	2
RRIM 937[M]	PB 5/51 × RRIM 703	2483	2	5	3	4	3	3	5	3
RRIM 2015[M]	PB 5/51 × IAN 873	2760	4	NA	NA	NA	4	4	4	3
PB 217[M]	PB 5/51 × PB 6/9	1778	4	4	4	2	2	3	4	1
PB 235[M]	PB 5/51 × PB S/78	2485	3	2	2	3	2	2	4	3
PB 255[M]	PB 5/51 × PB 32/36	2283	3	4	2	2	2	2	4	2
PB 28/59[M]	Primary clone	2023	1	3	3	2	2	2	4	2
PR 255[M]	Tjir 1 × PR 107	2018	3	4	3–4	3	1	3	4	3
PR 261[M]	Tjir 1 × PR 107	1838	3	4	3–4	3	1–2	4	3	3
IRCA 111[CD]	PB 5/51 × RRIM 600	1446	5	3	3	NA	NA	NA	NA	NA
IRCA 230[CD]	PB 5/51 × GT 1	1807	5	3	3	NA	NA	NA	NA	NA
RRIT 163[I]	PB 5/51 × RRIM 501	2086	2	NA	NA	NA	3	NA	3	NA
HAIKEN 1[C]	Primary clone	1500	3	4	3	2	NA	NA	NA	NA
REYAN 8-333[C]	SCATC 88/13 × SCATC 217	2187	3	3	3	NA	3	NA	NA	NA
IAN 873[B]	PB 86 × FA 1717	1920	4–5	3	4	NA	4	4	NA	NA
IAC 301[B]	RRIM 501 × AVROS 1511	2320	4	4	4	NA	4	4	NA	NA
IAC 40	RRIM 608 × AVROS 1279	2420	4	3	3	NA	2	3	NA	2
IAC 300	RRIM 605 × AVROS 353	1887	3	2	2	NA	3	2	NA	2
Fx 3864	PB 86 × PB 38	1755	4	3	3	NA	2	2	NA	3
IAN 4493[B]	FX 441 × Tjir 1	1711	3	3	2	NA	2	2	NA	2
RRIV 4[VN]	RRIC 110 × PB 235	2103[10Y]	2	2	4	3–4	2–3	2	NA	4

1 poor, *2* below average, *3* average, *4* good, *5* very good. *NA* not available, since the disease is not prominent
Under conditions of *M* Malaysia, *I* India, *C* China, *CD* Côte d'Ivoire, *B* Brazil
Tapping system = s/2 d/2 6d/7 86%; no. of tapping days per year = 158 ± 11 (with wide regional variation depending on weather); trees per hectare = 327 ± 34
IAN 873 exhibits good tolerance to SALB; tapping under Vietnamese (south east) conditions = s/2 d/3 6d/7; 6Y = average over 6 years; 10Y = average over 10 years; REYAN is new name for SCATC

Table 7.2 Comparison of polyclonal seedlings and multi-clonal populations in Tripura (10 years after field planting)

Attribute	Polyclonal seedlings	Multi-clonal population
Wind damage (%)	19.1	25.6
Uprooting (%)	Nil	1.3
Panel dryness (%)	2.5	3.8
Powdery mildew (%)	65	90
Girth (cm)	68.7 (30.5–100.6)	63.1 (38.5–89.5)
Mean yield (g tree^{-1} tap^{-1})	21.1 (2.6–70.3)	21.4 (9.3–35.5)
Mean yield (g tree^{-1} tap^{-1})		
Regime I (May–Sept.)	16.1	11.2
Regime II (Oct.–Jan.)	26.1	31.5

After Sasikumar et al. (2001)

This revolution is still to be achieved in Indonesia, where many smallholders still use seedlings.

Selection from seedling trees of commercial plantations gave 'primary clones' of unknown parental origin. A good example was provided by Gough (Prang Besar, Malaysia), who surveyed some million trees in the Kajang area of the Prang Besar Rubber Estate, from which he could select a dozen primary clones (Tan et al. 1996). Selected seedlings issued from polyclonal seed gardens were also used for selecting new clones. This possibility led to the establishment of other seed gardens not designed to produce commercial seed lots but specifically aimed at being a source of seedlings for selection. This process, based on natural pollination, has been called 'ortet selection' (the word 'ortet' is equivalent to 'mother tree'). A tree raised through bud grafting is a 'ramet' and not a 'clone'. However, since the term 'clone' is widely used in the rubber literature, this term will be used here. The concept of polyclonal seed gardens for the selection of clones is still considered interesting (Simmonds 1986), notably for the improvement of the wild Amazonian populations and more generally for population improvement in rubber (including Wickham populations). Breeding orchards without any plan are also used.

By 1930, the emphasis shifted to recombinant full-sib progenies from controlled hand pollination. Under this system, there was no selfing, and the crossing of related parents was avoided. The advantage of hand pollination was the possibility to cross two known parental clones carefully chosen for their high level of performance or their complementarities and to trace back the clones to their ancestors. With the development of biometry and quantitative genetics, this opened up new possibilities for evaluating the genetic worth of parents and progenies (for extensive reviews on breeding, see Tan 1987; Priyadarshan and Clément-Demange 2004; Priyadarshan et al. 2008).

A rubber recombination breeding programme is initiated by controlled hand pollination for the production of full-sib families, followed by three selection stages, viz. seedling evaluation trial (SET), small-scale clonal trial (SSCT) and large-scale clonal trial (LSCT). SSCT and LSCT sometimes are designated as preliminary proof clone trial and further proof clone trial, respectively. The process is cyclical, with the best clones becoming candidates for recombination in the next cycle.

From around 500 kg ha^{-1} in primary clones to more than 2500 kg ha^{-1} in the best current clones, rubber breeding has come a long way primarily due to recombination breeding and selection of clones under RRIM and PB series. RRIC 100 series released in Sri Lanka during the 1970s is yet another example. Much of the hybridization work in Malaysia (RRIM, Prang Besar), Indonesia, India, Côte d'Ivoire, Brazil, Thailand and Vietnam further strengthened the array of hybrid clones (see Priyadarshan and Clément-Demange 2004 for more details). These clones are known for their adaptability to specific hydrothermal/agroclimatic situations. At least 16 primary clones played a major role and can be considered as prime progenitors of many modern clones (see Priyadarshan and Clément-Demange 2004) (Fig. 7.1). Many valuable recombinants must have been lost during the course of assortative mating of primary clones and of hybrid clones followed by subsequent directional selection for yield under varied geo-climates (Priyadarshan 2003a). The breeding policy has been mainly to cross 'the best with the best'

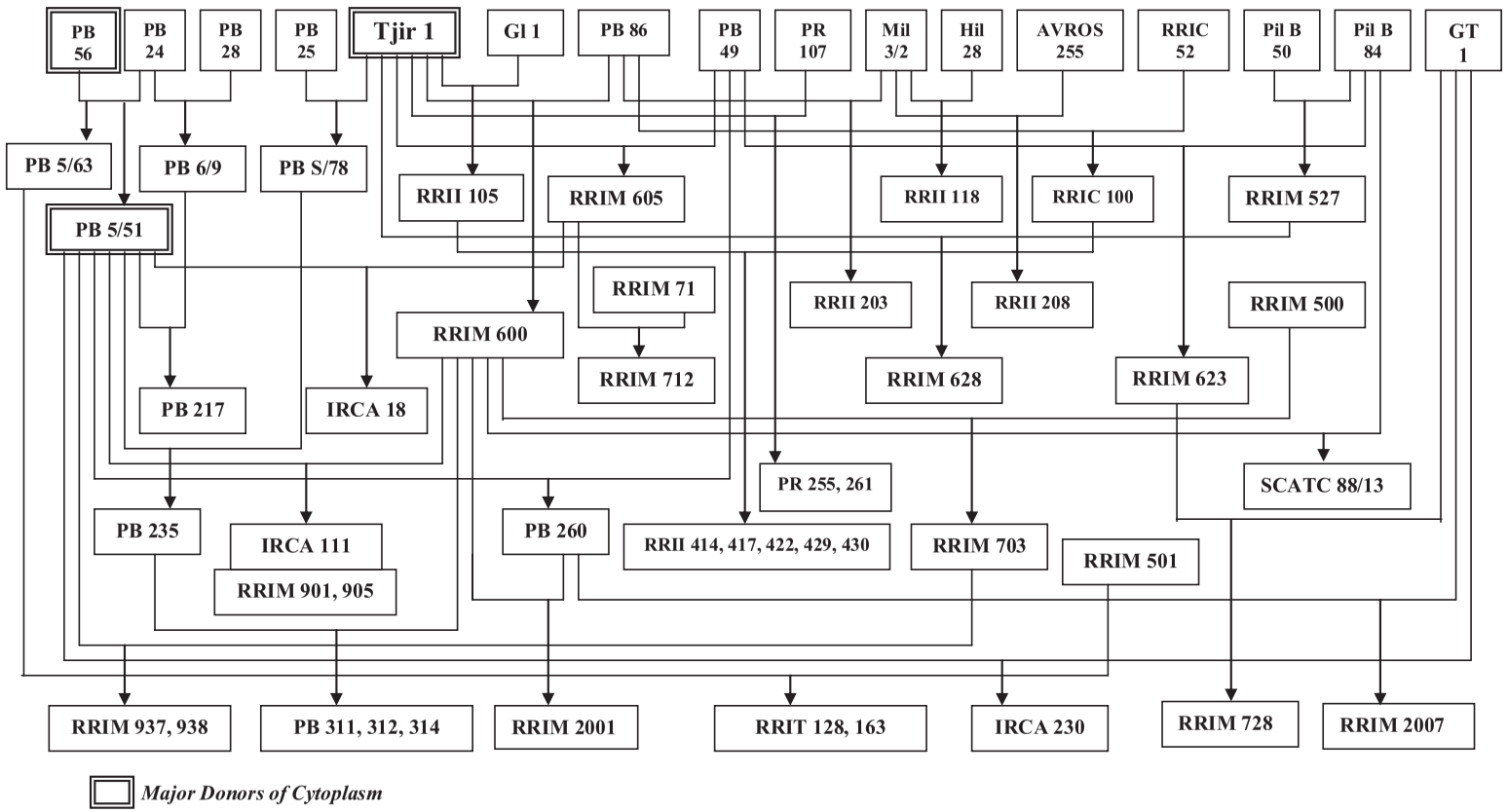

Fig. 7.1 Parentage of outstanding clones: *Gl* Glenshiel, Malaysia, *GT* Gondang Tapen, Indonesia, *IRCA* Institute de Recherches sur le Caoutchouc en Afrique, (Côte d'Ivoire), *Mil* Milakande, Sri Lanka, *RRIC* Rubber Research Institute of Ceylon (currently Sri Lanka), *RRII* Rubber Research Institute of India, *RRIM* Rubber Research Institute of Malaysia, *RRIT* Rubber Research Institute of Thailand, *Tjir* Tjirandji, Indonesia, *PB* Prang Besar, Malaysia

(GAM, generation-wise assortative mating), with strong emphasis on precocious yield in selection within Wickham material (Wycherley 1976). But it could be considered to take more advantage of genetic analysis and of quantitative estimation procedures, especially for the assessment of clonal general combining ability (GCA) for growth and latex yield improvement. Breeding for disease resistance has to take account of specific aspects related with host-pathogen interactions.

The aforesaid breeding scheme is of the past, and lately, *Hevea* breeding has been compelled to undertake drastic reduction in the years consumed for completing a breeding cycle and for derivation of clones. SSCTs and LSCTs are well avoidable. The scheme proposed here is to start with SETs comprising hybrid seedlings. The course normally followed is to evaluate through test tapping and select the best yielding seedlings. Presuming that a seedling can never reflect the yielding attribute during adult stage (see Sect. 7.5.1), this step needs to be suitably modified. The families of hybrid seedlings are to be raised in closer spacing (2 or 3 m) and allowed to attain tappable girth. These seedling trees are to be evaluated with a reference clone (selections from such evaluations are to be further laid under clonal nursery, only to reconfirm the yielding potential). The high-yielding seedling trees are to be made budwood points, only to have enough bud-grafted plants for block-level commercial evaluations. Once the yielding potential of the clonal derivative is confirmed under block trials, the clone is set for recommendation. When a normal breeding cycle takes 35–40 years, through skipping SSCTs and LSCTs, the scheme proposed can complete a breeding cycle in 17 years (Fig. 7.2). It takes 6 years for an LSCT to confirm yielding potential of clones (Chandrasekhar et al. 2007). However, the aforesaid scheme can confirm the yield, if the block trials are laid under multilocations.

7.4 Breeding Pattern

Latex yield and growth are polygenically or quantitatively controlled (Simmonds 1989). It is very clear that growth rapidity is made of many different physiological processes occurring at successive stages of the development of trees. As a result, most genetic populations are normally distributed for growth measurements. In contrast, the usual distribution of genetic populations for latex yield, notably of full-sib families, although continuous, is strongly dissymmetric with most genotypes having low yields and few of them having high yields. This was initially mentioned by Maas (1934) and could be an indication of a more important role of some genes with rare favourable alleles related to some specific physiological processes such as (i) the partition of assimilates, (ii) sucrose uptake by the laticifer cell or (iii) regulation of ethylene metabolism.

Initially, the highest yielding clones were empirically intercrossed, on the assumption that additive genetic variance was predominant and that the best clones would be the best parents. This was confirmed, to some extent, by a first genetic analysis showing that vigour and yield in seedlings were strongly additive (unpublished results cited by Wycherly 1969; Gilbert et al. 1973; Nga and Subramaniam 1974; Tan and Subramaniam 1975; Tan et al. 1975; Tan 1977, 1978, 1979, 1981a, b; Simmonds 1979). There was no significant dominance effect, and each parent would have to be assessed for its general combining ability (GCA), estimated from the evaluation of its progenies. For parents which had not undergone GCA assessment, their performance could be assumed as good, since their yield performance has been proved, with some possible exceptions (Simmonds 1989). However, it seems that the assumption of a good relationship between GCAs and their performance as potential clones has not actually been checked. Moreover, a possible decline in additive genetic variance over successive phases of breeding must be considered (Tan 1981a, b). Clearly, genetic parameters in the Wickham population would have to be re-estimated with more recent sets of parents and associated progenies.

It must be acknowledged that accurate GCA estimations of the clones, and more generally genetic studies, are not easy to achieve routinely, due to the limitations imposed by hand pollination and by the low fruit set in most genotypes.

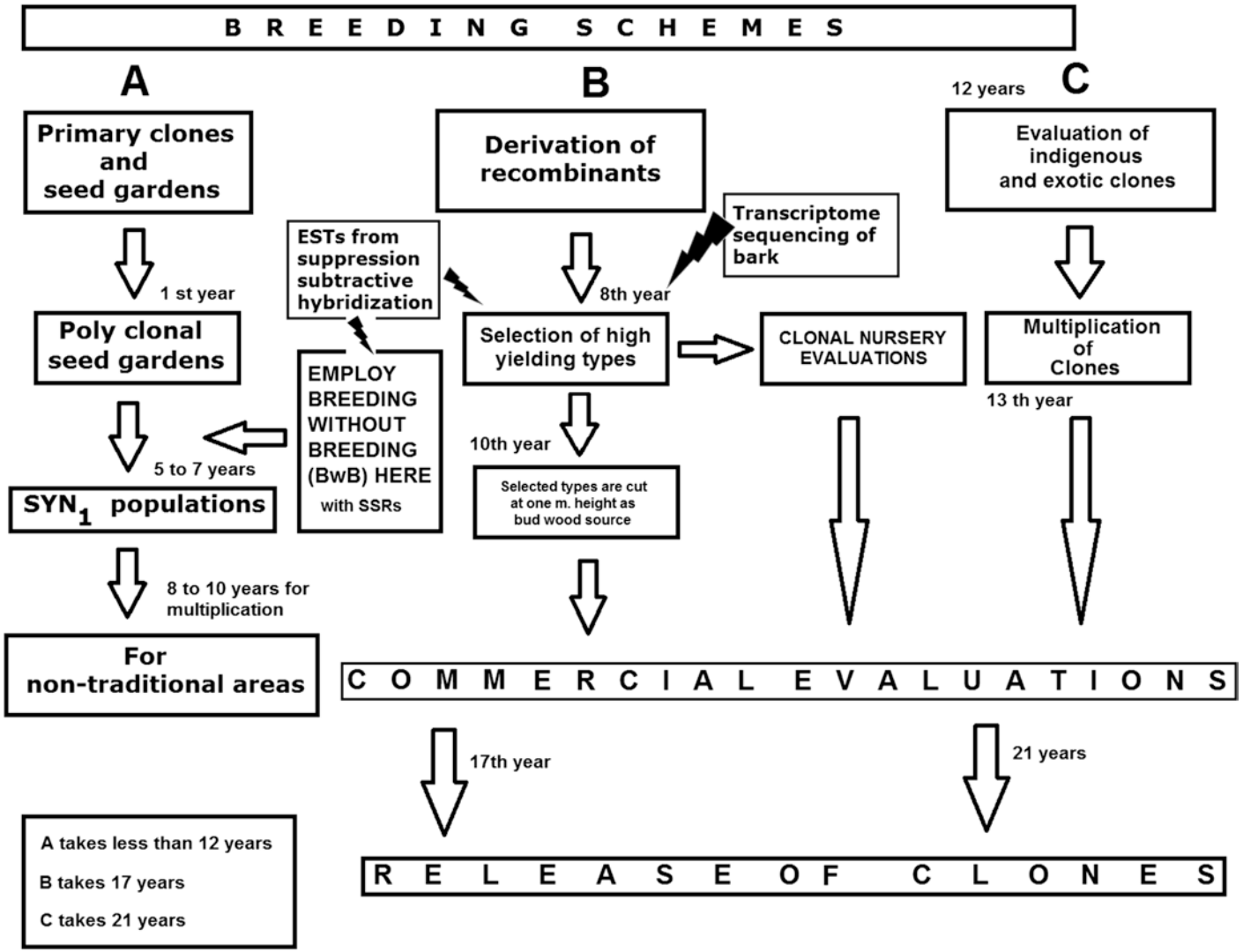

Fig. 7.2 Proposed breeding schemes for *Hevea*

Hand-pollination exercises usually end up with incomplete or partial diallel that will give only unbalanced data from the progenies. The yield data need to be analysed with special statistical tools like ASREML (a statistical software package) or with a programme specially written in statistical analysis system (SAS) for the purpose. This calls for a special statistical model meant for an incomplete diallel. Hence, the implementation of factorial mating designs in rubber necessitates more than one hand-pollination campaign for accumulating the required full-sib families which must then be planted together into the same trial. In contrast, the simple comparison of full-sib families can routinely provide rough estimations of each parent's worth, by accurate estimations of paired crosses, thus allowing family selection.

Observation of heterosis was never demonstrated in rubber, although this word was sometimes inadequately applied in two ways: (i) in the case of clones yielding more than their two parents, which is very usual at the upper tail of progeny distribution; and (ii) when the mean of the progenies of one cross is higher than the mean of the two parents. When the two parents are similar, the progenies may even exhibit a higher level than the best parent. If the number of progenies is large enough, the mean of the progenies actually exhibits the real level of the sum of gene actions contained by the two parents, whereas the specific combinations of the two parents exhibit only a partial view. As heterozygotic genotypes, most of rubber clones probably express a good amount of the possible heterosis in the Wickham population. The plant material which would be necessary for comparing doubled haploid lines and their hybrids has never been available for assessing heterosis. In the Wickham population, there are no known complementarities between two types of parents resulting systematically in superior progenies. Considering the existence of metabolic typology of Wickham clones, hypothetic complementarity between the two opposed metabolic types could be tested. Most of the Wickham × Amazonian crosses express low rubber yields, still far from the Wickham level; but their mean vigour is equivalent to that of Wickham × Wickham crosses. They generate many clones with faster growth. Rather than heterosis, this is because such crosses exhibit a higher variability than those of Wickham families. The breeding policy which was based on crossing 'the best with the best' generation-wise assortative mating (GAM) led to an intensive selection of clonal parents. The low female fertility of most of the clones often led to the preferential choice of fertile clones as seed parents, which contributed to the suspected reduction of the genetic base of the Wickham population. Tracing back to the ancestors of many cultivated clones shows that these ancestors are few in number. The list of the parents of clones bred in Malaysia since 1927 (Tan 1987) is made of 33 clones (PBIG, SR 1, PB 23, PB 186, PB 24, PB 25, PB 28/59, PB 28, PB 49, PB 56, PB 86, LCB 1320, PR 107, TK 14, AV 33, AV 49, AV 157, Tjir 1, WAR 4, GT 1, Lun N, Pil A44, Pil B16, Pil B50, Pil B58, Pil B84, Pil D61, Pil D65, RRIM 71, Ford 351, GL 1, BD 5 and BR 2), and most of the modern clones were bred from few clones. Some other clones like FX 25 and IAN 873 were recently added. Though this could seem well diversified enough, only four of these ancestors (PB 49, PB 86, Tjir 1 and Pil B84) played a major role; moreover, at an intermediate level, the clones PB 5/51, RRIM 501, RRIM 600, RRIM 605 and RRIM 623 were extensively used. As a consequence, many recent clones are more or less related, and this discouraged intercrossing. Although the Wickham population was shown to be homogeneous (Seguin et al. 2003), one solution could be to split it into two subpopulations by separating two groups of ancestors (based on molecular studies), which could help in managing the genetic variability of this population. Another feature is that not more than three or four generations have been produced by hand pollination since the original primary clones of the 1920s, further reducing the importance of recombination.

In order to achieve a good diversification of the crosses, many different crosses are usually made, resulting in rather small numbers of progeny per family (from 10 to 50 seeds per family) which is theoretically enough for a first comparison of the growth and yield levels of the families. The best families would then have to be made again in a

large size (towards 200 or more seeds per family) in order to extract elite clones from them. However, few results have been published on the comparisons of the families and on the identification of the best ones. A suggested explanation is that, with the benefit of bud grafting, most efforts are targeted towards the early identification of elite individual genotypes, which globally led to neglecting family selection. As a matter of fact, Simmonds (1996a) advised in favour of family selection in rubber as well as other crops.

7.5 Selection

Before World War II, for commercial seedlings, planters were confronted with the high tree variability. In 1928, Ashplant raised a controversy with his proposal to carry out a very early selection on seedlings and Cramer (1938) proposed to 'grade' the young rubber plants at field level, or even at the nursery stage, with a view to eliminating the lowest yielding plants. This first early selection procedure was carried out with a special tapping tool, the 'Testatex' knife. Although aware of the limited effectiveness of such a simple test, Cramer suggested that this method could also be used for selecting mother trees and creating new clones. Evers (1955) suggested planting seedlings at a high density and practising early heavy selective thinning on the basis of growth or of test tapping. This idea was tested with a thinning based only on vigour, but the only effect of thinning was to reduce the heterogeneity of the seedling stand and reach uniformity similar to that of bud-grafted clones. After the beginning of tapping, yield was not improved by this method (Wycherly 1969).

From the 1920s to the present day, clone selection has been carried out: (i) from seeds of the mother trees selected in commercial plantations; (ii) from natural pollination in polyclonal seed gardens (ortet selection); and (iii) increasingly from hand pollination. The best seedling trees could be bud grafted and multiplied as clones for further testing in different selection stages, with a gradually increasing number of trees per genotype and a decreasing number of genotypes.

The methodology of 'ortet selection', as developed by Prang Besar and the RRIM, has been described by Malaysian researchers (Ho et al. 1979). The main results of the programme initiated in 1972 by RRIM were presented by Tan et al. (1996). Shepherd (cited in Wycherly 1969) drew attention to this alternative source of new clones, and noted that 15% of the clones selected by Prang Besar since 1945 were obtained in this way. The seeds issued from ortet selection did not have the genetic properties of full-sib progenies. Due to the imbalance between pollinators in natural pollination and the uncertain and variable amount of selfing, family selection appeared to be inappropriate for these seeds, and individual selection was applied intensively. In the Prang Besar breeding programme in Malaysia, the small-scale trial was called the 'preliminary proof trial', and the large-scale trial, where clonal seedling families derived from seed gardens were also tested, was called the 'further proof trial'. On the basis of this scheme, many options were suggested and tested. Two contrasting cases can be considered: (i) a long process with 5 years in seedling evaluation trials (SETs), 10 years in small-scale clone trials (SSCTs) and 15 years in large-scale clone trials (LSCTs) (with a total of 30 years); and (ii) a short process with 3 years in SETs, 5 years in SSCTs and 12 years in LSCTs (with a total of 20 years). After this, block trials are to follow to ascertain the true potential under multilocations.

The last stage (LSCTs) is time-consuming, since the dynamics of latex yield, TPD, incidence of diseases and wind endurance are to be studied. Some attempts to find early predictors of susceptibility to TPD or to wind damage still remain unsuccessful. LSCTs are trials carried out in the planters' field. As each LSCT comprises a small number of clones (from 5 to 25), these clones are compared as simple units, with no reference to their familial origin and their genetic relatedness. Usually, these trials are carried out in a geographical network so that the clones can face various ecological conditions. Some multilocation monoclone blocks (clonal block trials, CBTs) can also be set up simultaneously, initiating a pre-recommendation process. Clone recommendations

are mainly based on the data from LSCTs and/or CBTs. There is no real rule concerning the number of trials and the number of testing years before one clone can be recommended. In Malaysia, LSCTs have been replicated so as to be exposed to the varied environments of the country. Clones were recommended for experimental-scale planting when the LSCTs were set up, and some clones could be recommended for large-scale planting after 10 years of tapping on virgin bark in LSCTs (17 years from establishing LSCTs). Large estates are often willing to take some risks and devote a few hundred hectares to 'prospective' clones. It can be suggested that a clone can be recommended and planted on a moderate scale after it was seen performing well during seven tapping years in two different LSCTs. Assessments from data on clone trials indicate that yield is seen to become stabilized after 7 years (Chandrasekhar et al. 2007).

The breeder's work is more focused on the first two stages: (i) SETs with one seedling per genotype and (ii) SSCTs with 10 to 40 budded trees per genotype. Research is devoted to the possible early measurements and to the correlations between early and adult traits or the correlations between traits measured at the two successive stages, to create a balance between the two stages. In any selection, two types of error are unavoidable but must be minimized: (i) discarding good genotypes and (ii) keeping bad genotypes (Simmonds 1985). With two selection stages, one stage is likely to discard the good genotypes taken from the preceding stage. Theoretically speaking, it is advisable to concentrate most of the selection intensity, for an index of the main traits, only on the stage that appears to be the most efficient globally.

Until 1960 at the RRIM, all the genotypes derived from hand pollination were tested simultaneously as seedlings in the nurseries (SETs) and as budded clones in SSCTs. Then, with the increasing number of seeds produced, a first selection was applied to seedlings planted at a high density in the nursery (SET), based on their vigour during the first year, and a reduced number of genotypes were then tested in SSCT (Wycherly 1969). Test tapping was applied to the nursery stage, using a system proposed by Hamaker (1914, cited in Morris and Mann 1938). Significant correlations between yields of individual seedlings and of the derived budded clones had been found by Brookson (1959). However, Ross (1965) found significant correlations between the mean yields for the first 5 years of tapping of the same clones in LSCTs and SSCTs, but not with those of the original seedlings. According to Wycherly (1969), selection to LSCT would require 3–5 years tapping in SSCT, and the duration of the SSCT would be around 10 years. The proportion of selection from SSCT to LSCT was 2–10%. Another process, with greater selection in the nursery and a second selection stage in 'promotion trials', was also tried in Malaysia (Ong et al. 1985). Such a system cuts out the SSCT stage, so reducing the selection period by about 10 years, but also reducing considerably the accuracy of selection.

At the Prang Besar Rubber Estate (in Malaysia), 2 years of test tapping were applied to the seedlings and a selection made of the genotypes to be tested as clones in a preliminary proof trial with 15–20 trees per clone. After at least 2 years of test tapping in the preliminary trial, clones were chosen for assessment in further proof trials with each clone replicated in blocks over 0.4 ha per clone (Shepherd 1969).

There has been some investigation of the possibility of using photosynthetic rate as a criterion in early selection for yield. Samsuddin et al. (1987a) made measurements in a hand-pollinated seedling population, derived from a breeding programme and grown in a nursery, but found no significant correlation between photosynthetic rate, nursery yield (early test tapping) and girth measurements. On the other hand, measurements of photosynthetic rate made on mature leaves of young bud-grafted plants of 23 clones grown in a controlled environment chamber were found to be significantly and positively correlated with the mean yields over 5 years of tapping of the same clones in a number of large-scale field trials in Malaysia (Samsuddin et al. 1987b). However, as the correlation coefficient was low (0.469) and only significant at $P = 0.05$, these workers concluded that further investigation was needed

before it can be decided whether the determination of photosynthetic rates in growth chambers is likely to be useful as a criterion for early culling of low yielders in a breeding programme. Although the leaf area index (LAI) of most commercial clones in mature stands may be similar, it is quite possible that differences between clones in crown architecture and in the partition of assimilates between growth and latex production may preclude any direct relationship between the photosynthetic rate of single leaves and the yield of mature trees (see Chap. 3 for more details).

At the Institut de Recherches sur le Caoutchouc en Afrique (IRCA) programme in Côte d'Ivoire, SETs lasted 2 or 3 years and were planted at a high density of 2000 trees ha^{-1} (2000 genotypes unequally distributed among around 40 full-sib families). Girth increment from 1- to 3 years old was measured, and test tapping applied to the trees during 2–8 weeks. About 100 genotypes were bud grafted to raise plants for SSCTs. The duration of SSCTs was limited to 8 years, because as time progressed there was increasing competition between the genotypes set up in small plots. Additionally, selection in SSCTs was made with test tapping at 3 or 4 years old (for clones with a trunk girth bigger than 25 cm) for 6 months. A 3-year tapping period was observed for 5–8-year-old trees. Selection of some three to five clones was made from both these sets, and LSCTs were raised immediately for further confirmation of their yielding potential (Odier 1983).

A method of very early yield assessment in SETs, based on leaf morphology, proved to be ineffective (Amand 1962). Assessing the density of stomata was proposed (Senanayake and Samaranayake 1970). A fast and simple method was developed to predict yield potential through quantifying latex oozing out of leaflets or petiolules during the first months of the nursery stage (Zhou et al. 1982). But, as simplification went too far, correlation between yields measured in SETs and in SSCTs fell below significant levels. Gnagne (1988) and Gnagne et al. (1990) studied the relationships between girth and latex yield measured on 2-year-old seedlings and on the corresponding 3-year-old bud-grafted clones in SSCTs but found no significant correlation for girth between SETs and SSCTs. However, significant correlation, although rather small, for latex yield between SETs and SSCTs (r = 0.2–0.3) was evident. Tan (1998) studied the relationships between selection in nursery and mature yield in SSCTs and concluded that nursery yield is the major selection criterion.

Although latex yield and girth are the main attributes, SETs and SSCTs demand selection for secondary attributes such as branching habit and resistance to leaf diseases. Physiological parameters were proposed as selection criteria, such as bursting index (BI), an indicator of susceptibility to early coagulation (Dintinger et al. 1981a, b), and photosynthetic rates (Samsuddin et al. 1987b). Anatomical parameters, such as bark thickness, number of latex vessel rings, latex vessel density and plugging index (PI), were also investigated (Tan 1998). PI, an indicator of the rate of coagulation after tapping, is negatively correlated with latex yield (Milford et al. 1969). The number of latex vessel rings showed a consistent correlation with yield (Sanderson and Sutcliffe 1929a, b; Frey-Wyssling 1930; Wycherly 1969; Huang et al. 1981), but counting the number of rings was difficult to carry out on young seedlings in the nursery. Considering vigour and yield in SSCTs, Wycherly (1969) suggested selecting clones that yielded more than predicted by the regression of yield on girth. Ho (1976) found that girth, number of latex vessels and PI accounted for 75% of the variation in yield between clones at the nursery phase, but only 40% at maturity. From the results of Hénon and Nicolas (1989), the thickness of the bark could not be considered a reliable attribute to predict yield, and it was assumed that the use of anatomical parameters as selection criteria is effective only if the genetic variation is large enough, which is not always the case in the selection of advanced Wickham clonal material (but it could assist in assessing the Amazonian and Wickham × Amazonian populations).

Correlations were found between the aforesaid physiological traits and latex yield, and the clonal nature of these traits was established (Eschbach et al. 1984). A method called 'latex diagnosis', based on four biochemical parameters

of the latex (dry rubber content, sucrose ratio, inorganic phosphorus ratio and thiol group ratio) was developed for optimizing the tapping systems and for monitoring commercial plots under tapping (Jacob et al. 1987). A clonal metabolic typology of clones was also established (Jacob et al. 1989; Gohet et al. 2003). The weak point of early tapping tests was that they tended to promote the genotypes having an easy flow with low viscosity. Latex diagnosis, applied to young bud-grafted 3–4-year-old trees which were tapped early with no ethephon stimulation in SSCTs, allowed the classification of the clones for the intensity of their metabolic activity and for the availability of sucrose in the latex (as a source of energy and of carbon for rubber biosynthesis). This method was used for selecting clones with active metabolism, fast and long latex flow and high initial yield, as well as clones with lower initial yield but with a high ratio of sucrose indicating the capacity to provide an important response to stimulated yield.

The experimental design of SETs can be: (i) a total randomization of the seedling trees, (ii) a total randomization of replicated familial plots (with 5–10 seedlings of the same full-sib family per plot) or (iii) a randomized block design with familial plots of 5–10 seedlings distributed in three to four blocks. One characteristic of these trials is that the number of seedlings per family usually varies widely from 10 to more than 100, and the trials are unbalanced. SSCTs are made of replicated clonal plots in randomized block or simple lattice designs. As each budded tree actually exhibits the performance of a stock-scion interaction, it is preferable to have a minimum of three trees per plot, so as to analyse average growth and yield data. The total number of trees per clone in an SSCT can vary from a minimum of 6 (two replications of 3 trees, a possible case for Amazonian germplasm improvement) to about 40 (4 replications of 10 trees). A large number of different bud-grafted clones can be tested in SSCTs (100 or more) in incomplete block designs called α-designs to achieve a better control of the environment (Patterson and Williams 1976).

Breeders have been aware of the limited effectiveness of selection in SETs, mainly due to the variation in environment applied to only one non-replicated tree per genotype (Evers 1959; Ho et al. 1979). Unsatisfactory attempts were made to multiply the seedlings as cuttings. As each of the two cotyledons of a rubber seed bears an axillary bud, a method was developed by Ramaer for splitting each seedling into two twin trees (Meyer 1938; Dijkman 1951) and, in this way, there were two replications of each genotype. But this method could never be developed routinely. As a matter of fact, selection in SETs must have a dual view: (i) genetic and (ii) biometric. Usually, the families in one SET may not have the required genetic structure of a nested or factorial design, which would enable the estimation of the genetic values of each seedling by the use of the equations based on the genetic relatedness between individuals, full-sibs or half-sibs (HS) (Falconer 1961). From a biometric point of view, the estimation of genetic value of the genotypes in a SET faces some critical limitations (Gnagne et al. 1998). The genetic variance between different full-sib families can be estimated but with no replication of the genotypes. While family selection is possible in a SET, a combined family-individual (genotypic) selection could be done only with a rough estimation of the necessary parameters. In contrast, bud grafting for SSCTs allows many replications of each genotype, so enabling real genotypic selection.

Criticism has been raised against phenotypic selection in SETs with the hope of picking up exceptional trees. Although full-sib families have been created, the range of variation is limited depending on the recombinant combinations possible and/or available. Such selections produce very limited exceptional individuals. Hence, the method of family selection was advocated (Jayasekera and Hettiarachi 1988; Simmonds 1996a). Considering the limitations imposed on the very early selection in a SET and the principle of concentrating on the selection of multiple traits in only one selection stage, Simmonds (1996a) proposed the following modifications: (i) carry out measurements during a maximum of 2–3 years and perform only a mild selection in a SET, by discarding the low-performing families as well as the low-performing genotypes within

the selected families; (ii) set up an SSCT with the selected families represented by 10–20 genotypes per family; (iii) concentrate the measurement efforts in SSCTs during the 8–10 years possible before competition between plots becomes too important; and (iv) analyse SSCT data as for a combined family-individual selection. When a routine tool for early selection is lacking, these proposals can only end up with the chances of losing more desirable high yielders.

There is a word of caution here: one cannot predict the potential of an 8-year-old tree when it is 1 or 2 years old. The capacity to attain increased yield develops with maturity and that depends on a number of physiological and morphological attributes coupled with the genetic nature of the seedling. Test tapping a seedling will never give an early indication of the true potential. There can be early yielders but there are late starters as well. To ascertain this, one has to wait till the tree attains tappable girth. Yet another vital point to be noted is that all the hybrids should be tapped and their potential assessed against the reference clone. A general error committed by breeders is that they test tap the hybrids that attain girth early and reject other hybrids. Such a selection can yield clones that attain girth early but need not always result in high-yielding clones. All the hybrids need to be assessed in due course. Taking into consideration all these constraints to successful early selection, Priyadarshan and Clément-Demange (2004) proposed a new scheme of deriving high-yielding clones. The proposal is: (i) the full-sibs are tapped upon attainment of girth (50 cm) and evaluated for yield against the reference clone; (ii) high yielders are selected, multiplied and are evaluated in a clonal nursery against the reference clone; and (iii) the final selections are multiplied and distributed for block-level trials. Nearly 8 years can be saved in this way. A clone can be derived in 20 years. While undertaking this scheme, it is advisable to conduct more hand pollinations in the desired crosses (based on the analysis of progeny yield data through statistical tools like ASREML/SAS) since the data are expected to be unbalanced. More full-sib families ensure greater chances of selecting desired genotypes. Figure 7.2 shows a comparison of breeding schemes by which clones of recommended genotypes can be derived, including a scheme that combines germplasm improvement with mainstream clone selection. This scheme would supersede the others that takes nearly 35 years to derive a clone (Priyadarshan et al. 2008).

7.5.1 Early Selection and Estimation of Genetic Value

Test procedures of new clones in a perennial crop like rubber are lengthy, still extending up to 20–30 years between pollination and yield assessment, distributed over three selection stages. This justifies efforts intended to improve early selection methods in order to optimize and shorten the cycle as much as possible. One component of early selection is made of the identification of traits that can be measured at young age and are predictive enough of behaviour at maturity. Another is optimization of available information and of combined management of the different selection stages in order to improve the accuracy of estimation of genetic value. The relationships between yield and parameters including girth, height, bark thickness, latex vessel number, latex vessel and sieve tube diameters, and rubber hydrocarbon in bark and petiole were inconsistent (Summers 1930; Gunnery 1935). This is probably because those simple relationships were not adequate enough to explain the whole-tree functioning. According to Hénon and Nicolas (1989), the thickness of the bark cannot be considered as a reliable attribute to predict yield but the number of latex vessel rings can help to differentiate poor yielders in both Amazonian and Wickham populations. But the variability of the anatomical traits of the bark appeared too narrow for undertaking selections within the Wickham population.

A method was developed in China to predict yield potential through quantifying latex oozing out of leaflets or petiolules (Zhou et al. 1982; 1983). Physiological parameters like plugging index (Ho 1976), bursting index (Dintinger et al. 1981a, b), photosynthetic rates (Samsuddin et al. 1987a, b), and morphological attributes

like number of stomata (Senanayake and Samaranayake 1970) were also used, but only plugging index and latex vessel number showed consistent and significant correlations with yield (Huang et al. 1981). On the other hand, correlations between yield of 2–3-year-old bud-grafted plants and mature yield could be demonstrated (Ho 1976; Tan and Subramaniam 1976). Gonçalves et al. (1998a, b) analysed the selection for growth of grafted clones at different ages in order to determine the younger possible age for efficient selection.

The first stage of selection is normally applied to seedlings (Fernando and De Silva 1971) that are full-sib progenies borne out of hand pollination and are evaluated in 'nursery' (SET, seedling evaluation trial). Information from this stage is used for selecting new clones to be evaluated as grafted trees in small-scale-clone trial (SSCT). Gnagne (1988) investigated different procedures for assessing yield on young seedlings at nursery stage such as the 'Mendes' test or the 'Hammaker-Morris-Mann' test (Dijkman 1951) and analysed the relationships (linear correlations) between SET and SSCT in order to determine some efficient selection rates at SET stage (Gnagne et al. 1990). It was found that yield measured at SET stage can be a predictor of mature yield of grafted clones evaluated at SSCT stage (with a moderate prediction ability), but growth before tapping appeared not acceptable as a predictor of growth or yield at SSCT stage. Tan (1997) confirmed those results and recommended to use mainly nursery yield as a yield predictor, and to apply a mild selection at the first nursery stage.

The combination of two early stages of selection (seedlings in SET, and grafted clones in SSCT) can be viewed only at a statistical level, based on correlations between the two stages for a set of genotypes. In this framework, Simmonds (1985) presented a theoretical approach in order to assist decision about the selection thresholds which can be applied at the first stage. But the seedlings in SET usually have the property of belonging to different full-sib families. Based on the theory of quantitative genetics, Jayasekera and Hettiarachchi (1988) underlined the importance of taking into account the 'family' value of the seedlings under selection and the rightness of 'family selection' was theoretically confirmed by Simmonds (1996a, b). Similarly, the case of nursery selection and the relationships between the two stages of early selection in rubber were examined by Gnagne et al. (1998) in order to have the best estimation of the genetic value of genotypes under selection. A combined family x individual selection was proposed in the form of a linear combination of family value and individual values. At nursery stage, with only one seedling tree per genotype, it will almost be impossible to directly assess the environment effect, so limiting the predictive efficiency of this first stage. Nursery selection could even be limited to a selection of the best families only. With this view, early selection does not aim priority to shortening the cycle but to improving the selection efficiency. Ong et al. (1986) proposed a modified selection scheme, based on 'promotion plot clone trials', combining SSCT and LSCT at the same stage, in order to reduce the time between hand pollination and the release of new clones.

As early selection is based on measurement of latex yield, this process introduces the risk to only select clones with greater early yield. Such clones were characterized by active metabolism and low level of sucrose in laticifers which was estimated by measuring inorganic phosphorus and sucrose ratios in the latex (Jacob et al. 1995). This type of latex diagnosis was applied to early selection of rubber, and a procedure was developed satisfactorily at the level of SSCT at CIRAD and CNRA (Gnagne et al. 1998). The structure and expression of laticifer-specific genes need to be studied in detail in order to detect molecular markers having correlation with yield or other traits. Since molecular genetic markers are independent of the environment, using them as predictors can contribute to improve the accuracy of genetic value assessment according to the concept of Marker-Assisted Selection or MAS (Lynch and Walsh 1998). This technique needs refinement towards operational level. Recently, Li et al. (2012) assembled and analysed de novo transcriptome sequencing data and reported the comprehensive

functional characterization of rubber tree bark. This research generated a substantial fraction of rubber tree transcriptome sequences, which are very useful resources for gene annotation and discovery, molecular markers development and microarrays development in rubber tree. One hundred and ten potential marker sites were randomly selected to validate the assembly quality and to develop expressed sequence tag-single sequence repeats (EST-SSR) markers. The profile of filtered differentially expressed (FDE) transcripts suggests that jasmonic acid (JA)- and linolenic acid (LA)-treated bark samples have a sufficient molecular basis for secondary laticifer differentiation, especially regarding secondary metabolites metabolism (Loh et al. 2016). Further, Nirapathpongporn et al. (2016) could derive a total of 10,321 EST sequences, generated from suppression subtractive hybridization-cDNA libraries of bark and latex that were used as sources for SSR searching. Such EST-SSR markers identified and developed could facilitate marker-assisted selection breeding that can be used for selection of high-yielding genotypes at juvenile stage. Breeders can utilize such innovative techniques during the years to come.

Bark is one of important agricultural and biological organs in *Hevea*. A thorough understanding of molecular mechanisms of bark formation is essential. Li et al. (2012) generated more than 30 million sequencing reads using Illumina paired-end sequencing technology. The similarity search indicated that 16,520 and 12,558 unigenes showed significant similarities to known proteins from NCBI non-redundant and Swissprot protein databases, respectively. When 22,756 unigenes were searched against the Kyoto Encyclopedia of Genes and Genomes Pathway (KEGG) database, 12,097 unigenes were assigned to 5 main categories including 123 KEGG pathways. Among the main KEGG categories, metabolism was the biggest category (9043, 74.75%), suggesting the active metabolic processes in rubber tree bark. The EST-SSR markers identified and developed could lead to a selection strategy during juvenile stage for high latex yield.

7.5.2 Paternity Identification and Breeding Without Breeding (BwB)

Pre-breeding of the Amazonian genetic groups has been considered to be carried out by recurrent selection based on recombination through seed gardens and natural pollination, and intensive selection. For methodological purposes, one seed garden made up of 50 Amazonian genotypes and the GT 1 clone, planted at CNRA (Côte d'Ivoire), was subjected to the analysis of gene flux and paternity identification with isozymes and microsatellites (Blanc et al. 2001; Lidah 2005). Paternity identification with microsatellites was carried out using the Cervus software (Marshall et al. 1998). A high level of confidence was found for paternity identification carried out using eight microsatellite markers. The distribution of the contribution of the different genotypes to pollination was found to be highly unequal, with four genotypes accounting for 40%, 14 genotypes accounting for 80% and 25 genotypes accounting for 95% of the total fertilization of the seed garden. The variation of selfing rate was assessed among the genotypes with an average of 5%, and no selfing was found on GT 1, as expected for a male-sterile clone. The isolation of the seed garden was confirmed since no allele other than those belonging to the parental population was found. Garcia et al. (2011) could adjudge the identification of 10 clones out of 12 with four microsatellites. The efficiency in paternity identification made possible by microsatellites suggests that a new method of selection may be possible by which the best trees are selected from seedlings resulting from natural pollination and their paternity is identified a posteriori. Transcriptome analysis of bark is yet another upcoming area for marker-assisted selection during juvenile stage that can revolutionize breeding *Hevea* rubber (Li et al. 2012). Also, Mantello et al. (2014) performed RNA sequencing (RNA-seq) of bark on the Illumina GAIIx that validated 78 SNPs in 36 genotypes. This new data set represents a powerful information source for rubber tree bark genes and will be an important tool

for the development of microsatellites and SNP markers for use in future genetic analyses such as genetic linkage mapping, quantitative trait loci identification, investigations of linkage disequilibrium and marker-assisted selection. Characterization and cross-amplification of microsatellites from wild *Hevea* species has augmented the possibility of transferability of these microsatellites to *Hevea brasiliensis* (Mantello et al. 2012).

The classical breeding methods used by tree breeders rely on pre-determined mating designs. However, El-Kassaby et al. (2006) has introduced a scheme of Breeding without Breeding (BwB) that allows the assemblage of full-sib (FS) and half-sib (HS) families from seed orchards' naturally pollinated offspring without conducting any crosses. This scheme circumvents artificial mating, focusing instead on a subset of randomly sampled, maternally known but paternally unknown offspring to delineate their paternal parentage. This method calls for highly informative molecular markers (e.g. SSRs) for pedigree reconstruction and to unravel the parentage (El-Kassaby and Lstibůrek 2009). SSRs are now in a development stage in *Hevea* rubber (Garcia et al. 2011; Li et al. 2012; An et al. 2013). But this situation shall improve with time. In *Hevea*, well-organized breeding orchards that permit pollen from only hetero-neighbours can be subjected for raising such FS and HS families. A two dimensional hetero-neighbours' layout as proposed by Simmonds (1986) can be well suitable for such an exercise (step 2 of Fig. 7.3). This can be used for both breeding full-sib offspring and for collection of polyclonal seeds. For this, large polyclonal orchards are necessary that can produce thousands of seeds every year. Alternately, a clone evaluation garden laid under completely randomized design (CRD) can also be used for collecting half-sib seeds (Fig. 7.3). CRD almost ensures a panmictic population. Such HS families shall be raised in closer spacing (3 m) that can be subjected for yield screening upon attainment of 50 cm girth. A mistake usually being committed by the breeders is to select the early yielding genotypes and reject the ones that are yet to be tapped. This exercise has indirectly culminated in the selection of clones with faster girth increment that reduces gestation period. However, a point to be remembered here is that the leftover set may contain high-yielding recombinants, which may attain maturity a little late. While exercising clone selection, both early yielding and late yielding clones are a necessity, to present before the planters an array of clones with vivid attributes to choose from. If SSRs that are linked to QTLs for high yield can be used, then, the exercise can minimize screening process to a great extent. In this way, this method allows the capture of 75–85% of the genetic response to selection attained through conventional programmes without the need to do any controlled pollination and simplified or possibly no experimental field testing: both considered to be the most resource-demanding activities in breeding programmes. The selections borne out of these HS evaluations can be further confirmed through clonal nursery trials following line RBD with a reference clone. Simultaneously, these selections can be propagated and given for trials in government-owned areas with a reference clone. In this way, a quick derivation of clone can be achieved. For all this, DNA profile of all available clones is a prerequisite to ascertain the parentage.

7.6 *Hevea* Clones

Rubber clones are denominated with a first part in letters (abbreviation of the origin) and a second part in numbers. Dijkman (1951) provides a list of denominations with their emblematic clones that were developed during the first half of the twentieth century, such as AVROS (AV 49, AV 255, AV 352, AV 2037), Bodjong Datar (BD 5, BD 10), Djasinga (Djas 1), Glenshiel (Gl 1), Gondang Tapen (GT 1), Kali Djeroek (KD 1), Landb. Mij. 'Oud Djember' (LMOD 53), Lands Caoutch Bedrijf (LCB 1320), Pataroeman (Pat 190), Pilmoor (Pil D65), Prang Besar (PB 186), Proefstation voor Rubber (PR 107 = LCB 510), Tjirandji (Tjir 1, Tjir 16), Waringiana (War 4), etc. In addition a denomination was established to indicate precisely the different types of seeds

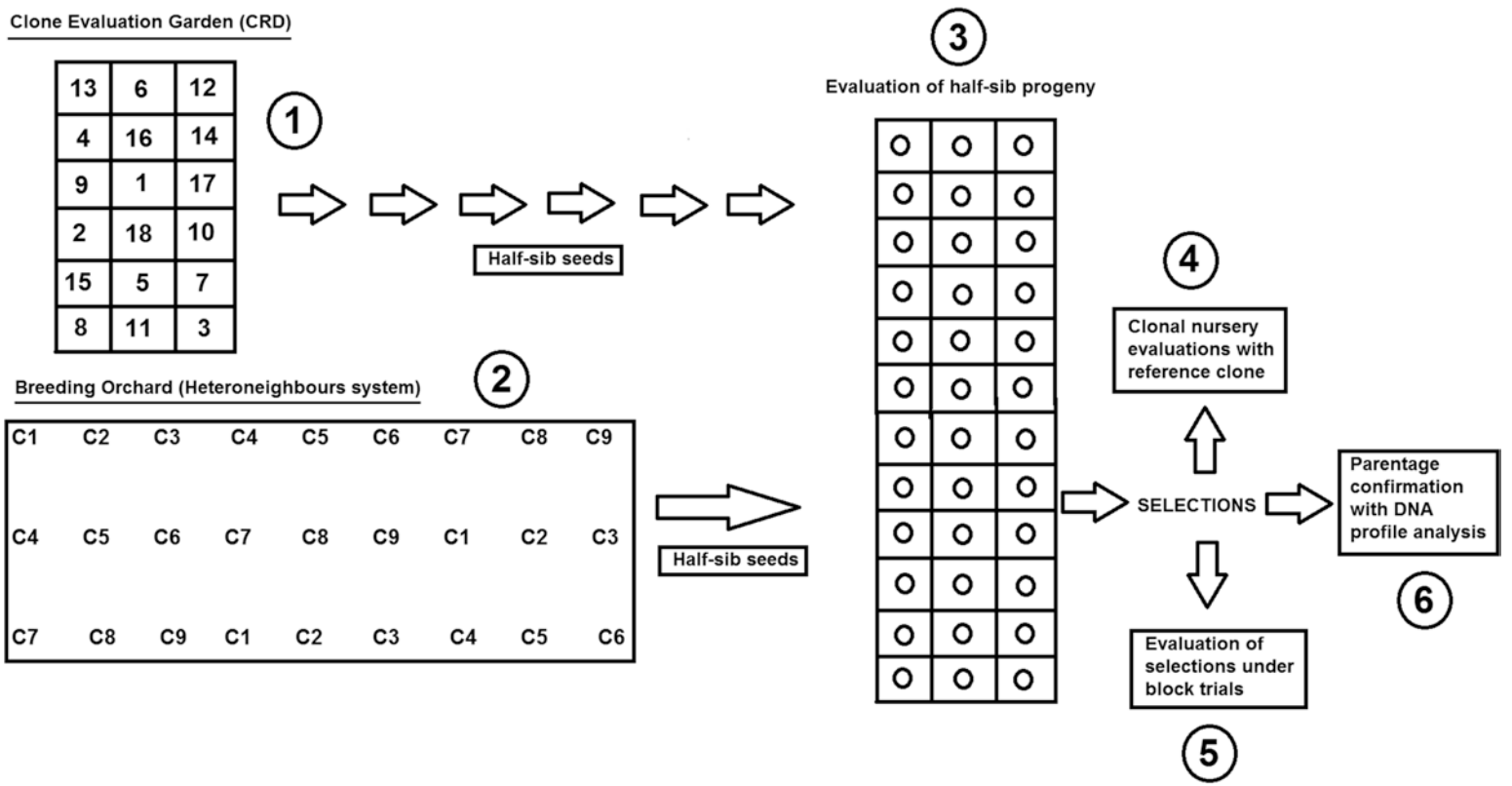

Fig. 7.3 Half-sib progeny analysis

or the genetic origin of clones issued from recombination: (i) 'illegitimate seedling families' (ill.) are issued from commercial plantings, with no known genetic origin; (ii) when only the mother parent of one clone is known (i.e. AV 163), the origin of the clone is denominated as AV163 ill.; and (iii) 'legitimate' full-sib seedling families issued from hand pollination are indicated by mentioning first the female (seed) and then the male (pollen) parent (i.e. PB 186 × Tjir 16).

The main denominations of some of the rubber clones are as follows:

AVROS: Algemeene Vereeniging van Rubberplanters ter Oostkust van Sumatra (Dutch) = General Association of Rubber Planters on the East Coast of Sumatra, Indonesia

F and FX: clones from the collections and recombinations of the Ford Company in Brazil

IAC: Brazil, Instituto Agronomico do Campinas

IAN: Brazil, Instituto Agronomico do Norte

IRCA: Côte d'Ivoire, clones created by the Ivorian-French breeding programme in Côte d'Ivoire since 1974

IRRI: Indonesia, Indonesian Rubber Research Institute (clones IR, but also LCB, PR and BPM)

MDF: Madre de Dios Firestone (clones collected in the Madre de Dios, Peru by Firestone)

PB: Malaysia, Prang Besar Rubber Estate

RRIC: Sri Lanka, Rubber Research Institute of Ceylon

RRII, India, Rubber Research Institute of India

RRIM: Malaysia, Rubber Research Institute of Malaysia (now integrated within the Malaysian Rubber Board); with also OS (clones issued from ortet selection) and PC (clones issued from Promotion Clone trials)

RRISL: Sri Lanka, Rubber Research Institute of Sri Lanka

RRIT: Thailand, Rubber Research Institute of Thailand

RRIV: Vietnam, Rubber Research Institute of Vietnam

SCATC: China, South China Academy of Tropical Crops (Hainan) – the code SCATC has now been changed to REYAN and

YITC: China, Yunnan Institute of Tropical Crops (Yunnan)

Cramer (1914) was the first to select the three 'Cultuurtuin' clones (primary) Ct3, Ct9 and Ct88 from 33 seedlings derived from the Penang Wickham trees (imported seeds), which were established at Buitenzorg (Bogor) in 1883 (Dijkman 1951). The most striking result from the mother-tree selection in Indonesia during the 1920s was the identification of GT 1 and PR 107, which are being cultivated even now. AVROS 49, recommended thereafter, was the standard clone in all the AVROS station experiments.

The ortet selection of Gough in Prang Besar (Malaysia) during the 1920s, based on a preselection of 618 seedlings from the Kajang area, produced important primary clones, among which is PB 86, one of the most important clones ever produced, as well as PB 23, PB 25 and PB 186 (Simmonds 1996b). Sanderson and Sutcliffe (cited in Dijkman 1951) obtained the primary clones Pil A44, Pil B16, Pil B84 (from the Pilmoor Estate in Malaysia) and Gl 1 with the same approach.

It is difficult to provide yield data for different clones which successively emerged in clone recommendations, because there has been no standard for characterizing the yield level of the clones over time in the successive experimental programmes. Tan (1987) suggested using the mean annual yield over 10 tapping years with 550 kg ha^{-1} for unselected Wickham seedlings, 1175 kg ha^{-1} for PilB 84 (selected in the 1920s), 1425 kg ha^{-1} for RRIM 501 (1928–1931), 2000 kg ha^{-1} for RRIM 600 (1937–1941) and 2125 kg ha^{-1} for RRIM 712 (1947–1958). Using the same criterion, Simmonds (1989) indicates 1890 kg ha^{-1} for the mean of 20 clones recommended in Malaysia, and 1330 kg ha^{-1} for PBIG seedlings. As a matter of fact, successive evaluations of sets of clones lead to a regular evolution of the clone recommendations without any global comparison.

Clone recommendations in Malaysia have evolved over time, and this indicates the diversity of clones that were prominent at different periods. From 1939 to 1988, 18 clones have been rec-

ommended in 'Class I', with the successive entries: Tjir 1, Tjir 16, PB 86, PilB 84, PB 25, Gl 1, RRIM 501, RRIM 513, PR 107, PB 5/51, RRIM 605, RRIM 623, GT 1, RRIM 600, PR 255, PR 261, PB 217 and RRIM 712. Most of these clones have also been used extensively as parents. For Indonesia, it is worth noting that PR 107 and GT 1, identified as mother trees in the 1920s and still cultivated now, got Class I status in Malaysia only in 1955 and 1967, respectively. PB 5/51 was withdrawn in 1977 because its yield was superseded by other clones. PR 107 was withdrawn in 1977 for low initial yield, although the mean yield over a long period is still among the best performances. GT 1 was withdrawn in 1992 due to its susceptibility to *Colletotrichum*, and because its yield appeared to be less competitive compared with that of other clones. In 1995, the recommendation system was deeply modified; however, the clones RRIM 600, PR 255, PR 261, PB 217 and RRIM 712 were maintained in the 'Group I' of the new system, and withdrawn only in 1998. PB 260 was elevated to 'Class I' in 1992, and is still in 'Group I', together with more recent clones of the RRIM 900 series and also PB 280, PB 350, PB 355, PB 359, PB 366 and PM 10. Clones of the RRIM 2000 series are in 'Group II'. The prominent role of RRIM 600, the most widely adaptable clone of the world, as well as GT 1, must be underlined. From 1986 to 1995, statistics of planting materials used in Malaysia show that the most important clones were PB 260, PB 217 and PB 235. PB 260 and PB 217 are still very important in many producing countries. From a physiological point of view, they have opposite behaviours. PB 260 exhibits a very active metabolism and a fast initial yield, but low sucrose reserves in the latex, a poor response to stimulation and a high susceptibility to dryness and brown bast (a physiological disease). In contrast, PB 217 is a slow starter but with a high level of sucrose reserves in the latex, a very good response to stimulation, a low susceptibility to dryness and brown bast and a very high-yield potential over a long tapping period. PB 235 is similar to PB 260 and it has a very fast growth and can be tapped at 4½ years in favourable conditions, whereas PB 260 can be tapped from 5 to 6 years, and GT 1 and RRIM 600 at 5½ years. PB 235 has a high initial yield but is prone to dryness and wind damage (PB 260 faces roughly the same problems). However, in non-traditional rubber-growing areas, these attributes are altered significantly with few or no symptoms of dryness (Priyadarshan 2003a).

Other important clones that have emerged from the breeding programmes of other countries include: (i) BPM 24 and BPM 1 in Indonesia, (ii) RRII 105 in India, (iii) RRIC 100 in Sri Lanka, (iv) RRIV 2 and RRIV 4 in Vietnam, (v) Haiken 1 and SCATC 88/13 in China and (vi) IRCA 18 and IRCA 230 in Côte d'Ivoire. Also, there are many other promising clones which are still under multilocation trials. Historically, Malaysia played a major role in rubber breeding, following the initial work of Dutch researchers in Indonesia. One main conclusion that can be drawn is that the turnover of the clones is slow but regular, which illustrates the fact that many years are necessary for accumulating the necessary observations and gaining a stabilized knowledge of the rubber clones. Deriving clones at definite intervals is challenging to the breeder, for which refinements in the methodology followed to reduce breeding cycle time and to assess the yielding potential are prime.

Genetics of Traits

8

Derivation of a high-yielding clone is the ultimate objective of the breeding programme. Most breeding is done with parents that show outstanding performance in field trials; however, information about the genetic worth of many of these parents is limited. Time required to release a clone is around 30 years, and due to this, the current strategy is to reduce breeding cycle through early selection through the use of secondary traits that show high correlations with latex production. Effective breeding strategies ensure detailed knowledge of the genetic architecture of the traits of interest, such as magnitude of heritability, GE interactions and genetic correlations between traits. Estimation of heritability is critical to identify relevant traits and to evaluate the scope for genetic gains. Clones with wider adaptability to several environments are prime for commercializing cultivation under contrasting environments. In addition, knowledge of phenotypic and genetic correlations between primary and secondary traits is key in predicting the effects of direct and indirect selection.

One of the first papers on genetics of *Hevea* is by de Paiva et al. (1994) that featured electrophoretic analysis of two natural populations for enzymatic systems malate dehydrogenase, shikimate dehydrogenase and leucine aminopeptidase by which four loci each were identified. Genetic distances between these two populations were similar. Though a few reports on heritability are available (Tan et al. 1975; Alika 1985; Jayasekera et al. 1994; da Costa et al. 2000; Furlani et al. 2005; Gonçalves et al. 2005, 2006; Verardi et al. 2012; Gouvêa et al. 2013; Silva et al. 2013), none of these studies comprehensively explore all critical elements of the genetic architecture. Silva et al. (2014) studied the genetic parameters such as narrow-sense heritability and additive genetic variance in single- and multisite analyses, genotype-by-environment interactions (type B correlations) and its effects on alternative selection strategies, additive genetic repeatability correlation for rubber yield based on three consecutive yearly measurements and trait-to-trait genetic associations (type B correlations) for all measured traits. Average rubber yield showed an estimated narrow-sense heritability of 0.31, with an estimated type B correlation of 0.84, indicating low levels of genotype-by-environment interaction. The trait survival and number of latex vessel rings (RG) showed larger genotype-by-environment interaction and the lowest heritability. High to moderate type B correlation was found in the foremost traits, with a value of 0.85 between girth and average rubber yield. On the other hand, de Oliveira et al. (2015) estimated genetic parameters in order to assess heritability, predict genetic gains and establish genetic and phenotypic correlations among yield-related traits of 22 families selected phenotypically for yield. Three early measurements were carried out for the traits like dry rubber yield, annual girth increment, number of latex vessel rings and bark thickness. Statistical analyses were carried out using the REML/BLUP (restricted maximum

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likelihood/best linear unbiased prediction) method. The additive genetic variance showed higher values than the residual variance, indicating high genetic variability in the population. The high values of heritability obtained indicated insignificant influence by the environment and high gains can be obtained by selection. High genetic correlation was found between girth increment and bark thickness indicating that selection of one of these traits would lead to gains for the other trait as well. Investigations by Silva et al. (2014) and de Oliveira et al. (2015) are to be meticulously analysed in view of dry rubber yield. Both these studies have not proven beyond doubt that any of the traits studied are having strong relation with dry rubber yield. Such studies only give indication of the probable traits that influence yield, but fail to prove their bearing on yield so that the trait in question can be used for undertaking selection at juvenile stage. So, any study on genetics of traits must be with the intention to improve early selection that is indispensable.

In Sri Lanka, Jayasekera et al. (1994) studied the nature and extent of genetic and environmental control of production traits in rubber trees, with nine clones in replicated trials at seven sites. The clones were both indigenous and imported, whereas the sites represented a range of environments. The analyses of girth data showed a consistent increase in heritability over 15 years from 12% to over 75% with a corresponding decrease in the effect of the environment (from 70 to 12%). With respect to yield, there was no such change from environmental control to genetic control of the character over the 5 years during which yield was monitored. Correlations between final tree size and earlier measurements indicate that different genes are involved in pre- and post-tapping growth.

To assess heritability, predict genetic gains and establish genetic and phenotypic correlations among yield-related traits, 22 families selected for yield were subjected for analysis (de Oliveira et al. 2015). Measurements on dry rubber yield, annual girth growth, number of latex vessel rings and bark thickness were made. Statistical analyses were carried out using the REML/BLUP (restricted maximum likelihood/best linear unbiased prediction) method. The additive genetic variance showed higher values than the residual variance, indicating high genetic variability in the studied population. The high values of heritability obtained indicated that the studied traits are little influenced by the environment and high gains can be obtained by selection in this population. High genetic correlation was found between annual girth growth and bark thickness suggesting that the selection of families on one of these traits would lead to gains for the other trait as well. The magnitude of genetic correlation indicated greater contribution from genetic factors than environmental factors in these correlations.

Silva et al. (2014) analysed data from 51 open-pollinated progenies tested on six sites in Brazil over several traits to estimate the following: genetic parameters such as narrow-sense heritability, additive genetic variance in single- and multisite analyses, type B correlations to determine the relevance of genotype-by-environment interactions and its effects on alternative selection strategies, additive genetic repeatability correlation for rubber yield based on three consecutive yearly measurements and type A correlations to evaluate trait-to-trait genetic associations for all measured traits. Average rubber yield showed an estimated narrow-sense heritability of 0.31, with an estimated type B correlation of 0.84, indicating low levels of genotype-by-environment interaction. The trait number of latex vessel rings showed larger genotype-by-environment interaction and the lowest heritability. High to moderate type B correlation was with a value of 0.85 between girth and average rubber yield. Open-pollinated seedlings always differ genetically, since *Hevea* exercises open pollination close to 79% (Pawsoi et al. 2013). Aforesaid of studies shall be feasible only in case of clones in comparison to their progenitors.

Heritability of tapping panel dryness (TPD) based on parent-offspring regression had been conducted (Narayanan and Mydin 2014). Incidence of TPD ranged from 3.6% in PB 5/51 to 46.4% in PB 235. Progenies of PB 5/51 × RRII 208 showed minimum TPD incidence (3.6%),

while those of RRIM 600 × PB 235 exhibited maximum incidence (29.6%). The study showed high narrow-sense heritability (h_2 = 0.50) for TPD. They concluded that TPD could be governed by heritable gene action indicating that breeding for TPD resistance shall be a good option. TPD is governed by anatomical, physiological and environmental parameters including stock-scion interactions in addition to genetic causes. TPD is the least understood melody in *Hevea* rubber that cannot be attributed to a single reason alone. Hence, studies on regression may give some lead to its occurrence but cannot in any way give a wholesome explanation to the occurrence of TPD, either in the progeny or in the progenitors.

A synthesis of the studies on genetics of traits gives a general impression that considerable and feasible investigations have not been held so far. This could be due to (a) long breeding cycle, (b) the lack of large hybrid progenies, non-synchronous flowering and (c) low seed set. A diallel can never be achieved in *Hevea* rubber and such programmes always end up with unbalanced diallel. Seed set under controlled pollination is around 11.8% (Priyadarshan and Clément-Demange 2004). Natural cross-pollination is around 79% estimated through microsatellite markers (Pawsoi et al. 2013). Estimated outcrossing rate varied from 58.62 to 98.36 with 21% selfing rate. With these constraints, conducting genetic studies in *Hevea* rubber will be a task unless microsatellite markers are utilized in traits governing yield. As such, traits governing yield are not concretely established. *Hevea* rubber is an excellent example for soil-plant-atmosphere interactions, and multitude of factors like latex vessel rows, girth, feeder roots, canopy area and stock-scion interactions (to name a few) are responsible for yield which is a multichannel end point. Hence, genetic studies on yield will be cumbersome.

Environmental Constraints and Adaptation to Global Changes

9

The Great Amazonian basin is flat, between equator and 15°S with altitudes not exceeding 200 m with a wet equatorial climate (Strahler 1969). The climate is characterized by a mean monthly temperature of 25–28 °C and abundant rainfall of more than 2000 mm/year. The attributes ideal for rubber cultivation are (a) 2000–4000 mm rainfall distributed over 100–150 rainy days/annum (Watson 1989), (b) mean annual temperature of around ±28 °C with a diurnal variation of about 78 °C (Barry and Chorley 1976) and (c) sunshine hours of about 2000 h/year at the rate of 6 h/day in all months (Ong et al. 1998). In a study with hydrothermal index, Rao et al. (1993) rationalized Senai of Malaysia (1°36'N; 103°39'E) to be the most suitable for rubber cultivation and production.

The increased global demand for rubber, as also the extension in cultivation of other agricultural crops, prompted the countries outside the hitherto traditional zone to focus their attention on the cultivation of rubber (Pushparajah 1983, 2001). Such a tendency often extended rubber to sub-optimal environments. Specific areas of China, Thailand, Vietnam, India, Côte d'Ivoire and southern plateau of Brazil fall under non-traditional zones that experience one or more stress situations, viz. drought, low temperature, high altitude, diseases and strong winds. The mean annual temperature decreases when moved away from the equator with more prominent winter conditions during November–January. Northeastern states of India and China lying between 18°N and 24°N are regions well recognized as inhospitable for the crop, exhibiting stress situations like low temperatures and typhoons (Zongdao and Yanqing 1992). It may also be worthwhile to note that rubber areas of China and Tripura fall under the same latitude range, though climatic conditions in vivid pockets of China shall vary since China's tropical and subtropical regions are undulating and diversified (Priyadarshan and Gonçalves 2003; Priyadarshan 2003a). Four main climatic zones are prominent in Brazil, viz. Amazonian basin, Brazilian plateau, coastlands within tropics and southern states. Rubber inhabits all areas except southern states. Southern Brazilian plateau (450–500 m MSL) especially São Paulo is being experimented for rubber cultivation. This move to areas seasonally affected by dry and cold conditions is mostly motivated in Brazil to escape from the climatic conditions congenial to SALB. These areas, apart from high altitude, offer high rainfall that often exceeds the basic requirements. A geo-climatic comparison of various environments such as Tripura, China, Côte d'Ivoire, Indonesia, Vietnam and Thailand would amply reveal a spectrum of climatic conditions over which rubber is grown (Table 9.1). Climatic conditions in these countries also exhibit considerable variation (Table 9.2). In India, marginal areas delineated as non-traditional zones, spread over the states of Maharashtra, Orissa, Tripura, Assam, West Bengal, Meghalaya and Mizoram, pose a multitude of hazards, viz. moisture stress, low temperature, wind, high altitude

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Table 9.1 Spectrum of climatic features of rubber-growing countries

Country	General climatic features
Malaysia	Tropical, annual southwest (April to October) and northeast (October to February) monsoons
Thailand	Tropical; rainy, warm, cloudy southwest monsoon (mid-May to September); dry, cool northeast monsoon (November to mid-March); southern isthmus always hot and humid. North and northeast areas are non-traditional for rubber
India	Tropical monsoon type with winter (November to January), summer (March to May), southwest monsoon season (June to September) and post-monsoon or northeast monsoon season (October to December). Most of the rainfall is brought by southwest monsoon. Because of the geographical diversity of India, regional climate conditions in the extreme north, east and west vary from the general conditions given here. Specific areas of west, east and northeast are non-traditional for rubber
Sri Lanka	Tropical monsoon; northeast monsoon (December to March); southwest monsoon (June to October)
Indonesia	Tropical climate even all year-round. Heavy rainfall usually between December and January. The equatorial position of the country makes opposite climates in the north and the south
China	Extremely diverse, tropical in south to subarctic in the north, with great climatic differences resulting from the monsoon, the expanse of the land mass and the considerable differences in altitude. Typhoons are prudent in southeast China between July and September. China is a non-traditional zone for rubber
Vietnam	Tropical in south; monsoonal in north with hot, rainy season (mid-May to mid-September) and warm, dry season (mid-October to mid-March). Diverse range of latitude, altitude and weather patterns produces enormous climatic variation. North Vietnam like China has two basic seasons: a cold humid winter from November to April, and warm, wet summer for the remainder of the year. The northern provinces share the climate of the north, while the southern provinces share the tropical weather of the south. South Vietnam is relatively warm. Central highlands and the coastal regions are non-traditional areas for rubber
Côte d'Ivoire	Tropical along coast, semi-arid in far north; three seasons—warm and dry (November to March), hot and dry (March to May) and hot and wet (June to October); three main climatic regions: the coast, the forest and the savannah. Low rainfall areas in north (less than 1300 mm) are non-traditional experimental zone for rubber
Nigeria	Varies; equatorial in south, tropical in centre, arid in north. Two principal wind currents affect Nigeria; the *harmattan*, from the northeast, is hot and dry and carries reddish dust from the desert and causes high temperatures during the day and cool nights. The southwest wind brings cloudy rainy weather
Liberia	Tropical; hot, humid; dry winters with hot days and cool to cold nights; wet, cloudy summers with frequent heavy showers
Brazil	Range: equatorial, tropical, semi-arid, highland tropical and subtropical. Annual average temperature in the Amazon region is 22–26 °C. Brazil is in the south of the equator; seasonal changes are vice versa compared to north of the equator. Plateau of São Paulo is a non-traditional area for rubber

Source: www.britannica.com; www.worldatlas.com; www.wmo.ch; www.usda.gov; www.iwmi.org

and disease epidemics, apart from altered soil physical properties.

Nevertheless, even if breeding can be efficiently involved, adaptation of rubber cropping to non-traditional environments will not be able to overcome constraints towards growth and latex yield compared to traditional zones. So, this issue is related to socio-economic conditions (rubber price, cost of labour, availability of land). Moreover, adaptation to new areas needs more ecophysiological research with a better evaluation of existing clones in various environments rather than on creation of new clones that can be considered only on a long-term perspective.

9.1 *Hevea* Under Marginal Conditions

Climatic factors play a pivotal role in the establishment and development of any crop. Ambient temperature, wind, rain fall, vapour pressure deficit, sunshine hours, etc. are some of the factors that govern such establishments (Table 9.3). The manifestations over the plant body are the real reflection of its interaction to such factors, and the evaluation of various genotypes under such varied environments will give an idea on which genotype would be suitable for a new environment in question.

Table 9.2 Climatic details of traditional and non-traditional areas

Attributes	Bogor (Indonesia)[a]	Pindorama (Sao Paulo, Brazil)[b]	Kourou (French Guiana)[a]	Odienne (Côte d'Ivoire)[b]	Nong Khai (Thailand)[b]	Hainan (China)[b]	Agartala (Tripura, India)[b]	Senai (Malaysia)[a]	Dak Lak (Vietnam)[b]
Temperature (°C) (mean)	27.4	22.9	26.3	25.6	26.8	22.6	25.4	26.9	21.5
Daily temperature range (°C)	9.1	11.8	7.8	12.7	10.2	7.8	9.9	7.2	7.9
Relative humidity (%)	79	67	81.5	67	74	79.9	76.8	82.3	75.7
Sunshine (% h)	61	55.1	49.9	59.2	58.1	46.8	50.8	47.8	48.8
Wind run (m/s)	2.4	1.6	1.35	1.3	1.2	2.7	1.38	2.1	2.5
Rainfall (mm/annum)	1791.5	1117.6	2573.53	1297.9	1455.96	1431.29	1960.1	2282.2	1669.31
No. of rainy days	159	117	193	119	128	151	93	182	163
Moisture availability index	0.78	0.49	1.4	0.67	0.7	0.6	1.1	1.2	0.8
Penman ET_o (mm/day)	4.4	3.87	3.78	4.3	3.97	3.48	3.39	3.9	3.57
Latitude	5°9′S	20°25′S	5°7′N	9°30′N	17°51′N	19°2′N	23°49′N	1°36°N	14°55′N
Longitude	106°58′E	49°59′W	52°56'W	7°34′W	102°44′E	109°30'E	91°16′E	103°39°E	108°10′ E
Altitude (m)	16	505	48	451	164	671	31	13	655

Source: www.iwmi.org
Senai (Malaysia) is considered as the area offering optimum environment
[a]Traditional
[b]Non-traditional

Table 9.3 Climatic factors influencing growth and yield of rubber

Attribute	Manifestations	Reference
Ambient temperature (°C)		
Less than 0	Severe cold damage	Jiang (1984)
<5	Cold damage	Zongdao and Xueqin (1983)
10	Mitosis occurs but photosynthesis discontinues	-do-
18	Plant cells divide normally just for survival (crucial temp. for tissue differentiation)	-do-
<18	Yield decreases with late dripping	-do-
18–24	Optimum for latex flow	Shuochang and Yagang (1990)
22–28	Favourable for latex flow	Shangpu (1986), Jiang (1988)
27–30	Optimum range for photosynthesis	Shangpu (1986), Shamshuddin (1988)
34–40	Respiration exceeds photosynthesis; retardation of growth and scorching of young leaves	Lee and Tan (1979), Chandrashekar et al. (1990), Ong et al. (1998)
Annual temp. 20–28 °C	Optimum for growth, latex production	Shamshuddin (1988)
Diurnal variation (7–10 °C)	Optimum	Rao and Vijayakumar (1992)
Monthly temp. 20 °C	Negligible growth	Jiang (1988)
Rainfall (mm)		
1300–1500	Optimum for growth and production	Pushparajah (1983)
1800–2000	Optimum for growth and production	Pakianathan et al. (1989)
9–11 mm/day	Congenial	Liyanage et al. (1984)
Rainy days		
100–125 days/year @ 125 mm/month	Optimum	Ong et al. (1998)
Water requirement 3–5 mm/day	Optimum	Monteny et al. (1985), Haridas (1985)
Wind (m/s)		
1.0	Favourable	Zongdao and Xueqin (1983)
1.0–1.9	No evident hindrance	Oldeman and Frere (1982)
2.0–.9	Growth and latex flow retards	Yee et al. (1969)
>3.0	Severe inhibition of growth and latex flow	Zongdao and Xueqin (1983)
8–13.8	Leaf laceration	Zongdao and Yanqing (1992)
17.2	Branch breaks, trunk snaps	-do-
24.5	Uprooting	-do-
Sunshine		
2000 h/year	Optimum	Ong et al. (1998)
Ambient vapour pressure deficit (mb)		
>12	Decrease in latex flow	Paardekooper and Sookmark (1969)
28	Initiation of stomatal closure	Rao et al. (1990)
35	Stomata closes	-do-

Clones that attain required girth (50 cm) early are preferred since yield can be retrieved from them especially under a new environment. Accordingly, girth increment under immature phase becomes a crucial attribute in *Hevea*. In a comparison of girth increment of RRIM 600 in traditional and non-traditional areas, Sethuraj et al. (1989) reported 4.3 cm less girth in the northeastern region of India compared to traditional belt. While RRII 105 is counted as one of the best

suitable clones for the traditional areas, PB 235, RRIM 600, RRII 208 and Chinese clone Haiken 1 are seen to be adaptable in the northeast region of India (Priyadarshan et al. 2000a, b; Mondal et al. 1999). In a study with 7 clones and 5 hybrids, Meenattoor et al. (2000) rationalized the hybrid clone 82/29 to attain higher girth in non-traditional environments. Girth increment is seen to be minimum during winter months (Meenattoor et al. 1991; Priyadarshan et al. 1998a), which is over 20% of the total annual girth (Vinod et al. 1996). These preliminary evaluations amply rationalized that clones, which perform well under traditional areas, do not behave similarly under non-traditional environments.

Though genotype-environment interaction studies have been undertaken at several sites earlier also (Jayasekera 1984), the environment had not been bifurcated into climatic and edaphic factors. In the studies with seven clones of Indonesian (GT1, PR 261, PR 255), Malaysian (RRIM 701, PB 235, RRIM 600) and Brazilian (IAN 873) origin, Gonçalves et al. (1998a, b) could bifurcate the climatic and edaphic factors affecting the interactions. This was done by exercising clone × site interactions (four test locations) through calculation of estimated heritability (h^2 b) and genetic gains (GG_s) that showed PB 235, IAN 873 and RRIM 600 with greater values under different sites. Yet another study with half-sib progenies of 22 Asian clones evaluated under three test sites demonstrated genotype-site interactions were significant for rubber production and girth increment (Costa et al. 2000). However, these studies never rationalized clones suitable for a specific location. The aforesaid discussion amply proves that growth trends of clones are location specific and clones exhibiting better growth need to be evolved for a specific environment.

9.1.1 Abiotic Stress Factors

In China, two types of cold damages (chilling injury) have been identified, viz. radiative and advective (Zongdao and Xueqin 1983). In radiative type, the night temperature falls sharply to 5 °C, whereas the day temperature ranges between 15 and 20° C or above; in advective type, the daily mean temperature remains below 8–10 °C, with a daily minimum of 5 °C. In both these types, under extreme circumstances, complete death of the plant is the ultimate outcome. An analogous atmosphere prevails in northeastern states of India also. Reports from China point out that while clones GT 1 and Haiken 1 can withstand temperatures as low as 0 °C for a short span, SCATC 93-114 can endure temperature as low as −1 °C. The cold wave conditions in Tripura state (northeast India) can be conveniently classified as relating to the radiative type. Chinese clones like Haiken 1, SCATC 88-13 and SCATC 93-114 are being evaluated in Tripura. Initial yielding pattern shows Haiken 1 to be a high yielder among Chinese clones, as compared with RRIM 600 which is used as a local check. Though SCATC 93-114 is proclaimed with greater cold endurance under Chinese conditions, it never shows considerable yield potential under the conditions of Tripura, at least during the initial stages on B0-1 panel (Priyadarshan et al. 1998a, b). Interestingly enough, as temperature goes down, yield increases, but when it goes below 18 °C, yield also goes down (Fig. 9.1). China has also developed the clone Zhanshi 86, which is more cold endurant than SCATC 93-114; this clone is issued from seedlings out of a random cross between SCATC 93-114 and Wuxing I_3 (Senyuan 1990). Further, clones like Zhanshi 306-15 (RRIM 600 x Guangxi 6-68) give around 10 kg of dry rubber per tree. But these contentions are to be proved at the block level. IAN 873, a SALB-resistant high-yielding clone developed in Brazil, shows resistance to cold weather in China (Senyuan 1990). RRIM 600 appeared to be the most tolerant to chilling stress, since it showed no cellular lysis or leaf necrosis and the best recovery as revealed by net photosynthesis, stomatal conductance, and optimal and effective photochemical efficiencies, and PB 260, IRCA 317, PR 107 and PB 217 were seen to be sensitive to chilling (Mai et al. 2010). Efficient reactive oxygen species elimination is crucial in determining the chilling tolerance in *Hevea*.

The areas between 15°N and 20°N of western and eastern India have been identified as non-

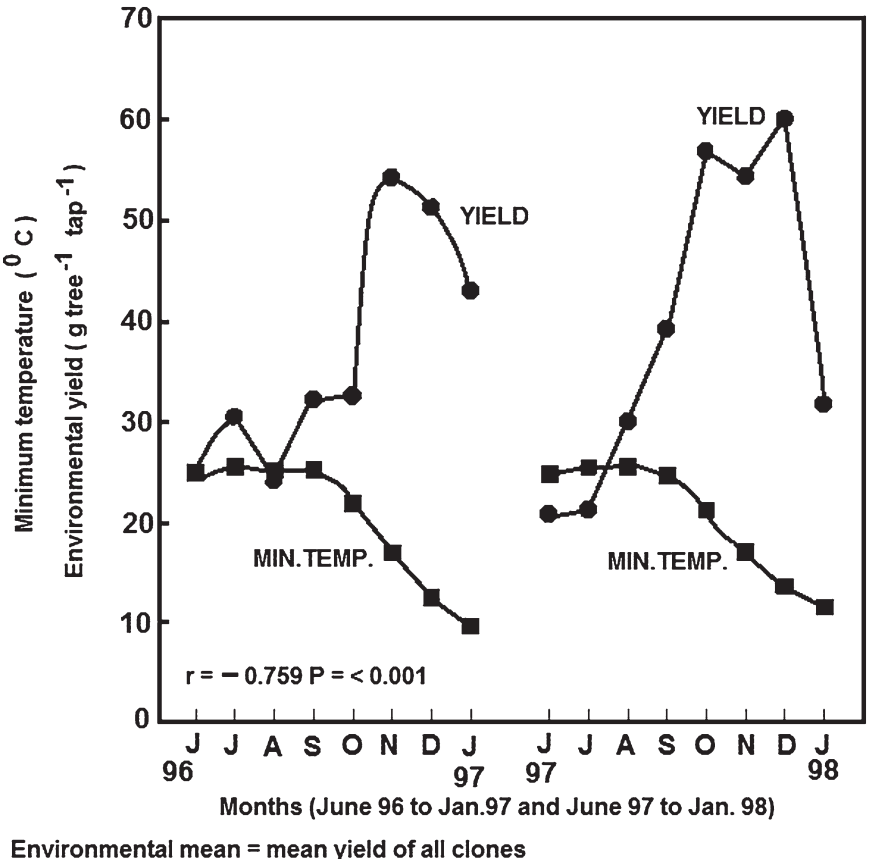

Fig. 9.1 Depiction of minimum temperature and yield over 2 years (1996–1998)

traditional zones for rubber cultivation. For instance, the Konkan region experiences long dry periods, high temperatures, low atmospheric humidity and zero rainfall between September and May; daytime temperatures range at 38–41 °C during summer months, with occasional days getting as hot as 47 °C; though it gets rainfall of 2430 mm, the distribution of that rainfall is uneven (Devakumar et al. 1998). The atmosphere during summer results in high vapour pressure deficit. Almost an analogous situation prevails in the eastern part of India.

Wind is yet another abiotic stress influencing establishment and growth of rubber. One impact is a contribution to the drying effect of drought conditions, especially with regimes of long-lasting steady winds such as met during the dry season in highlands of Vietnam. It is argued that wind speeds of 2.0–2.9 m per second retard rubber growth and latex flow and that speeds of 3.0 m per second and above severely inhibit normal growth (Table 9.3). Winds over Beaufort force 10 (more than 24.5 m per second) play havoc with branch breaks, trunk snaps and uprooting of trees, mainly prevalent in China, during June to October (Watson 1989). Studies in China revealed that clones PR 107 and Haiken 1 can be wind enduring, and PB 5/51 is wind enduring in Tripura (Priyadarshan 2003a, b). Establishment of shelter belts, consisting of fast-growing and wind-resistant species, is one remedial measure being followed in China (Zongdao and Xueqin 1983). But this exercise needs proof taking into account their effects on rubber stand as well as the economic cost of their implementation and land occupation. Alternatively, adoption of judicial pruning of branches and induction of branches at lower height can reduce wind damage from 25.3% to 13.7% (Zongdao and Xueqin 1983; Bernardes et al. 1995). In Côte d'Ivoire,

rubber plantations often experience wind damage due to storms occurring at the onset of the rainy season (April–May). Though hypotheses have been made on the relationship between architecture and wind susceptibility of the clones, no clear factual validation is available (Combe and du Plessix 1974; Hofmann 1981). The quality of rubber wood related to resistance to wind has also been investigated (Ahoba 1985). Tapping is heavily limiting growth rate, to some extent in height but mainly at the level of trunk girth increment, by comparison with non-tapped trees (Clément-Demange et al. 1995). Following these observations, a large-scale design comparing two levels of girth standard (50 and 65 cm) for the initiation of tapping was conducted in Côte d'Ivoire. Although bigger size of the trunk improves the stiffness of the tapped trees, opening after the attainment of 65 cm delays the productive period. A mechanical experiment consisting in artificially bending and breaking trees of different clones also confirmed the prominence of the relationship between the trunk section and the stiffness of the trees, irrespective of the clone (Fourcaud et al. 1999).

9.1.2 Regions of India, Thailand and Vietnam

Climatologically India has five main zones, viz. tropical rain, tropical wet and dry, subtropical rain, temperate and desert (Fig. 9.2). Of these, former three are identified to be suitable for rubber cultivation. Several locations of these zones are counted as non-traditional due to latitude and altitude changes. Northeast India (23–25°N and 90–95°E) offers suitable non-traditional environment for rubber. A low temperature period during November–January, complete defoliated period during February–March, brief moisture stress during March, tropical storms during monsoon (June–August) and *Oidium* infestation during refoliation are the constraints in these states. Rubber is a prominent species in the states of Tripura, Assam, Meghalaya, Mizoram and Arunachal Pradesh. Tripura (22° 56′ and 24° 32′ N and 91° 10′ and 92° 21′ E) is a representative environment of these states and owns a maximum area under rubber. The climate is subtropical (mediocre) with moderate temperature (summer 36.6°–17.9 °C, winter 28.9°–7.17 °C) and high humid atmosphere.

The areas between 15°N and 20°N of western and eastern India also have been identified as non-traditional zones for rubber cultivation. For instance, the Konkan region of western India experiences long dry periods, high temperatures, low atmospheric humidity and zero rainfall between September and May. Daytime temperatures range at 38–41 °C during summer months with occasional days getting as hot as 47 °C. The region gets rainfall of 2430 mm, but with an uneven distribution (Devakumar et al. 1998). High solar radiation coupled with high temperature and low relative humidity results in high vapour pressure deficit between the leaf and the surrounding atmosphere, and this subsequently increases the evapotranspirative demand of the atmosphere. Thus, rubber trees in this region are subjected to prolonged periods of both soil and atmospheric drought stress. Irrigated plants showed 32% increment in leaf area index (LAI) leading to 52% more shoot biomass/tree (Devakumar et al. 1998). Water deficit in the dry period is 1070 mm, whereas in traditional areas it is 350 mm (Jacob et al. 1999). Reduction in girth of trees (0.2–0.5 mm) was observed during summer months. Towards the end of summer, moisture level falls below permanent wilting point (PWP) (17.5%). The atmosphere during summer results in high vapour pressure deficit between the leaves. The high intensities of sunlight, more than what is required to saturate photosynthesis, can aggravate the harmful effects on *Hevea* leaves (Devakumar et al. 1998). Almost an analogous situation prevails in the eastern part of India also. Similarly, the non-traditional areas of Thailand (13–18°N), viz. Chachoengsao (east), Nong Khai and Chiang Mai provinces (northeast), experience a marked dry season for 6 months and severe moisture deficit (temperature 14–39 °C) with a minimum temperature of 5 °C during January (Saengruksowong et al. 1983). Rainfall (1110–1550 mm) is confined to mainly June–September (Watson 1989).

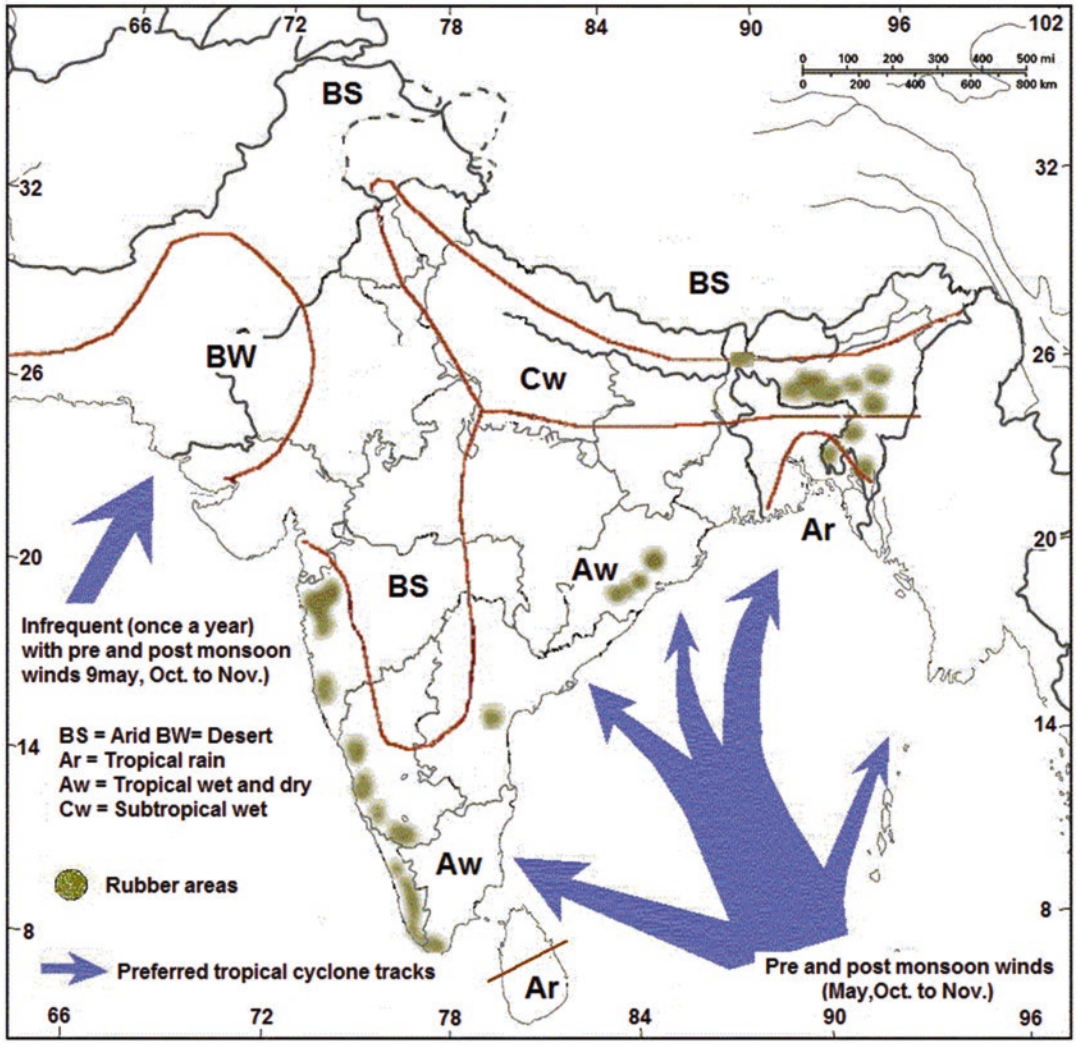

Fig. 9.2 Rubber areas and cyclone tracks of India

The rubber areas of Vietnam are scattered between 12°N and 21°N (Fig. 9.3). The research and development of rubber in non-traditional areas are streamlined depending on altitude, viz. high lands of 450–600 MSL, high lands of 600–700 MSL and coastal regions. Southeast area is the traditional region for rubber where nearly 3 million hectares are under rubber. While southeast region is with relatively flat terrain, highlands and coastal regions are <550 m. The highlands and coastal regions that are non-traditional experience low temperatures (5.5 °C), regular strong winds, rain fall lasting for several days, lesser sunshine and higher number of misty days (Hoa et al. 1998; Tuy et al. 1998). The highlands are predominantly ferrallitic and belong to a major family of red or yellowish-red soils. They are clayey, deep and basalt (Eschbach et al. 1989). Ever since rubber was introduced in 1897, Vietnam has taken steps to extend the area to 500,000 hectares including expansion to marginal areas. Rubber is a second priority crop for Vietnam (Chapman 2000).

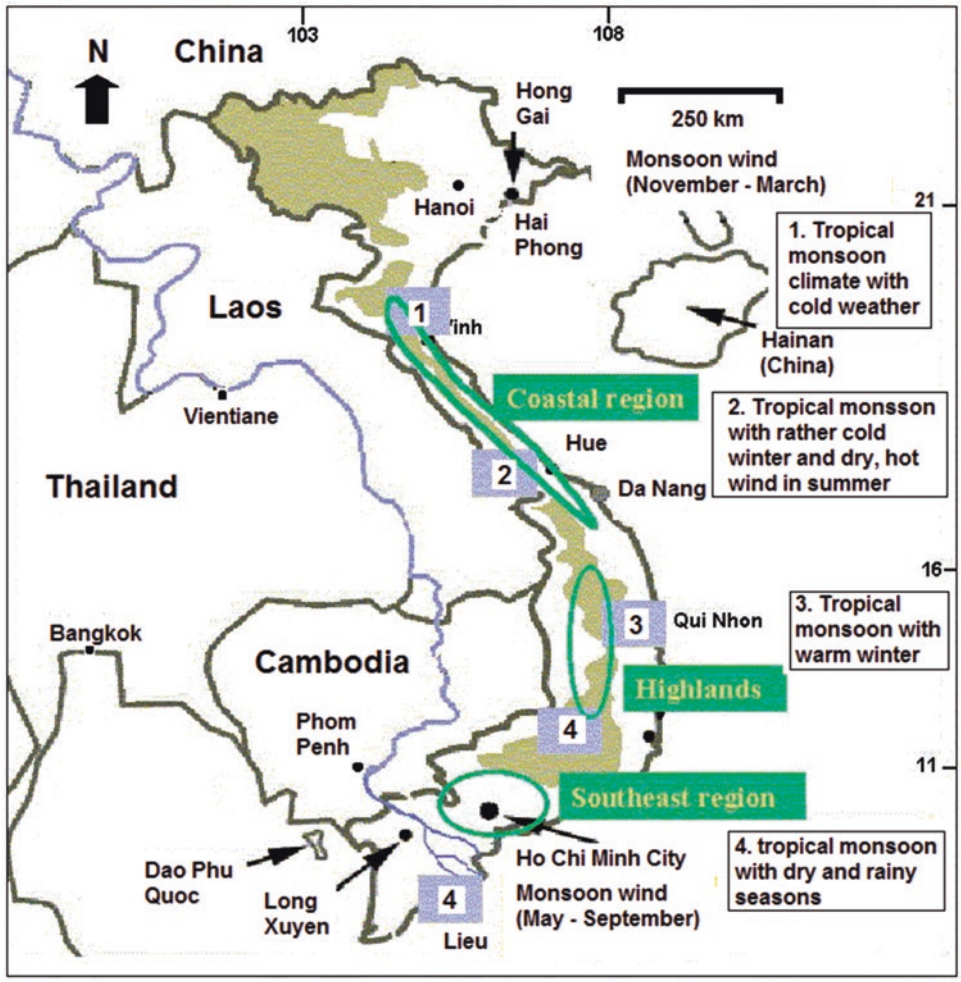

Fig. 9.3 Rubber areas and climatic details of Vietnam

9.1.3 Chinese Conditions

China has been divided into six climatological zones, viz. tropical wet and dry, subtropical wet, subtropical summer rain, temperate, desert and temperate continental (Fig. 9.4). Of these, the former three are being experimented with rubber. The rubber-growing areas of China fall under 18–24°N and 97–121°E, spread over to five provinces of south China, viz. Hainan, Guangdong, Fujian, Yunnan and Guangxi. These areas are under tropics and subtropics having monsoonal climate. Pronounced monsoon and dry seasons prevail from May to November and December to April, respectively. Two types of cold regimes have been identified, viz. *radiative* and *advective* (Zongdao and Xueqin 1983). In *radiative* type, the night temperature falls sharply to 5 °C and the day temperature ranges between 15 and 20 °C or above, while in *advective* type, the daily mean temperature remains below 8–10 °C, with a daily minimum of 5 °C. In both these types, under extreme circumstances, complete death of the plant is the ultimate outcome. Reports from China point that clones GT 1 and Haiken 1 can withstand temperatures up to 0 °C for a short span, while SCATC 93-114 can endure a temperature of even −1 °C. The cold wave conditions prevailing other than China can be conveniently classified as radiative type.

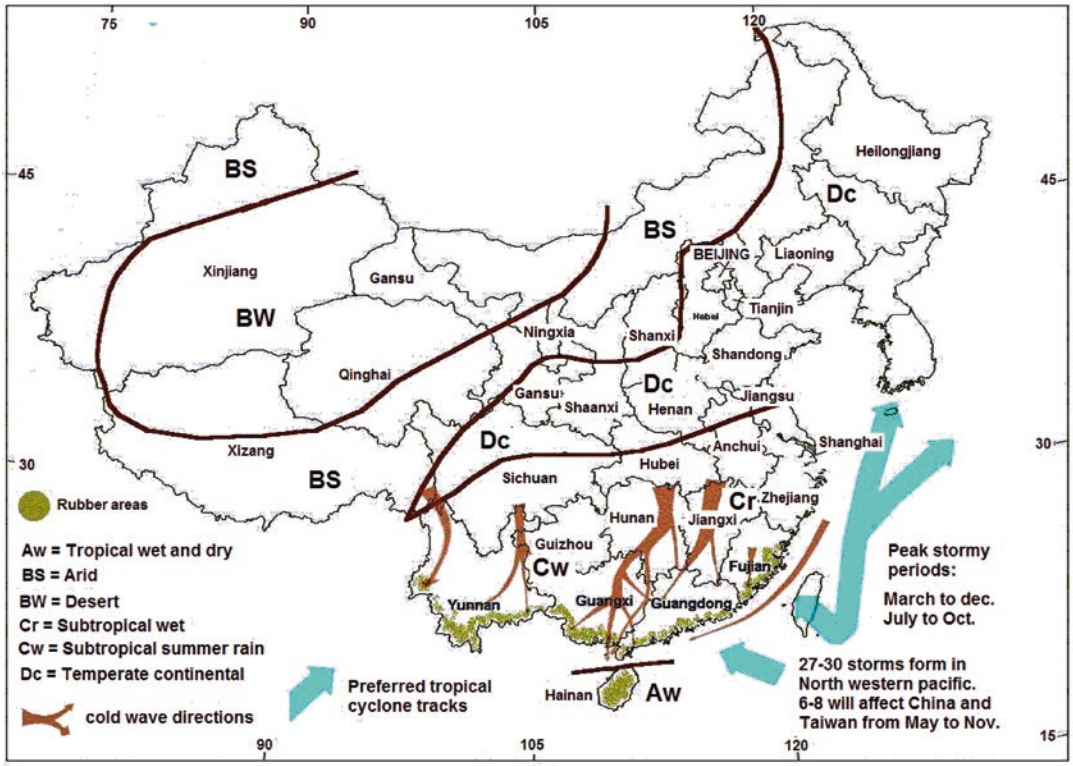

Fig. 9.4 Rubber areas and climatic details of China

9.1.4 Conditions in West Africa

Countries in West Africa (Côte d'Ivoire, Liberia, Ghana, Nigeria, Guinea and Sierra Leone) are suitable for rubber. Rainfall is confined to April to October as southwest monsoon which winds over the Gulf of Guinea, resulting in high rainfall in the coastal region and diminishes steadily northwards. The presence of Mount Cameroon acts as a great barrier for rain-bearing winds to settle and to give the second highest rainfall in the world (1000 cm). These areas also experience an average annual temperature of 25 °C with least diurnal temperature range. Northern parts of the rubber-growing countries experience dry wind popularly known as *harmattan* during November to April, originating in Sahara desert. Cameroon experiences tornadoes during rainy season. Soils are derived from sedimentary rocks, which have been weathered, leached, eroded and deposited. They are naturally deep and poorly supplied with nutrients. But soils of west Cameroon are more fertile and have a tendency to fix nutrients. The coastal areas are densely forested and suitable for rubber.

Côte d'Ivoire, prominent rubber producer, is located between latitudes 5°N and 6°N and at longitudes 3°W and 8°W. Though the areas fall under the tropical belt, water is the limiting factor due to low rainfall. Considering isobar of 1500 mm, and dry season not exceeding 5 months (monthly rainfall below 100 mm), 20% of the area is suitable for rubber cultivation (Dea et al. 1997). Areas towards north are identified as marginal, where rainfall is below 1300 mm. Even under moderate conditions, in spite of favourable rainfall and short dry season, areas having gravelled elements in soil profile impose 20–30% weak growth in rubber (Dea et al. 1997).

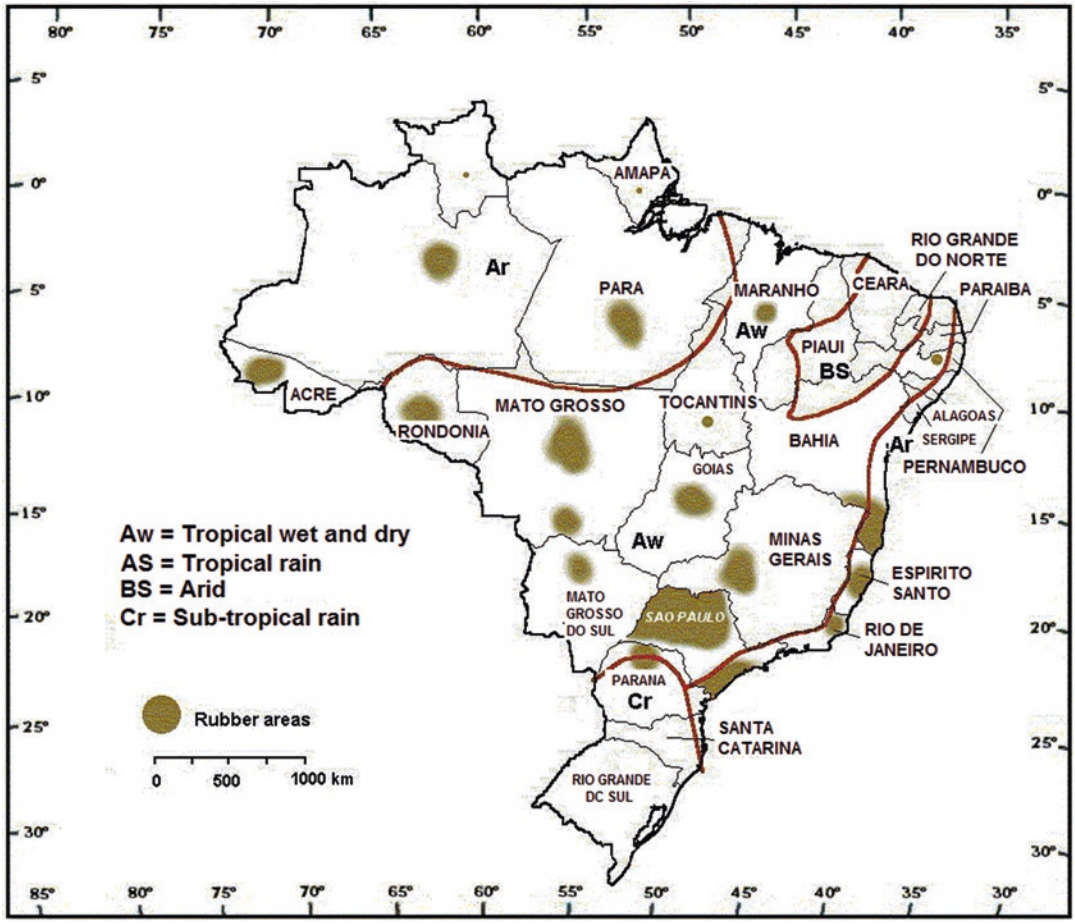

Fig. 9.5 Rubber areas and climatic details of Brazil

9.1.5 Situation in South America

Brazil has four main climatic zones, viz. tropical rain, tropical wet and dry, subtropical rain and temperate (Fig. 9.5). Though the former two are congenial for rubber, the southern plateau of São Paulo (20–24°S, 44–52°W) with tropical wet and dry climate is the main production area, due to the absence of epidemic of SALB (South American Leaf Blight—*Microcyclus ulei*). The most important production region is the northwestern region, where the climate is tropical of altitude type with a summer rainy season from October to March and a cold dry winter from June to August with temperature reaching 15–20 °C. The yearly total rainfall ranges from 1000 to 1400 mm. The ideal altitude for rubber is 350–900 m above sea level. The undulating flat areas are with podzolic and latosolic soils, deep and well drained both with autotrophic and dystrophic types. A few plantations are located in volcanic red soils of high fertility. The low leaf wetness duration and relative low temperature in the winter reduce the epidemics of SALB (Gonçalves et al. 1999).

9.2 Phenology Under Differential Geo-Climates

Hevea exhibits significant changes in phenology under differential geo-climates (Table 9.4). The shift in cultivation towards north and south of equator induces phenological changes amply.

Table 9.4 Seasons and phenological attributes expressed during various periods in Tripura and São Paulo

Phenology	Tripura[a]	São Paulo[b]
Defoliation	December–January	August–September
Refoliation	February–March	September–October
Flowering	March–April	October–November
Lean yield	May–September	August–January
Peak yield	October–December	February–July
Rainy season	May–October	October–March
Winter	November–January	June–August

[a]Priyadarshan et al. (2000a)
[b]Ortolani et al. (1998)

A comparison is made here in relation to Tripura (northeast India) and São Paulo (south Brazil). Rubber in Tripura experiences wintering during December–January, and reflushing commences by February, followed by flowering (Priyadarshan et al. 2001). The yielding pattern of clones in Tripura shows a clear delineation of low- and high-yielding regimes (Priyadarshan et al. 1998a). There is a multitude of factors that induce a low-yielding period, viz. low temperature, utilization of carbohydrate reserves for refoliation, flowering, fruit development during February–April, low moisture period during February–March and powdery mildew (*Oidium heveae* Stein.) infestation. These factors together impose an ensuing low-yielding period during May–September (Priyadarshan et al. 2000a, b). A photoperiodic stimulus, operating because a slight change in the proportion of light to dark in the day length is sufficient enough to trigger flowering, has been very well established in several tropical tree species (Baker et al. 1983). However, the fall in temperature during October–November stimulates yield while the mean temperature is 28 °C, making the atmosphere most ideal for latex flow and production. Minimum temperature experienced early morning shall be 15–18 °C, and after 10 a.m., the temperature shoots to 27–28 °C. While the former is congenial for latex flow, the latter is ideal for latex regeneration through accumulation (Ong et al. 1998). The plateau region of São Paulo state, an escape area for SALB, has been referred to as the most important rubber-growing region of Brazil (Costa et al. 2000). While Tripura lies at 22–24°N, São Paulo is at 20–22°S (400–500 m MSL) making these areas non-traditional. Unlike Tripura, trees are exploited throughout the year in São Paulo. Reflushing, flowering and seed fall are experienced once a year in São Paulo (Ortolani et al. 1998).

Plants react to different environments through phenological changes. For instance, defoliation is a phenomenon to circumvent moisture and low-temperature stresses through minimizing transpiration so as to ensure reproduction, seed dispersal and perpetuation of generations. Flowering and fruit formation utilize large amount of carbohydrate reserves. Hence, flowering and fruit formation precede a low-yielding phase in rubber both in Tripura and São Paulo (Figs. 9.6 and 9.7). The environmental conditions inducing defoliation, flowering, and low- and high-yielding periods are analogous. The peak yielding period in São Paulo is January–May, followed by winter and defoliation, while in Tripura January–May is the low-yielding period. The latitudinal changes towards south and north of equator induce phenological changes in *Hevea* in vice versa fashion. It is noteworthy that São Paulo is a disease-free zone for South American leaf blight (SALB). Similarly, Tripura experiences only *Oidium heveae.* But the traditional regions experience the incidence of at least two other diseases (*Corynespora* and *Phytophthora*). It may be pertinent to believe that low temperature and dry spell extend an antagonistic effect over the environment to reduce disease incidence in a particular zone. Moreover, the non-existence of alternate hosts shall be another reason for disease-free atmosphere. Since the peak yielding periods in both south and north of equator are in a vice versa fashion, it is evident that rainfall and minimum temperature in a region are the major factors influencing yield and phenology of rubber. It is also known that dry period increases and mean temperature decreases when one moves away from the equator. Minimum temperatures in the range of 15–20 °C influence the

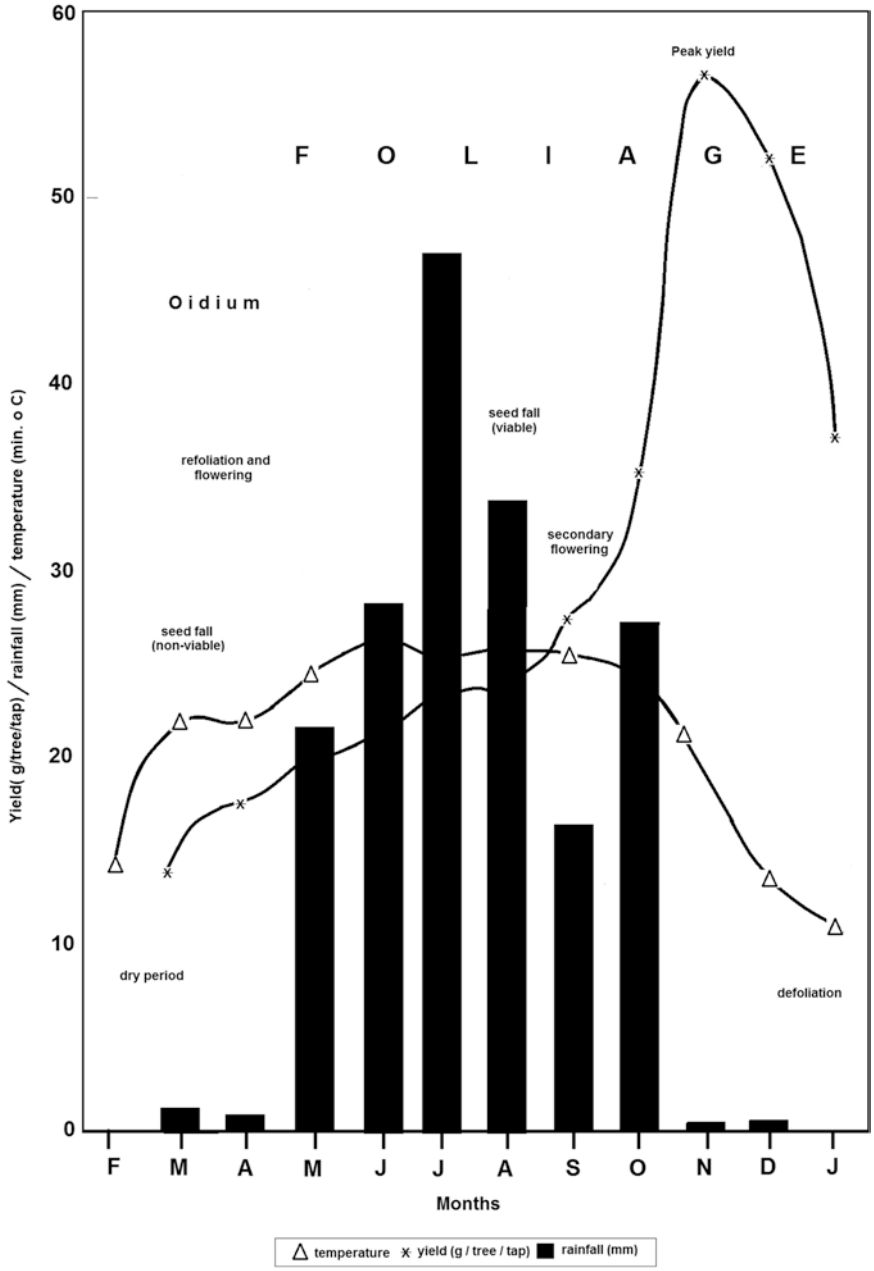

Fig. 9.6 Phenology under Tripura (northeast India)

yield in north and south of the equator. The effect of rainfall assumes significance only after 2 months in increasing yield. The differential phenology in *Hevea* on both sides of the equator might be advantageous towards shortening the testing cycle. For instance, Sumatra island (Indonesia) has two conspicuous flowering seasons, since the equator passes through its centre (Priyadarshan et al. 2001). This needs to be exploited fully by the breeders. The annual cycle of solar radiation intensity is shown to correspond closely with the flowering near the equa-

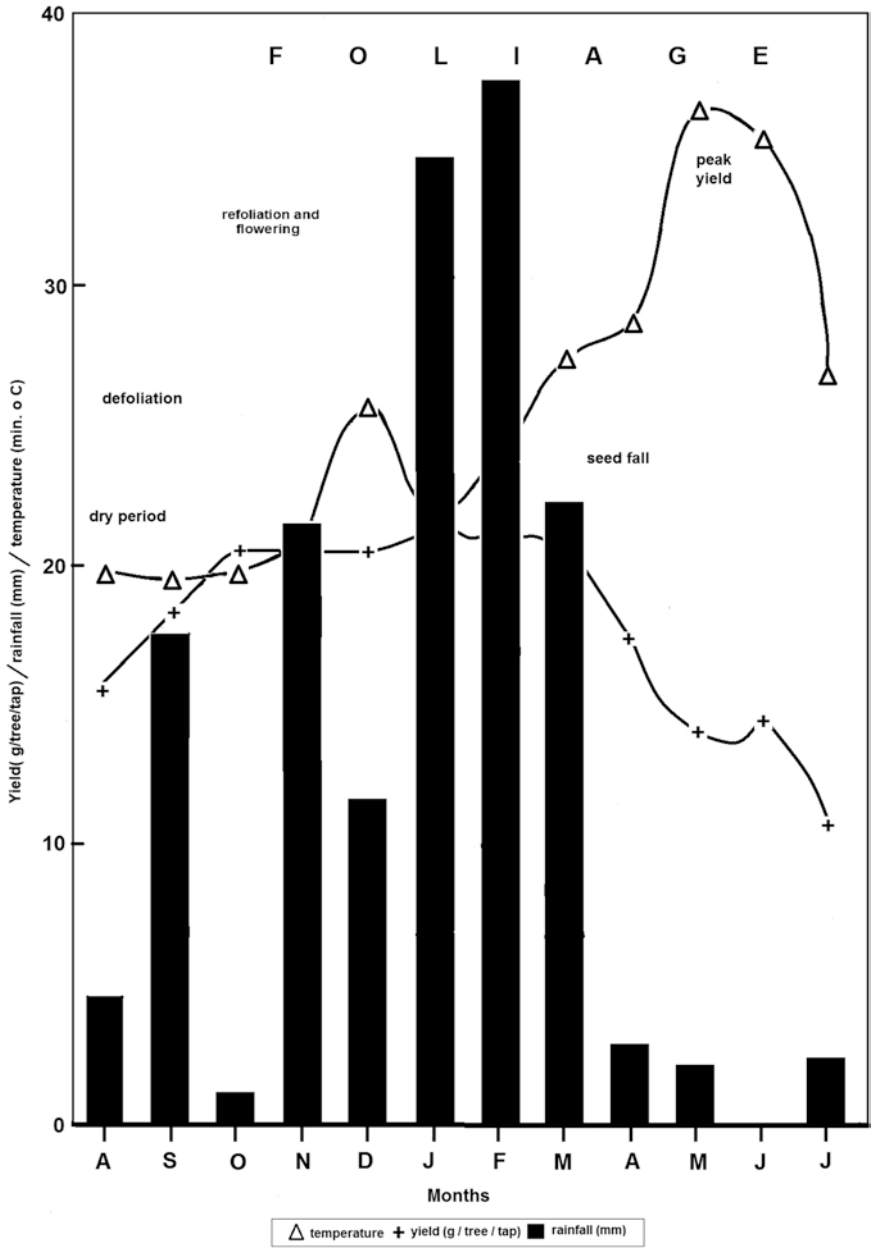

Fig. 9.7 Phenology under São Paulo (Brazil)

tor and in the subtropics. While in temperate regions the incoming solar radiation (insolation) is dependent on both day length and radiation intensity, the insolation at the equator is entirely due to radiation intensity. High solar radiation and bright sunshine induce synchronous anthesis around spring and autumn equinoxes at the equator (Yeang 2007).

9.3 Immature Phase Under Sub-optimal Environments

Clones that attain required girth early (50 cm) are preferred under a new environment, since that is the first sign of adaptation. In a comparison of girth increment of RRIM 600 in traditional and

non-traditional areas of India, Sethuraj et al. (1989) reported 4.3 cm less girth in the northeast region of India compared to traditional zone. While RRII 105 is counted as one of the best suitable clones for the traditional areas, PB 235, RRIM 600, RRII 208 and Chinese clone Haiken 1 are seen to be adaptable in the northeast region of India (Priyadarshan et al. 2000a, b; Mondal et al. 1999). Girth increment is seen to be minimum during winter months (November to January; Priyadarshan et al. 1998a). These preliminary evaluations amply rationalized that clones, which perform well under traditional areas, do not behave similarly under non-traditional environments. In the water-limiting environment of Konkan region (west India), shrinkage of tree stems has been observed during moisture deficit period (March–June). The monsoon period (July–August) though experiences cloudy and low sunshine hours, girth increment indicated trees received adequate photosynthetically active radiation. Also, a full potential irrigation during dry period gave maximum growth that is 50% less than the growth observed in the preceding monsoon period (Mohanakrishana et al. 1991), presuming that *Hevea* prefers low vapour pressure deficits for growth. Clonal differences were evident in stomatal characteristics in trees grown under moisture stress (Chandrashekar et al. 1997). While Konkan region experiences active girth increment between July and September, in northeast India, May–August is the congenial period for better growth (Priyadarshan et al. 1998a). Both the regions require 8–9 years for the trees to attain maturity. In a comparative study involving 15 clones of Indian, Malaysian, Sri Lankan and Indonesian origin, RRII 208, RRIC 52, RRII 6, RRIC 100 and RRIC 102 were seen to exhibit better growth in the Konkan region of India. Even in low-temperature affected northeast India, RRII 208 showed better growth in addition to PB 235, RRIM 600, RRII 118 and SCATC 93/114. Evidently, these clones were developed under hydrographic environments specific to each location. However, RRII 105, a potential clone for traditional region, was not adjudged as drought/low temperature tolerant and hence not adapted to these conditions. However, Rao et al. (1990) reported that RRII 105 responded well to dry weather of traditional areas through higher values of stomatal resistance, leaf water potential and lower transpirational water loss. This differential performance needs to be studied in depth with physiological tools. In a comparative stability analysis of girth in Tripura, Haiken 1, PR 107 and SCATC 93/114 were seen to be more stable. However, higher contribution towards girth increment was seen in RRII 208 followed by Haiken 1 and SCATC 93/114. Clones with higher stability were with wind endurance also (Priyadarshan et al. 1998a).

In an analysis with clones of vivid geographical origin (GT1, AVROS 2037, RRIM 600, PB 217 and PB 235) under different locations in Côte d'Ivoire, Dea et al. (1997) demonstrated growth is influenced by the availability and extent of rainfall. Rainfall in these areas varied from 1090 to 1600 mm with 4–6 dry months. Trees took 7–8 years to attain maturity. A similar exercise was done in Vietnam, where non-traditional areas imposed immaturity period of 1.5–2 years more compared to traditional zones (Tuy et al. 1998). Immaturity period increased with altitude. GT 1, RRIC 110, RRIC 121, PB 235 and VM 515 were seen to be with higher girth increment.

9.4 Yield Depression, Patterns, Regimes and Specific Adaptation

Like immature phase, the mature phase of rubber also exhibits differential performance of clones under various non-traditional environments with single or multitude of stresses. Yield depression during a specific period is the main setback when we examine the phenotypic expression of this attribute of *Hevea* under marginal conditions. This is evident when yield profiles are taken

from Tripura (India), São Paulo (Brazil) and highlands of Vietnam, where two yielding regimes are prudent in a year (Fig. 9.8). Months preceding the low temperature period seldom experience depression in yield. In India, in the northeastern states, May–September used to experience a low-yielding period. This is the carried-over effect of stress periods that is not prudent in traditional areas. There are multitude of factors that induce a low-yielding period, viz. low temperature (November–February), utilization of carbohydrate reserves for refoliation (February–March), flowering and fruit development after refoliation (April–August), low moisture period (March) and incidence of leaf diseases during refoliation (February–March). These factors together impose an ensuing low-yielding period (Priyadarshan et al. 2000a). An analogous situation prevails in the non-traditional areas of Brazil (southern plateau), but in a vice versa fashion (Ortolani et al. 1998; Priyadarshan et al. 2001). However, fall in temperature during November stimulates yield. The daily temperature range in non-traditional areas of northeast India during winter is around 8–12 °C, making the atmosphere most ideal for latex flow and production. Minimum temperature experienced in the early morning during tapping is 15–18 °C, and after 10 a.m., the temperature shoots to 27–28 °C. While the former is congenial for latex flow, the latter is ideal for latex regeneration through accumulation of rubber particles (Ong et al. 1998). The rubber-growing areas of Vietnam fall under the same latitude range and experience the same trend. However, areas of China are diversified and hence exhibit a trend depending on the temperature and altitude.

Chinese clones Haiken 1, SCATC 88-13 and SCATC 93-114 are being evaluated in Tripura. Initial yielding pattern shows Haiken 1 to be a high yielder against RRIM 600 as a local check (Priyadarshan et al. 1998b). SCATC 93-114 is proclaimed as cold endurant under Chinese conditions (Zongdao and Xueqin 1983) and shows the same trend in Tripura also (Priyadarshan et al. 1998b). There are clones that show consistency in yield over months, viz. PB 235, RRII 203, and RRII 208. Among these, PB 235 has been evaluated under differential conditions. PB 235 shows consistency under stressful conditions of Tripura (low-temperature area), Côte d'Ivoire (high minimum temperature) and Vietnam (high altitude; Priyadarshan et al. 2000b; Dea et al. 1997; Thanh et al. 1998). Its latex contains low sucrose concentrations implying rapid utilization of the precursor. Its biosynthetic activity is also seen to be intense with higher values of latex yield, dry extract (dry rubber yield) and high inorganic phosphorus (Pi) with a rapid regeneration between two tappings (Serres et al. 1994; Jacob et al. 1995). PB 235 does not tend to increase yield significantly at longer tapping intervals. Such observations were made under warm climatic conditions of Côte d'Ivoire (Serres et al. 1994), which amply conform to the inferences drawn from our studies on yielding trends (Priyadarshan et al. 2000a, b). The aforesaid attributes of PB 235 amply suggest its utility for Tripura, Côte d'Ivoire and Vietnam, which can be confirmed through on-farm trials. The low wind tolerance of PB 235 shall be circumvented through induction of branches at a lower height (2 m), high-density planting and commencement of tapping upon attainment of 60 cm girth instead of the usual 50 cm (Clement-Demange et al. 1998). GT 1 is yet another clone that deserves special mention, since it is counted as a high-yielding clone in China (Zongdao and Xueqin 1983; Zongdao and Yanqing 1992). GT 1 has not been counted as a high yielder in Tripura, though Tripura and rubber-growing areas of South China fall under the same latitude range. This disparity in yielding potential could be attributed to diverse climatic and edaphic factors. A comparison of yield and secondary attributes of clones evaluated in Tripura and São Paulo would reveal their differential performance (Tables 9.5 and 9.6).

In Vietnam, clones are being evaluated under different altitude ranges. While PB 312, PB 280, RRIC 101 and RRIC 130 gave 100–146% more yield than GT 1 under altitudes 650 m, PB 235, VM 515 and PB 255 exhibited 72–93.5% yield

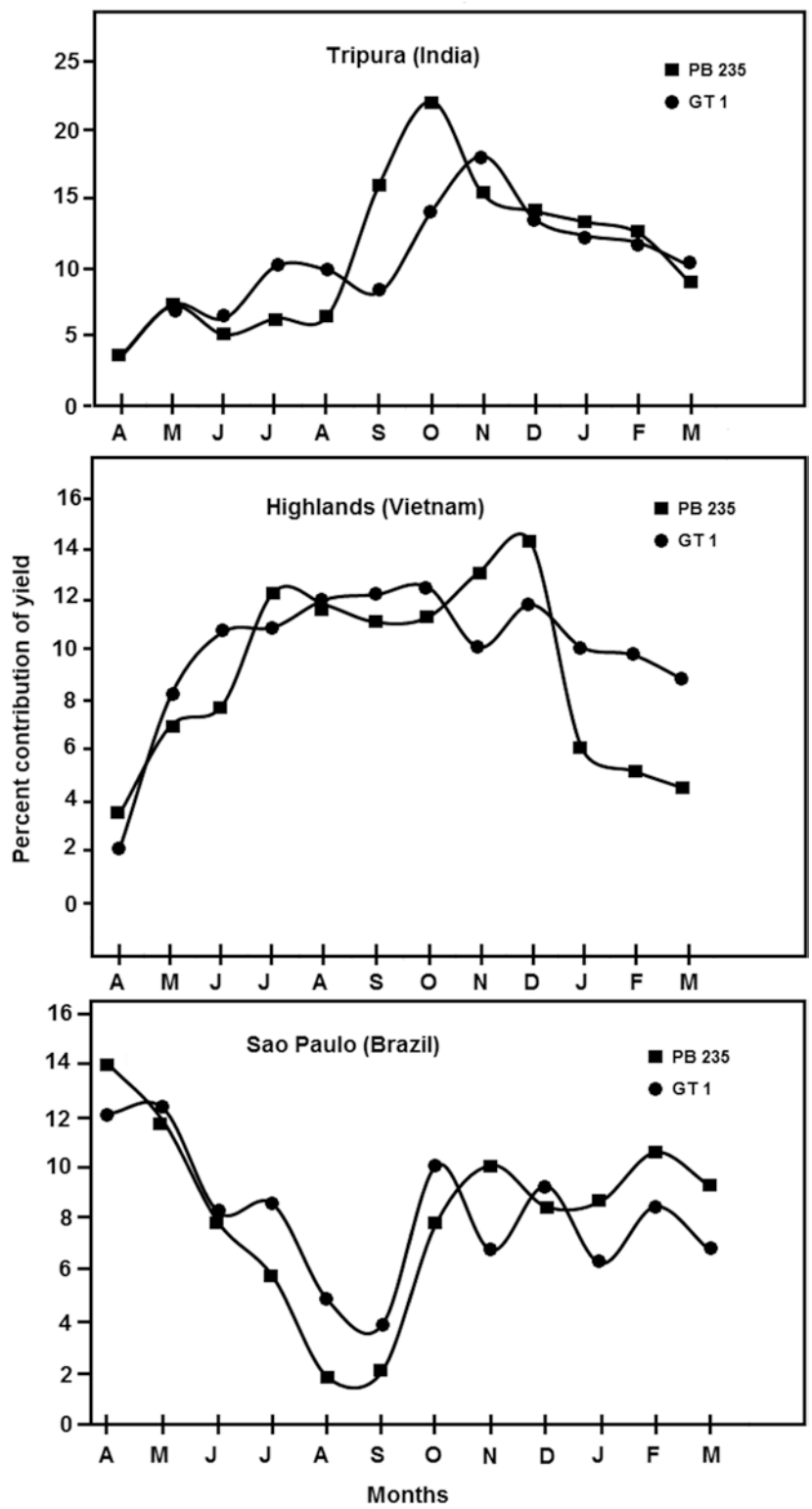

Fig. 9.8 Yielding trends over different months in Tripura (northeast India) and highlands of Vietnam and São Paulo (Brazil). All are non-traditional areas for rubber

Table 9.5 Yield and secondary attribute of 20 clones evaluated in Tripura

Clone	Stand (initial)	Girth (mature)	Yield (projected) kg/ha	Crop efficiency[c]	Wind damage	TPD	*Oidium* incidence
RRII 5	Average	Low[a]	1118[e]	0.85	Moderate	Low	Severe
RRII 105	Good	Moderate[a]	1297[e]	1.00	Moderate	Low	Severe
RRII 118	Good	High[a]	1389[e]	1.07	High	Mild	Moderate
RRII 203	Good	Moderate[a]	1647[e]	1.14	Low	Low	Mild
RRII 208	Good	Moderate[b]	1597[f]	0.93	High	Very mild	Severe
RRIM 600	Good	Moderate[a]	1499[e]	0.99	Low	Moderate	Severe
RRIM 605	Good	Moderate[a]	1074[e]	0.74	Moderate	Moderate	Moderate
RRIM 703	Average	Moderate[a]	1426[e]	1.21	Moderate	Low	Mild
RRIC 52	Average	Moderate[a]	992[e]	0.51	High	Low	Mild
RRIC 105	Average	High[a]	1093[e]	0.59	High	Low	Low
PB 5/51	Good	Low[a]	984[e]	0.74	Low	Mild	Very severe[d]
PB 86	Good	Low[a]	1083[e]	0.77	Moderate	Low	Moderate
PB 235	Good	High[a]	1858[e]	1.34	Moderate	Moderate	Severe
GT 1	Good	Moderate[a]	1077[e]	0.85	Low	Mild	Moderate
Gl 1	Good	Low[a]	557[e]	0.44	Mild	Low	Severe
HARBEL 1	Average	Low	752[e]	0.58	Low	Low	Severe
PR 107	Good	Good[b]	878[f]	0.29	Very low	Mild	Very severe[d]
SCATC 88/13	Good	Good[b]	910[f]	0.67	Low	Moderate	Severe
SCATC 93/114	Good	Good[b]	822[f]	0.24	Medium	Very mild	Low
Haiken 1	Good	Good[b]	1011[f]	0.68	Medium	Mild	Moderate

Projected yield = g/tree/tap × no. of tappings × total stand (350)
[a]Over 13 years
[b]Over 7 years
[c]g/cm of the tapping cut
[d]With secondary infection
[e]D panel
[f]B panel

increase under altitudes of 450–600 m (Tuy et al. 1998). This evidently indicated that the performances of clones are not complimentary under differential altitudinal climates (Table 9.7). In Thailand, nearly 2.6 million hectares are delineated in the north and northeast regions, which has been divided into three zones depending on soil profile and climatic information. GT1, PB 28/59, RRIM 600 and PB 5/51 are the prominent clones adapted to these regions (Krisanasap and Dalkit 1989; Watson 1989). An insight into the impact of climate would amply rationalize the role of certain attributes over the yielding ability of clones. Minimum temperature, wind velocity and evaporation are seen to have negative correlation with monthly mean yield (Priyadarshan et al. 2000a). The rationale is that fall in temperature along with reduced evaporation and low wind speeds prevails upon the microenvironment to influence yield stimulation during cold period. It is evident that turgour pressure in laticiferous system is vital for the flow of latex and yield. Turgour pressure as high as 10–14 atmospheres is seldom observed before sunrise, and studies on diurnal variations in latex yield gave a correlated response between latex yield and variations in atmospheric vapour pressure (Moraes 1977). The atmospheric vapour pressure is very high during cold months thus increasing the latex flow. But there are clones like PB 235 and

Table 9.6 Yield and secondary attributes of 20 clones being evaluated in the plateau region of São Paulo state (data provided by P. de S. Gonçalves)

Clone	Stand (initial)	Girth[a] (mature)	Yield[a] (projected) kg/ha[e]	Crop efficiency[b]	Wind damage	TPD	*Oidium* incidence
RRIM 600	Good	Moderate	2100[c]	0.83	Low	Moderate	Low
PB 235	Good	High	1834[c]	1.10	Moderate	Mild	Severe
GT 1	Good	High	1679[c]	0.95	Moderate	Moderate	Moderate
PR 255	Good	Moderate	1700[d]	0.93	Moderate	Moderate	Mild
PR 261	Average	Moderate	1973[d]	1.21	Moderate	Low	Mild
IAN 873	Good	Moderate	1890[c]	1.02	Moderate	Low	Moderate
Fx 3864	Good	High	1755[c]	1.07	Moderate	Low	Moderate
PB 330	Good	Moderate	1980[c]	0.99	Low	Mild	Moderate
PB 217	Average	Low	2100[c]	0.68	Moderate	Mild	Mild
PR 107	Good	Moderate	1870[c]	0.55	Low	Mild	Moderate
IAC 35	Good	Moderate	2100[c]	0.82	Low	Moderate	Low
IAC 40	Good	High	2400[c]	1.20	Moderate	Moderate	Low
IAC 56	Average	Moderate	1900[c]	0.95	Moderate	Moderate	Low
IAC 300	Good	High	2200[c]	0.82	Moderate	Moderate	Moderate
IAC 301	Good	Moderate	2100[c]	0.65	Low	Moderate	Low
IAC 302	Good	Moderate	1800[c]	0.80	Moderate	Low	Moderate
IAC 303	Good	Moderate	5191[c]	0.82	Low	Low	Low
IAN 4493	Average	High	1711[c]	0.93	Moderate	Moderate	Low
RO 45	Average	High	1500[c]	0.70	Moderate	Low	Low
IAN 3156	Low Average	Moderate	2500[c]	0.60	Moderate	Moderate	Moderate

[a]over 7 years
[b]g/cm of the tapping cut
[c]Tapping system 1/2S d/3 6d/7 (with ethephon stimulation 2.5%)
[d]Tapping system 1/2S d/2 6d/7
[e]Prospected yield = g/tree/tap × number of tapping × total stand (400)

RRII 208 that show less stimulation towards the onset of cold period. Studies conducted revealed clones, especially PB 235 and GT 1, as less responsive to Ethrel stimulation (Gohet et al. 1995). From these observations, it can very well be presumed that PB 235 is less responsive to stimulation irrespective of the stimulant, which is a positive attribute. PB 235 owns a specific adaptive mechanism, whereby it yields more when ambient temperature ranges from 22 to 28 °C. When all clones continue with a higher yield in combination with descending temperature, PB 235 recedes yield during January when the ambient temperature gets below 15 °C. Studies conducted in China with few other clones endorse the same trend in GT 1 (Zongdao and Xueqin 1983). Ambient temperatures ranging from 18 to 24 °C are conducive for latex flow (Zongdao and Yanqing 1992) (see Table 9.8 for yield and secondary attributes in China). Evidently, the existence of genetic homeostasis and their subsequent expression in the changed environment might be the reason for the near-uniform yielding trend in these clones. Through homeostasis, perhaps, yield is reduced and the source–sink relations are brought to equilibrium to ensure the survival during cold/stimulated period.

The trend shown by clones under sub-optimal environments is in sharp contrast to that of traditional areas of India where RRII 105 and RRIM 600 are prominent yielders when evaluated separately (Nazeer et al. 1991; Mydin et al. 1994). In Tripura, a comparison of yielding trends of PB

Table 9.7 Main characteristics of clones under marginal areas of Vietnam (data provided by T.T.T. Hoa)

Kontum Province (highlands—550 m a.s.l. grey soil)						
Clone	Girth at opening	Girth (mature)	Yield over 10 years in kg/ha (g/tree/tap)	*Oidium* infestation	*Phytophthora* leaf fall	TPD
GT 1	Moderate	Moderate	1191 (46.4)	Moderate	Moderate	Moderate
PB 235	High	Moderate	1607 (59.8)	Severe	Low	Moderate
PB 255	Moderate	Moderate	1174 (56.2)	Moderate	Moderate	High
PB 310	Moderate	Moderate	1659 (52.8)	Low	Low	Moderate
PR 255	Low	Moderate	1191 (49.8)	Moderate	–	Moderate
PR 261	Low	Moderate	1197 (64.2)	Moderate	–	High
RRIC 110	High	Moderate	1558 (66.3)	Low	Moderate	High
RRIM 600	Moderate	Moderate	1177 (57.9)	Low	High	Moderate
VM 515	Moderate	Moderate	1539 (63.4)	Moderate	High	High
Dak Lak Province (highlands—700 m a.s.l., basaltic soil)						
Clone	Girth at opening	Girth (mature)	Yield over 7 years (kg/ha)	*Oidium* infestation	*Phytophthora* leaf fall	TPD
GT 1	Moderate	Moderate	1005	Moderate	Moderate	Moderate
PB 235	Moderate	Moderate	998	Severe	Low	Moderate
PB 310	Moderate	Moderate	1065	Low	Low	Moderate
PR 107	Low	Moderate	669	Severe	–	Moderate
RRIC 110	High	Moderate	1422	Moderate	Moderate	Moderate
RRIM 600	Moderate	Moderate	1153	Low	High	Moderate
RRIM 712	Moderate	Moderate	1170	Moderate	Moderate	Moderate
VM 515	Moderate	Moderate	1056	Moderate	High	High
RRIV 1	Moderate	Moderate	1236	Low	Moderate	Low
Quang Tri Province (coastal region—50 m a.s.l., basaltic soil)						
Clone	Girth at opening	Girth (mature)	Yield over 5 years (kg/ha)	*Oidium* infestation	*Phytophthora* leaf fall	TPD
GT 1	Moderate	Moderate	966	Low	–	–
PB 235	High	High	1368	Low	–	–
PB 310	High	High	1005	Low	–	–
RRIM 600	Moderate	Moderate	1355	Low	–	–
LH 82/92	High	Moderate	1281	Low	–	–

TPD tapping panel dryness
RRIV 1, LH 82/92 = clone bred by RRIV

235 and RRIM 600 rationalized that these clones under a specific environment express 'cross-over' type of GE interactions, wherein 28 g represents the threshold level below which these clones are expected to experience stress (Priyadarshan et al. 2000a) (Fig. 9.9). Presumably, a clone giving more than 28 g/tree/tap shall not experience any stress. Clones of varied geographical origin could be delineated into three groups, viz. high-, moderate- and low-yielding clones. RRII 203 and PB 235 exhibited least depression in yield (18.6% and 31.9% respectively) during regime I (Priyadarshan et al. 2000b). In general, dry rubber yield and minimum temperature showed a negative relationship with minimum temperature (see Sect. 9.1.1). However, PB 235 lacked any such relationship when considered individually, indicating thereby that it is less sensitive to yield stimulation towards the onset of cold season (Priyadarshan et al. 2000b). Also, in these environments, PB 235 has been adjudged as a high-yielding clone. Performances of *Hevea* clones

Table 9.8 Yield and secondary attributes of some clones in China (data provided by H. Huasun)

Clone	Site	Girth	Yield *kg/ha*	Years of tapping	Wind damage	Cold damage	*Oidium* incidence	TPD	Stand
GT1	Yunnan	Moderate	1257.2	9	–	Low	Moderate	Moderate	Commercial
RRIM600	Yunnan	Moderate	1190.3	10	Moderate	Moderate	Moderate	Moderate	Commercial
PR107	Yunnan	Moderate	1007.9	10	very low	Moderate	Severe	Low	Commercial
GT1	West Guangdong	Low	994	9	–	Low	Moderate	Moderate	Commercial
93–114	West Guangdong	Low	980.3	9	–	Very low	Moderate	Low	Commercial
YUNYAN 77-2	Yunnan	Moderate	1874.5	9	–	Low	Severe	Mild	Advanced trial
REYAN 88-13[a]	Hainan	Moderate	1700	8	Moderate	Moderate	Severe	Moderate	Advanced trial
REYAN 7-33-97	Hainan	Moderate	1910	9	low	Low	Moderate	Moderate	Advanced trial
REYAN 8-333	Hainan	Moderate	2187	7	Moderate	Low	Moderate	Moderate	Advanced trial
DAFENG95	Hainan	Moderate	1509.6	8	Low	Low	Moderate	Low	Advanced trial
WENCHANG11	Hainan	Low	1953.5	10	Very low	Moderate	Low	Moderate	Advanced trial
HAIKEN 1	Hainan	Low	886.6	10	Very low	Moderate	Severe	High	Advanced trial

Tapping systems:
The first three tapping years: s/2·d/3, and without ethylene stimulation, about 75 tapping days per year
After first 3 years of tapping: s/2·d/2, and without ethylene stimulation, about 110 tapping days per year
[a]Erstwhile SCATC

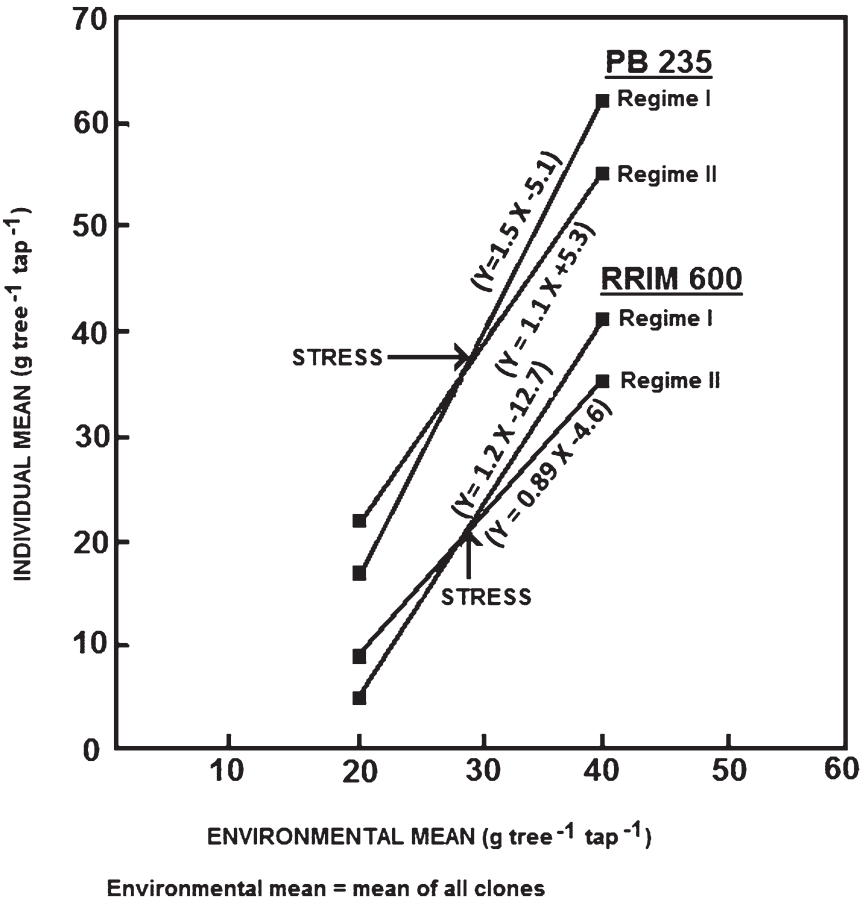

Fig. 9.9 Regression of yield of PB 235 and RRIM 600 over environmental yield under two regimes

under immature and mature phases are different, and the clone that attains maturity early need not necessarily be the best yielding clone. This is due to a lack of significant relationship between girth increment and yield.

9.5 Tree Physiology Under Stressed Environments

The rapid and widespread expansion of rubber plantations in Southeast Asia calls for a greater understanding of rubber tree physiology and the potential impacts of high water consumption on local hydrology (c.f. Guardiola-Claramonte et al. 2008). Kobayashi et al. (2014) undertook sap flow measurements to study the intra- and inter-annual variations in transpiration rate (Et) in a rubber stand in the low-elevation plain of central Cambodia. Mean stand sap flux density (JS) indicated that rubber trees actively transpire in the rainy season but become inactive in the dry season. A sharp, brief drop in JS stand sap flux occurred simultaneously with leaf shedding in the middle of the dry season in January. Although the annual maxima stand sap flux of JS were approximately the same in the two study years, the maximum daily stand Et transpiration of ~2.0 mm day^{-1} in 2010 increased to ~2.4 mm day^{-1} in 2011.

Canopy-level stomatal response was influenced by changes in solar radiation, vapour

pressure deficit, soil moisture availability, leaf area and stem diameter. After 2 years of growth in stem diameter, transpiration potential was comparable to other species. The sensitivity of canopy conductance (gc) to atmospheric drought indicated isohydric behaviour (maintenance of a constant leaf water potential at midday, which is similar in well-irrigated plants and under drought conditions) of rubber trees. Modelling also predicted a relatively small sensitivity of canopy conductance to the soil moisture deficit and a rapid decrease in canopy conductance under extreme drought conditions. However, annual observations suggest the possibility of a change in leaf characteristics with tree maturity and/or initiation of latex tapping. The estimated annual stand transpiration rate was 469 mm $year^{-1}$ in 2010, increasing to 658 mm $year^{-1}$ in 2011.

Subsequently, Giambelluca et al. (2016) investigated the effects of expanding rubber cultivation on water cycling in Mainland Southeast Asia (MSEA). Evapotranspiration (*ET*) was measured within rubber plantations at Bueng Kan, Thailand, and Kampong Cham, Cambodia. After energy closure adjustment, mean annual rubber *ET* was 1211 and 1459 mm $year^{-1}$ higher at the Thailand and Cambodia sites, respectively. These rates were higher than that of other tree-dominated land covers in the region, including tropical seasonal forest (812–1140 mm $year^{-1}$) and savannah (538–1060 mm $year^{-1}$). The mean proportion of net radiation used for *ET* by rubber (0.725) was similar to that of tropical rainforest (0.729) and much higher than that of tropical seasonal forest (0.595) and savannah (0.548). Plant area index (varies with leaf area changes) explains 88.2% and 73.1% of the variance in the ratio of latent energy flux (energy equivalent of *ET*) to potential latent energy flux (LE/LE_{pot}) for midday rain-free periods at the Thailand and Cambodia sites, respectively.

High annual rubber *ET* resulted from high late dry season water use, associated with rapid refoliation of the *Hevea* trees, facilitated by tapping of deep soil water and by very high wet season *ET*, a characteristic of deciduous trees (Giambelluca et al. 2016). Spatially, mean annual rubber *ET* increases strongly with increasing net radiation (R_n) across the three available rubber plantation observation sites, unlike non-rubber tropical ecosystems, which reduce canopy conductance at high R_n sites with high net radiation. High water use by rubber raises concerns about potential effects of continued expansion of tree plantations on water and food security in MSEA (cf. Fox et al. 2014b; Ahrends et al. 2015).

The eddy flux measurements over a 3-year period in two rubber plantation sites of northeastern Thailand and central Cambodia, sites having distinct dry seasons, give more additional ecophysiological aspects (Kumagai et al. 2015). They used a combination of actual evapotranspiration (*ET*) flux measurements and an inverted version of a simple two-layer E_T model for estimating the mean canopy stomatal conductance (g_s). There was less sufficient stomatal regulation at the Thailand site, where there might be the little risk that water stress-induced hydraulic failure was lower because of its comparatively higher annual rainfall amount. In comparison, at Cambodian site, where annual potential water balance (precipitation − potential evaporation: $P - E_{T\text{-}POT}$) was negative, there was stricter stomatal regulation, preventing excessive xylem cavitation (Kumagai et al. 2015). Collectively, the three studies demonstrate *Hevea* behaves differently under water stress conditions. Guardiola-Claramonte et al. (2010) argued that rubber evapotranspiration (*ET*) is energy limited during the wet season, but during the dry season, water consumption is mostly governed by environmental variables that directly affect rubber phenology, namely, vapour pressure deficit, temperature and photoperiodicity. In a hill slope-based hydrologic model to predict the basin-scale hydrologic consequences of rubber replacing native vegetation, simulations suggested greater annual catchment water losses through ET from rubber dominated landscapes compared to traditional vegetation cover.

Under conditions of drought-prone northeast Thailand, Ayutthaya et al. (2011) studied the

effects of soil and atmospheric drought on whole-tree transpiration, leaf water potential and whole-tree hydraulic conductance in mature RRIM 600. Under well-watered soil conditions, transpiration was tightly regulated in response to high evaporative demand (i.e. evapotranspiration of ~2.2 mm day^{-1} or maximum vapour pressure deficit ~1.8 kPa). When the trees experienced intermittent soil drought, whole-tree evapotranspiration decreased sharply when relative extractable water in the top soil was <0.4. The midday leaf water potential (Ψ (md)) on sunny days did not change as a function of soil drought and remained stable at approximately –1.95 MPa, i.e. displaying isohydric behaviour. The decrease in whole-tree transpiration was mainly due to the change in whole-tree hydraulic conductance. Whole-tree hydraulic conductance remained constant over a wide range of environmental conditions and decreased sharply at low soil water availability. Sopharat et al. (2014) measured tree transpiration, evaporative demand and soil water every day over 15 months. The xylem vessels of rubber trees under drought stress are vulnerable to cavitation, particularly in the leaf petiole. By closing, the stomata play an essential role in limiting cavitation. Clones differ in their susceptibility to cavitation, which occurs at xylem water potentials in the range of −1.8 to −2.0 MPa (Carr 2012). Investigations on tree transpiration, evaporative demand and soil water availability of RRIM 600 in a drought-prone area (Khu-Mueang, Buriram province in northeast Thailand) on every day over 15 months showed that basic relationships with evaporative demand, leaf area index and soil water availability were globally supported (Sopharat et al. 2015). Experimental data showed a strong regulation of transpiration under non-limiting soil water at high evaporative demand, when the evapotranspiration (*ET*) was approximately above 2.3 mm d^{-1}. The analysis of the corresponding canopy conductance confirmed a dramatic decrease above vapour pressure deficit values equal to 1 kPa. This result supports the previous analysis of Ayutthaya et al. (2011) where such a response was related to isohydric behaviour with a stable maximal value of the whole-tree hydraulic conductance and a stable minimum value of the leaf water potential. Sopharat et al. (2015) made two conclusions: (a) there is a need of taking into account the direct regulation of transpiration vs. high evaporative demand which often is omitted in simple agroclimatic models and (b) there is a need to provide a first diagnosis of water constraints on transpiration, in order to help the evolution of cultural choices and practices towards greater sustainability.

Jinagool et al. (2015) evaluated clones without subjecting plant materials to drought stress, making it useful in large-scale screening for drought tolerance in the near future. They first compared the most widely used techniques for measuring vulnerability to cavitation (air pressurization and Cavitron), and the effect of sample conditions (size, age and sunlight exposure), in order to ensure reliable analysis. Ten rubber clones (BPM 24, PB 217, PB 235, PB 260, PB 5/51, RRII 105, RRII 118, RRIM 600, RRIT 251 and RRIT 408) were compared for their xylem vulnerability to cavitation in branches and petioles, and for other traits related to drought response, including stomatal response and leaf shedding occurring during a simulated drought. They also tested the plasticity of vulnerability to cavitation on two clones (RRIM 600 and RRIT 251) grown in three locations with contrasting precipitation regimes, where no clonal variability was found. However, clonal differences in xylem vulnerability to cavitation were found in petioles, and clones also showed differences in stomatal response and in leaf shedding behaviour in response to a simulated drought. A genetic canalization for vulnerability to cavitation in organs is critical for survival, such as branches; there are clonal differences for traits related to drought avoidance: vulnerability to cavitation of petioles, leaf shedding behaviour and stomatal response.

Under the conditions of southwest China (Xishuangbanna), Li et al. (2013) assessed seasonal water-use strategies of rubber trees over the course of a rainy/dry season cycle. Stable isotope compositions of water in xylem, soil, rain and groundwater were sampled on seasonally distinct dates, and soil water content, root distribution and leaf water potential on sunny days were measured

in order to determine the proportion of water derived from different soil layers. Midday leaf water potential of rubber trees was relatively stable throughout the year and did not drop significantly during the late dry season, displaying isohydric behaviour. Soil and stem water isotope signatures along with rooting distributional patterns revealed that rubber trees extracted their water mostly from the top 30 cm and less from below 70 cm of the soil profile during the late rainy season when soil water was plentiful. During the late dry season, as the moisture in the middle soil layers (30–70 cm) was gradually depleted, the depth of water uptake shifted to deeper soil levels. Model calculations showed that the proportion of water uptake from the shallow soil layer (<30 cm) increased markedly after the most recent rainfall in the late dry season and the early rainy season (varying between 65% and 71%), indicating significant plasticity in sources of water uptake in this dimorphic-rooted species. This ability to take up a large proportion of shallow soil water after rainfall is likely the key feature enabling rubber trees to thrive through the period of greatest water demand. These results suggest that rubber trees are able to adjust the allocation of resources and thus acclimate to the spatiotemporal changes to water conditions in the soil profile.

Swidden agriculture (slash-and-burn or shifting cultivation) has long been the dominant farming system in Montane Mainland Southeast Asia (MMSEA). Today the ecological bounty of this region is threatened by the expansion of settled agriculture, including the proliferation of rubber plantations (Ziegler et al. 2009; Ahrends et al. 2015). The issue is further complicated as rubber is now being increasingly cultivated in physical environments that are marginal in terms of long-term viability (Ahrends et al. 2015). In the current conception of REDD+ (reduce emissions from deforestation and forest degradation), landscapes involving swidden qualify almost automatically for replacement by other land-use systems because swiddens are perceived to be degraded and inefficient with regard to carbon sequestration (Ziegler et al. 2009). However, swiddening in some cases may be carbon-neutral or even carbon positive, compared with some other types of land-use systems (Ziegler et al. 2012). Fox et al. (2014a) described how agricultural policies and institutions have affected land use in the region over the last several decades and the impact these policies have had on the livelihoods of swiddeners and other smallholders. Fox et al. (2014a) further argued for a deeper and more systematic analysis of the multiple consequences for the design of successful REDD+ policies in MMSEA and other areas of the developing world. REDD+ policies should be structured not so much to 'hold the forest boundary' but to influence the types of land-use changes that are occurring so that they support both sustainable livelihoods and environmental services, including (but not limited to) carbon.

Meta-analysis of over 250 studies reporting above- and below-ground carbon estimates for different land-use types indicated great uncertainty in the net total ecosystem carbon changes that can be expected from many transitions, including the replacement of various types of swidden agriculture with oil palm, rubber or some other types of agroforestry systems (Ziegler et al. 2012; Yuen et al. 2013). These transitions are underway throughout Southeast Asia and are at the heart of REDD+ debates. As some transitions may negatively impact other ecosystem services, food security and local livelihoods, the entire carbon and non-carbon benefit stream should also be taken into account before prescribing transitions with ambiguous carbon benefits (Ziegler et al. 2012).

9.6 *Hevea* and Clean Development Management

United Nations Framework Convention on Climate Change (UNFCCC) says 'the Clean Development Mechanism (CDM), defined in Article 12 of the Protocol, allows a country with an emission-reduction or emission-limitation commitment under the Kyoto Protocol (Annex B Party) to implement an emission-reduction project in developing countries. Such projects can earn saleable Certified Emission Reduction (CER) credits, each equivalent to one tonne of CO_2,

which can be counted towards meeting Kyoto targets'. It also says 'the mechanism stimulates sustainable development and emission reductions, while giving industrialized countries some flexibility in how they meet their emission reduction or limitation targets'.

The Kyoto Protocol was adopted on 11 December 1997 but came into force on 16 February 2005 after undergoing complex ratification process. There are 192 parties to the Kyoto Protocol that commits industrialized countries to stabilize greenhouse gas (GHG) emissions based on the principles of UNFCCC. On 12 December 2015, the Conference of the Parties to the UNFCCC (COP 21) adopted the Paris Agreement by decision. The Paris Agreement's central aim is to strengthen the global response to the threat of climate change by keeping a global temperature rise this century well below 2 °C above pre-industrial levels and to pursue efforts to limit the temperature increase even further to 1.5 °C. Additionally, the agreement aims to strengthen the ability of countries to deal with the impacts of climate change.

The Copenhagen Accord, established during the 15th Conference of the Parties (COP-15) in Copenhagen in 2009, mentioned the *Copenhagen Green Climate Fund*. During COP-16 in Cancun, the matter of governing the GCF was entrusted to the newly founded Green Climate Fund Board, and the World Bank was chosen as the temporary trustee. During the 17th COP at Durban, it was decided that the GCF would become an operating entity of the financial mechanism of the UNFCCC. GCF is expected to be the centrepiece of long-term financing under UNFCCC with a goal of raising $100 billion per year by 2020. This resource mobilization has to become a reality during the years to come.

A CDM project must provide emission reductions that are additional to what would otherwise have occurred. The projects must qualify through a rigorous and public registration and issuance process (Fig. 9.10). Approval is given by the

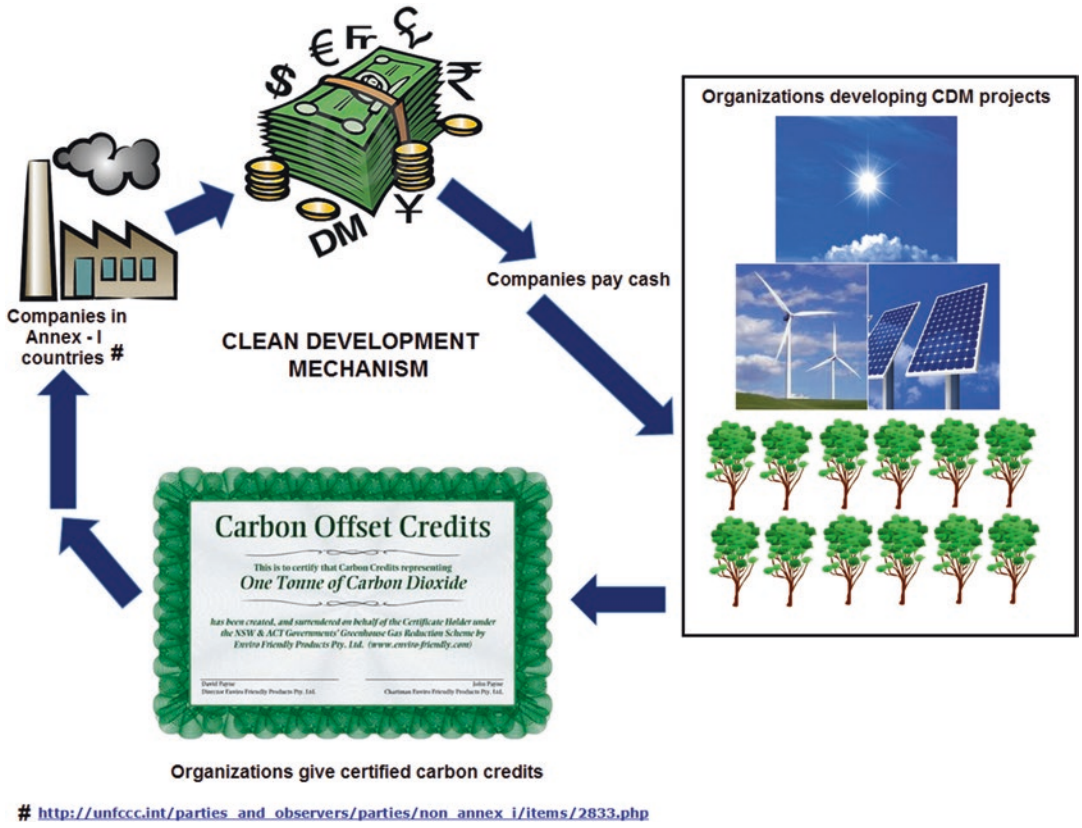

Fig. 9.10 Diagrammatic representation of Clean Development Management

designated national authorities. Operational since the beginning of 2006, the mechanism has already registered more than 1650 projects and is anticipated to produce CERs amounting to more than 2.9 billion tons of CO_2 equivalent in the first commitment period of the Kyoto Protocol, 2008–2012 (kindly see http://unfccc.int/kyoto_protocol/items/2830.php for further details).

Accumulation of greenhouse gases in the upper atmosphere is leading to changes in climate, particularly in temperature. The average global surface temperature increased by 0.6 ± 0.2 °C over the twentieth century and is projected to rise by 0.3–2.5 °C in the next 50 years and 1.4–5.8 °C in the next century. Global warming changes the earth's atmospheric circulation that leads to altered patterns of precipitation and also ushers in extreme climate events. Though the economic and ecological consequences of global warming will vary by region, in the tropics it may threaten the production of crops and may even become a major cause of species extinction. Carbon dioxide (CO_2) emanating from fossil fuel combustion, cement production and land-use change is the highest bidder among the greenhouse gases (GHGs) that prevent radiation from being reflected into the space and causes warming of the atmosphere (Kelkar 2006). Over the last 100 years, and more strikingly in the past couple of decades, the rise in temperature has exceeded the natural variation recorded until then. The same was the trend for CO_2 also (Fig. 9.11a, b). Significant evidence has been accumulated to show the direct relationship between the accumulation of GHGs and rising mean temperatures (Jacob 2005). The concentration of CO_2 in the atmosphere has been rising at the rate of approximately 1.6 ppm/year during the 1980s and 1990s. This increased amount of atmospheric CO_2, if fixed, can be an advantage to tide over the alarming trend towards global warming. Going back to the 1980s, of the total of 7.1 ± 1.1 Pg C/year, about 3.3 ± 0.42 Pg C/year remained in the atmosphere, which would come to 1.6 ppm rise in the atmospheric CO_2 (1 Pg of C ~ 0.48 ppm of CO_2), and the remaining 1.8 ± 1.6 Pg C/year remains unaccounted (Jacob 2006). This is termed as 'terrestrial missing sink' that needs to be absorbed by the terrestrial ecosystems. Though all these arguments have sent alarm bells ringing for the developed and developing countries as well, the counterargument is that there are real holes in the climate science that challenge the logic of climate change arguers (Schiermeier 2010).

Under the Kyoto Protocol of the United Nations Framework Convention on Climate Change (UNFCCC), signatory countries must decrease emissions of CO_2 to the atmosphere or increase rates of removal and storage. The Protocol's Clean Development Mechanism (CDM) allows a country that emits C above agreed-upon limits to purchase C offsets from an entity that uses biological means to absorb or reduce GHG emissions. The CDM is currently offered for afforestation and reforestation projects, and is expected to be extended to C sequestration in agricultural soils. Markets for soil and plant C sequestration are also developing outside the protocol in addition to those promoted by CDM. The interest in C sequestration and trading as mechanisms for both environmental protection and poverty alleviation in developing countries has increased considerably in the last decade. CDM could result in enhanced income and conservation of natural resources in the developing world. Two types of payments are anticipated, namely for C capture and C storage. Moreover, technological practices that are known to slow down soil C oxidation and increase C fixation are also being adopted in rubber plantations. Such strategies include improved soil and water conservation practices like leguminous cover cropping, application of organic manure, mulching, intercropping, etc., which are known to have helped in the increased enrichment of soil organic C by about 30–50% from about 1.9% to 2.39% in the lower depth of soils and to 2.9 C% in the top soil.

A mature rubber plantation would qualify as 'forest' as per the CDM definition (Kurian 2006). Mean annual leaf litter fall for a mature *Hevea* rubber ecosystem which included falling branches, twigs and fruits was estimated to be around 3.7–7.7 ton/ha. Total biomass accumulated in a tree over 33 years is 1.8 metric tons, which amounted to 596 metric tons per hectare. The total amount of carbon sequestered in 1 hectare of rubber plantation made up of tree biomass,

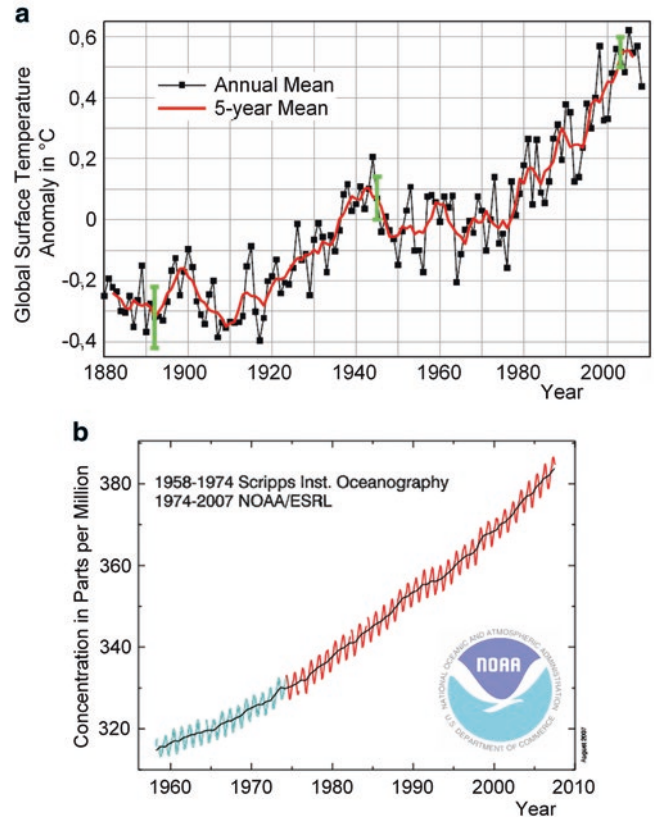

Fig. 9.11 (**a**) Global temperature anomaly at 10 m above land and ocean surface between 1880 and 2008, with the mean temperature in the period of 1951–1980 as a reference (0 °C temperature anomaly). *Black dots* represent mean temperatures in the respective year, and the red line denotes a moving average over the annual temperatures. Green error bars denote the uncertainty of data. From 1951 to 1980, there was no remarkable trend in temperature increase. This was due to increasing atmospheric pollution with aerosols that reflected sunlight efficiently back to space, thus reducing the energy input at visible wavelengths. Later on, waste regulations have led to a reduction of atmospheric pollution (Source: NASA). Time series of monthly averaged atmospheric carbon dioxide concentrations (green and red curves) and moving average (*black line*) measured from 1960 to 2007 on the Northern Hemisphere. Oscillations seen in the green and red curves are from seasonal variations of vegetation productivity. The *black curve* is a moving annual average where the seasonal fluctuations are no longer visible (Source: NOAA)

latex produced and contribution from leguminous cover crops is 680 metric tons. The possible credit revenue entitlement per hectare at the end of 33 years at the rate of US$12 per metric tons is about US$8160. However, C trading will not be able to function without government's firm backing. Since technical and institutional conditions are not yet in place to make C sequestration as a successful business venture, it is more practical for resource-limited rubber industry to pursue C sequestration initially as 'long-term pilot projects' in partnership with global carbon trading ventures.

In a study with 21-year-old rubber trees, Jacob (2006) estimated 66.47 tons of C/acre being sequestered. More than 77% of the carbon pool was in the above-ground biomass and more than 22.6% was from below ground. Timber constituted the single largest carbon pool (35.7%). Maximum rate of carbon sequestration occurred during seventh and eighth years, and thereafter it decreased a little but remained constant till the 18th year. After that, it will be well below the 3.17 tons of C/acre/year. The CO_2 equivalent of the total carbon sequestered during 21 years would be 243.7 tons/acre/ or 11.6 CERs/year (certified emission reduction). On the

other hand, the net carbon assimilation rates through leaf photosynthesis can be in the range of 7.01–9.1 μ mol CO_2 m $^{-2}$ s^{-1} (Jacob 2006). Cultivation of rubber trees on non-forested land could act as a carbon sink by sequestering carbon in biomass and indirectly in soils. In a study with 67 trees under Barak Valley area of Assam (India), Brahma et al. (2016) estimated total biomass (above and below ground) to be increasing from 41 kg $tree^{-1}$ under 5–10 years to 307 kg $tree^{-1}$ under 30–40 years age group of plantations. Total vegetation carbon stock (mega gramme—Mg ha^{-1}; above and below ground) ranged from 16.00 (5–10 years) to 105.73 (30–40 years), which, they argue, is more than many tropical forestry and agroforestry systems. Carbon sequestration rate was to the tune of 2.56 mega gramme C ha^{-1} $year^{-1}$, which is equivalent to 9.39 tons of CO_2 per year. Considering the capability to stock high-biomass carbon, restoring degraded and secondary forests through *Hevea* rubber, shall improve livelihood security and advance climate change mitigation strategies.

To measure the biomass in plantations of different ages and to determine the organic carbon content and $\delta^{13}C$ in the soils, Maggiotto et al. (2014) conducted a study in Paraná state of Brazil. Biomass accumulation was evaluated using destructive method. While total carbon stock in the top 60 cm of the pasture soil was 63.4 Mg C ha^{-1}, 4- and 15-year-old rubber tree plantations showed 66.8 Mg C ha^{-1} and 79.3 Mg C ha^{-1}, respectively, indicating thereby the rubber tree plantations have untapped potential to sequester carbon over a relatively short time period. Sone et al. (2014) measured trunk diameter, tree height and biomass of PB 260 aged twenty two years in Sumatra, Indonesia. The tree biomass growth and rubber yield rates peaked at 8 and 10 years after planting, respectively. The tree growth rate declined rapidly than rubber yield. Hence, overall biomass consistently increased from 5% in a 3-year-old tree to 40% in a 20-year-old. The values of estimated annual carbon sequestered as tree biomass and as rubber were 4.2 t C ha^{-1} $year^{-1}$ and 1.9 t C ha^{-1} $year^{-1}$, respectively.

International political and economic interests, following the Kyoto Protocol, require estimates of carbon sequestration. Wauters et al. (2008) assessed the carbon stock in rubber under two contrasting climatic areas: western region in Ghana (2–14-year-old) and Mato Grosso (14–25-year-old) in Brazil. Trees with a range of stand ages and clone types were felled and partitioned into log, live lignified branches, dead branches, non-lignified fine branches, leaves, taproot and lateral roots. Allometric relationships (log-transformed power functions) based on trunk circumference at a height of 170 cm (C_{170}) were used to predict the tree foliage, above ground, below ground and total carbon content (kg C $tree^{-1}$) (r^2 = 0.86–0.99). Predicted tree carbon stock for 14-year-old stands was higher in Ghana (76.3 t C ha^{-1}) than in Mato Grosso (41.7 t C ha^{-1}), which was partially explained by a difference in tree height growth. In the framework of the Kyoto Protocol, these results could be useful when drafting a Project Design Document (PDD) for Afforestation and Reforestation Clean Development Mechanism (AR-CDM). Though the aim of these two studies is complimentary, the results are significantly different, probably because of the methodologies used. UNFCCC needs to acknowledge the methodology used for calculating the CERs, before embarking into the process of carbon credit transfers.

In the biodiverse-rich Xishuangbanna Prefecture, Yunnan Province, China, there is a rapid increase in rubber plantation from 8% to 22% of total area between 2002 and 2010 (Fig. 9.12). Zomer et al. (2014) could delineate four bioclimatic zones and nine strata, overlaid with protected areas, and associated with ongoing land-use change. Significant changes in the areal extent and distribution of all zones and strata could be projected, with an averaged mean annual temperature increase ranging from 1.6 to 2.4 °C. The expectation is that by 2050, there could be significant geographical shifts in all identified strata, with an average upward shift of 309 m of elevation for all strata. On average, more than 75% of Xishuangbanna is predicted to shift to a different zone, with 96% shifting to a different stratum. The area conducive to rubber plantations, currently limited by climatic conditions, expands to nearly 75% of the total area. Zomer et al. (2014) predict that the climatic change potentially

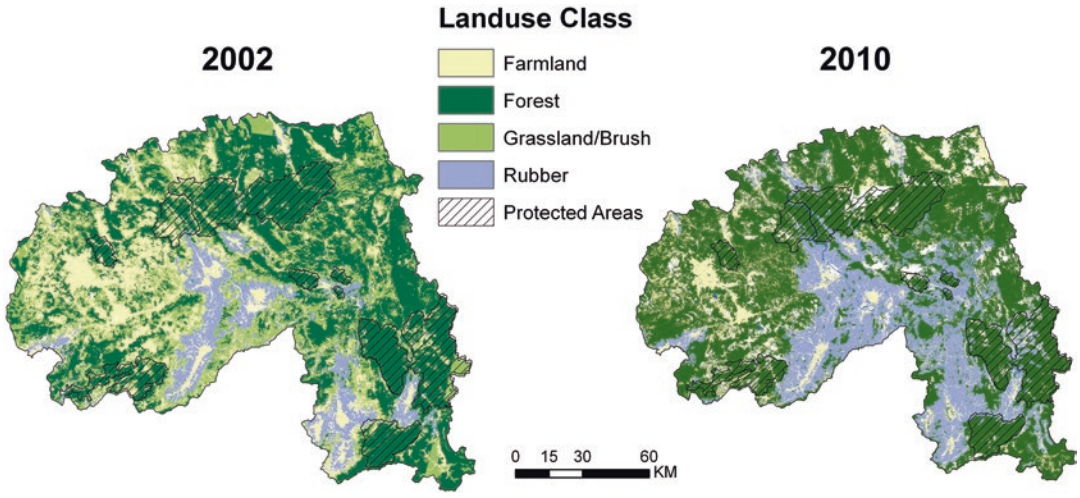

Fig. 9.12 Land use in Xishuangbanna Prefecture in 2002 and 2010, showing expansion of forest, expansion of rubber plantation, loss of grassland and loss of farmland (After Zomer et al. 2014) (Photo courtesy: Robert John Zomer, Centre for Mountain Ecosystem Studies, World Agroforestry Center, East Asia, Kunming, China)

removes the bioclimatic barriers to further expand rubber plantations within the area and increases pressure on remaining biodiversity both within and outside of protected areas.

The essential decrease in mean carbon biomass density from 61.5 to 30–35 Mg (mega gramme) ha^{-1} occurs when rubber is planted at an elevation range higher than 800 m a.s.l. in subtropical conditions of Xishuangbanna, China. Even lower values (15.3 Mg ha^{-1}) were reported for rubber plantations at an elevation >1000 m (Li et al. 2008). A correct comparison of carbon biomass density can be done based on time-averaged C stocks (taCs) (Blagodatsky et al. 2016), and in this case, the different ages of plantations and different development rates of trees do not matter as the whole rotation cycle is taken into consideration. The time span of an economic rubber tree life cycle (25–30 years) depends on the tree diameter recommended for tapping (usually 16–18 cm) and not on actual biomass accumulation. Therefore, C stocks (and taCs) at higher elevations are lower due to a lower carbon accumulation rate. Blagodatsky et al. (2016) argue that if tapping is done later at higher elevation and tapping intensity decreases along with a lower tree growth rate and hence lower latex production, the rotation time will increase (e.g. from 25 to 35 years) as well, with a corresponding increase in taCs (from 19 to 37 Mg C ha^{-1}; Fig. 9.13). Rubber yield varies depending on environmental conditions, management and clone (Golbon et al. 2015; Priyadarshan et al. 2005; Priyadarshan 2011), resulting in cumulative C stocks of 14–33 Mg C ha^{-1} during 20 years of tapping of rubber trees in one rotation (Blagodatsky et al. 2016). Jawjit et al. (2010, 2015) estimated annual greenhouse gas emissions from rubber plantations in Thailand to be about a 6.4 Mg CO_2 equivalent per Mg of fresh latex per year. Time-averaged carbon stocks (Cta) of lowland and highland rubber plantations of Xishuangbanna, China, were 58 Mg C ha^{-1} and 28 Mg C ha^{-1}, respectively, which have larger carbon sequestration potential than non-forest land-use types (Yang et al. 2016). However, natural forest had a Cta of 156–185 Mg C ha^{-1}. Sensitivity analysis of Cta variability showed that forest C stocks have the largest influence on landscape carbon balance. Time series analysis of land-use and land-cover maps (1989, 2007, 2012) demonstrated that during 23 years, the whole landscape of the nature reserve (26,574 ha) gained 0.644 Tg C (Yang et al. 2016).

Enhanced remote sensing techniques can greatly improve C stock estimates. An analysis clearly shows that current efforts to conserve forests,

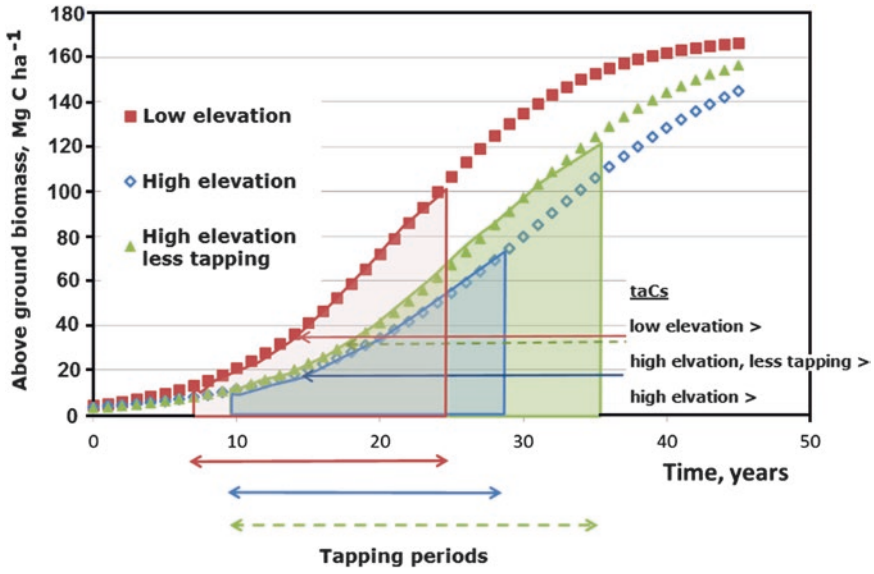

Fig. 9.13 Proposed effect of the rubber plantation location management strategy on above-ground biomass dynamics and time-averaged C stocks (*taCs*). Data for the calculation of rubber development were taken from Yang et al. (2005). Delay in plant development and a later start of tapping are typical for plantations located at higher elevations (e.g. 500 m a.s.l. versus 900 m a.s.l.) for regions with sub-optimal growth conditions (e.g. Xishuangbanna, China). Tapping frequencies are two times per week or once per week (less tapping) (Figure courtesy: Sergey Blagodatsky, Institute for Plant Production and Agroecology in the Tropics and Subtropics, Stuttgart Germany)

biodiversity and traditional land-use systems require an improved understanding of both the projected climatic changes and the responses of biodiversity and traditional agricultural systems to changing conditions. *Hevea* rubber can play a pivotal role in this judgement and could be used meticulously to successfully mitigate adverse effects due to climate changes.

Genotype-by-Environment Interactions

10

The penultimate success of a plant breeding programme depends on its ability to provide farmers with genotypes/clones with guaranteed superior performance (phenotype) in terms of yield and/or quality across a range of environments. While there can be clones that do well across a wide range of conditions (widely adapted genotypes), there are also clones that perform well exclusively under a restricted set of environments (specifically adapted genotypes). As in widely adapted genotypes, specific adaptation of genotypes is also closely related to the phenomenon of genotype-by-environment interaction. Information about phenotypic stability and adaptability assessed through GE interaction studies is prime for the selection of crop varieties/clones. Since phenotypic performance of a genotype is not necessarily the same under diverse agro-ecological conditions, the concept of stability has been defined and assessed in several ways and several biometrical methods including univariate and multivariate analyses (Lin et al. 1986; Becker and Leon 1988; Crossa 1990). The most widely used is the regression method, based on regressing the mean value of each genotype on the environmental index or marginal means of environments (Romagosa and Fox 1993). A good method to measure stability was proposed by Finlay and Wilkinson (1963) and was later improved by Eberhart and Russell (1966). They were followed by AMMI model (Gauch and Zobel 1996) and GGE biplot (Yan and Kang 2003). All these merely tried to group genotypes and environments and do not use other information than the two-way table of means. Further, factorial regression was introduced as an approach to explicitly utilize genotypic and environmental covariates for describing and explaining GE interactions. Finally, QTL modelling was put forth as a natural extension of factorial regression, where marker information is translated into genetic predictors. Tests for regression coefficients corresponding to these genetic predictors are tests for main effect QTL expression and QTL by environment interaction (QEI). QTL models for which QEI depends on environmental covariables form an interesting model class for predicting GEI, for new genotypes and new environments. QTL technology has not been efficient for predicting complex traits affected by a large number of loci. Recent delineation of high-density markers has been useful to predict genomic breeding values, thus increasing the precision of genetic value prediction over that achieved with the traditional use of pedigree information (Crossa 2012). Genomic data also allow assessing chromosome regions through marker effects and studying the pattern of covariability of marker effects across differential environmental conditions. For realistic modelling of genotypic differences across multiple environments, sophisticated mixed models are necessary to allow for heterogeneity of genetic variances and correlations across environments. Models like (a) additive model, (b) regression on the mean model, (c) additive main effects and multiplicative interactions model, (d) factorial regression

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models, (e) mixed models for genetic variances and covariances and (f) modelling main effect QTLs and QTL-by-environment interaction are some of the strategies being highlighted for the study of GE interactions (Malosetti et al. 2013).

10.1 G × E Interactions and Specific Adaptation

Tan (1995) could study GE variation using joint regression analysis. The results showed that differences in linear regression accounted for only a small proportion of the total G × E interaction effects, suggesting the non-linear nature of the G × E interaction for most of the characters in *Hevea* and the involvement of more than one major environmental factor causing the G × E interaction. Tan (1995) argued that regression technique needs caution due to the non-linearity of the G × E interaction, and simple statistical parameters such as CV or variance and mean of genotypes over different trial sites can serve as practical and useful guides in clonal selection.

As succinctly explained in Chap. 9, cultivation of *Hevea* has been extended to sub-optimal environments by many natural rubber-producing countries. *Hevea* rubber yields differently under traditional and non-traditional areas (Priyadarshan 2003a). This is evident from the analysis of overall yield and yielding trends in Tripura (India), Vietnam, China and São Paulo (Figs. 10.1, 10.2, 10.3 and 10.4). Under the hydrothermal situations of Tripura, in a study involving 15 clones of vivid geographical origin, all clones showed an increment in yield towards the onset of cold season i.e. during October to November. It is implicit that the cold weather (18–20 °C) is very favourable for latex flow by pushing the coagulation time to a later period of the day, and the onset of cold season renders a stimulatory effect to maximize yield and the trend continues till the temperature falls below 15 °C during January. The clones are classified under two categories: (a) one showing a slow escalation in yield from April onwards, reaching the maximum during November, and receding sharply during December and January and (b) the other with a low-yield regime during April to October and with the peak yield during November and December (high-yield regime), and then receding during January. In São Paulo (southern hemisphere), the low- and high-yield regimes are vice versa. While PB 235 comes under the first category, all the other clones come under the second. The trend shows that the first category is appreciable since the clones give considerable yield during Regime I, which ensures better returns to the planter. The rationale is that fall in temperature along with reduced evaporation and low wind speeds prevail upon the microenvironment to influence yield stimulation during October to December (Priyadarshan 2003b). PB 235 seems to own an adaptive mechanism, whereby it yields higher when ambient temperature ranges between 22 and 28 °C. When all clones continue with a higher yield in combination with descending temperature, PB 235 recedes yield during January when the ambient temperature gets below 18 °C. Studies conducted with RRII 208 endorse the same trend (Priyadarshan and Nair 2002). Evidently, the existence of genetic homeostasis and their subsequent expression in the changed environment might be the reason for the near uniform yield in these clones. In São Paulo, RRIM 526 showed higher yield during low regime in comparison to RRIM 600, RRIM 614, AVROS 1328 (Goncalves, IAC, São Paulo, personal communication). These observations clearly rationalize the selection to be in favour of consistent yielder (Priyadarshan et al. 2005).

Data on overall means of 15 clones, low- and high-yielding periods for 11 years (1990–2001), were separately analysed via GGE biplot to judge specific adaptation of clones (Priyadarshan et al. 2008). Stability estimates viz., ecovalence, stability variance, s^2, Huehn's non-parametric statistics and Lin and Binn's superiority measure were also calculated. These estimates were comparable except for Huehn's non-parametric statistics. GGE biplot analysis was conducted under symmetrical, entry-focused and tester-focused procedures. While entry-focused scaling for overall means kept years towards a single cluster presuming that all clones were performing comparably over the years, under tester-focused

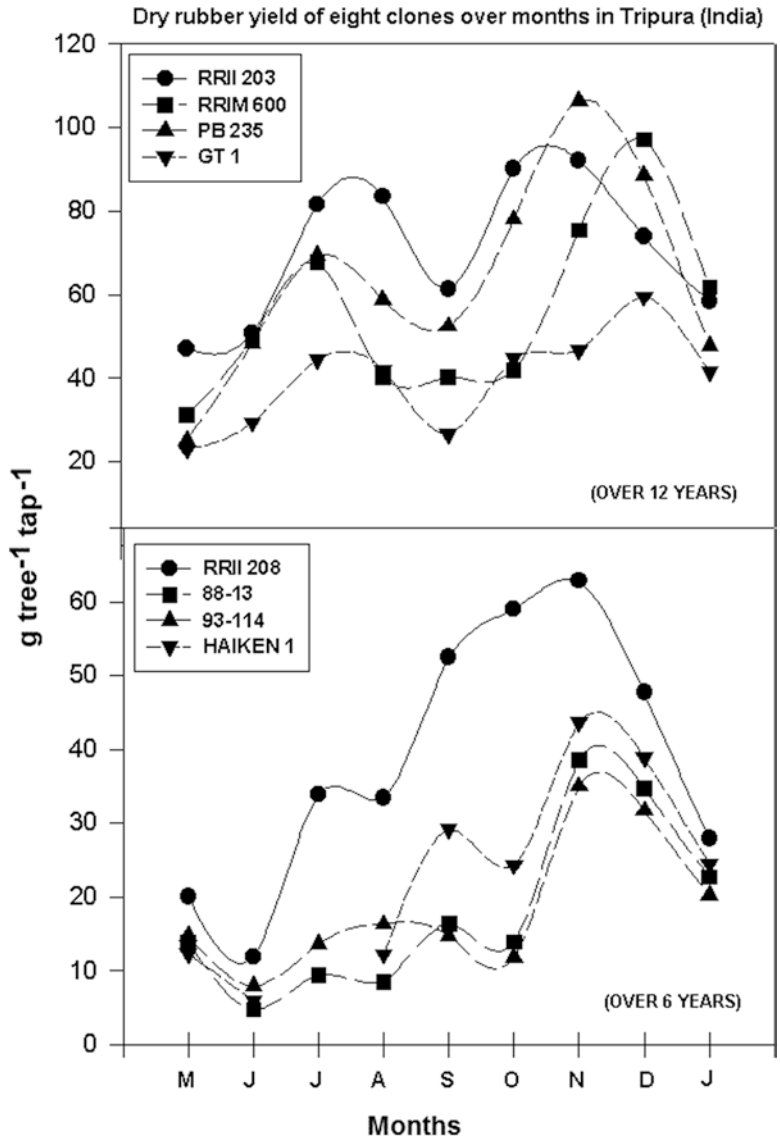


Fig. 10.1 Dry rubber yield of eight clones over months in Tripura (northeast India)

scaling, the years were well spread, indicating the climates of all the years were not same. Overall genotype means were high for PB 235, RRII 203, RRIM 600, RRII 118 and RRIM 703. The symmetrical scaling of data of regime II showed 1999 and 2000 exhibiting higher principal component analysis (PCA) 1 values. Clones RRII 105, RRII 118, RRIM 703 and PB 235 were high yielders under this regime. The performance of clones was different when mean values were plotted against PCA values (Fig. 10.5). While RRII 105, RRIM 703, RRIM 600 and RRII 118 were most stable under regime I, RRIM 605 had a higher stability under regime II. Under both circumstances, PB 235 and RRII 203 were high yielders. When overall means were plotted, RRII 105, RRIM 703, RRII 118 and PB 235 were noted to be most stable over 11 years(Fig. 10.6). Under all these years, PB 235, RRII 203 and RRIM 600 were high yielding.

The weather variables and environmental index were also used as covariates while analysing the yield data to determine the variable that contributed to heterogeneity in the GE interactions.

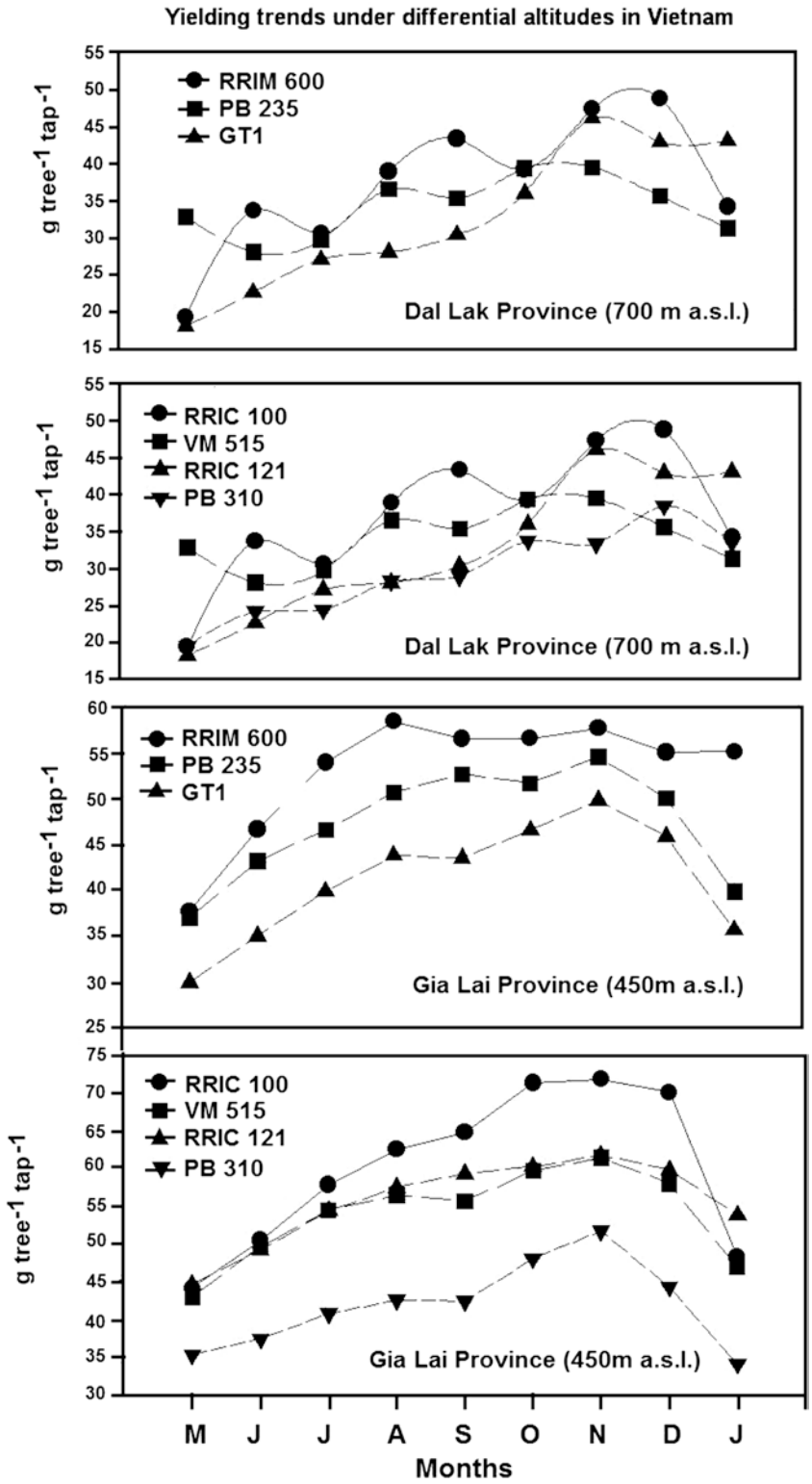


Fig. 10.2 Yielding trends under different altitudes in Vietnam

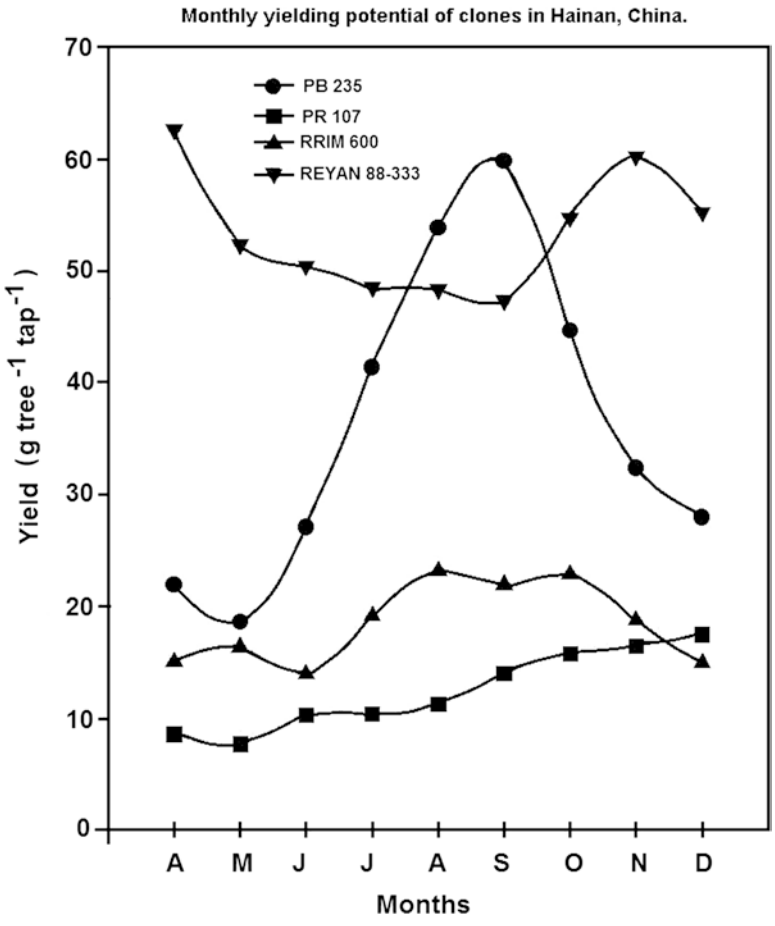


Fig. 10.3 Monthly yielding potential of clones in Hainan (China)

The test of heterogeneity for environmental index showed high significance, indicating that the high stability values of few clones (s^2i_j over the years were due to the linear effect of the environment (Priyadarshan 2003a, 2014) (see Table 10.1). However, under Malaysian conditions, Tan (1995) accounted GE interactions with a non-linear effect of wind damage and disease. In fact, these hazards play a prominent role in differentiating the adaptation of clones to one or another location. Grouping of clones with high mean and low coefficient of variation is proved to be dependable in selecting better performers in a new environment (Tan 1995; Priyadarshan et al. 2002). GE interactions were also significant for rubber production and girth increment under the conditions of São Paulo (Gonçalves et al. 1998a, b; da Costa et al. 2000). This need of identifying specific adaptation of clones to the diversity of rubber planting tracts might lead to emphasize ecophysiological research that can provide functional and predictive models for characterising these adaptations.

In a stability analysis for girth increment and rubber yield of seven clones from five comparative trials in São Paulo (Brazil), Gonçalves et al. (2003) delineated that year by location and location variability were the dominant sources of interactions. The stability analysis identified GT 1 and IAN 873 as the most stable clones for girth increment and rubber yield, respectively, since their regression coefficients were almost the unity ($\beta = 1$), and they had one of the lowest deviations from regressions. Their coefficient of determination (R^2) was as high as 89.5% and 89.8% confirming their stability. In contrast, clones such as PB 235, PR 261 and RRIM 701 for girth increment and clones such as GT 1 for

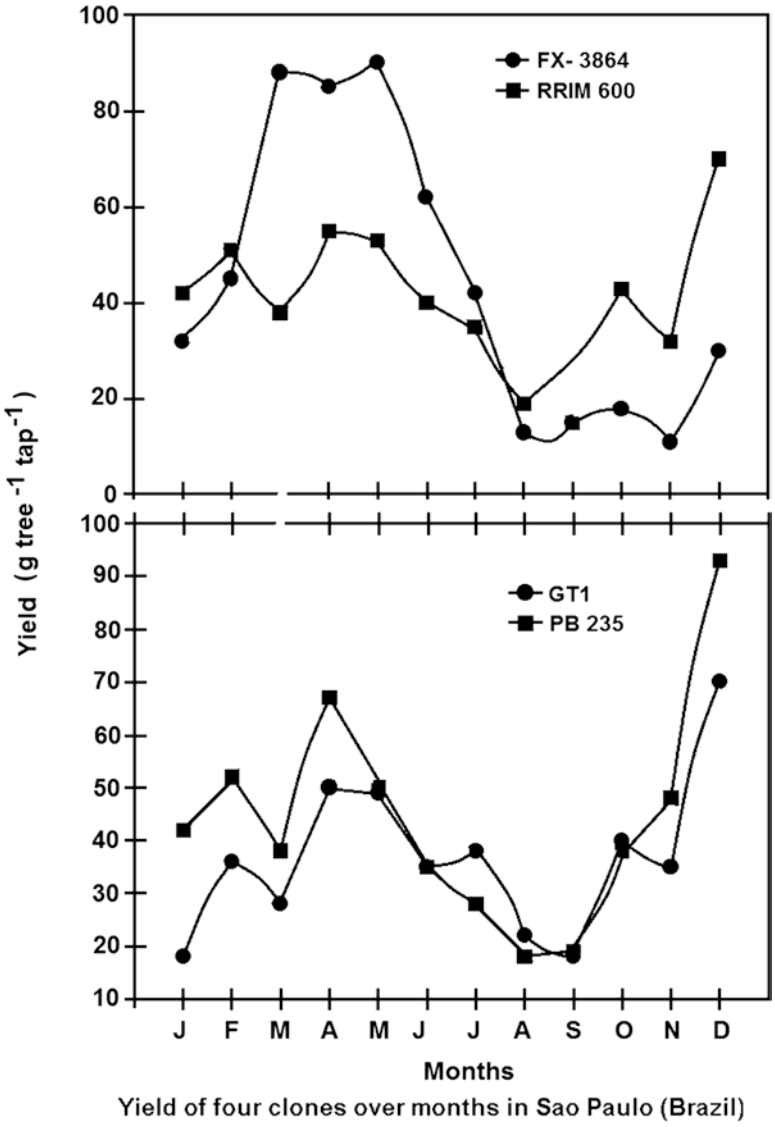
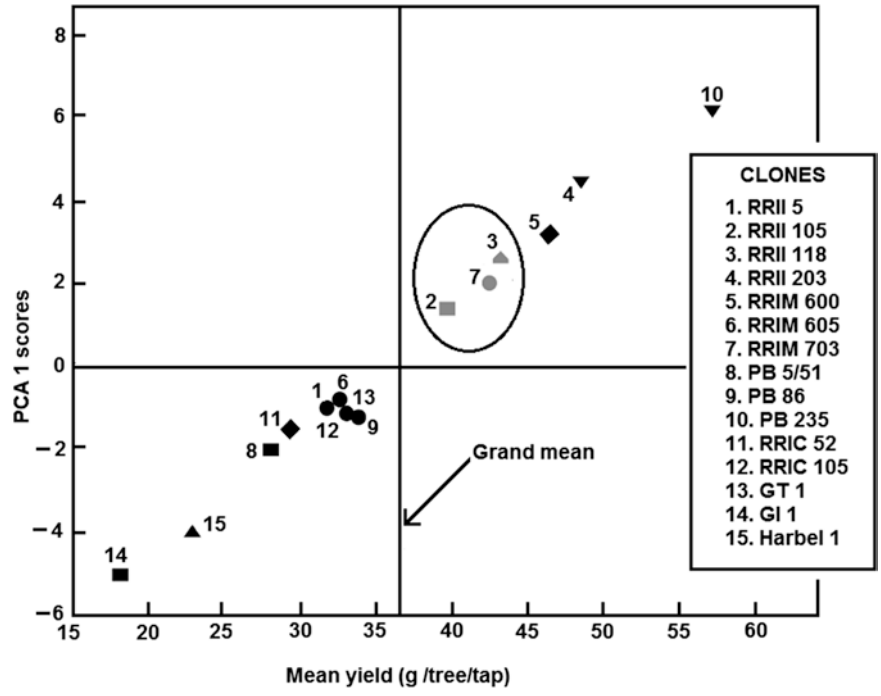


Fig. 10.4 Yield of four clones over months in São Paulo

rubber yield with regression coefficients greater than one were regarded as sensitive to environment changes. Withanage et al. (2005) could get almost the same results in clones bred for Sri Lanka with significant genetic, environmental and genotype x environment interactions from the fifth-year girth measurement.

The association among different methods of stability analysis were also assessed (Gouvêa et al. 2016) using different traits and different groups of genotypes for the same trait. Two open-pollinated progeny populations and a group of 25 clones were analysed following stability analyses like Wricke; Eberhart and Russell; Lin and Binns; AMMI (Principal Additive Effect and Multiplicative Interaction) and HMRPGV (Harmonic Mean of the Relative Performance of the Genetic Values) predicted by BLUP (Best Linear Unbiased Prediction). The Spearman correlation was used to verify the association between the stability parameters. Gouvêa et al. (2016) argued that the association among these stability parameters varied according to the trait and/or the group of genotypes analysed.

The stability status of 24 Hevea clones along with GT1 and PB 235 as checks under traditional



Depiction of mean yield against PCA 1 values.

Fig. 10.5 Depiction of mean yield over PCA 1 values

Biplot analysis of yield of 15 clones over eleven years.

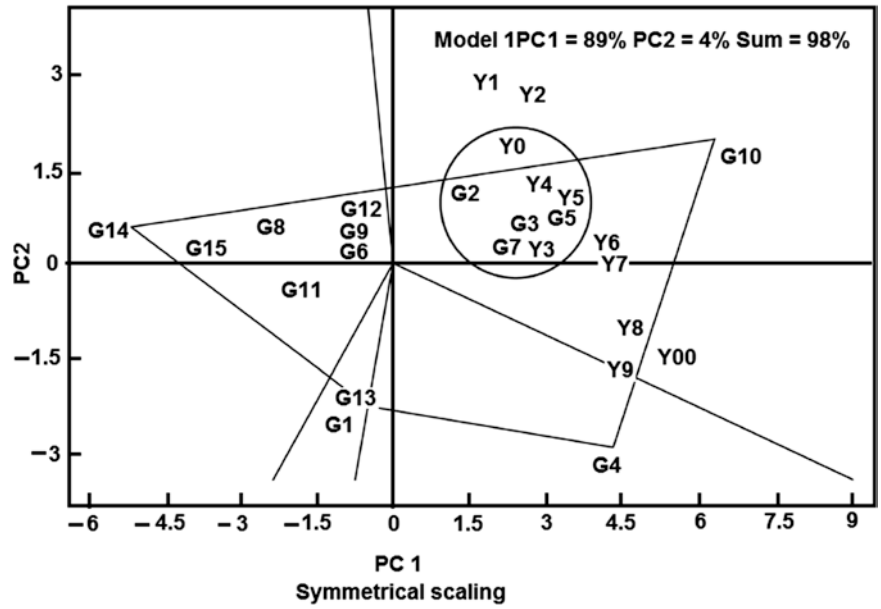


Fig. 10.6 Biplot analysis of 15 clones over 11 years

Table 10.1 Simultaneous selection for yield and stability during 1998–1999 using Kang and Magari's STABLE programme (after Priyadarshan 2003b)

Clone	Yield	Yield rank	Adjusted to rank	Adjusted rank	Stability Y	Stability variance	YS (i) $ rating #
RRII 5	51.2	7	−1	6	1185.3	−8	−2
RRII105	55.8	10	1	11	2335.0	−8	3+
RRII118	57.1	11	1	12	274.2	0	12+
RRII 203	80.1	14	3	17	3838.4	−8	9+
RRIM 600	64.2	12	3	15	1220.2	−8	7+
RRIM 605	42.6	3	−3	0	2069.5	−8	−8
RRIM 703	64.3	13	3	16	1225.7	−8	8+
PB 5/51	42.6	4	−3	1	526.9	0	1
PB 86	51.9	8	−1	7	1179.9	−8	−1
PB 235	81.3	15	3	18	1773.9	−8	10+
RRIC 52	48.8	6	−2	4	1245.6	−8	−4
RRIC 105	48.5	5	−2	3	1115.0	−8	−5
GT 1	52.0	9	−1	8	603.2	0	8+
Gl 1	27.0	1	−3	−2	1734.0	−8	−10
Harbel 1	38.1	2	−3	−1	633.9	0	−1
Mean	53.7						1.8

LSD ($p = 0.05$) = 3.4; key: + selected genotype; # −4, −8 σ^2_i at $p = 0.05$ or 0.1; 0 = non-significant; $ selected clone must have a value of more than the mean YS_i value

(Binh Duong) and non-traditional (Nghe An) environments of Vietnam was conducted by Thanh et al. (2016). Eberhart and Russell model was applied for the analysis. Genotype × environment interactions were found to be highly significant for both latex yield and girth, indicating that they were significantly affected by the environments. Stability analyses (linear regression coefficient and mean square deviations) indicated that LH 94/359, LH 91/579 and LH 94/481 showed wider adaptability.

A synthesis of aforesaid GE analysis clearly indicates that *Hevea* clones exhibit attributes of specific adaptation to varied environments. A number of techniques to delineate stability had been applied and most of them provided comparative results. The technique/analysis is not the question, but the varied performance of *Hevea* under a spectrum of environments is intriguing and need to be addressed more precisely with better statistical tools. All those tools that are applied for annual crops cannot be made applicable for *Hevea*, but tools need appropriate refinement because unlike annual crops, *Hevea* is a continuous yielder from the same stand over days, months and years.

Biological Constraints 11

It is noteworthy that unlike other clonal species, *Hevea* is not affected by viral diseases (Simmonds 1989). Apart from South American leaf Blight (SALB), other diseases of economic importance are the Gloeosporium leaf disease (*Colletotrichum gloeosporioides*), powdery mildew (*Oidium heveae*), Corynespora leaf disease (*Corynespora cassiicola*) and the Phytophthora leaf fall (*Phytophthora* spp.). Clonal specificity is evident towards resistance to these diseases (Wycherly 1969). A study with Gloeosporium showed that clones from Malaysia and Indonesia are fairly resistant, while clones from Sri Lanka and China are less resistant. But clones from South America are seen to be highly resistant indicating local adaptation rather than breeding is the cause for the resistance (Simmonds 1989). Ho (1986) gives a good narration of the breeding implications of diseases in *Hevea*. It is imperative that too much susceptible genotypes are rejected at the first instance and the survivors are seen to be moderately resistant.

11.1 South American Leaf Blight

South American Leaf Blight (SALB—caused by *Microcyclus ulei*) that is singularly devastating is a stress factor limiting the yield of *Hevea.* It has played and still plays a major role in the history and in the geographic distribution of rubber industry in the Americas. On the one hand, it prevents Latin America from developing rubber cropping in all the otherwise favourable climatic conditions, and on the other hand, it represents a permanent major threat to the crop in Asia and Africa (Dean 1987; Davies 1997). da Hora et al. (2014) used six genomic regions (LSU rRNA, mtSSU, MCM7, EF-1 α, Act and ITS) for reconstructing the molecular phylogeny of the SALB fungus based on material collected throughout Brazil. The analyses support the classification of the fungus in the family *Mycosphaerellaceae* s. str. and place it firmly within the clade *Pseudocercospora* s. str., now accepted as one of the distinct genera within *Mycosphaerellaceae*. da Hora et al. (2014) proposed new combination of *Pseudocercospora ulei* and the life cycle of the fungus is confirmed, based on both experimental and phylogenetic evidence. The epidemiology and genomics of this fungus needs to be investigated further by the mycologists to reconfirm the taxonomic status of the fungus.

Some amount of breeding work, mainly based on back-cross technique, has been undertaken in the past to incorporate resistance to these diseases in high-yielding clones. However, the efforts were in vain due to unknown polygenic nature of the attributes, high variability of the pathogen and multiple interactions between fungus strains and rubber clones (Rivano 1997a, b). Simmonds (1990, 1991) argue that the pathotype-specific resistance (vertical resistance-VR) has resulted in catastrophic failures. Horizontal resistance (HR) should be more effective and durable (Rivano et al. 1989; Simmonds 1990). Resistance sources appear to be absent in high-yielding Wickham

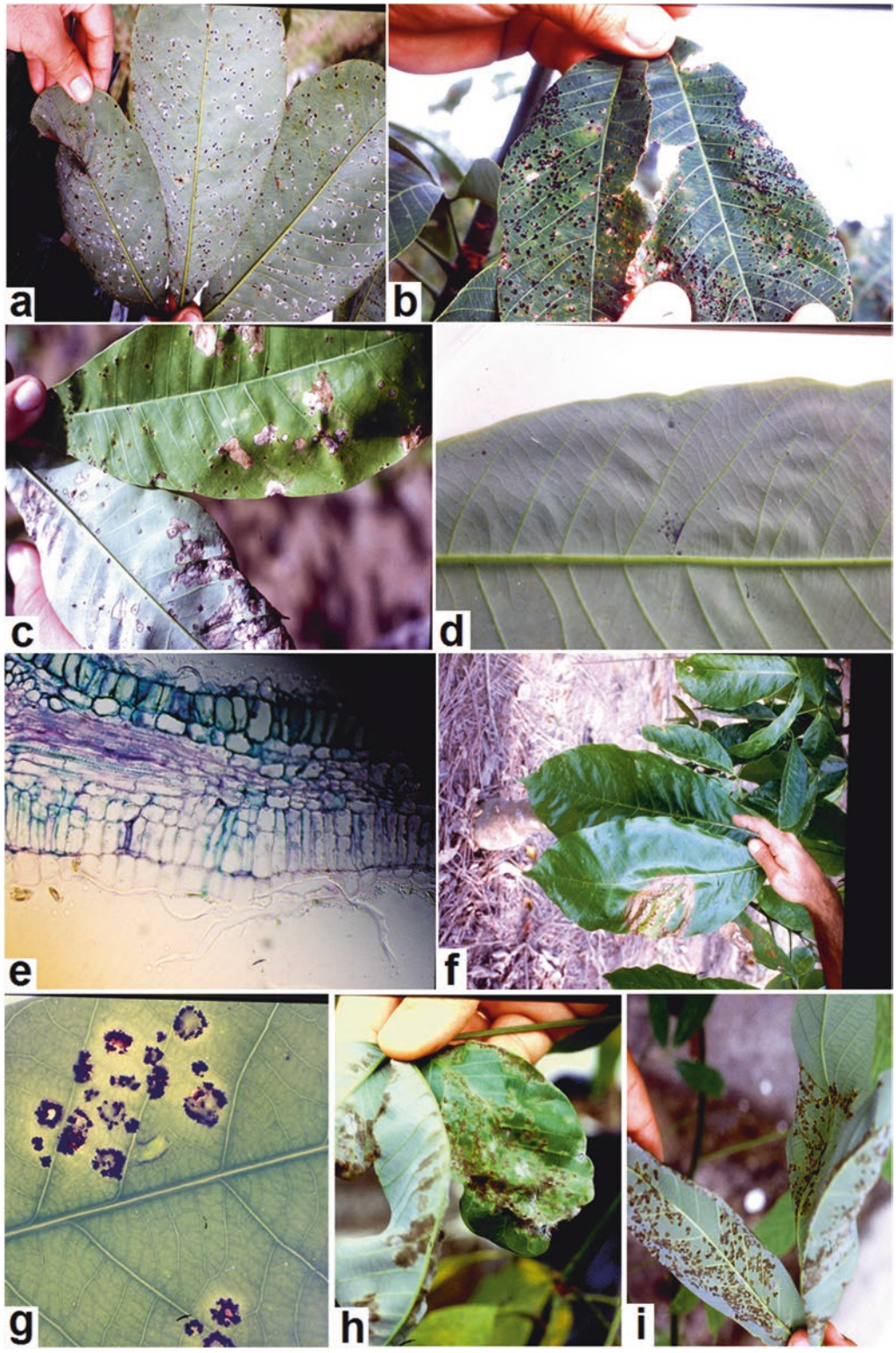
P.M. Priyadarshan, *Biology of* Hevea *Rubber*, DOI 10.1007/978-3-319-54506-6_11

population, but rather frequent within the Amazonian germplasm. However, the wild population is yet to be improved for yield. With these views, efforts have been reoriented towards the analysis of partial resistance components (Junqueira et al. 1990). Recently, the genetic determinism of the resistance source of *H. benthamiana* (F 4542), widely used in many former back-cross programmes, has been characterized by a genetic map (Lespinasse et al. 2000b). A Cirad-Michelin common research and breeding programme is currently carried out in Brazil for reducing the incidence of SALB on rubber cropping. SALB is present in Bolivia, Brazil, Peru, Ecuador, Colombia, Venezuela, Guyana, Surinam, French Guiana, Trinidad, Panama, Costa Rica, Nicaragua, EI Salvador, Honduras, Guatemala, Haiti and Mexico (Holliday 1970; Compagnon, 1986) and has caused the abandonment of ambitious programmes of extensive rubber cultivation in the South American humid tropics.

Under the CIRAD-Michelin-Brazil (CMB) breeding programme of Brazil set up in 1992 to breed SALB-resistant clones, the programme developed CMB genotypes through combining family and individual selections (Rivano et al. 2013). Thirteen genotypes were selected for evaluation of their resistance, girth and rubber production in a trial network covering eight sites in Brazil and Ecuador. Resistance was confirmed after several years and promising yields were obtained for three clones (CDC 312, FDR 5788, PMB 1) against resistant clone MDF 180. FDR 5788 gave an estimated yield of 1.8 ton/ha per year. There were significant differences between clones, sites and clone-site interactions. Nine *Hevea* genotypes resistant to SALB (TP875, FDR5788, MDX608, PMB1, CDC429, CDC312, FDR4461, MDF180, SIAL893) were isolated and evaluated from among 960 accessions in a 12-year trial located in the Michelin plantation (Bahia, Brazil) having quantitative resistance to SALB (Cardoso et al. 2014). They were characterized by partially sporulating lesions (anamorph stage). No sexual form of *M. ulei* (stromata, teleomorph stage) was found in FDR5788, MDF180, CDC312, PMB1. FDR5788, MDX608, CDC312 and PMB1 were with estimated yields of 2.6, 2.0, 1.8 and 1.2 t year^{-1} ha^{-1}, respectively.

Conidia and ascospores cause infection and both are equally important in completing the disease cycle (Langford 1945; Chee 1976a, b, 1977). Rain plays an important role in the spread of leaf blight. It is believed that rain is the most effective disseminator of large masses of spores and wind is the chief means of dispersal. Brookson (1963) observed that conidia survived for 2 weeks under normal laboratory conditions. However, the longevity of conidia decreases as RH increases. Under high humidity, conidia survive for 3 weeks, and at 100% RH, they are killed within 1 week. It seems probable that leaf blight could be spread by conidia carried on plants, plant parts or man himself. Outbreaks of leaf blight occur when the daily temperature is below 22 °C for longer than 13 h, RH over 92% for a period longer than 10 h and rainfall above 1 mm per day for the previous 7 days (Holliday 1969; Chee 1976a). The fungus can affect petioles, green stems, inflorescences and fruits. But the most obvious infection is on young leaves on the abaxial surface of 4–9-day-old, expanding tender leaves. They appear as greyish-black lesions covered with olive-green powdery sporulating masses (Lieberei 2007) (see Fig. 11.1). On the young infected leaves, lamina distortion, growth arrest, crinkling

Fig. 11.1 (**a**) Stromata of *Microcyclus ulei* on *Hevea brasiliensis* leaves, superinfected with *Hansfordia pulvinata*, a potential biocontrol organism. (**b**) Stromata of *M. ulei*, upper leaf surface. (**c**) Mixed infection of rubber tree leaves with *Thanatephorus cucumeris*, causal agent of target leaf spot and small black necrotic spots of partially resistant *Hevea brasiliensis* leaves in a resistance reaction against *M. ulei penetrationta* of *M. ulei.* (**d**) Initial infection step of attack of young rubber tree leaf by *Thanatephorus cucumeris*. Oxidizing latex drops develop at the penetration site. (**e**) cross-section through *Hevea brasiliensis* leaf (young leaf). Violet staining: hyphae of *M. ulei* parallel to vascular bundle and hyphae growing down to lower leaf surface to form conidiophores. (**f**) Leaf of *Hevea pauciflora* with typical lesion or target leaf spot (*Thanatephorus cucumeris*). (**g**) Ring-forming lesions caused by fusion of small globular stromata of *M. ulei*. (**h**) Conidiospore layers on highly susceptible young *Hevea brasiliensis* leaves. (**i**) Conidiospore layers on susceptible young *Hevea brasiliensis* leaves. The small conidiophore layers are not confluent but slightly separated. This is caused by a control factor of the leaves, which might be developed into a resistance component (Photo courtesy: Reinhard Lieberei)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and shrivelling of leaflets, blackening, drying and abscission are the common symptoms. The secondary stage develops on the adaxial surface of the leaves as it hardens. In Trinidad, the conidia have maximum dispersal in June and July and peak ascospore concentration occurred from August to November during the wet season (Chee 1976b). In a mature stand of rubber, a fresh disease cycle probably starts when ascospores are released from leaves which fall due to wintering and also from infected leaves remaining on the trees. As infection builds up on the newly emerging flushes, conidia take over the spread during the wet season to complete the disease cycle.

After penetration of the leaf surface, the hyphae colonize the underlying tissue by intercellular growth (Fig. 11.2). They often enter the tissue layers adjacent to the leaf vascular bundles and spread rapidly along the veins into the leaves. In this biotrophic phase, compatible combinations do not show cell death. However, in resistant clones, the cells in direct contact with the penetration hyphae collapse. Hashim et al. (1978) ascribed this to a hypersensitive reaction and to pre-formed resistance factors (Blasquez and Owen 1957; Figari 1965), and proof was also given for induced defence compounds such as scopoletin (Tan and Low 1975; Giesemann et al. 1986; Garcia et al. 1995). This early detection process of the fungal presence in the attacked tissue of resistant plants leading to a hypersensitive response is a typical defensive reaction (Breton et al. 1997). This reaction is regarded as an indicator for complete or vertical resistance, but this concept is applicable only to mature leaves of *H. brasiliensis* and occurs with most genotypes of the previously uninvestigated host species *Hevea pauciflora* (Junqueira et al. 1988). At the biochemical level of host reactions, a hypersensitive response is often associated with well-described defence reactions such as formation of reactive oxygen-type compounds (Garcia et al. 1999), deposition of autofluorescent compounds in the cell wall (Mevenkamp 1992), synthesis of callose, occurrence of scopoletin as phytoalexin (Giesemann et al. 1986; Garcia et al. 1995), and finally cell death in a restricted area surrounding the penetrating hyphae. Detailed and quantified descriptions have been given by Garcia et al. (1999). Rubber tree leaves are formed in a flush growth pattern. Directly after bud burst, the leaves are thin, have a high respiration rate, no net photosynthesis (Lieberei et al. 1996) and are devoid of any resistance against the virulent isolates of *M. ulei.* In the course of maturation, rubber tree leaves change from susceptible to completely resistant (Chee 1980). This maturation requires 12–20 d after bud burst and the maturation time is genotype dependent.

The causal fungus *Microcyclus ulei* (P. Henn.) von Arx and E. Muller (*Dothidella ulei* P. Henn.) is specific to *Hevea* species only. The pathogen has been recorded on four species, viz. *H. brasiliensis, H. benthamiana, H. guianensis and H. spruceana.* SALB infection results in repeated defoliation, die-back of the shoots and even death of the mature trees (Stahel 1927; Holliday 1970; Rao 1973). An examination of the morphology and an updated taxonomic description of this species has appeared elsewhere (Chee and Holliday 1986). In the South American plantations, it reduced the yield by over 90%. More than 90% of the world's natural rubber requirement is being met by production from the Far East (Holliday 1970). All the planted African and Asian rubber is extremely susceptible and the climatic conditions present in the rubber-growing areas of Asian and African countries are comparable to that of the American tropics. Hence introduction of SALB into these regions could destroy the existing plantations. This has prompted rubber-growing countries to implement quarantine regulations (Altson 1950; Edathil 1986).

Eleven physiologic races (plus an avirulent one) of the pathogen have already been detected. Many clones which were reported to be tolerant/resistant to *M. ulei* have succumbed later with the appearance of more virulent strains. Six species in which natural infection was not reported include H. *camporum, H. microphylla, H. nitida, H. paudflora, H. camargoana* and *H. rigidifolia.* The clones belonging to these species are being used in Brazil for crown bud grafting on high-yielding susceptible clones. Some resistant clones which are being used for crown budding are PA 31, IAN 717, IAN 6486, IAN 7388, IAN 7657,

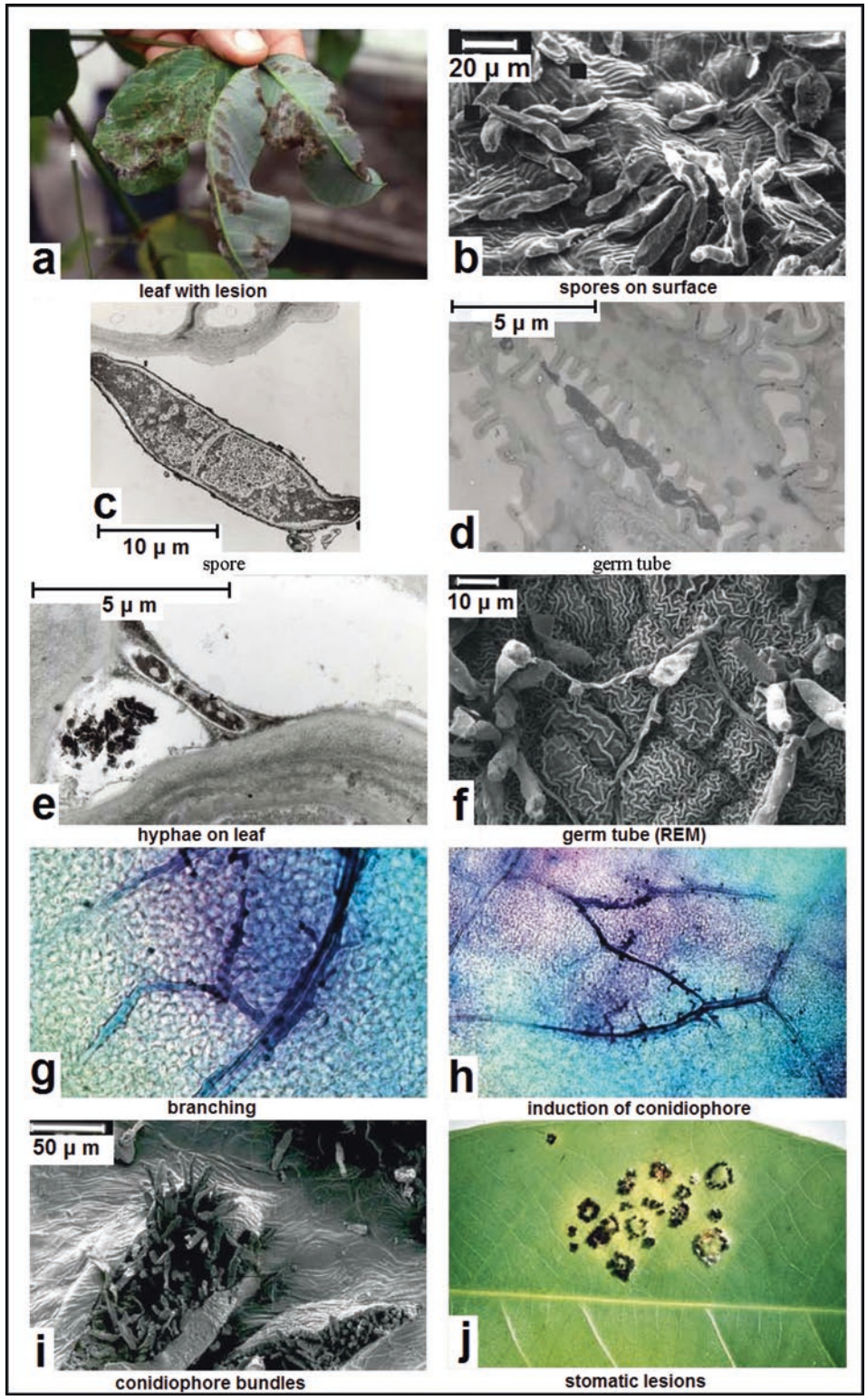

Fig. 11.2 Habit and habitat of South American Leaf Blight (SALB)

FX 25, FX 614 and FX 636 (Holliday 1970; Pinheiro et al. 1982).

Biological control of *M. ulei* using *Hansfordia pulvinata,* a hyperparasite which grows well on conidial lesions, has been attempted (Lieberei et al., 1989). It was reported by Feldman (1990) that mycorrhizal fungi can cause an increase of resistance of the rubber tree against *M. ulei.* The generation period of spores was increased and the sporulation of the pathogenic fungus was decreased. The diameter of lesions was also decreased.

Of late, molecular studies are being pursued on resistance to SALB. According to detailed studies by Seguin et al. (1996) and Lespinasse et al. (2000b), *Hevea brasiliensis* has a diploid genomic organization with rare duplicated loci. These studies led to the identification of 18 basic linkage groups in a rubber genome of 2150 cM total map length. Using the 195 progenies of the population derived from crossing of PB 260 × FX 3899 and their response to six isolated strains of *M. ulei*, eight quantitative trait loci (QTL) with respect to resistance were identified on seven independent linkage groups. Le Guen et al. (2003) and Lespinasse et al. (2000b), on the basis of their molecular data, prepared the mapping of genes conferring field resistance to SALB. For the first time, it was shown that both factors for partial resistance and for complete resistance were quantitatively expressed in the progeny and could be correlated with five loci. The molecular approach to this plant–pathogen combination has greatly enhanced the possibilities of proceeding with marker-assisted breeding and selection. Le Guen et al. (2011) attempted a QTL mapping of MDF 180 for resistance towards SALB. The resistance of progeny from a cross between PB 260 (susceptible) and MDF 180 (resistant) was assessed under controlled conditions with the inoculum of three *M. ulei* isolates and under natural conditions. Microsatellite marker mapping showed no QTL in the susceptible parent. In the resistant parent, Le Guen et al. (2011) identified a qualitative resistance gene against isolates from French Guiana and a major quantitative resistance factor determining the resistance against isolates from the state of Bahia (Brazil). The qualitative resistance gene was denominated *M15md* and was located in the linkage group g15. Two of the four minor resistance QTLs showed an epistatic interaction with *M15md*. RNA-seq high-throughput sequencing technology was used to analyze the differential expression of FX 3864 clone transcriptome at 0 and 48 h post infection (hpi) with the *M. ulei* isolate GCL012 (Páez et al. 2015). They could identify 86 differentially expressed genes associated with the defence response of FX 3864 to GCL012. Seven putative gene members of the AP2/ERF ethylene (ET)-dependent superfamily were found to be downregulated. An increase in salicylic acid (SA) was associated with the up-regulation of three genes involved in cell wall synthesis and remodelling. The defence response of FX 3864 against the GCL012 isolate was associated with the antagonistic SA, ET and jasmonic acid (JA) pathways. According to Fang et al. (2016), in the course of maturation, leaves of *Hevea* become more resistant to leaf diseases, including the South American Leaf Blight (SALB). They sequenced the *Hevea* leaf transcriptome at four developmental stages (I–IV) by Illumina sequencing to understand the underlying mechanisms of this defence and to identify the candidate genes involved (Fig. 11.3). Of the 3905 differentially expressed genes identified for leaf development, 67.8% (2651) were during the transition to leaf maturation that are meant for cyanogenic metabolism, lignin and anthocyanin biosynthesis for developing leaves (stages I–III) and mature leaves (stage IV). Such studies are really beneficial in devising strategies to engineer resistance to leaf diseases.

Several plant protection operations are being carried out for controlling this disease. Aerial spraying (8–10 rounds) is done using benomyl 300 g, thiophanate methyl 200 g or mancozeb 2 kg in 30 L water per ha at intervals of 7–10 days. For fogging 200 g thiophanate methyl or 1 kg mancozeb are being used in 6–8 L of agricultural spray oil per ha at intervals of 4–7 days (Martins and Silva 1979; Chee and Wastie 1980). Systemic fungicides like chlorothalonil (Daconil), triforine (Saprol) and triadimefon (Bayleton) are found promising in small-scale trials (Chee and Wastie 1980; Dos Santos et al.1984). In case of an accidental entry

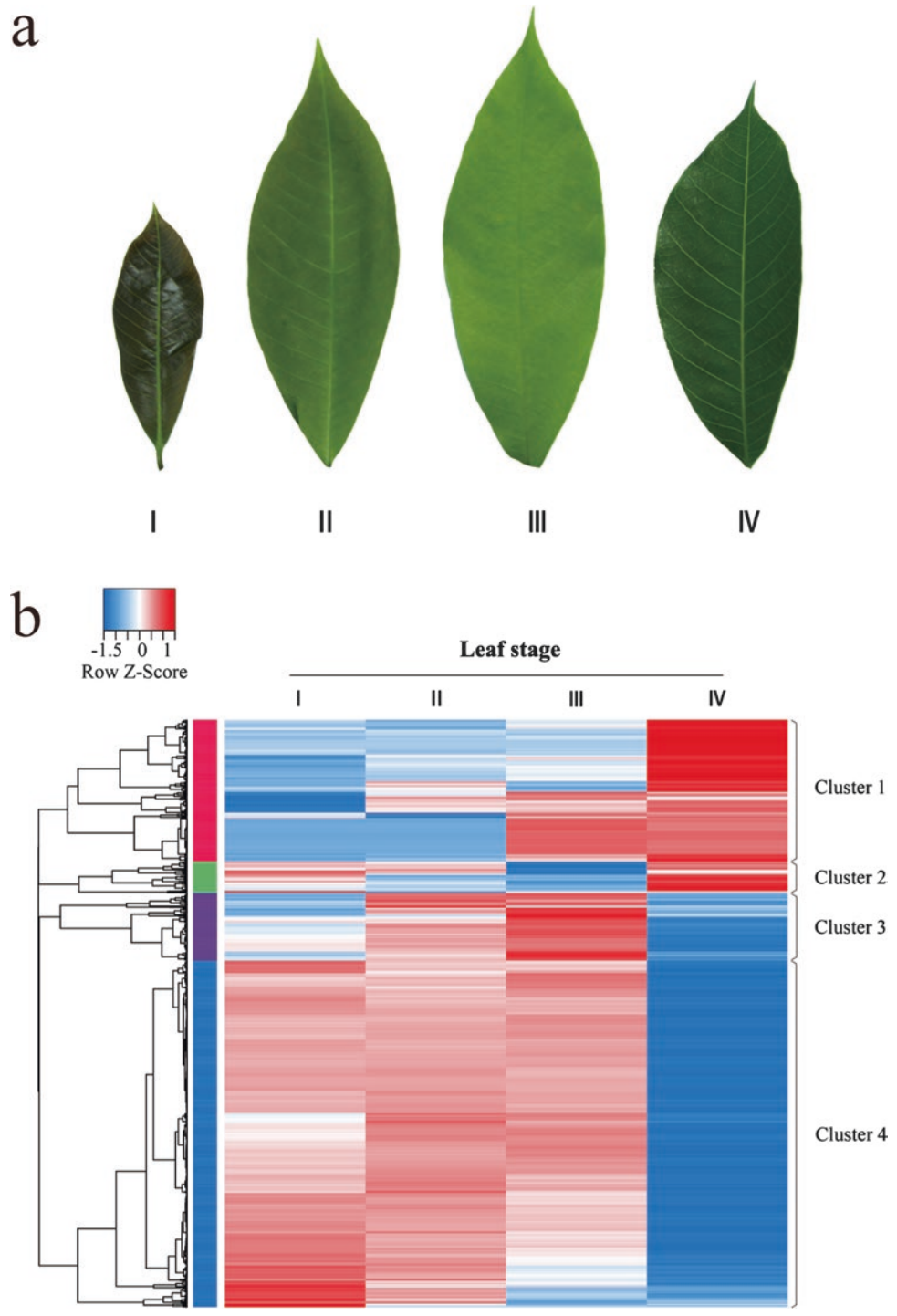

Fig. 11.3 Relationship between exponential leaf growth, transition phase from completely susceptible leaf stage to completely resistant stage. (**a**) Four representative stages of *Hevea* leaf development. I, bronze; II, colour change; III, pale green; IV, mature; (**b**) Expression profile and clustering of 3095 differentially expressed genes across four developmental leaf stages (After Fang et al. 2016) (Photo courtesy: Chaorong Tang, Rubber Research Institute, Chinese Academy of Tropical Agricultural Sciences, Danzhou 571737, Hainan, China)

of this disease, despite the phytosanitary measures, immediate adoption of eradication procedures should receive top priority. First two to three rounds of spraying (aerial application) with protectant chemicals such as mancozeb, benomyl or thiophanate methyl are given, and then the entire area is defoliated using n-butyI2,4,5-T, folex, cacodylic acid or ethephon, so that the trees remain leafless for about 2 months (Abdul Aziz 1976; Lim and Hashim 1977).

11.2 Abnormal Leaf Fall

Abnormal leaf fall is the most destructive disease in India and occurs during the southwest monsoon months of June, July and August. It infects pods, leaves and tender shoots causing heavy defoliation and die-back of tender twigs. The first report on this disease from India was in 1910 from estates near Palapilly, in Trichur district, Kerala state (McRae 1918). In due course, the disease spread to all other rubber-growing districts. Later, the disease was reported from Sri Lanka and Burma (Petch 1921). Subsequently pod rot and leaf fall were also reported from Cambodia, Vietnam, Liberia, Ghana, Nigeria, Cameroon, Congo, Brazil, Peru, Nicaragua, Costa Rica and Venezuela. In Malaysia, a serious outbreak of this disease was noticed during 1966 (Chee et al. 1967; Chee 1969). Pod rot and leaf fall due to *Phytophthora* attack have been reported from Thailand also (Chee and Greenwood 1968). Though this disease occurs in several countries, severe incidence necessitating adoption of control measures every year is observed only in South India.

Rainfall is the most important predisposing factor for the initiation and spread of the disease. In the traditional rubber-cultivated areas in India, a continuous spell of 250–350 mm rain for 7–10 days without intermittent hot sunshine, with minimum and maximum temperatures within the range of 22–25 °C and 26–30 °C, respectively, and relative humidity (RH) above 90% is most congenial for the outbreak of the disease. Under such conditions of low temperature and very high atmospheric humidity, the disease spreads rapidly and assumes epidemic proportions. Under normal monsoon, the disease starts by the middle of June and reaches the peak by the middle of July. However, when monsoon is late, very heavy incidence is noticed from the middle of July to middle of August.

Different species of *Phytophthora* are reported to be causing pod rot, bark rot, patch canker and leaf fall diseases of rubber in various countries. In India, four species of *Phytophthora*, viz. *P. palmivora* (Butler) Butler, *P. meadii* McRae., *P. nicotianae* var. *parasitica* (Dastur) Waterhouse and *P. botryosa* Cheewere are isolated from infected specimens (Thankamma et al. 1968; Edathil and George 1976, 1980). However, the species most common in the traditional areas is *P. meadii.*

Hyphae of the fungus are found to ramify inside the tissues of the infected portions intercellularly or intracellularly. Sporangia are found emerging externally through the stomata. The shape and size of the sporangia vary according to the species. During favourable climatic conditions, the pathogen resorts to the production of profuse asexual sporangia, which aid in quick dispersal and rapid spread of the disease. The sporangia liberate binucleate, biciliate and reniform zoospores, which swim in the available water and on contacting the green tissues, produce germ tubes, thus establishing a fresh infection on the host. Sporangia may also germinate directly producing germ tubes which also causes fresh infection. The pathogen gains entry into the host tissue through stomata (Thankamma et al. 1975). In general, all high-yielding clones and clonal seedlings are susceptible to abnormal leaf fall disease under Indian conditions. Clones like PB 86, PB 235, PB 260, PB 311, PB 28/59, RRIM 600, RRIM 628, RRIM 703, RRII 5, PR 255, PR 261 and Tjir 1 are observed to be susceptible to the disease.

Prophylactic spraying of rubber plants with 0.75% Bordeaux mixture is the very popular method (Ashplant 1928). Later experiments revealed that 1% Bordeaux mixture was more effective for the control of this disease and is being adopted extensively by rubber planters. It was noticed that addition of 0.5% zinc sulphate

to 0.5% Bordeaux mixture could give adequate protection to the clones RRIM 600 and RRII 105 and reduced the cost of spraying by about 35% when compared to spraying with 0.75% or 1% Bordeaux mixture (Idicula et al. 1994). As an alternative to Bordeaux mixture, Copper oxychloride (COC) dispersed in agricultural spray oil sprayed through low-volume applicators proved effective for the control of this disease.

11.3 Powdery Mildew

Powdery mildew disease was first reported from Indonesia. Subsequently, it was reported from Uganda (Small 1924) Sri Lanka (Stoughton-Harris, 1925) and Malaysia (Sharples 1926). In India, the disease was reported in 1938 (Mitra and Mehta 1938). Since then, the disease has been reported from almost all rubber-growing countries. The disease affects the immature leaves of rubber when the trees refoliate after annual wintering and causes leaf fall (Fig.11.4). Tender leaves at the brown or light green stage are highly susceptible. The presence of dull cool weather with intermittent light showers during refoliation predisposes the plants to severe disease attack. Prevalence of mist, dew and cloudy days with 75–80% relative humidity is favourable for disease development. Early wintering clones usually escape from the disease because the climatic conditions during their refoliation period are not favourable for the disease development. Late wintering clones are usually severely affected. Dry weather conditions during wintering period encourage early and rapid wintering and consequent escape from the disease. In India, the disease is severe in Kanyakumari, Idukki and Wayanad districts of South India and in the north-eastern states.

The optimum temperature for germination, infection and sporulation ranges from 25 to 30 °C (Liyanage et al. 1985). The fungus is disseminated by air-borne conidia. The peak sporulation is around noon. *Oidium heveae* Steinm, an obligate parasite, is responsible for the disease. The fungus produces superficial, branched, hyaline and septate hyphae. The hyphae are anchored on the host tissue with haustoria which help in deriving nutrients. The fungus has simple erect conidiophores which bear elliptical or barrel-shaped vacuolated conidia with round ends. The sexual stage has not yet been reported.

Leaf fall due to powdery mildew adversely affects the growth and yield of rubber trees. Wastie and Mainstone (1968) have reported a crop loss of 8.1% in the clone PB 5/51 over a period of 9 months, in Malaysia. Increased bark renewal and girth increment of trees protected against powdery mildew compared to unprotected trees were also observed. Tan et al. (1985) have reported 6.3–10.3% yield increase by controlling

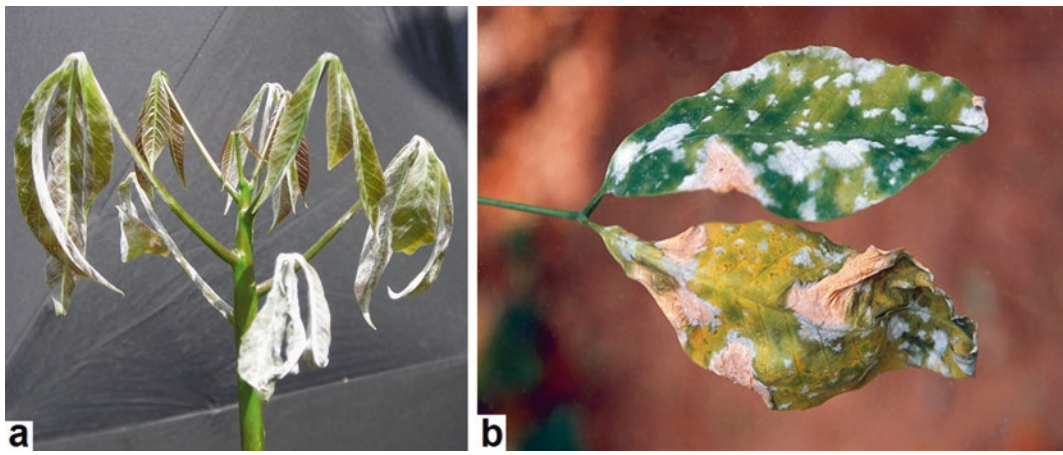

Fig. 11.4 Infestation by *Oidium heveae* in nursery and mature leaves

powdery mildew disease. In India, it was observed that in clone PB 86, 8–12% more disease in unprotected plots when compared to protected resulted in 21–32% crop loss. Similarly, 8–18% more disease in unprotected RRIM 600 caused 14–29% crop loss. The disease caused reduction in yield throughout the year. Disease resistance has been reported only in the low-yielding clone LCB 870 from Sri Lanka. In India, clones PB 86, GT 1, Gl 1, PR 107, RRIM 703, RRII 208 and PB 310 show some tolerance. The clones Tjir 1, PB 5/51, RRIM 605, RRII 105, RRII 118, RRII 300, PR 261, PB 21.7, PB 235, PB 280 and PB 311 are susceptible.

Chinese clone Reyan 7-33-97 is susceptible to powdery mildew. To study the benzothiadiazole (BTH)-induced resistance at gene level, Luo et al. (2013) constructed differentially expressed cDNA library by suppression subtractive hybridization (SSH). There were 23 cDNA sequences matching the function of basic metabolism, signal transduction and secondary metabolism selected randomly from the cDNA library and comparison to nucleic acid sequences in *GenBank*. Seven expressed sequence tags (ESTs) were logged in *GenBank,* and accession numbers were GW873071 and GW874604-GW874610. Their results implicated that BTH could effectively induce resistance to powdery mildew through increasing expression of defence-related genes in leaves. Such approaches should provide new insights for rubber disease management.

Wang et al. (2014) studied the effects of powdery mildew infection on the mitochondrial and chloroplast functions. Powdery mildew damaged the structure and function of mitochondria prior to chloroplasts, causing inner and outer membranes disruption. The intact rate of mitochondria membrane was reduced from 70% in control leaves to 23.1% in the leaves at 5 days after inoculation (dai). Significant decreases in the activities of cytochrome c oxidase, NADH oxidation and malate dehydrogenase (MDH) were observed in the powdery mildew-infected leaves. Tricarboxylic acid cycle (TCA) and electron transfer capacity were seriously impaired after powdery mildew invasion. Chlorophyll contents, maximal photochemical efficiency (Fv/Fm), actual photochemical efficiency of photosystem II (ΦPSII) and electron transport rate (ETR) were dramatically decreased in the infected leaves from 10 days after infection.

Dusting with sulphur gives effective control of powdery mildew disease. Spraying wettable sulphur is preferred only in the nurseries and young rubber plantations as repeated spraying in mature areas is expensive and impracticable. Sulphur dust having a minimum of 70% sulphur is generally used for dusting. The dust should be dry, free flowing and should pass through 325 mesh-sieve (particle size 40 microns). Dusting is done at the rate of 11–13 kg/ha at an interval of 7–10 days. Three to six rounds of dusting are usually required. First round of dusting is done when 10% of the trees start refoliation. Micron duster is employed for this purpose. The duster should be carried along every fourth row of trees at a speed of 3–4 km/h. With one duster, nearly 10–12 ha can be covered in a day. Sulphur dusting should preferably be done early in the morning so that the dew on the leaves helps in sticking of the dust. The still air in the morning hours also helps to raise the dust to reach the canopy. An integrated approach using tridemorph and sulphur in dust form was found to be more effective (Edathil et al. 1992). Carbendazim (Bavistin) 1.5% dust has also proved to be effective and could be used in integration with sulphur.

11.4 Corynespora Leaf Disease

Corynespora cassiicola (Berk. & Curt.) Wei is an important plant pathogenic Ascomycete causing the damaging corynespora leaf fall (CLF) disease. A small secreted glycoprotein named cassiicolin was previously described as an important effector of *C.* cassiicola. *Corynespora* causes leaf spot and leaf fall diseases. First reported in India from seedling nurseries (Ramakrishnan and Pillay 1961b), it was then reported from Malaysia (Newsam 1963), Nigeria (Awodern 1969), Indonesia (Soepena 1983), Sri Lanka (Liyanage et al. 1986) and Thailand (Kajornchaiakul et al. 1987). The disease has now been found in almost

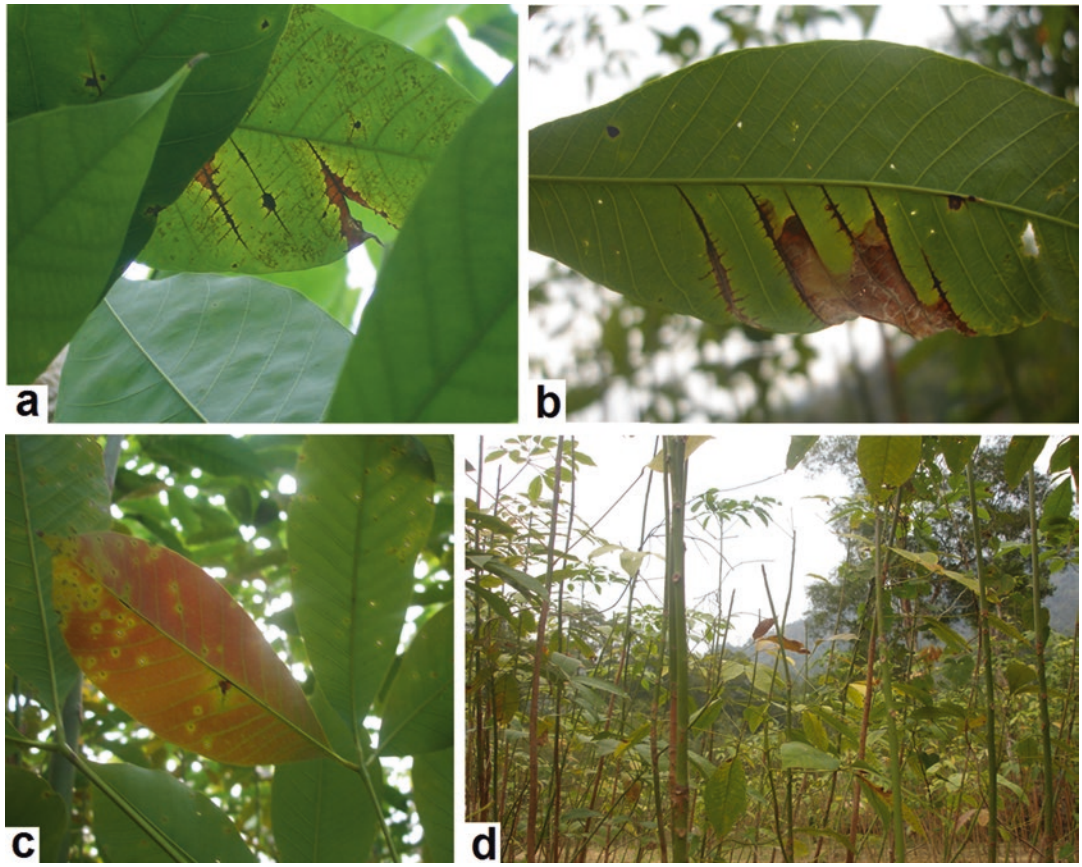

Fig. 11.5 Corynespora leaf disease in Hainan, China (Photo courtesy: Pu Jinji, EPPI, CATAS, China)

all rubber-growing regions (Chee 1988). Severe leaf fall was reported from Malaysia (Tan 1990) and Indonesia (Sinulingga et al. 1996). The disease appears in mature plantation during refoliation period infecting young leaves. The environmental factors favouring disease development are high humidity, a temperature of 28–30 °C, humid air and cloudy weather (Situmorang et al. 1996). The conidia of the fungus, produced abundantly on infected leaves, are carried by wind and cause rapid spread of the disease. The spore release increases steadily from morning and reaches the peak by noon and thereafter falls to very low levels (Chee 1988). The spore load in air has been negatively correlated to rainfall (Radziah et al. 1996). Conidia remain viable for about a month. Although the host range of *Corynespora* is wide (Liyanage et al. 1986), cross infectivity is doubtful (Chee 1988). In the severe form of the disease, a characteristic browning and blackening of veins gives a 'fishbone'- or 'railway track'-like appearance (Fig. 11.5). Even a single leaf spot can cause defoliation. Severe infection on the midrib causes leaf blight. When leaf petioles are infected, greyish-black lesions are formed causing defoliation without any symptoms on the lamina. Repeated defoliation and refoliation lead to shoot die-back. However, there is a relationship between exponential leaf growth, transition phase from completely susceptible leaf stage to completely resistant stage (Fig.11.6).

M. Déon, et al., Diversity of the cassiicolin gene in Corynespora cassiicola and relation with the pathogenicity in Hevea brasiliensis, Fungal Biology (2013) studied the diversity of the cassiicolin-encoding gene in *C. cassiicola* isolates sampled from various hosts and geographical origins. A cassiicolin gene was detected in 47%

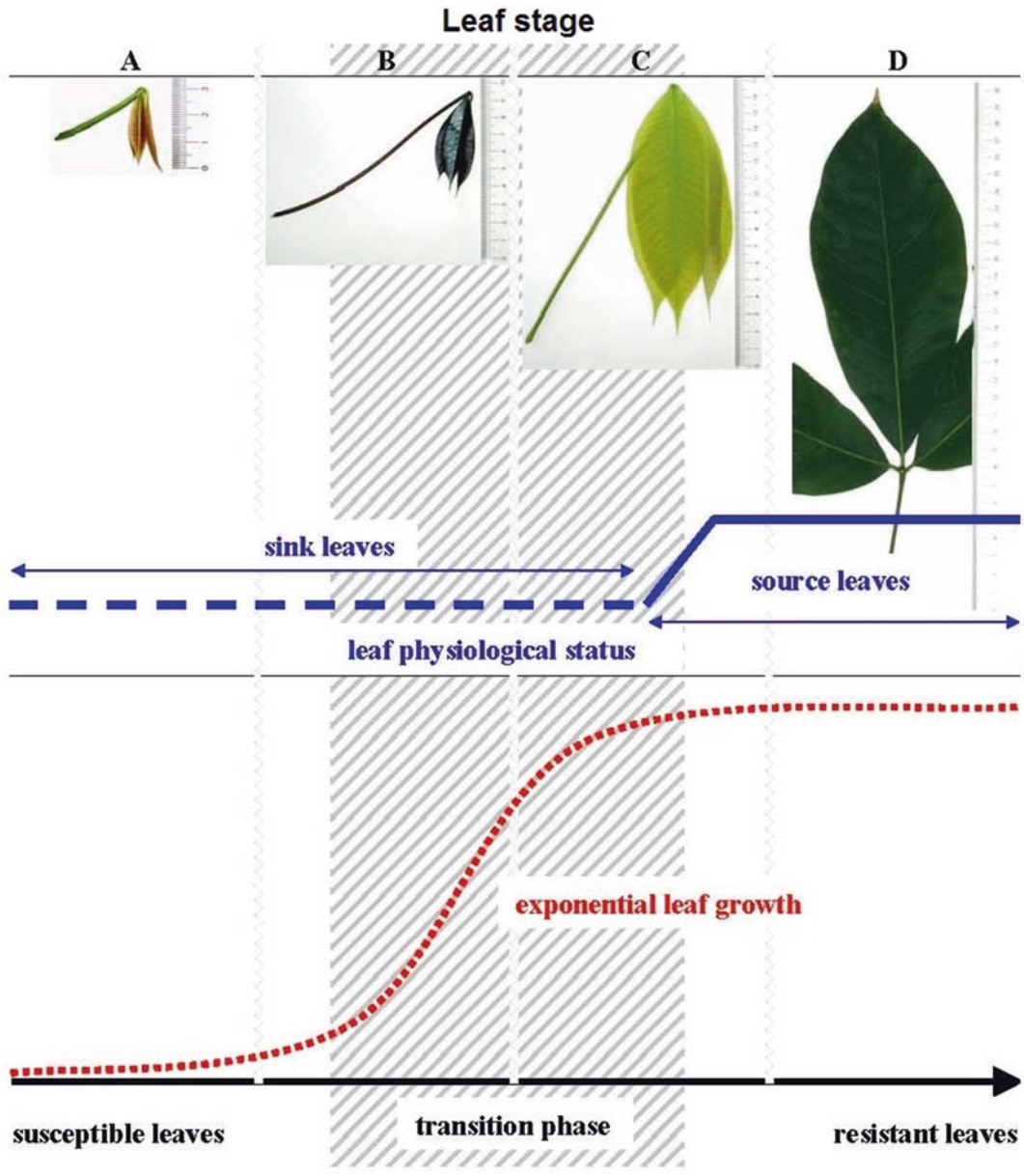

Fig. 11.6 Relationship between exponential leaf growth, transition phase from completely susceptible leaf stage to completely resistant leaf stage and the short physiological step from sink to source leaves. This attempts to he different developmental processes that turn a susceptible leaf into completely age resistant. The hatched area shows the resistance factors that interact in various ways and do not allow the correlation of the QTLs developed with the leaf properties in transit

of the isolates, encoding up to six distinct protein isoforms. In three isolates, two gene variants (Cas2 and Cas6) encoding cassiicolin isoforms were found in the same isolate. A phylogenetic tree based on four combined loci and elucidating the diversity of the whole collection was strongly structured by the toxin class, as defined by the cassiicolin isoform. The isolates carrying the Cas1 gene (toxin class Cas1) were all grouped in the same highly supported clade and were found

the most aggressive on two rubber tree cultivars. The study of Déon et al. (2013) provides a platform for future studies of *C. cassiicola* population biology and epidemiological surveys. RRIC 103, RRIC 104, RRIM 600, RRIM 725, Tjir I, RRIC 110, RRIC 133, RRIM 600 GT 1, PB 5/51, PB 217, PB 235, PB 260, PR 107, RRIM 901, RRIM 905 and Tjir 1 are seen susceptible (Tan 1990; Jayasinghe and Silva 1996). AVROS 2037, BPM 24 and RRIC 100 are reported as tolerant from Indonesia (Azwer et al. 1993). Studies conducted in France indicated PB 260 to be highly susceptible and GT 1 to be tolerant (Breton et al. 1997).

Several fungicides have been recommended for the control of *Corynespora* leaf disease. Spraying of benomyl, mancozeb, captan or propineb is recommended for affected nursery plants (Jayasinghe and Silva 1996; Hashim 1994). Four to five rounds of spraying with tridemorph (Calixin 0.6/ha) or mancozeb (Dithane M45 1.5–3 kg/ha) are recommended for *Corynespora* control in Indonesia (Soepena et al. 1996).

11.5 Shoot Rot

Initial symptom of this disease is noticed on the terminal portions, especially on the purple-coloured leaflets. Within 24–48 h, the leaflets become dark-coloured and the rotting extends up to the petiole. In a short time, infection spreads to other leaflets also. Subsequently, infection spreads to the stem and progresses from apex to downwards. The affected portions of the stem are initially dark brown but later turn black and shrunken. The rotting of the shoot may extend from 15 to 75 cm in length. The diseased portion dries up and later new branches arise from below the infected portion. Clones that are susceptible to abnormal leaf fall are also susceptible to shoot rot.

The disease could be controlled by prophylactic spraying with copper fungicide for mature and immature plants in the field. Repeated spraying with 1% Bordeaux mixture or 0.5% Bordeaux mixture +0.5% zinc sulphate at an interval of 7–10 days is required to protect the young plants in the nursery and field during the monsoon period. Phosphorous acid at 0.16% and metalaxyl MZ at 0.2% are also effective in checking the disease (Idicula et al. 1998).

11.6 Gloeosporium Leaf Disease

Even though the disease is not a serious problem in mature rubber, it has been observed throughout the rubber-growing regions. Though confined to seedling and budwood nurseries, immature plants are also being seriously affected. The disease is generally noticed during April to May, before the onset of southwest monsoon and in August, September and October or whenever wet weather is prevailing. High humidity is a pre-requisite for the formation of sporocarp. Free water is necessary for optimum germination of the fungus. Germination of spores occurs in a few hours at 100% humidity and longer time is taken at lower levels of humidity (Wastie 1972).

Tender leaves produced soon after bud burst are more susceptible to infection. Under extensive damage, leaves become distorted, turn black, shrivel and fall off, leaving the petioles on the stem. The infection usually starts at the tip of the leaf and spreads towards the base. If the leaf gets infected at a later stage, it becomes either highly spotted or may be partially damaged along the tip and margin. As the leaf ages, the margins of the leaf spots become thick and raised above the surface as conical projections, this being the most important diagnostic feature of the disease. The pathogen is *Gloeosporium alborubrum* retch. The hyphae of the fungus penetrate the tissues of the affected part. Intraepidermal or subepidermal stromata are formed on the infected region.

A severe incidence in the immature plants in the field may lead to heavy defoliation and shoot die-back resulting in the girth retardation and extension of immaturity period. In Indonesia, the persistence of this disease over a long period resulted in loss of yield up to 50% and delay in maturity up to 3 years (Basuki 1992). After 3 years of continuous artificial defoliation to control SLF, a yield increase exceeding 30% was achieved in Malaysia (Radziah and Hashim 1990).

PB 217, PB 260 and RRIM 600 are the clones having some tolerance.

Copper oxychloride spraying carried out as a prophylactic measure in April/May keeps this disease under check. Mancozeb (0.2%), carbendazim (0.05%), bitertanol (0.025%) and Bordeaux mixture (1%) were found effective in controlling the disease in young rubber plantations (Joseph et al. 1994). Mechanical fogging of captafol in oil (0.6 kg/ha) thrice at weekly intervals during refoliation gave good control of the disease in Malaysia (Tan et al. 1985). In Malaysia, artificial defoliation by aerial spraying of several chemical defoliants mixed in water is practiced (Radziah and Hashim 1990). In Cameroon, ethephon (3 l/ha)-induced defoliation and early refoliation helps in avoiding secondary leaf fall (Senechal and Gohel 1988).

Biotechnology

12

The attainment of yield plateau and prevalent intra-clone variations in yield of *Hevea* prompted researchers to tackle these problems through employing the modern tools of biotechnology. However, higher yield alone would not encourage cultivation of *Hevea*, since the species is sensitive to biotic and environmental attributes and physiological disorders. The long breeding cycle and the large size of the crop also make breeding time-consuming. Biotechnology applied to *Hevea* can be discussed under two headings: (i) in vitro culture and (ii) molecular breeding. While in vitro culture deals mainly with regeneration and propagation, molecular breeding includes identification, characterisation, introduction and expression of novel genes (see Chap. 13). The emergence of genomics has overshadowed the relevance of in vitro culture. The conspicuous reason for this is that in vitro culture was attempted by several laboratories worldwide, but progress had been very minimal as the techniques envisaged could not be commercialised successfully. On the other hand, genomics worked meticulously that could make inroads into the intricacies of *Hevea* culture and development faster than any other branch of science.

12.1 In Vitro Culture

Experimentation with in vitro culture of rubber commenced during the 1960s with Chua (1966) attempting to derive callus cultures from the plumule tissues of seedlings. The effects of osmotic concentration, carbohydrates and pH of the culture media were also studied. Later, the RRIM took the initiative of undertaking large-scale tissue culture work through maintaining callus cultures from various explants (Paranjothy and Gandhimathi 1976). It expanded to somatic embryogenesis and micropropagation through stem explants. While anther culture was employed to achieve pure lines first and exploitation of heterosis thereafter, micropropagation and somatic embryogeny were used to generate homogeneous populations. Although research on in vitro culture commenced nearly 45 years ago, even after rigorous experimentation, these areas are still in their infancy due to shortcomings towards commercial applicability. Expectations of better performance of these multiplication techniques are based on three considerations: (i) cloning the root system would generate new and more homogeneous rootstocks or monogenetic clones; (ii) selection of clonal roots would improve the exploitation of existing genetic variability; and (iii) use of rejuvenated clonal plant material would potentially provide important agricultural attributes towards higher growth, latex yield and resistance to wind and dryness.

Carron et al. (1989, 1995a, b, 2001, 2005) has amply reviewed in vitro approaches applied to the rubber tree (including tissue culture, haplogenesis, microcutting, somatic embryogenesis, protoplast culture, germination of immature embryos and cultivation of laticiferous tissue).

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Microcuttings and somatic embryogenesis were studied in *Hevea* in order to achieve rapid clonal propagation as an alternative to the drawbacks of the use of cuttings and bud grafting techniques. Somatic embryogenesis as a means of regeneration opens up possibilities for transgenic technology. In vitro culture is made up of the application of many laboratory protocols involving hormones, nutrients and culture medium and of histocytological controls; details can be found in the works of Chen et al. (1982), Chen (1984), El Hadrami et al. (1991), Etienne et al. (1991, 1993, 1997a), Carron et al. (1992), Housti et al. (1992), Montoro et al. (1993), Veisseire et al. (1994a, b), Wang and Chen (1995), Seneviratne and Wijesekara (1996), Cailloux et al. (1996), Linossier et al. (1997), Wang et al. (1998), Sushamakumari et al. (2000a) and Kumari Jayashree et al. (2001). Bouychou (1953), Chua (1966), Wilson and Street (1975), Paranjothy and Gandhimathi (1976) and Audley and Wilson (1978) were the first rubber researchers to develop callus and tissue cultures derived from epicotyl, green stem or plumule tissues of young seedlings. The aim was to use calli to study the laticiferous system and the action of ethephon, but they encountered problems of ploidy instability. The RRIM took the initiative of maintaining callus cultures from various explants that later expanded to somatic embryogenesis and micropropagation through stem explants (Paranjothy and Gandhimathi 1976).

12.2 Anther Culture

The Rubber Research Institute of Ceylon (RRIC) was the first to carry out the culture of anthers to raise haploid plants (Satchuthananthavale and Irugalbandara 1972). However, the first plants from *Hevea* pollen were made available during 1977 at the Baoting Institute of Tropical Crops, Hainan, China (Chen et al. 1979). Since then, at least four laboratories in China took the lead in researching the production of haploid plants in vitro (Carron et al. 1989). In addition, attempts were made to produce plants through gynogenesis (Guo et al. 1982; Yang and Fu 1997).Carron et al. (1989) enumerated three phases for the production of haploids from anther culture. In the first phase, production of callus and embryos takes nearly 50 days. Here, the media formulation is vital since the balance between callus development and initiation of embryos needs to be maintained (Chen 1983). The modified MB (microbouturage) medium (Chen 1984) is widely used with the addition of naphthalene acetic acid (NAA) and coconut water, which regulate development of microspores, and a judicial concentration of sources of N, K and sugar leads to the production of calli and embryos. The somatic callus then degenerates and the embryos develop from microspores. Subculture must be carried out at this stage into differentiation medium in order to avoid degeneration of embryos (Chen et al. 1982). Maturity of embryos is the crucial factor in the second phase. The cultures need 2–3 months for the apical bud to develop. Coconut water at this stage will be substituted with gibberellic acid (GA3) for better development of cotyledons. In the third phase, progressive increment of GA3, gradual withdrawal of other growth regulators, addition of 5-bromouracil and reduction of sugar result in the development of plants from embryos. Cytological investigations of callus, embryos and plantlets showed mixoploidy (Qin et al. 1979). However, when the plants develop in vitro, there is a progressive tendency towards diploidy (Carron et al. 1989). Above all, the developmental stage of the anther is vital for the right results. The anthers from male flowers that have a yellow corolla should not be selected, for the microspores will be in a binucleate stage. Such anthers will repress callus development and embryogenesis. Only uninucleate pollen is ideal for haplogenesis, which can be obtained from greenish-yellow flowers (Chen 1984; Shije et al. 1990). (see Chap. 4 for somatic embryogenesis and meristem culture).

12.3 Protoplast Culture and Embryo Rescue

Attempts towards protoplast culture and fusion were carried out using young immature leaves (Cailloux and Lleras 1979; Wilson and Power 1989), using discs of pith in the apical part of young green shoots or anther calli (Othman and Paranjothy 1980). Subsequently, Cazaux and d'Auzac (1994) obtained microcalluses from embryogenic callus-derived protoplasts of *H. brasiliensis*, but without plant regeneration. Recently, Sushamakumari et al. (2000b) reported successful plant regeneration from embryogenic cell suspension-derived protoplasts of *Hevea*. Protoplasts were isolated from immature inflorescence-derived cell suspensions and produced microcolonies on 'KPR' medium (Kao and Michayluk 1975). Protoplast-derived cell colonies proliferated, upon subculture on MS-based regeneration medium, with 40% of the protoplast-derived calli developing somatic embryos. Subsequently, they germinated into plants on the same medium. Fusion of protoplasts was aimed at hybridising different *Hevea* species for breeding resistance to SALB.

In vitro germination of mature and immature zygotic embryos issued from hand pollination has been considered as a way of improving the success rate of genetic recombination in rubber (Muzik 1956; Paranjothy and Gandhimathi 1976; Carron 1981). Good results (90% success in germination) were achieved only for immature embryos that were at least 3–3.5 months old after fertilisation. It also appeared that immature seeds of this age could be germinated in vivo under controlled conditions. However, this procedure, which is expensive, did not appear to guarantee increased efficiency, nor was it a means for rescuing rare progenies.

12.4 Direct Gene Transfer

Somatic embryogenesis in rubber is becoming standardised in different laboratories worldwide as an efficient system for plant regeneration from cells. At the same time, efforts have been made to transform *Hevea* cells in order to increase genetic variation in a targeted way (a new form of mutagenesis) and complement plant breeding efforts with the possibility of modifying already selected high-performing clones with specific genes (addition or inactivation) while avoiding meiotic recombination. However, in the short term, genetic transformation is becoming a powerful tool for investigating how the rubber genome functions with the assistance of targeted mutations.

The first transgenic rubber trees were reported by Arokiaraj et al. (1994, 1996, 1998), who used the particle bombardment method and then the *Agrobacterium tumefaciens* system on anther-derived calli from clone GL 1, with a view to in vivo production of high-value recombinant proteins (Yeang et al. 1998). The first experiments were carried out with plasmid vectors harbouring the strong and non-specific cauliflower mosaic virus (CaMV) 35S promoter and β-glucuronidase and *nptII* reporter genes encoding neomycin phosphotransferase. Plant regeneration rates are strongly affected by genetic modification and require improvement. However, fluorometric assays and ELI-SAs were performed to prove the expression of *gus* and *nptII* genes, respectively, in calli and embryoids (Arokiaraj et al. 1996). The expression of foreign proteins in *Hevea* latex was demonstrated by Arokiaraj et al. (1998). This transformation appeared to be stable even after three vegetative generations with no chimeras, indicating that a single transformed plant is sufficient to achieve a population through bud grafting. Lately, a gene for an antibody fragment against the coat protein of the bacterium *Streptococcus sanguis* (Yeang et al. 2002), a gene coding for 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR, involved in rubber biosynthesis) and a gene for human serum albumin (Arokiaraj et al. 2002) have been expressed in rubber latex through genetic transformation. At the present time, these transformation experiments are based on a limited number of regenerated trees.

Stable transgenic callus lines starting from integument-derived friable embryogenic calli of the clone PB 260 have been transformed by *A. tumefaciens* (Montoro et al. 2000, 2003),

and more than 200 transformed plantlets, incorporating the *uidA* reporter gene, have been transferred to soil in a greenhouse (Pujade-Renaud et al. 2005). Recently, Jayashree et al. (2003) achieved *A. tumefaciens*-mediated genetic transformation and the regeneration of transgenic plants in *H. brasiliensis*. This was the first report of the production of transgenic plants with a superoxide dismutase gene (*HbSOD*) under the CaMV 35S promoter using an *A. tumefaciens*-mediated gene transfer system for *H. brasiliensis*. The morphology of the transgenic plants was similar to that of untransformed plants. Histochemical *gus* assay revealed the expression of the *uidA* gene in embryos as well as leaves of transgenic plants. The presence of the *uidA*, *nptII* and *HbSOD* genes in the *Hevea* genome was confirmed by PCR amplification and genomic Southern blot hybridisation analyses. Subsequently, an efficient and reproducible protocol for *A. tumefaciens*-mediated genetic transformation and plant regeneration of *Hevea* with the gene coding for superoxide dismutase under the control of figwort mosaic virus (FMV) 34S promoter has been reported by Sobha et al. (2003a, b). Further, the authors claimed that the transgenic plants are being grown in poly bags and they will be bud grafted for the evaluation of oxidative stress later. Plants of RRII 105 were integrated with the Manganese superoxide dismutase (MnSOD) gene through *Agrobacterium*-mediated genetic transformation events. The SOD activity was 35% and 31% higher under normal and drought conditions, respectively, in one of the transgenic plants than that of bud-grafted plants. Derivation of transgenic plants integrated with genes like MnSOD is a significant step forward to cultivate rubber under marginal conditions (Jayashree et al. 2011).

Evidence on the functionality of introgressed genes and identification of genes of interest for research investigation or for clonal improvement are issues relating to the field of genomics (discussed in the next section). To complement this process, research has been under way for identifying, cloning and characterising specific promoters to be associated with genes of interest in a plasmid vector, in order to optimise the gene expression, especially at the level of the latex cells of the tapped rubber tree. Cloning of ethylene-inducible and/or laticifer-specific promoters from the rubber tree has been undertaken (Pujade-Renaud et al. 2000, 2001). Glutamine synthetase (*gs*) and hevein (*hv*) gene promoters were targeted, based on the fact that *gs* overexpression was observed in latex after ethylene treatment (Pujade-Renaud et al. 1997), and the *hevein* protein has been found only in laticifers (Broekaert et al. 1990). Genomic clones of genes were obtained and partially sequenced (*hv1*, *hv2* and *gs1*, *gs2*, *gs3*). Unfortunately, the promoter region of *gs1* was lacking. It was not possible to distinguish *gs2* from *gs3*, or *hv1* from *hv2*, as these genes were highly homologous, including in their non-coding regions. Gene expression analysis revealed that (i) both *gs1* and *gs2*/*gs3* were responsive to ethylene in latex, with *gs1* apparently strictly induced and *gs2*/*gs3* overexpressed; (ii) *hv* gene expression in latex was very strong but not significantly responsive to ethylene; (iii) *gs1* and *gs2*/*gs3* were differentially expressed in tissues derived from in vitro culture at various stages of development; (iv) both *gs* and *hv* genes were highly expressed in undifferentiated tissue; and (v) *hv* gene expression increased with embryo development, according probably to the laticifer differentiation stage. Sub-cloning of *hv1*, *hv2*, *gs2* and *gs3* promoter regions in a vector for transformation, in fusion with the *gus* reporter gene, was undertaken in order to analyse the functionality of these promoters. As a preliminary result, the *gs3* promoter-*gus* construct was introduced into rubber tree callus tissue by particle gun bombardment.

Transient *gus* activity was detected, which demonstrated functionality of the isolated *gs3* promoter. As *gs* genes revealed differential expression during the embryogenesis process, isolated *gs* promoters combined with a fluorescence reporter gene could become, under non-destructive conditions, a potential marker of embryogenesis. As *hevein* belongs to a multigene family, different *hevein* precursor genes were cloned and compared by sequence alignment, revealing a divergence between two groups (*Hev1* and *Hev2*) in their promoter regions. One representative in each group was chosen

(*Hev1.1* and *Hev2.1*) and promoter-*gus* constructs were introduced into rice callus tissues by *A. tumefaciens* for functional analysis in a heterologous host (Pujade-Renaud et al. 2005). The two promoters were found to be functional and, to some extent, inducible, but *Hev1.1* expression level was very low, adding to other observations (P. Arokiaraj, unpublished results; P. Montoro, unpublished results), suggesting that the range of tissues and organs expressing the *hevein* promoters may be larger and not restricted to latex cells. *Hev2.1* was activated by wounding in rice, confirming Northern blot expression profiles observed in rubber, and was also induced by pathogen infection (*Magnaporthe grisea*) in rice. Functional analysis of these promoters is now continuing in the rubber tree itself; however, the *Hev2.1 hevein* gene promoter is assumed to be able to drive efficient overexpression of genes transferred to the rubber tree at the level of latex cells. New molecular constructs have recently been prepared with promoter *Hev2.1* and genes *Cu*/Zn-*SOD* and *GCL* (codes for glutamyl cysteine ligase, involved in resistance to oxidative stress).

Conventional rubber breeding takes more than 25 years to develop a new clone, but genetic transformation is the quick alternate method to introduce desirable genes. The first transformation report in *Hevea brasiliensis* was published in 1991 (Arokiaraj and Wan 1991) through *Agrobacterium*-mediated transformation. The first transgenic *Hevea* plants, using anther-derived callus as the explant of the clone Gl1, were successfully developed by Arokiaraj et al. (1994) following biolistic transformation method. Subsequently, transgenic plant was developed using *Agrobacterium*-mediated gene transfer of anther-derived calli (Arokiaraj et al. 1996, 1998). Inner integument tissue of the immature fruit of the clone PB260 was used as the explant for genetic transformation (Montoro et al. 2003). Transgenic plants of *H. brasiliensis* PB260 were developed through *Agrobacterium*-mediated transformation by Blanc et al. (2005). Earlier transformation events were only with various marker genes. Later the experiments were focused on transferring various agronomically important genes into *Hevea* with enhanced tolerance to abiotic stresses, production of recombinant proteins etc. Subsequently, attempts were made to increase the SOD enzyme activity by overexpression of the same genes in *Hevea*. Transgenic plants were developed with SOD gene under the control of CaMV 35S and FMV 34S promoters (Jayashree et al. 2003; Sobha et al. 2003a). Biochemical analysis of the transgenic embryogenic callus of *Hevea* with SOD indicated significant increase in the activity of superoxide dismutase, catalase and peroxidase as compared to the control (Sobha et al. 2003a, b). Jayashree et al. (2003) reported successful development and establishment of transgenic rubber plant with SOD gene for their further evaluation. Genetic transformation experiment to overexpress *hmgr1* gene, involved in latex biosynthesis, in *Hevea* was performed by Arokiaraj et al. (1995). They could generate transgenic embryos, which failed to produce any transgenic plant. However, they showed enhanced *hmgr* activity in the transformed calli. A significant achievement towards antibiotic marker-free *Hevea* transgenic development avoiding the constraints of GMO regulations was made by Leclercq et al. (2010). They developed an efficient genetic transformation procedure for PB260 using a recombinant green fluorescent protein (GFP). They showed GFP selection is less time-consuming in terms of callus subculturing and offered the possibility of producing antibiotic-resistant marker-free transgenic plant. Unfortunately, till date, none of these transformed genotypes has been taken to the planter's field for commercial utilisation.

Genomics and Molecular Breeding 13

Scientific developments over the last two decades have led to a new phase of genetics—the plant genomics. This is the application of a wide range of novel methods and technologies for analysing the structure of the genome and its interaction with cell metabolism for protein synthesis and the use of this knowledge for better understanding of the functioning of plants or improving them into new varieties. Plant genomes are more complex than other eukaryotic genomes, and analysis reveals many evolutionary flips and turns of the DNA sequences over time. One spectacular result achieved at the beginning of this century was the entire genomic DNA sequencing of *Arabidopsis* (The Arabidopsis Genome Initiative 2000) (see Bevan and Walsh 2005) and *Oryza* (rice—International Rice Genome Sequencing Project (IRGSP)) (Eckardt 2000; Jackson 2016), the main dicotyledon and monocotyledon plant models used due to their small genomes. Some recent powerful technologies are:

(i) automatic DNA sequencing, where one machine can read two million base pairs a day (Egan et al. 2012);
(ii) microarrays and DNA chips where tens of thousands of genes can be scanned for activity levels at the same time (Pflieger et al. 2001); and
(iii) automated genotyping machines that can assay tens of thousands of DNA diagnostic points a day (Li et al. 2001).

In fact, it will soon be possible to monitor whole genomes by the use of DNA molecular genetic markers (MGMs) or analysis of gene expression on single chips. Two main fields must be distinguished: (i) MGMs, which are non-coding DNA fragments independent from the variation of the environment, and (ii) expressed genes. Genomic technologies were taken up by various research groups working with *Hevea*, in order to increase knowledge and also to identify new targets for breeding and/or complement genetic transformation and assist rubber breeders in various strategies. The group led by Huasun at the Rubber Research Institute, Chinese Academy of Tropical Agricultural Sciences (CATAS) (in collaboration with Beijing Institute of Genomics, Chinese Academy of Science), Danzhou, China (see Tang et al. 2016) and Centre for Chemical Biology, Universiti Sains Malaysia (Rahman et al. 2013) and the group led by Sithichoke Tangphatsornruang at the National Center for Genetic Engineering and Biotechnology, Bangkok, are the prominent schools working on *Hevea* genomics. Tun Abdul Razak Research Centre (TARRC) (Malaysian Rubber Board) and The Genome Analysis Centre (TGAC), Norwich Research Park, UK, are also actively participating in this research. The latest advancement was during 2013, when Rahman et al. reported the draft genome sequence of *H. brasiliensis*. The assembly spans to ~1.1 Gb of the estimated 2.15 Gb haploid

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genome. Overall, ~78% of the genome was identified as repetitive DNA. This was refined by Lau et al. (2016).

13.1 Non-expressed Molecular Genetic Markers (MGMs)

In conventional plant breeding, many morphological traits are used as markers for analysing genetic traits and identifying cultivars, but specific genetic information on Mendelian traits has been rare in *Hevea*. In the 1980s, isozymes, the expressions of which are not modified by the environment, have been used in rubber as proteic genetic markers for (i) cultivar identification, (ii) genetic diversity analysis, (iii) control of progenies originating from hand pollination and (iv) reproductive biology (Chevallier 1988; Leconte et al. 1994; Paiva et al. 1994). Analysis of isozymes was developed at CIRAD with a set of 13 polymorphic isozymic systems to formulate a diagnostic kit associated with a clonal identification database. This kit is proved to be able to differentiate a large set of cultivated clones (Leconte et al. 1997). However, the analyses are to be carried out near the field sites due to the fragility of isozyme molecules exposed to normal temperature, or otherwise the samples need to be freeze-dried before transportation to the laboratory. Moreover, isozyme-based analyses are limited by the rather small number of marker loci available and a general lack of polymorphism for these loci.

MGMs are the ideal means for identifying genotypes and tracing the segregation and inheritance of alleles related to economically important characters. MGMs are powerful tools that could enhance the speed and effectiveness of rubber breeding as is already the case for maize and some other plants. General advantages of DNA markers include (i) their ability to reveal the sites of variation in DNA segments among many individuals, (ii) their abundance and distribution over the whole genome and (iii) their independence from the variations of the environment. There is a growing arsenal of MGMs that are currently used, notably in identifying quantitative trait loci (QTLs). The process of using such markers as selection criteria is called marker-assisted selection (MAS), the methodology of which is still at research level in rubber. There are several types of MGMs currently used: (i) restriction fragment length polymorphism (RFLP), (ii) random amplification of polymorphic DNA (RAPD), (iii) amplified fragment length polymorphism (AFLP), (iv) single sequence repeats (SSR) or sequence-tagged microsatellite sites (STMS), (v) DNA amplification fingerprinting (DAF), (vi) microsatellite-primed PCR (MP-PCR) and (vii) single nucleotide polymorphism (SNP).

RFLP involves the use of restriction enzymes to cut chromosomal DNA at specific short restriction sites; polymorphism results from variations in length of the fragments due to duplications or deletions between the sites or mutations at the restriction sites. RFLP provided the basis for most early work but requires a relatively large amount of DNA and is rather expensive in a large screening programme. RAPD utilizes low stringency PCR amplification with single primers of arbitrary sequence to generate strain-specific arrays of anonymous DNA fragments. The method requires tiny DNA samples and analyses a large number of polymorphic loci. AFLP requires digestion of cellular DNA with a restriction enzyme and then using PCR and selective nucleotides in the primers to amplify specific fragments.

The method measures up to 100 polymorphic loci and requires a relatively small DNA sample for each test. SSR analysis is based on DNA microsatellites, (short-repeat) sequences that are widely dispersed throughout the genome of eukaryotes, which are selectively amplified to detect variations in the number (and length) of simple sequence repeats. SSR analysis requires tiny DNA samples and has a low cost per analysis. SNPs are detected using PCR extension assays that efficiently pick up point mutations. The procedure requires little DNA per sample and costs little per sample once the method is established. MGMs, independent from the environment such as isozymes, have been used in the same way to determine the degree of variability within plant populations. They allow direct

access to the coding and non-coding regions of the genome, making their number potentially unlimited. Among different characteristics, they can be easily visualized and identified (such as by probes and Southern blots, PCR amplification and electrophoresis, radio-labelling, fluorescence). The more efficient MGMs generally assign one sole locus, and the diversity of their possible alleles determines the level of their polymorphism. The first applications to rubber, on nuclear and cytoplasmic genomes, were made with RFLPs. Then, when a PCR technique was developed with random primers, RAPDs were used, as well as AFLPs, which combine restriction enzymes and PCR. Microsatellites (SSRs), of PCR developed from specifically designed primers, appeared very powerful due to their high polymorphism (between 15 and 20 alleles per marker). In comparison with isozymes or mRNA, the high stability of DNA makes it possible to send leaf samples from any remote plantation site to a laboratory by normal mail for analysis.

Although RFLPs are powerful tools for studying genetic diversity and mapping, this technology is not preferred now since it is labour intensive and requires large DNA samples. Its marker index value (expressed as the number of polymorphic products per sample) is also low with only 0.10 compared with PCR-based marker systems like RAPDs (0.23), SSRs (0.60) and AFLPs (6.08) (Low et al. 1996). Ever since isozymes were utilized for clonal identification (Chevallier 1988), tools like minisatellites (Besse et al. 1993a), RFLPs (Besse et al. 1993b, 1994), mitochondrial and chloroplastic RFLPs (Luo et al. 1995), RAPDs and DAFs (Low et al. 1996; Varghese et al. 1998; Venkatachalam et al. 2001, 2002), AFLPs (Lespinasse et al. 2000a) and SSRs (Besse et al. 1993a; Atan et al. 1996; Low et al. 1996) were developed and used in detection, increasing the number of molecular markers in *H. brasiliensis*. Polymorphism in microsatellites was detected also in *H. pauciflora*, *H. guianensis*, *H. camargoana* and *H. benthamiana* (Low et al. 1996), and cross-species amplification was also done in these species (Souza et al. 2009). Microsatellite-enriched libraries were produced and led to the identification of large numbers of microsatellite markers (Atan et al. 1996; Seguin et al. 2003; Saha et al. 2005); sequences of 472 of them are currently accessible on the European Molecular Biology Laboratory (EMBL)/GenBank databases. CIRAD has also developed a database for rubber clonal identification by the use of ten microsatellite markers; microsatellite markers from rubber pathogens can also be used for distinguishing the genetic differences between the races, as has been done with 11 markers for *Microcyclus* (Le Guen et al. 2004).

Although considerable progress has been made to increase the yield in *Hevea* clones in the recent past, satisfactory resistance to biotic and abiotic stress has not been achieved because of limited genetic resources within the *Hevea* gene pool. Wind damage is one of the serious problems in rubber-growing countries; each year there is a considerable loss of rubber trees due to wind damage in rubber plantations. The incorporation of the dwarf character into high-yielding *Hevea* clones would be useful for generating a high-yielding tree with a desirable architecture (dwarf stature) (Venkatachalam et al. 2004). Information on the genetic and molecular basis of the dwarf character in this species could provide insights on the development of high-yielding dwarf clones that would eventually lead to decreasing the negative consequences of wind damage (abiotic stress) and high-density planting but might have negative effects on rubber wood productivity. The identification of molecular markers for the dwarf character would be important for isolating true-to-type high-yielding dwarf hybrid lines in the early stage of plant breeding programmes. Venkatachalam et al. (2004) identified a dwarf genome-specific RAPD marker in the rubber tree. The primer OPB-12 generated a 1.4 kb DNA marker from both natural and controlled F1 hybrid progenies (dwarf stature) derived from a cross between a dwarf parent and a normal cultivated clone as well as from the dwarf parent; it was absent in the other parent (RRII 118). To validate this DNA marker, 22 F1 hybrids (13 with a dwarf stature and 9 with a normal stature) were analysed; the dwarf genome-specific 1.4 kb RAPD marker was present in all dwarf-stature hybrids and absent in all

normal-stature hybrids. This DNA marker was cloned and characterized. DNA marker locus specificity was further confirmed by Southern blot hybridization.

13.2 Expressed Genes in *Hevea*

Non-expressed MGMs and also markers of expressed genes (single-strand conformation polymorphism (SSCP)), based on PCR, aimed at mutation detection in expressed genes, were studied by Lekawipat (2004) in 66 Amazonian and 40 Wickham accessions. It was found that microsatellites could detect higher polymorphism than gene-specific primers of SSCP in rubber accessions, although markers of expressed genes can be assumed to be more related with some putative breeding objectives. SSCP markers could not differentiate the Wickham and the Mato Grosso accessions. By the use of reverse genetics from mRNA to cDNA libraries (RT-PCR), the fields of functional genomics and molecular physiology are being developed in rubber by different teams, predominantly working on latex cells, on such themes as rubber biosynthesis, latex-cell functioning, the latex coagulation process, ethylene biosynthesis and metabolism, oxidative stress, tapping cut dryness and brown bast (the reversible or irreversible forms of TPD), allergenic proteins in the latex, heterologous genes to be expressed in the latex, drought tolerance, leaf fungus diseases, cyanogenesis metabolism or defence proteins and photosynthesis.

In reproductive biology, rubber flower and inflorescence development has been characterized: one important gene regulating flower induction and development (*leafy/floricaula*) was cloned and its expression was analysed and localized by in situ hybridization (Dornelas and Rodriguez 2005). In the field of post-germination changes in rubber seeds, proteomics (2D-PAGE and mass spectrometry methods) were implemented for examining the changes in protein expression from the mature seed to the germinated seed (Wong and Abubakar 2005). The suppression subtractive hybridization (SSH) technique is currently widely implemented between different pairs of mRNA samples for the production of molecular resources by RT-PCR in the form of subtracted cDNA libraries; microarrays or sequencing and comparison with entries from the databases will then assist in searching the functions of these expressed genes (candidate gene approach) (see Sathik et al. 2009). Expressed sequence tags (ESTs, or small and partial 5′-end sequences of expressed genes) related to various metabolic aspects are being collected to create EST banks that broadly represent the genes expressed in one tissue, such as latex cells, and this assists in the study of gene function and regulation. Entries of these banks are compared with public databases of already known genes for identifying the putative functions of the corresponding genes. These EST banks will also create the way for macroarray- or microarray-based studies of *Hevea* gene expression. The 'Latex Lambda Triplex' EST-cDNA library (Ko et al. 2003) published in the EMBL/GenBank databases (858 entries) showed that about 16% of the database matched ESTs encoding rubber biosynthesis-related proteins. Rubber biosynthesis-related genes appeared to be mostly expressed, followed by defence-related genes and other protein-related genes (Han et al. 2000). Published DNA sequences of the latex allergens were matched against these ESTs, so indirectly providing a ranking of the allergens depending on their concentration in the latex; more than 1000 ESTs matched with the sequences of *REF* (gene for rubber elongation factor, or *Hev.b.1*) and *SRPP* (gene for small rubber particle protein, or *Hev.b.3*). Cubry et al. (2014) retrieved EST sequences from public database, and these sequences were trimmed and microsatellite motifs searched using an ad hoc bioinformatic pipeline. Pairs of primers for the amplification of candidate markers were generated. A total of 10,499 unigenes from both sources of sequences and 673 microsatellites motifs were detected using the default parameters of the pipeline. Two-hundred and sixty-four primer pairs were tested and 226 (85.6%) were successfully amplified. Out of the amplified candidate markers, 164 exhibited polymorphism. A striking expansion of

the REF/SRPP (rubber elongation factor/small rubber particle protein) gene family and its divergence into several laticifer-specific isoforms seem crucial for rubber biosynthesis. Tang et al. (2016) presented a high-quality genome assembly of *Hevea* clone Reyan 7-33-97 (1.37 Gb, scaffold N50 = 1.28 Mb) that covers 93.8% of the genome (1.47 Gb) and harbours 43,792 predicted protein-coding genes (Table 13.1). The REF/SRPP family has isoforms with sizes similar to or larger than SRPP1 (204 amino acids) in 17 other plants examined, but no isoforms detected with similar sizes to REF1 (138 amino acids), the predominant molecular variant. The expansion of vast repetitive elements makes the genome size of *Hevea* apparently larger than its closely related species, such as cassava, castor oil, cottonwood and flax. REF1 is special for its smaller size and is highly expressed in latex than the others, indicating its predominant role in rubber production (see Fig. 13.1). REF1 gene expression was reported to correlate with yield levels of *Hevea* cultivars (Priya et al. 2007). There are two classes of rubber particles in *Hevea* latex: the large particles (LRPs) with REF located on their surfaces and the small particles (SRPs) that are coated with SRPP (Dennis and Light 1989; Berthelot et al. 2014). SRPs are far superior in number, accounting for 94% of all rubber particles in the latex, whereas LRPs constitute only the remaining 6% of the rubber particles. However, it is precisely this 6% of rubber particles by number that makes up 94% of the rubber by volume in the latex (Yeang et al. 1995). Tang et al. (2016) argue that the emergence of REF1, which is located on the surface of large rubber particles that account for 94% of rubber in the latex (despite constituting only 6% of total rubber particles, large and small), is pivotal to *Hevea* evolution.

Table 13.1 Details of *Hevea* genome and gene annotation

Estimate of genome size	1.46 Gb
Number of scaffolds	7453
Total length of scaffolds	1.37 Gb
N50 of scaffolds	1.28 Mb
Longest scaffolds	6.41 Mb
Number of contigs	84,285
Total length of contigs	1.29 Gb
N50 of contigs	30.6 kb
Longest contigs	312.7 kb
GC content	34.84%
Number of genes	43,792
Percentage of gene length in genome	12.47%
Mean gene length	3913 bp
Gene density	31.9 Mb^{-1}
Transcripts number	46,631
Mean transcript length	1483 bp
Mean coding sequence length	1123 bp
Mean exon length	308 bp
Exon GC content	41.54%
Mean intron length	677 bp
Intron GC content	32.61%
Masked repeat sequence length	977.5 Mb
Repeats percentage of total scaffolds	71.18%
Repeats percentage of total contigs	75.69%

After Tang et al. (2016)

Expressed sequence tags, derived as simple sequence repeats (microsatellites = EST-SSRs), are different from traditional genomic SSR (gSSR) markers. They are more likely to be embedded in the functional gene sequences, less costly and time effective, and may provide abundant information. By analysis of 10,018 expressed sequence tags (ESTs) out of 10,829 for rubber tree (*Hevea brasiliensis*) available in public domain DNA databases, 799 SSR loci were found in the 643 non-redundant SSR-ESTs (SSR-containing ESTs), corresponding to 1 SSR in every 2.25 kb of the ESTs in rubber tree transcriptome (Feng et al. 2009). Of the total 799 SSRs in these ESTs, 673 (84.2%) contained simple repeat motifs, while 126 (15.8%) represented compound motif types. Of the total EST-SSRs, 45.3% (362/799) were mononucleotide repeats (MNRs), 42.2% (337/799) were dinucleotide repeats (DNRs), 11.9% (95/799) were trinucleotide repeats (TNRs) and 0.6% (5/799) were tetranucleotide repeats (TTNRs) and hexanucleotide repeats (HNRs). The repeat motifs AAG and AG were the most abundant without regard to single nucleotide repeat. A total of 184 primer pairs were designed based on the non-redundant SSR-ESTs. Using 55 °C as annealing temperature, 110 primer pairs successfully amplified 12 *H. brasiliensis* cultivated varieties and 4 related species. Analysis on 74 alleles amplified by 30

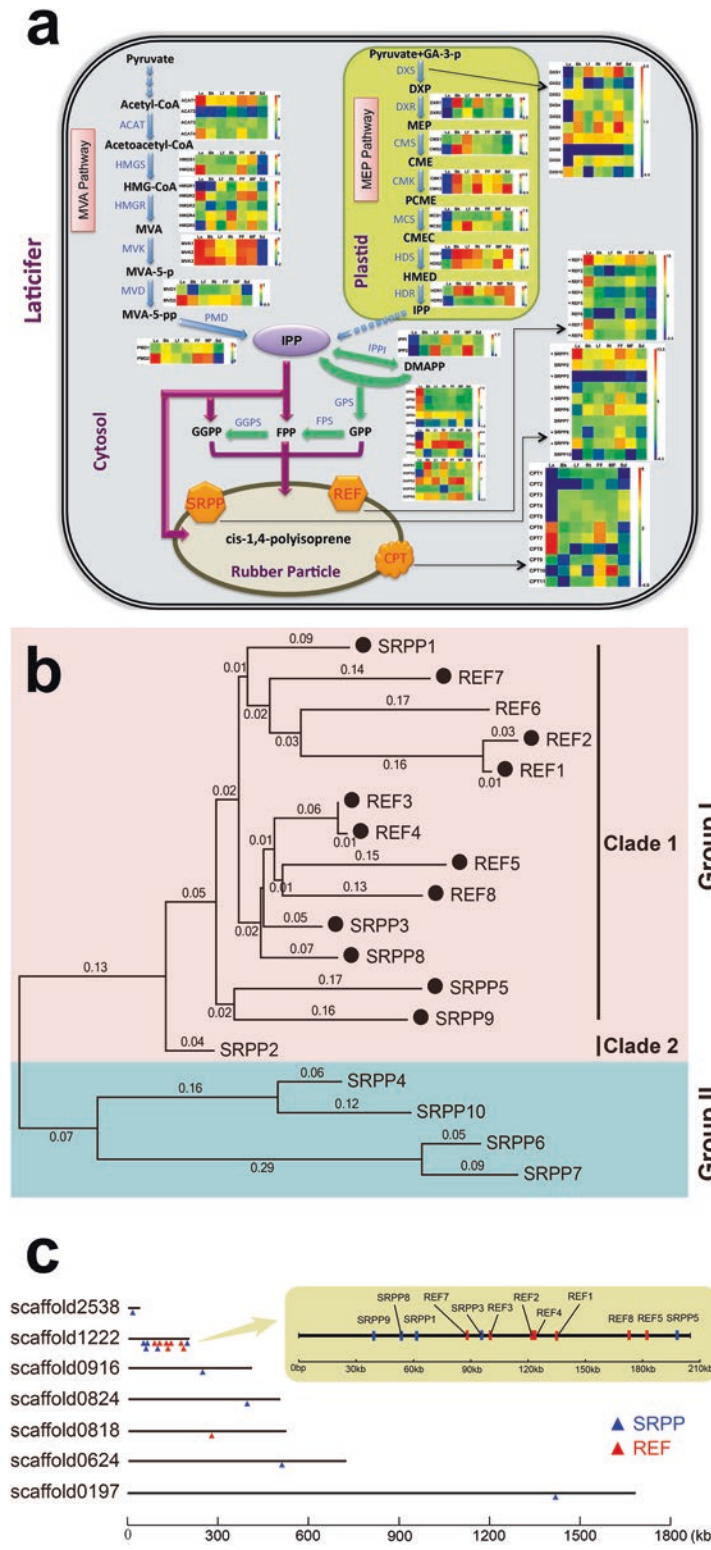

Fig. 13.1 (**a**) The rubber biosynthesis pathway and expression profiles of genes involved (*Lx* latex, *Bk* bark, *Lf* leaf, *Rt* root, *FF* female flower, *MF* male flower). (**b**) Phylogeny of REF/SRPP gene family. The tree is constructed by using MEG A5.1 with neighbour-joining model and bootstrap test with 1000 replicates. (**c**) Genomic location of the REF/SRPP genes. Scaffolds are represented as solid bars with their names on the left and length on the bottom. Note that most of the REF/SRPP genes including the four laticifer-specific ones (REF1, SRPP1, REF3 and REF7) are located in a single scaffold (scaffold 1222) (After Tang et al. 2016)

randomly selected primer pairs indicated the medium polymorphism of the EST-SSRs developed. Based on 272 alleles detected by 87 EST-SSR markers, an assessment of genetic diversity was carried out on 12 *H. brasiliensis* clones and 4 related species. In addition, investigation based on five selected EST-SSRs by cloning and sequencing across some cultivated species and related species provided evidence for cross-species/genera transferability of the EST-SSR markers (Feng et al. 2009).

Development and characterization of new genomic microsatellite markers in *H. brasiliensis* and the evaluation of their transferability to other *Hevea* species were attempted in the recent past (Mantello et al. 2012). They constructed di-and tri-nucleotide enriched libraries. From these 2 libraries, 153 primer pairs were designed and initially evaluated using 9 genotypes of *H. brasiliensis*. A total of 119 primer pairs had a good amplification product, 90 of which were polymorphic. We chose 46 of the polymorphic markers and characterized them in 36 genotypes of *H. brasiliensis*. The expected and observed heterozygosities ranged from 0.1387 to 0.8629 and 0.0909 to 0.9167, respectively. The polymorphism information content (PIC) values ranged from 0.097 to 0.8339, and the mean number of alleles was 6.4 (2–17). These 46 microsatellites were also tested in 6 other *Hevea* species. The percentage of transferability ranged from 82% to 87%. Locus duplication was found in *H. brasiliensis* and also in 5 of other species in which transferability was tested. This study reports new microsatellite markers for *H. brasiliensis* that can be used for genetic linkage mapping, quantitative trait loci identification and marker-assisted selection. The high percentage of transferability may be useful in the evaluations of genetic variability and to monitor introgression of genetic variability from different *Hevea* species into breeding programmes.

Genes responsible for the synthesis of rubber transferase, the key enzyme for polymerization of polyisoprene (natural rubber), appeared to be among the most abundantly expressed genes in the latex. *Hevein*, a chitin-binding protein, one of the defence proteins that play a crucial role in the protection of wound sites from fungal attack, is also involved in the coagulation process; it belongs to a multigene family, and the specificity of its expression in the latex is under investigation (Broekaert et al. 1990; Pujade-Renaud et al. 2005). Nearly 12.6% of the proteins available in the latex are defence related (Han et al. 2000). Among 200 distinct polypeptides (Posch et al. 1997), mainly three rubber synthesis-related genes are expressed in the latex: (i) *REF* (Dennis and Light 1989; Goyvaeerts et al. 1991), (ii) *HMGR* (Chye et al. 1992) and (iii) *SRPP* (Oh et al. 1999). The most abundantly expressed gene is *REF* (6.1%) and then *SRPP* (3.7%) (Han et al. 2000). References and partial- or full-length sequences of these cloned genes can be found in the EMBL/GenBank databases.

Unlike photosynthetic genes, transcripts involved in rubber biosynthesis are 20–100 times greater in laticifers than in leaves (Kush et al. 1990). On the other hand, transcripts for chloroplastic and cytoplasmic forms of glutamine synthetase are restricted to leaves and laticifers, respectively (Kush et al. 1990), indicating thereby that the cytoplasmic form of glutamine synthetase plays a decisive role in amino acid metabolism of laticifers. The transcript levels of hydrolytic enzymes, namely, polygalacturonase and cellulase, might be taken as indicators for a better development of the laticifers. Genes expressed in the latex of *Hevea* can be divided into three groups based on the proteins they encode: (i) defence-related proteins such as hevein, chitinase, β-1,3-glucanase and HEVER; (ii) rubber biosynthesis-related proteins such as rubber elongation factor (REF), HMGR and 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMGS), *cis*-prenyltransferase (CIS), geranyl-geranyl diphosphate (GGPP) synthase, small rubber particle protein (SRPP) and isopentenyl diphosphate (IPP) isomerase; and (iii) latex allergen proteins such as Hev.b.3, Hev.b.4, Hev.b.5 and Hev.b.7. The biological functions of the allergenic proteins are largely unknown (Oh et al. 1999).

Genes expressed under stressed conditions is yet another topic that received due attention recently. Silva et al. (2014) studied leaf, panel

and latex ESTs under cold-stressed conditions. For panel and latex libraries, samples were collected from 16-year-old tree clones of PB217, PR255, GT1, PB235, RRIM701 and IAN873, and leaves of the same clones were collected from the rubber tree germplasm. PB217, PR255, GT1 and IAN873 were subjected to a 24-h cold treatment in a growth chamber and maintained at 8 °C with a 12-h photoperiod. This treatment was performed to promote the expression of genes involved in cold response and for the development of molecular markers related to this stress condition. A total of 8263 reads were assembled, generating 5025 unigenes that were analysed; 912 expressed sequence tags (ESTs) represented new transcripts, and 2 sequences were highly upregulated by cold stress. These unigenes were scanned for microsatellite (SSR) regions and single nucleotide polymorphisms (SNPs). In total, 169 novel EST-SSR markers were developed, of which 138 loci were polymorphic. Nearly 98% of this presented transferability to six other *Hevea* species. Locus duplication was observed in *H. brasiliensis* and other species. Additionally, 43 SNP markers in 13 sequences that showed similarity to proteins involved in stress response, latex biosynthesis and developmental processes were characterized. cDNA libraries are a rich source of SSR and SNP markers and enable the identification of new transcripts. Such markers will be a valuable resource for linkage mapping and QTL identification.

13.3 Transcriptome Analysis

Transcriptome analysis is one of the main approaches for identifying the complete set of active genes in a cell or tissue for a specific developmental stage or physiological condition. Transcriptome analysis is a second-generation sequencing technology offered by companies such as Illumina, Roche and Life Technologies. Salgado et al. (2014) reported on the sequencing, assembling, annotation and screening for molecular markers from a pool of *H. brasiliensis* tissues. A total of 17,166 contigs were successfully annotated. Then, 2191 single nucleotide variation (SNV) and 1397 simple sequence repeat (SSR) loci were discriminated from the sequences. This is the first study of the *Hevea* transcriptome, covering a wide range of tissues and organs, leading to the production of the first developed SNP markers. Transcriptome studies have to come a long way to yield meaningful results to tag such gene expression studies to vivid genes responsible for QTLs, resistance and other quality traits.

By assembling and analysing de novo transcriptome sequencing data, Li et al. (2012) reported the comprehensive functional characterization of rubber tree bark. This research generated a substantial fraction of rubber tree transcriptome sequences, which were very useful resources for gene annotation and discovery, molecular markers development, genome assembly and annotation, and microarrays development in rubber tree. The EST-SSR markers identified and developed in this study will facilitate marker-assisted selection breeding in rubber tree. Moreover, this study also supported that transcriptome analysis based on Illumina paired-end sequencing is a powerful tool for transcriptome characterization and molecular marker development in non-model species, especially those with large and complex genomes.

To obtain more information on the *Hevea brasiliensis* genome, Triwitayakorn et al. (2011) sequenced the transcriptome from the vegetative shoot apex yielding 2,311,497 reads. Clustering and assembly of the reads produced a total of 113,313 unique sequences, comprising 28,387 isotigs and 84,926 singletons (see Box 13.1). Also, 17,819 expressed sequence tag (EST)-simple sequence repeats (SSRs) were identified from the data set. To demonstrate the use of this EST resource for marker development, primers were designed for 430 of the EST-SSRs. Three-hundred and twenty-three primer pairs were amplifiable in *H. brasiliensis* clones. Polymorphic information content values of selected 47 SSRs among 20 *H. brasiliensis* clones ranged from 0.13 to 0.71, with an average of 0.51. A dendrogram of genetic similarities between the 20 *H. brasiliensis* clones using these 47 EST-SSRs suggested two distinct groups that correlated well with clone pedigree. These novel EST-SSRs together with

the published SSRs were used for the construction of an integrated parental linkage map of *H. brasiliensis* based on 81 lines of an F1 mapping population. The map consisted of 97 loci, consisting of 37 novel EST-SSRs and 60 published SSRs, distributed on 23 linkage groups and covered 842.9 cM with a mean interval of 11.9 cM and ~4 loci per linkage group. Although the numbers of linkage groups exceed the haploid number (18), but with several common markers between homologous linkage groups with the previous map indicated that the F1 map in this study is appropriate for further study in marker-assisted selection.

Box 13.1 Isotigs, Singletons and Contigs

Isotigs: Normally one gene should have one transcript, but due to splice variation one gene can have many transcripts. Suppose a gene is made of three exons, exon1, exon2 and exon3. It will generate three contigs in newbler, contig 1, contig 2 and contig 3. Due to splice variation, the final transcript can consist of exon1 + exon2 + exon3 or exon1 + exon3, etc. Thus, we get two types of variation here; these two are called isotig 1 and isotig 2. isotig 1 consists of contig 1 + contig 2 + contig 3 and isotig 2 consists of contig 1 + contig 3. These two isotigs are variation of one transcript. So these two isotigs combinedly fall into one isogroup.

Singletons: All reads are not provided as input in the final assembly. The unused reads, also called singletons, are often contaminants or insufficiently trimmed reads from the genome.

Contig: contig (originated from contiguous) is a set of overlapping DNA segments that together represent a consensus DNA.

Scaffold and contig N50s: The most widely used annotations for describing the quality of a genome assembly are its scaffold and contig N50s. A contig N50 is calculated by first ordering every contig by length from longest to shortest. Next, starting from the longest contig, the lengths of each contig are summed, until this running sum equals one-half of the total length of all contigs in the assembly. The contig N50 of the assembly is the length of the shortest contig in this list. The scaffold N50 is calculated in the same fashion but uses scaffolds rather than contigs. The longer the scaffold N50 is, the better the assembly is. However, it is important to keep in mind that a poor assembly that has forced unrelated reads and contigs into scaffolds can have an erroneously large N50. Note too that scaffolds and contigs that comprise only a single read or read pair—often termed 'singletons'—are frequently excluded from these calculations, as are contigs and scaffolds that are shorter than ~800 bp. The procedures used to calculate N50 may therefore vary between genome projects.

Reads: reads refer to somewhat digital information obtained from the sequencing machine (e.g. Illumina MiSeq) and stored in the fastq file with quality scores per base. From a single run one can get millions of reads, where each read will have a set bp size, e.g. 100 bp long.

TSS (Transcription Start Site): It is where the RNA polymerase sits down on the DNA and starts to make an RNA copy of the DNA.

CAGE (cap analysis gene expression): This is a technique used in molecular biology to produce a snapshot of the 5′ end of the mRNA population. The small fragments (usually 27 nucleotides long) from the very beginnings of mRNAs (5′ ends of capped transcripts) are extracted, reverse-transcribed to DNA, PCR amplified and sequenced.

Construction of linkage maps is crucial for genetic studies and marker-assisted breeding programmes. Recent advances in next-generation sequencing technologies allow for the generation

of high-density linkage maps, especially in non-model species lacking extensive genomic resources. Pootakham et al. (2015) constructed a high-density integrated genetic linkage map of rubber tree (*H. brasiliensis)*, the sole commercial producer of high-quality natural rubber. We applied a genotyping-by-sequencing (GBS) technique to simultaneously discover and genotype single nucleotide polymorphism (SNP) markers in two rubber tree populations. A total of 21,353 single nucleotide substitutions were identified, 55% of which represented transition events. GBS-based genetic maps of populations P and C comprised 1704 and 1719 markers and encompassed 2041 cM and 1874 cM, respectively. The average marker densities of these two maps were one SNP in 1.23–1.25 cM. A total of 1114 shared SNP markers were used to merge the 2 component maps. An integrated linkage map consisted of 2321 markers and spanned the cumulative length of 2052 cM. The composite map showed a substantial improvement in marker density, with one SNP marker in every 0.89 cM. This is the most saturated genetic map in rubber tree to date. This integrated map allowed us to anchor 28,965 contigs, covering 135 Mb or 12% of the published rubber tree genome. Pootakham et al. (2015) further demonstrated that GBS is a robust and cost-effective approach for generating a common set of genome-wide SNP data suitable for constructing integrated linkage maps from multiple populations in a highly heterozygous agricultural species.

In an attempt to develop additional microsatellite markers in rubber tree, Pootakham et al. (2012) employed a 454 pyrosequencing technology to obtain genomic shotgun sequences and subsequently identified over 24,000 putative simple sequence repeats (SSRs). A total of 418 potential SSR loci were chosen for an empirical validation. Two-hundred and twenty-four primer pairs yielded successful amplification, and 90 SSR markers exhibited polymorphism among the 18 rubber tree accessions evaluated. The number of alleles per locus ranged from 2 to 7, with an average of 3.44 alleles per SSR. The gene diversity of individual microsatellite loci displayed a broad range of values from 0.104 to 0.901 with a mean of 0.612. The polymorphism information content also varied greatly from 0.099 to 0.893 with an average of 0.577. We also identified a set of five highly informative markers that were able to unequivocally distinguish 18 rubber tree genotypes examined in this study.

In an effort to facilitate biological, biochemical and molecular research in rubber biosynthesis, Xia et al. (2011) reported the use of next-generation massively parallel sequencing technologies and de novo transcriptome assembly to gain a comprehensive overview of the *H. brasiliensis* transcriptome. The sequencing output generated more than 12 million reads with an average length of 90 nt. In total 48,768 unigenes (mean size = 436 bp, median size = 328 bp) were assembled through de novo transcriptome assembly. Out of 13,807 *H. brasiliensis* cDNA sequences deposited in GenBank of the National Center for Biotechnology Information (NCBI) (as of February 2011), 11,746 sequences (84.5%) could be matched with the assembled unigenes through nucleotide BLAST. The assembled sequences were annotated with gene descriptions, Gene Ontology (GO) and Clusters of Orthologous Group (COG) terms. In all, 37,432 unigenes were successfully annotated, of which 24,545 (65.5%) aligned to *Ricinus communis* proteins. Furthermore, the annotated unigenes were functionally classified according to the GO, COG and Kyoto Encyclopedia of Genes and Genomes databases. Xia et al. (2011) claim that this is the most comprehensive sequence resource available for the study of rubber trees as well as it demonstrates effective use of Illumina sequencing and de novo transcriptome assembly in a species lacking genomic information.

Mantello et al. (2014) performed RNA sequencing (RNA-seq) of *H. brasiliensis* bark on the Illumina GAIIx platform, which generated 179,326,804 raw reads on the Illumina GAIIx platform. A total of 50,384 contigs that were over 400 bp in size were obtained and subjected to further analyses. A similarity search against the non-redundant (nr) protein database returned 32,018 (63%) positive BLASTx hits. The transcriptome analysis was annotated using the clusters of orthologous groups (COG), gene ontology (GO),

Kyoto Encyclopedia of Genes and Genomes (KEGG) and Pfam databases. A search for putative molecular marker was performed to identify simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs). In total, 17,927 SSRs and 404,114 SNPs were detected. Finally, we selected sequences that were identified as belonging to the mevalonate (MVA) and 2-C-methyl-D-erythritol 4-phosphate (MEP) pathways, which are involved in rubber biosynthesis, to validate the SNP markers. A total of 78 SNPs were validated in 36 genotypes of *H. brasiliensis*. This new data set represents a powerful information source for rubber tree bark genes and will be an important tool for the development of microsatellites and SNP markers for use in future genetic analyses such as genetic linkage mapping, quantitative trait loci identification, investigations of linkage disequilibrium and marker-assisted selection.

Molecular mechanisms underlying high yield are not well understood. Li et al. (2015) reported the sequencing, assembly and comparative analyses of latex transcriptome from RRIM600 and RY 7-20-59. In total, 33,852 unigenes were generated with de novo assembly. The blastx results indicated that 27,886 and 15,704 unigenes showed significant similarities to known proteins from NCBI nr and Swissprot databases, respectively. Among these annotated unigenes, 21,841 and 9010 were separately assigned to Gene Ontology (GO) functional categories and Clusters of Orthologous Groups (COGs). Of 126 KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways, metabolic pathway was the biggest one, suggesting that active metabolic processes happen in rubber tree latex. In contrast to RRIM600, 2513 and 1391 genes were separately up- and down-regulated in RY 7-20-59. The expression profiles of 25 unigenes were further confirmed by real-time PCR, suggesting that the differently expressed genes (DEGs) identified by RNA-seq were accurate and reliable. The DEGs between RRIM600 and RY 7-20-59 were significantly enriched in plant-pathogen interactions, phenylalanine metabolism, ubiquinone and other terpenoid-quinone biosynthesis, biosynthesis of secondary metabolites and photosynthesis. Interestingly, the genes involved in rubber biosynthesis pathway, such as CPT, GPPS, HMGR, HMGS, FPPS and DXS, were differently expressed between RRIM600 and RY 7-20-59. Such studies really help to provide new insights into understanding latex transcriptome and molecular mechanisms underlying high yield.

13.4 Rubber Biosynthesis

Rubber biosynthesis in *Hevea* laticifer cells has become a major field of research applied to the expression and regulation of genes, with a view to possibly opening the way to genetic manipulation of the isoprenoid biosynthesis pathway enzymes. Rubber molecules (1,4 *cis*-polyisoprene) are formed from polymerization of molecules with five carbons, IPP, and aggregated as rubber particles packaged within a membrane which protects them from oxidation, in latex vessels. The general metabolic pathway of rubber biosynthesis is as follows. Sucrose from photosynthesis is actively transported into laticiferous cells through the plasmalemmic membrane and is then hydrolysed into glucose and fructose by invertase. These sugars are then converted into acetyl-coenzyme A (acetyl-CoA) through glycolysis. Three molecules of acetyl-CoA are condensed into mevalonic acid and then IPP. Polymerization of thousands of molecules of IPP leads to dimethylallyl diphosphate (DMAPP) and GGPP, with the action of the enzyme rubber transferase associated with REF, a molecule fixed on the rubber particles' membranes.

Although natural rubber is synthesized and made almost entirely of isoprene units derived from IPP, an allylic disphosphate is also required as the priming co-substrate to initiate the subsequent extensive prenyl chain elongation process for the formation of rubber macromolecules. Both the HMGS and the HMGR have been shown to be involved in the early steps of rubber biosynthesis by forming 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) using HMGS. The HMGS catalyses the condensation of acetyl-CoA and acetoacetyl-CoA to form HMG-CoA (Suwanmanee et al. 2002, 2004; Sirinupong et al. 2005).

In plants, HMG-CoA is reduced by HMGR to mevalonate (MVA) and is subsequently converted into IPP. Chye et al. (1992) reported that there are three genes encoding HMGR in *Hevea*, namely, *hmg1*, *hmg2* and *hmg3*. The *hmg1* gene is likely to be involved in rubber biosynthesis, whereas the *hmg3* is possibly involved in isoprenoid biosynthesis of another nature. Gene expression analysis by type of tissue indicated that MVA-pathway genes were highly expressed in latex, as compared with other types of tissue, and that HMGS and HMGR exist in multiple copies and have different expression patterns (Sando et al. 2008).

The first step in rubber biosynthesis is the isomerization of IPP to DMAPP by IPP isomerase. The successive head-to-tail condensation reactions of the five-carbon intermediates, catalysed by the enzyme rubber transferase, have been assumed to yield rubber. Oh et al. (2000) have isolated and characterized a cDNA clone encoding IPP isomerase from *Hevea* and showed in an in vitro rubber assay that the recombinant IPP isomerase was required for rubber biosynthesis. In order to examine possible participation of GGPP synthase in the enzymatic prenyl chain elongation in natural rubber biosynthesis, Takaya et al. (2003) studied the GGPP synthase gene from *Hevea*. Based on their investigation, GGPP synthase would catalyse the condensation of IPP with allylic diphosphates to give GGPP. Therefore, GGPP is one of the key precursors in the biosynthesis of biologically significant isoprenoid compounds. Natural rubber is thought to be made almost entirely of *cis*-isoprene units derived from IPP, and the enzyme responsible for polymerization is believed to have characteristics similar to the *cis*-prenyl diphosphate synthases. The gene responsible for the *cis*-1,4-polymerization of isoprene units has been isolated and characterized in *Hevea* recently by Asawatreratanakul et al. (2003). It was suggested that rubber biosynthesis in *Hevea* is mediated by the association of a soluble *trans*-prenyltransferase with the REF, a 14.6 kDa protein, tightly bound to the rubber particles in the laticifers (Dennis and Light 1989). Farnesyl diphosphate (FPP) is a key intermediate in the biosynthesis of at least 20,000 isoprenoids. FPP is also the allylic diphosphate initiator of rubber biosynthesis in plants. FPP is synthesized by the enzyme farnesyl diphosphate synthase (FPS), which has been cloned and characterized from *Hevea* by Adiwilaga and Kush (1996). FPP formed by FPS is assumed to be the initiating substrate for rubber formation. The REF protein was isolated and studied extensively, and the results indicate that the FPS and REF complex was responsible for the *cis*-1,4-polyprenol condensations observed in isolated rubber particles (Light and Dennis 1989). The *REF* gene was isolated and the involvement of REF on rubber formation was analysed by Attanayaka et al. (1991) and Goyvaeerts et al. (1991). Oh et al. (1999) reported a novel *Hevea* cDNA sequence associated with SRPP, and the sequence analysis revealed that this protein is highly homologous to the REF. It is possible that the rubber biosynthesis pathway is coordinately regulated by these enzymes. However, the precise mechanism for the biosynthesis of rubber molecules has not yet been established. Moreover, the exact site of the formation of new rubber molecules still remains unknown.

The genes uniquely or preferentially expressed in the latex may be important for rubber biosynthesis (Han et al. 2000). They constructed cDNA libraries from the latex of *Hevea* to investigate the genes expressed in the latex by single-run partial sequencing of the cDNA clones. Sequence analyses identified 245 expressed sequence tags (ESTs), of which 57% showed homology to previously described sequences in public databases. About 16% of the database-matched ESTs encode rubber biosynthesis-related proteins such as rubber elongation factor (REF) and small rubber particle protein (SRPP). The second most frequent transcripts next to rubber biosynthesis-related genes were defence genes and protein metabolism-related genes (12.6% each). About 27% of the database-matched ESTs had sequence homology with genes of unknown function. Among the redundantly expressed genes, REF was the most frequently expressed (6.1%), followed by SRPP (3.7%) and HbLAR (2.9%). Northern blot analyses showed that ten (71%) of the 14 ESTs studied were expressed at a higher level in latex than in leaves.

13.5 Gene Mapping

To gain a better understanding of miRNAs and their relationship with rubber tree gene regulation networks, Kanjanawattanawong et al. (2014) generated large genomic DNA insert-containing libraries to complement the incomplete draft genome sequence and applied as a new powerful tool to predict the function of interested genes. Bacterial artificial chromosome and fosmid libraries (a fosmid library is prepared by extracting the genomic DNA from the target organism and cloning it into the fosmid vector), containing a total of 120,576 clones with an average insert size of 43.35 kb, provided approximately 2.42 haploid genome equivalents of coverage based on the estimated 2.15 Gb rubber tree genome (Rahman et al. 2013). Based on these library sequences, the precursors of 1 member of rubber tree-specific miRNAs and 12 members of conserved miRNAs were successfully identified. A panel of miRNAs was characterized for phytohormone response by precisely identifying phytohormone-responsive motifs in their promoter sequences. Furthermore, the quantitative real-time PCR on ethylene stimulation of rubber trees was performed to demonstrate that the miR2118, miR159, miR164 and miR166 are responsive to ethylene, thus confirming the prediction by genomic DNA analysis. The *cis*-regulatory elements identified in the promoter regions of these miRNA genes help augment the understanding of miRNA gene regulation and provide a foundation for further investigation of the regulation of rubber tree miRNAs.

Past several years have witnessed major advances in our understanding of plant genomes and genomic information through whole genome sequencing. The increasing availability of data from several plant genome sequencing projects provides a promising direction for investigating genes and their functional and sequence homologs involved in plant development (Avraham et al. 2008). Although genome sequencing projects lead to the identification of the complete catalogue of genes of an organism, they don't consider the gene expression patterns. Large-scale end sequencing of cDNA library generates ESTs, representing genes expressed in particular tissues or under particular developmental or environmental conditions. They have also been the target of sequencing in many of the projects and found invaluable for genome assembly and annotation. Whole genome sequence information helps in many aspects of plant trait improvement through gene discovery to transgenesis and use of molecular markers in breeding. *Hevea* genome sequencing project has already been launched jointly by Tun Abdul Razak Research Centre (TARRC) of the Malaysian Rubber Board and the newly established Genome Analysis Centre at Norwich, UK, and probably RRIM600 was sequenced and draft sequence has been made available (Rahman et al. 2013). Quantum of *Hevea* genome sequencing work is a monumental task as the haploid genome size is enormous (~ 4 x 10^3 Mbp as per calculation based on the DNA content measured by Leitch et al. (1998)) and also rubber possesses a high-complexity genome with >60% repetitive sequences. Taking RRIM 600 as an example, Lau et al. (2016) assembled the genome based on ~155-fold combined coverage with Illumina and PacBio sequence data and has a total length of 1.55 Gb with 72.5% comprising repetitive DNA sequences. A total of 84,440 high-confidence protein-coding genes were predicted. Comparative genomic analysis revealed strong synteny between *H. brasiliensis* and other Euphorbiaceae genomes. Production of high levels of latex can be attributed to the expansion of rubber biosynthesis-related genes, their high expression in latex (Fig. 13.2).

The genome assembly consists of 189,316 scaffolds with an N50 size of 67.2 Kb. Using high-coverage sequence data and the application of PacBio long reads, the N50 size of *Hevea* genome assembly was increased 23-fold, and the number of scaffolds was decreased threefold compared to the previously published assembly of Rahman et al. (2013). Anchoring the genome assembly to the linkage groups of *H. brasiliensis* with 196 RFLP markers from the published genetic map (Lespinasse et al. 2000a) was done by Lau et al. (2016). In total, 189 scaffolds that account for 43.6 Mb in length (3% of the assembly size) were anchored. The assembly was anchored to 18 linkage

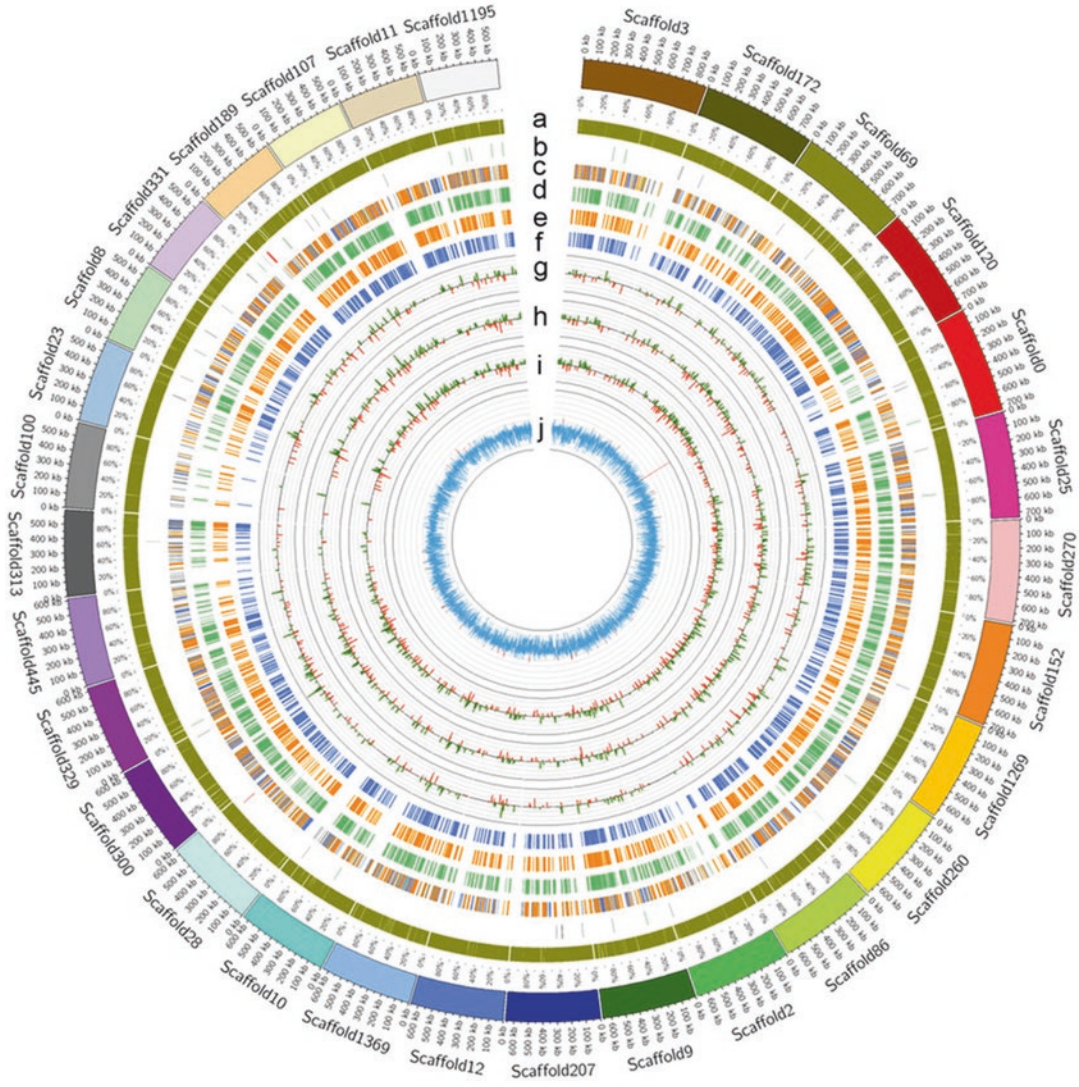

Fig. 13.2 Characterization of the *Hevea brasiliensis* genome. Circos plot of the 30 longest scaffolds. (**a**) Repeats. (**b**) Non-coding RNAs (rRNAs, tRNAs and other ncRNAs are represented by red, green and grey lines, respectively). (**c**) Gene model annotation against the NCBI non-redundant protein database (BLAST matches to *R. communis*, *M. esculenta*, *J. curcas* and other organisms are represented by blue, green, orange and grey lines, respectively). (**d**) Orthologous genes in *M. esculenta*. (**e**) Orthologous genes in *J. curcas*. (**f**) Orthologous genes in *R. communis*. (**g**) CAGE (cap analysis gene expression) tags per million (TPM) in latex (TSSs mapped to the sense and antisense strand are represented in green and red, respectively). *TSS* transcription start site. (**h**) CAGE TPM in leaf (TSSs mapped to the sense and antisense strands are represented in *green* and *red*, respectively). (**i**) CAGE TPM in bark (TSSs mapped to the sense and antisense strands are represented in *green* and *red*, respectively). (**j**) GC content (values of >50% and < = 50% are represented in *red* and *blue*, respectively) (After Lau et al. 2016) (Figure courtesy: Nyok-Sean Lau, Alexander Chong Shu-Chien, Centre for Chemical Biology, Universiti Sains Malaysia)

groups (Lau et al. 2016) (Fig. 13.3 a, b, c). The GC content of the assembled *Hevea* genome was 34.17%, similar to those of the sequenced genomes of *R. communis* (32.5%), *J. curcas* (33.7%) and *M. esculenta* (34.86%) from the Euphorbiaceae family. In addition, Lau et al. (2016) also identified 483 disease resistance genes that constitute about 0.57% of all *Hevea* genes.

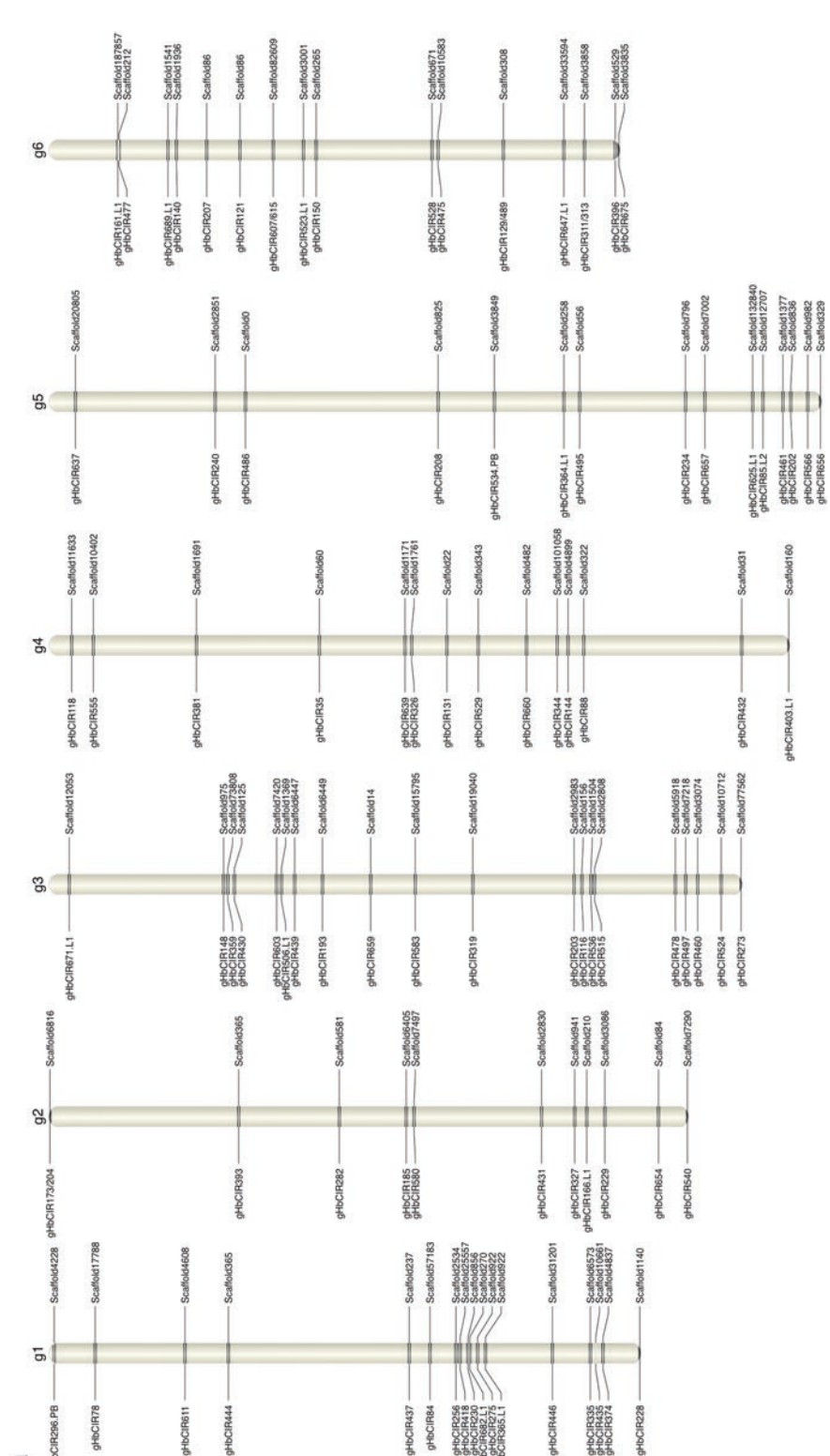

Fig. 13.3 (**a**–**c**) Anchoring the genome assembly to the linkage groups of *H. brasiliensis*. One-hundred and ninety-six RFLP markers from the published genetic map (Lespinasse et al. 2000a) were used for anchoring the scaffolds to the linkage groups. In total, 189 scaffolds that account for 43.6 Mb in length (3% of the assembly size) were anchored (After Lau et al. 2016) (Figure courtesy: Nyok-Sean Lau, Alexander Chong Shu-Chien, Centre for Chemical Biology, Universiti Sains Malaysia)

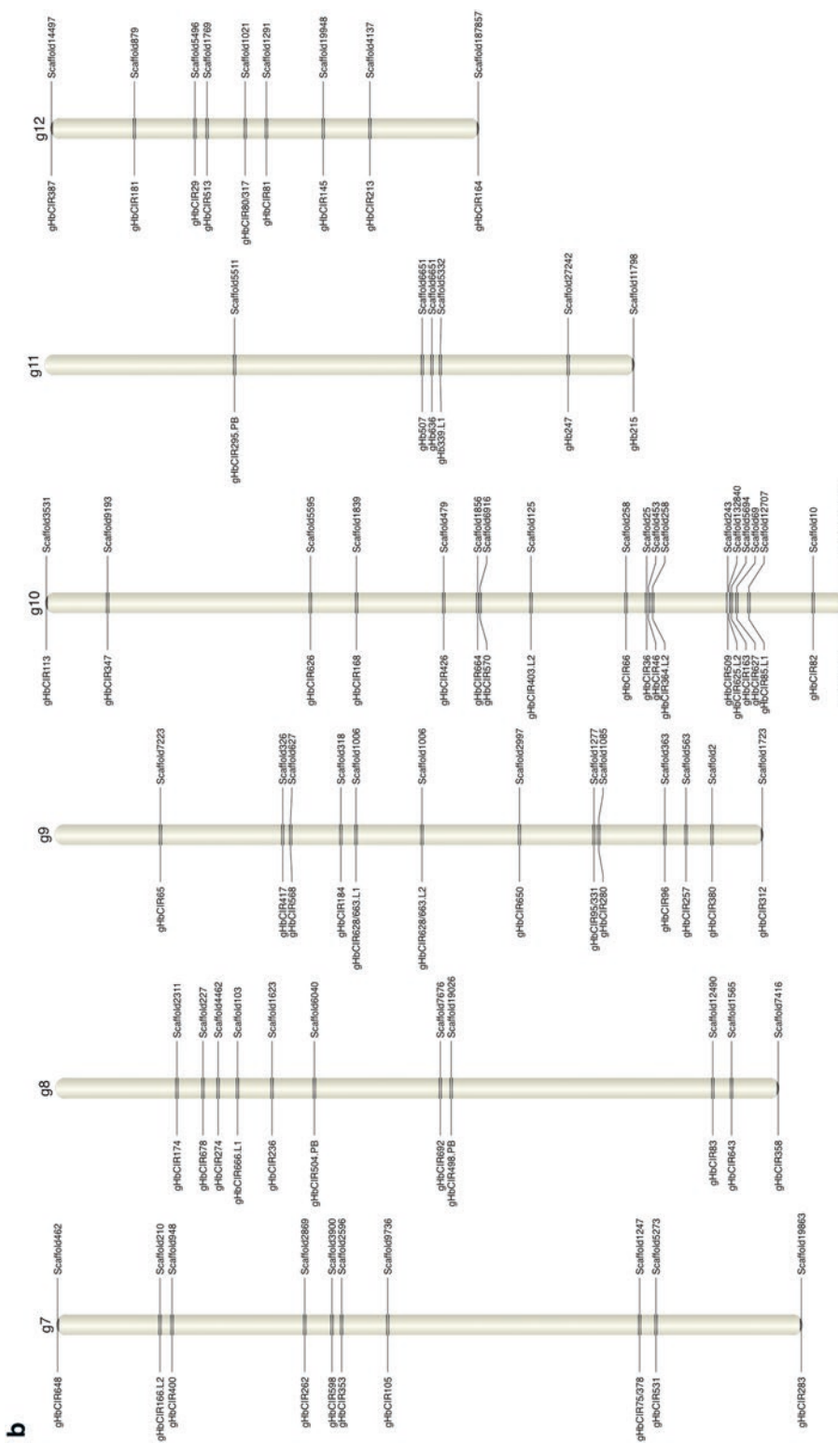

Fig. 13.3 (continued)

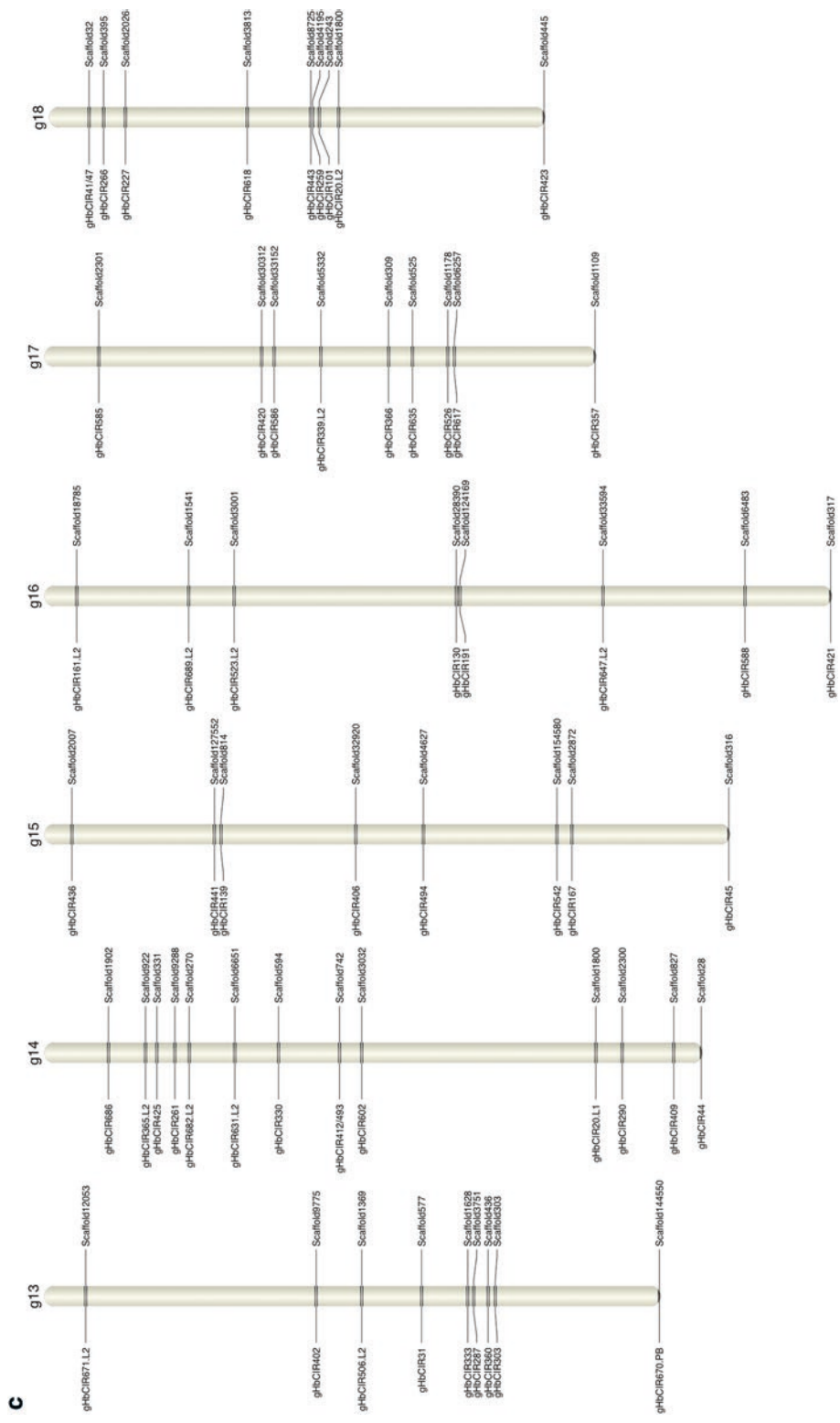

Fig. 13.3 (continued)

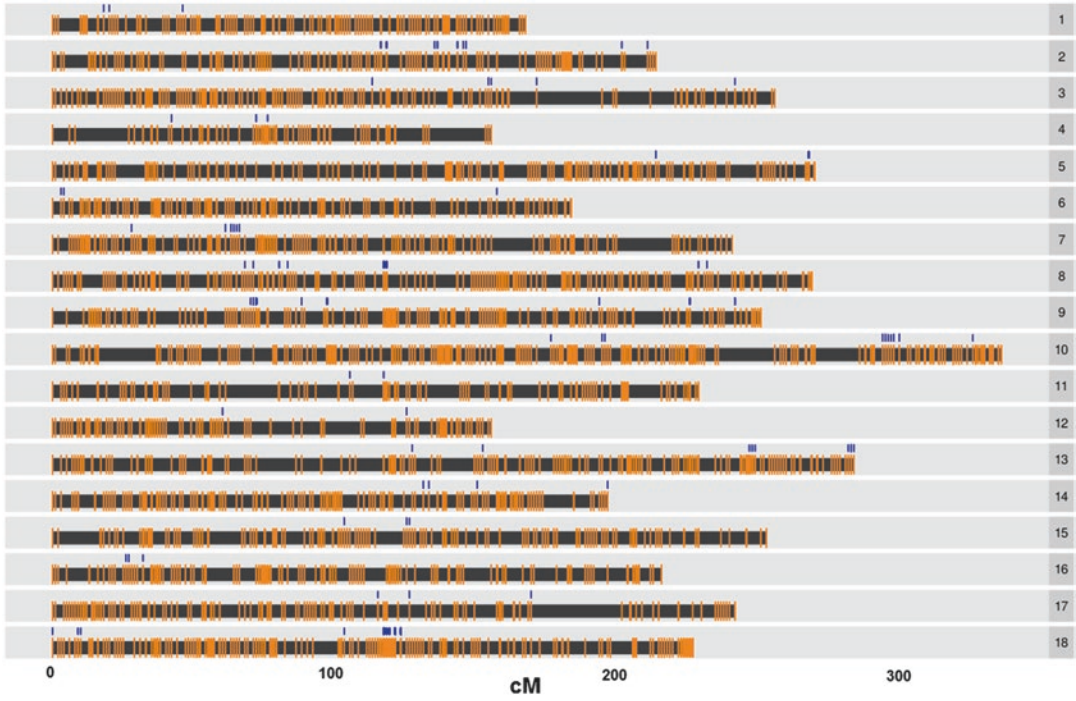

Fig. 13.4 Rubber linkage group ideograms with positions of non-redundant markers indicated as orange vertical bars and markers from within scaffolds indicated as vertical *blue* bars (After Shearman et al. 2015) (Figure courtesy: Sithichoke Tangphatsornruang, National Center for Genetic Engineering and Biotechnology, Bangkok)

According to Shearman et al. (2015), the genome assembly consists of 1,150,326 scaffolds ranging from 200 to 531,465 bp and totalling 1.1 Gb. Only 143 scaffolds, totalling 7.6 Mb, were placed into linkage groups (Rahman et al. 2013). Shearman et al. (2015) performed RNA-seq on six clones of *Hevea* (BPM24, RRII105, RRIC110, PB235, RRIT251 and RRIM600) to identify SNPs and InDels (insertion and deletion of bases in DNA) and performed target sequence enrichment to genotype a set of SNPs in 149 off-springs from a cross of RRIM600 x RRII105. They used this information to generate a linkage map by anchoring 24,424 contigs from 3009 scaffolds, totalling 115 Mb or 10.4% of the published sequence, into 18 linkage groups (Fig. 13.4). Each linkage group contains between 319 and 1367 SNPs, or 60 to 194 non-redundant marker positions, and ranges from 156 to 336 cM in length. This linkage map includes 20,143 of the 69,300 predicted genes from rubber tree and will be useful for mapping studies and improving the reference genome assembly. de Souza et al. (2016) could delineate 143 putative polymorphic positions assembled into 10,071 contigs (N50 = 3078) by a de novo assembly strategy.

Li et al. (2016a) sequenced and analysed the transcriptomes of two parent clones (RRIM600 and PR107) and their hybrids (RY 7-33-97 and RY 7-20-59) to understand their SNPs and small insertions/deletions (InDels). They could gather >31,000 genetic variations in 112,702 assembled unigenes. Their results showed that the higher yield in F_1 hybrids was positively associated with their higher genome heterozygosity in terms of SNPs and InDels. SNPs and InDels have a bearing on ethylene- and jasmonic acid–responsive genes at the transcription level.

13.6 Molecular Biology of Tapping Panel Dryness (TPD)

The two forms of TPD (reversible simple 'tapping cut dryness' related to over-exploitation of the laticifer tissue and the quite irreversible 'brown

bast' related to the development of necrosis within the bark of tapped trees) are currently addressed at the level of gene expression and protein synthesis. Dian et al. (1995) showed that (i) the latex from trees displaying tapping cut dryness exhibited five proteins specific to the cytosolic compartment of the latex cells and these were related to the disease, (ii) major changes consisted of a dramatic increase of a 14.5 kDa protein and a 26 kDa protein in diseased plants and (iii) the 26 kDa protein was linked to the coagulation process. Then Sookmark et al. (2002) observed that the two main polypeptides (here called P15 and P22) were found to accumulate in the cytosol of the TPD-affected trees; P15 and P22 were identified as *REF* (*Hev.b.1*) and *SRPP* (*Hev.b.3*), respectively. Specific molecular probes were designed for a detailed characterization of *REF* and *SRPP* gene expression and RFLP mapping. This allowed the demonstration that *REF* and *SRPP* display very similar expression profiles. They are highly overexpressed by the tapping-induced metabolic activation, although not by wounding per se, or ethylene or abscisic acid. In addition to this similarity in gene expression, they were found to share one common locus in the genome. Eventually, no significant difference in *REF* and *SRPP* gene expression was observed between healthy and TPD trees, indicating that their TPD-related accumulation in the cytosol was not transcriptionally regulated. Western blot analysis demonstrated that osmotic lysis of the sedimentable organelles (lutoids) in vitro caused the release of *REF* and *SRPP* from the rubber particle membrane into the cytosol. A mechanism of cellular delocalization as a consequence of the lutoid instability was proposed to explain *REF* and *SRPP* accumulation in the cytosol of TPD trees (Sookmark et al. 2002). In recent years, studies aimed to identify genes associated with TPD have also been carried out. Chen et al. (2003) reported that the expression of *HbMyb1* was likely to be associated with TPD syndrome. At RRII, the TPD research was focused on identification of TPD-responsive genes by SSH technology applied to mRNA isolation from latex (Venkatachalam et al. 2005). The goal of this study was to identify genes whose mRNA levels are differentially expressed in the rubber tree during TPD development. To identify the genes involved in this process, two SSH cDNA libraries were constructed. For the forward-subtracted cDNA library, healthy RNA was used as the tester and TPD RNA served as the driver, whereas TPD RNA was the tester and healthy RNA was the driver for the reverse subtracted cDNA library. A total of 1079 putatively positive clones were screened from these two libraries; 352 of these clones were positive by differential screening with forward and reverse subtracted probes and were selected for sequencing analysis. The putative functions of clones were predicted by BLASTX/BLASTN analysis. Among these, 64 were genes whose function had been previously identified while the remaining clones were genes with either unknown protein function or insignificant similarity to other protein/DNA/EST sequences in existing databases. Differentially expressed genes selected by subtractions were classified into 12 broad categories according to their putative functions generated by BLAST analysis. The possible links between the identified regulated genes and TPD syndrome were considered by dot-blot analysis and compared where two unique genes were strongly down-regulated under the TPD condition. Two genes, *Myb transcription factor* and *translationally controlled tumour-induced protein* (*TCTP*), that were unigenes to the forward-subtracted cDNA library (up-regulated) were selected for expression analysis. The expression of two selected gene transcripts was examined by Northern blot analysis using plant tissues of both healthy and TPD trees. Results from Northern analysis confirmed that the expression of these two genes was down-regulated in TPD trees. This was the first study reporting a set of suppressed genes in tapping cut dryness-affected trees by the SSH technique. Some other known genes identified in this study might provide new insights into TPD development in the rubber tree (Venkatachalam et al. 2005). Similar research based on SSH is currently being developed on brown bast (Kongsawadworakul et al. 2005). Apart from genetic engineering, studies on laticifer-specific gene expression could have important implica-

tions for selection and breeding. The use of mRNA transcript levels as molecular markers for early selection could be considered (Kush et al. 1990). It is also felt that extensive studies on the expression of genes and the regulation systems in different fields may open new paths for rubber breeding. Functional genomics in rubber will develop faster and faster, taking advantage of research developed on other species (through comparison with the information of public databases) and by focusing on specific areas of interest in order to gain a good understanding of the functioning of the network of interacting genes and regulating factors.

Eliathe et al. (2012) conducted a study to identify protein markers with yield potential and susceptibility to tapping panel dryness (TPD). Yield and susceptibility to TPD were compared in 11 clones (stimulated and non-stimulated). Their lutoid fraction polypeptides were analysed using one- and two-dimensional electrophoresis. Susceptibility to TPD appeared as a clonal trait which is not related to yield potential. TPD can occur either in stimulated or non-stimulated clones, but overstimulation increases TPD symptoms. While PB235, PB260 and IRCA130 were seen highly susceptible to TPD, IRCA41, PB217, AF261, AVROS2037 and GT1 were less susceptible. Eliathe et al. (2012) analysed 32 KDa and 35 KDa lutoidic proteins. High yielding clones with less TPD were characterized by abundant quantity of 35 KDa lutoidic polypeptide. On the contrary, clones susceptible to TPD were characterized by abundant quantity of 32 KDa polypeptide. In low-yielding clones (RO38, Tjir 1), 32 KDa protein was more abundant than 35 KDa. Overstimulation induces a decrease of 35 KDa protein intensity. This study demonstrates the utility of 32 and 35 KDa polypeptides detects yielding potential and susceptibility to TPD. Li et al. (2010) could construct forward and reverse cDNA libraries from the latex of healthy and TPD trees using suppression subtractive hybridization (SSH) method to identify the genes related to TPD. Of the 1106 clones obtained from the two cDNA libraries, 822 clones showed differential expression in two libraries by reverse Northern blot analyses. Sequence analyses indicated that the 822 clones represented 237 unique genes; and most of them have not been reported to be associated with TPD in rubber tree. The expression patterns of 20 differentially expressed genes were further investigated to validate the SSH data by reverse transcription PCR (RT-PCR) and real-time PCR analysis. According to the Gene Ontology convention, 237 unique genes were classified into 10 functional groups, such as stress/defence response, protein metabolism, transcription and post-transcription, rubber biosynthesis, etc. Among the genes with known function, the genes preferentially expressed were associated with stress/defence response in the reverse library, whereas metabolism and energy in the forward one. The genes associated with TPD were identified by SSH method in this research. Systematic analyses of the genes related to TPD suggest that the production and scavenging of reactive oxygen species (ROS), ubiquitin proteasome pathway, programmed cell death and rubber biosynthesis might play important roles in TPD. Therefore, such results not only enrich information about the genes related to TPD but also provide new insights into understanding the TPD process in rubber tree.

Trunk phloem necrosis (TPN) is known as a physiological disorder since 1980s. Distinguished from rubber tree tapping panel dryness (TPD), by its macroscopic symptoms and presumed origin, little attention has been paid to its microscopic features. de Faÿ (2011) has come out with some evidence that both syndromes could be linked to an impaired cyanide metabolism. In order to characterize TPN and compare it with TPD microscopically, the inner phloem of tapping panels was investigated by light and transmission electron microscopy in healthy trees and TPN-affected trees. TPN-affected phloem presented numerous and varied structural and ultrastructural features. Signs of cellular deterioration could be seen in a great number of specialized cells, i.e. laticifers and sieve tubes, but not in very specialized cells, i.e. parenchyma cells and companion cells. There were also signs of cellular dedifferentiation in other parenchymatous cells, e.g. in tylosoids and hyperplasic cells. These cells were derived from parenchyma cells

that ensheath laticifers in which the latex coagulated. Numerous structural features of TPN are common to TPD, notably tylosoids associated with in situ coagulated latex, which are also known to be early structural markers of TPD and cyanide-induced. de Faÿ (2011) therefore concluded that TPN is identical to or a variant of TPD and is a degenerative disorder of rubber tree trunk phloem resembling plant stress response, programmed cell death and plant tumourigenesis in some aspects.

13.7 Genomics for Changed Climates

Plant species are the best experimental material to study changes in climate since they express their reactions to such changes through significant manifestations. There will be perceptible indications in terms of morphological, physiological and genomic expressions towards such changed climates. As rubber spreads to new areas where drought and cold are conspicuous stress factors, employing genomic tools can largely help in advancing knowledge about the ability of rubber to adjust such environments. The work of Silva et al. (2014) stems promise in this direction. They studied leaf, panel and latex ESTs under cold-stressed conditions. For panel and latex libraries, samples were collected from 16-year-old tree clones of PB217, PR255, GT1, PB235, RRIM701 and IAN873, and leaves of the same clones were collected from the rubber tree germplasm. PB217, PR255, GT1 and IAN873 were subjected to a 24-h cold treatment in a growth chamber and maintained at 8 °C with a 12-h photoperiod. This treatment was performed to promote the expression of genes involved in cold response and for the development of molecular markers related to this stress condition. A total of 8263 reads were assembled, generating 5025 unigenes that were analysed; 912 expressed sequence tags (ESTs) represented new transcripts, and 2 sequences were highly up-regulated by cold stress. These unigenes were scanned for microsatellite (SSR) regions and single nucleotide polymorphisms (SNPs). In total, 169 novel EST-SSR markers were developed, of which 138 loci were polymorphic. Nearly 98% of this presented transferability to six other *Hevea* species. Locus duplication was observed in *H. brasiliensis* and other species. Additionally, 43 SNP markers in 13 sequences that showed similarity to proteins involved in stress response, latex biosynthesis and developmental processes were characterized. cDNA libraries are a rich source of SSR and SNP markers and enable the identification of new transcripts. Transcriptome analysis is one of the main approaches for identifying the complete set of active genes in a cell or tissue for a specific developmental stage or physiological condition.

13.8 Perspectives on Genomics

Application of molecular tools in rubber tree improvement was lagging behind because of limited knowledge of the genome. Initially, hybridization-based RFLP markers, providing co-dominant information, were used to characterize *Hevea* germplasm. RFLP technique was proved to be useful for genetic diversity study in wild and cultivated *Hevea* accessions using low-copy-number nuclear probes (Besse et al. 1994). RFLP analysis of organelle genomes of *Hevea* was also performed for establishing evolutionary relationships as these two genomes could reflect true evolution because of their uniparental inheritance (Luo et al. 1995). Mathew et al. (2005) studied the phylogenetic relationship among three species of rubber, *Hevea brasiliensis, H. benthamiana* and *H. spruceana*, employing different molecular marker techniques, namely, RAPD, chloroplast DNA, PCR-RFLP and heterologous chloroplast microsatellites. RAPD analysis clearly indicated a high degree of polymorphisms among the three species. For the first time, Low et al. (1996) detected microsatellites in the *Hevea* genome through the database search of some *Hevea* gene sequences. The construction of a microsatellite-enriched library in *Hevea brasiliensis* was reported by Atan et al. (1996). The detection of high degree of polymorphism indicates a way to introduce desirable variation into *H. brasiliensis* either through introgression or transformation.

Genetic linkage map presents the linear order of markers (genes and other identifiable DNA sequences) in their respective linkage groups depicting the relative chromosomal locations of DNA markers by their patterns of inheritance. The linkage map allows revelation of more and more restricted segments of the genome and undoubtedly enhances our understanding in many areas of plant systematics. A genetic map for *Hevea* spp. was constructed using a population derived from an interspecific cross between PB260 (*H. brasiliensis*) and RO38, an interspecific hybrid clone (*H. brasiliensis* × *H. benthamiana*), following the pseudo-testcross strategy (Lespinasse et al. 2000a). The markers were assembled into 18 linkage groups, thus reflecting the basic chromosome number, and covered a total distance of 2144 cM. A total of 717 loci constituted the synthetic map, including 301 restriction fragment length polymorphisms, 388 amplified fragment length polymorphisms, 18 microsatellites and 10 isoenzymes. Homologous linkage groups between the two parental maps were merged using bridge loci. Average marker density was 1 per 3 cM. Lespinasse et al. (2000b) mapped quantitative trait loci (QTLs) for resistance to South American leaf blight (SALB), a disease of the rubber tree caused by the fungus *Microcyclus ulei* using the same cross combination (PB260, a susceptible clone, and RO38, a SALB-resistant clone). Eight QTLs for resistance were identified on the RO38 map, whereas only one QTL was detected on the PB260 map. New linkage maps were added by Lau et al. (2016) and Shearman et al. (2015).

Transcriptome sequencing and development of microarrays have been undertaken recently in *Hevea* rubber (Triwitayakorn et al. 2011; Salgado et al. 2014). Sequencing of transcriptomes of bark that leads to EST-SSR markers is also of prime importance (Li et al. 2012; Cubry et al. 2014) that calls for rigorous research. Such developments are certainly welcome that elevates *Hevea* rubber research on par with other tropical tree crops. However, such innovations must help to find answers to intriguing issues like tapping panel dryness (TPD), stock-scion interactions and yield differences exhibited among trees raised through bud grafting, molecular markers for selecting high yielders at juvenile stage, delineation of parents of open-pollinated seedlings, production of natural somatic seeds and so on.

Transcriptomes of CATAS8-79 and PR107 have been sequenced to dissect the molecular mechanism for the regulation of latex regeneration and duration of latex flow (Chao et al. 2015). More than 26 million clean reads were generated and 51,829 all-unigenes were totally assembled. A total of 6726 unigenes with differential expression patterns were detected between CATAS8-79 and PR107. Expression pattern of genes upon successive tapping was analysed by quantitative PCR. Several genes related to rubber biosynthesis, cellulose and lignin biosynthesis and rubber particle aggregation were differentially expressed between CATAS8-79 and PR107. The level of endogenous jasmonates, carbohydrate metabolism, hydroxymethylglutaryl-CoA reductase (HMGR) and *Hevea* rubber transferase (HRT) are pertinent in mevalonate (MVA) pathway for latex regeneration. On the other hand, level of endogenous ethylene (ETH), lignin content of laticifer cell wall, antioxidants and glucanases are pivotal for duration of latex flow (Chao et al. 2015) (Fig.13.5). In RRIM600 alone, approximately 10,000 DNA sequences representing genes expressed in the latex have been delineated through DNA sequencing technology (Mat-isa et al. 2009). They developed *NRESTdb* (Natural Rubber EST database) to provide easy access and rapid analysis of such data as the first publicly available EST database for *H. brasiliensis*. Such studies are to be augmented further to draw feasible answers for questions on latex production, laticifer-specific gene expression and tapping panel dryness.

Lately, there was a comparative evaluation between self-rooting juvenile clones (JCs) and bud-grafted (donor) clones (DCs) at transcriptome level (Li et al. 2016b). Genes, especially encoding epigenetic modifications, are differentially expressed in JCs and DCs. Genes involved in carbohydrate metabolism, hormone metabolism and reactive oxygen species scavenging were up-regulated in JCs of CATAS7-33-97 and Haiken 2, indicating that the JCs provide

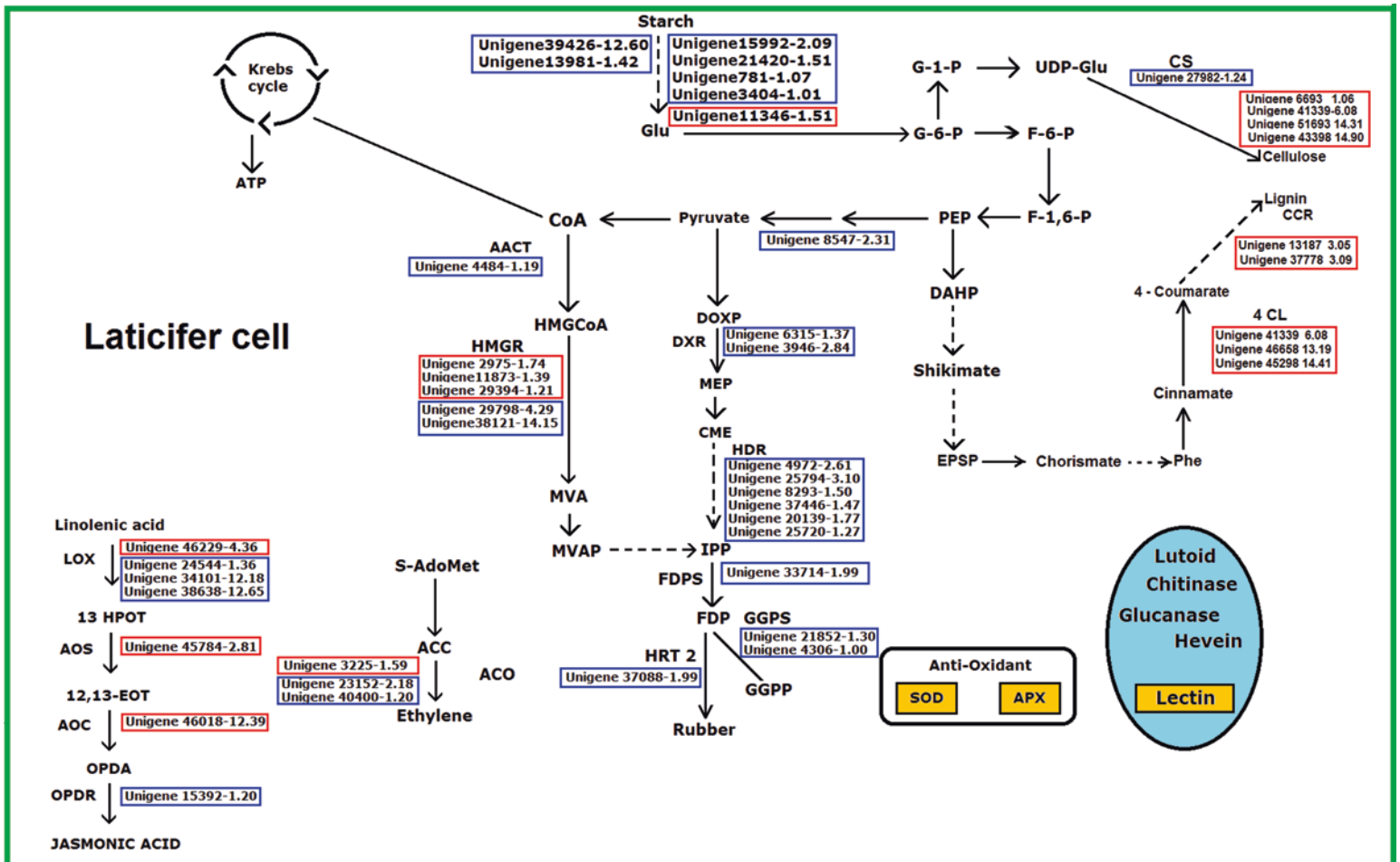

Fig. 13.5 Schematic representation of differentially expressed unigenes related to latex regeneration and duration of latex flow in laticifer cell. Unigenes up-regulated in PR107 are shown in red boxes but down-regulated in CATAS8-79. Those in *blue* boxes are unigenes up-regulated in CATAS8-79 but down-regulated in PR107. Numbers represented Log2 values in DGE data. The *dashed arrows* indicate multiple steps of enzymatic reactions (After Chao et al. 2015)

sufficient molecular basis for the increased rubber yield. Comparative trial between self-rooting JCs and DCs, self-rooting JCs exhibited better performance in rubber yield (Li et al. 2016b). Such investigation clearly indicates the intricateness of stock-scion interactions. Decoding the genes responsible for apomixis or parthenocarpy (Hand and Koltunow 2014) and their introduction into Hevea genome to achieve somatic seeds is the futuristic goal in *Hevea* genomic research.

The *Hevea* genome has now been published three times over. Yet not everyone comes up with the same findings. Rahman et al (2013) do not offer the newest *Hevea* genome, and the honour goes to the RIKEN team of Japan, working in collaboration with Universiti Sains Malaysia (USM) for a more comprehensive genome analysis (Lau et al. 2016). However, the most comprehensive genome to date comes from Chinese Academy of Tropical Agricultural Sciences (CATAS) group in China (Tang et al. 2016). CATAS declares the genome size as 1.47 Gb (Tang et al. 2016), whereas USM and RIKEN/USM both give the figure of 2.15 Gb. Despite this discrepancy, it is noteworthy that the CATAS assembly captures practically all the USM sequences contained in a purportedly larger draft genome. It appears that CATAS has done a better job of fitting contiguous sequences into a smaller number of scaffolds (Tang et al. 2016). Thus, while USM has a scaffold N50 of only 67.2 kb, CATAS weighs in with a massive scaffold N50 of 1.28 Mb. Despite three published *Hevea* genomes now in the public domain, the discrepancies between the reports have the logic that the last word is still not yet in.

Genomics, no doubt, is a science that can make inroads into the intricacies of gene actions in *Hevea*. The only care to be observed is to continue the studies with a set goal so that the plant breeder will get ample assistance to derive new clones at definite intervals, as also in tandem with circumstantial demands.

Ancillary Income Generations

14

Hevea honey and wood are the two supplementary income-generating products that can raise significant income from rubber plantations. A brief description is given here on the potentialities of these tools in rubber plantations, even though dealing with such subjects is beyond the scope of this book.

14.1 *Hevea* Honey

Neither male nor female flowers secrete nectar, but much is secreted by the extrafloral nectaries (on young leaf petioles and fleshy scales of young shoots) (Parkin 1900). Over the years, honey bee rearing saw major vicissitudes due to non-availability of bees many a times. In India, there was a rehabilitation measure through the introduction of *Apis mellifera* with a reported average yield of 60 kg/hive/year compared to 19.46 kg/hive/year for the popular Indian honey bee, viz. *Apis cerana indica* (Haridasan et al. 1987). On an average, 15–20 *A. cerana indica* hives can be placed per ha, and the results of a recent survey showed an average yield of 12.1 kg/hive/year for the Indian honey bee (Chandy et al. 1998). Therefore, the mature rubber plantations in India have the potential to produce 67,886 ton of rubber honey annually.

The honey-flow period of rubber plants ranges from January to March, and during which honey bees collect large quantities of nectar from the extrafloral nectarines. Lack of honey flow in rubber during the prolonged dearth period from April to December necessitates alternate bee flora for offseason bee management in rubber plantation-based apiaries (Nehru et al. 1990). *A*ccording to the Bureau of Indian Standards (BIS) specifications, rubber honey is medium grade (Grade A) with an average moisture content of 22%. The important properties of rubber honey are given in Table 14.1. Apart from honey, other principal hive products are pollen (bee bread), propolis, beeswax and bee venom which also have industrial uses. The major consuming industries of honey in the domestic market are the Ayurvedic and allopathic pharmaceuticals, bakery, confectionery, dairy and tobacco manufacturing. Under the current rates, with 15 hives/ha, a production potential of 182 kg can be realized. With a farm gate price of 2 US$/kg for unprocessed honey, the estimated net income is around US$ 100/ha/annum.

14.2 *Hevea* Wood

Rubber wood, which can be transformed into furniture by sawing, and also into plywood, particleboard, medium-density fibreboard (MDF) and fuel wood after felling the old rubber plots, has become a second product of rubber cropping; it may represent about 15% of the total income of the farmers, and it generated a profitable industry mainly in Malaysia and Thailand but also in India, Vietnam, Indonesia and Cambodia. It has

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become a new challenge for breeders, and this was first addressed by the Rubber Research Institute of Malaysia (RRIM) (Othman et al. 1995). Many clones of RRIM 2000 series are considered latex-timber clones. The price of timber rubber wood at field level, initially very low, has now reached the average current level of 5000 US$ per hectare in Malaysia and Thailand. The average lignous biomass at felling of the aerial parts of the trees (excluding roots) is roughly around 180 m^3 per hectare, from which 90 m^3 can be sawn, providing the industry with around 20–50 m^3 sawn wood. Allied species and Amazonian germplasm accessions have great potential as a source of rubber wood (Table 14.2).

The emergence of rubber wood as a basic renewable source (Fig. 14.1) is an outcome of the sustained research and development efforts since 1970s through standardization of preservative treatment and drying procedures. Rubber wood is now extensively used in furniture manufacture, structural applications and interior decoration. The estimated world market size of rubber wood-based furniture and other items is around US$ 1500 million and the export earnings of Malaysia alone was US$ 655 million in 1995 (MTIB 1996). Malaysia and Thailand are the leading countries in terms of commercial production, consumption and export.

Hevea rubber timber is whitish yellow when freshly cut and turns pale cream after drying. The air-dry specific gravity is 0.557 with an average weight of about 515 kg/m^3 at 12% moisture content (Sekhar 1989). The growth rings are absent or ill defined (Silva 1970), and the growth ringlike structures displayed in the cross-sectional view of the timber are merely false rings which are formed by the distribution pattern of tension wood fibres (Reghu et al. 1989a). The sapwood is not differentiated from heartwood due to lack of deposition of

Table 14.1 Properties of rubber honey

Properties	Range	Average
Viscosity (in centipoises) at 27 °C	550–3800	1358
Specific gravity at 27 °C	1.40–1.34	1.38
Moisture (%)	21.5–25.5	22.0
Reducing sugars Levulose (%) Dextrose (%)	69.08–74.8 34.88–40.70 33.57–37.97	72.80 37.14 35.98
Non-reducing sugars (%)	0.78–3.14	1.71
Acidity (%)	0.06–0.20	0.13
Ash (%)	0.09–0.39	0.22
Protein (%)	0.05–0.25	0.14
Yeast (million/g)	103.9–158.0	139.39

After George et al. (2000)

Table 14.2 Estimated wood volume from potential clones, accessions of Brazilian Amazonian and allied species

Clone	Parentage	Age (year)	Clear bole volume (m^3/tree)	Canopy wood volume (m^3/tree)	Total wood volume (m^3/tree)
RRIM 910	PB 5/51 × RRIM 623	22	0.76	0.57	1.33
RRIM 912	-do-	22	0.75	0.75	1.50
RRIM 931	PB 5/51 × RRIM 713	20	0.68	0.68	1.36
PB 235	PB 5/51 × PB S/78	20	0.80	0.80	1.60
PB 355	PB 235 × PR 107	22	0.93	2.32	3.25
RRIM 2008	RRIM 623 × PB 252	14	0.33	0.99	1.32
RRIM 2014	RRIM 717 × PR 261	14	0.53	0.80	1.33
Clones of Brazilian Amazonian					
RO/OP/4–20/125	–	13	1.259	1.159	2.518
AC/F/5–21/197	–	13	1.403	1.052	2.455
MT/C/5–12/137	–	13	1.054	1.318	2.372
AC/F/21–64/221	–	13	1.137	1.364	2.501
Allied species					
H. pauciflora	–	24	1.13	0.41	1.14
H. guianensis	–	24	1.45	2.18	3.64
H. nitida	–	24	1.04	1.04	2.08

After Arshad et al. (1995)

Fig. 14.1 Processed *Hevea* rubber wood

pigmented extraneous materials that usually occur during heartwood formation in other hardwood timber species. Though reserve metabolites in the form of soluble sugar, starch, etc., are abundant in rubber wood, conversion of these materials into heartwood substances through long-term ageing process and necrobiosis of storage cells does not take place, mainly due to the fast-growing nature of rubber trees. Hence heartwood formation is virtually absent in rubber trees, and the storage tissue is always filled with soluble sugar and starch which in turn is easily attacked by fungi, insects, borers, beetles, termites, etc. (Kadir and Sudin 1989). Early wood and late wood differentiation is not possible in rubber due to the long and continuous cambial activity associated with the fast-growing tendency. Also, cambial activity involves production latex vessels, which is a constant process during growth of a tree.

Rubber wood is composed of fibres, vessel elements (pores) axial parenchyma and rays in different proportions similar to that of other hardwood species. The fibres are lignified or partially lignified and are 1.1–1.5 mm in length (Bhat et al. 1984) and about 22 μm in thickness (Silva 1970), and the vessels are small to moderately large with 1–4 pores/mm^2. The structure and distribution patterns of pores enhance the chemical impregnation capacity of rubber wood during preservative treatments. The lumen of the pores is usually filled with balloon-like parenchymatous outgrowths called tyloses which are a characteristic feature of rubber wood. The nature and extent of tylosis formation and their impact on preservative impregnation are still obscure. Tension wood formation is considered as a natural abnormality which creates various problems (Harlow 1970). Tension wood is characterized by its unlignified gelatinous fibres. Sharma and Kukreti (1981) observed 15–65% tension wood fibres that create a variety of drying, wood working and finishing problems (Ipe et al. 1987). Rubber wood is lignocellulosic and its density is not uniform throughout (Midon 1994).

14.2.1 Processing

Processing of rubber wood consists of preservative impregnation and drying. The basic objective of preservative treatment is to protect rubber wood from biodeterioration caused by various biological agents. There are short-term and long-term protections. For temporary protection, a dip treatment and a number of insecticides and fungicides are carried out. In long term, the wood preservatives are allowed to penetrate deep into the timber for complete preservative penetration either through dip diffusion process or pressure impregnation process.

The deep diffusion or boron diffusion process is done only on freshly sawn timber having more than 50% moisture content. Here, immersion of freshly sawn timber in a mixture of boric acid and borax in water is done (Gnanaharan 1982; Tam and Singh 1987; Gnanaharan and Mathew 1982; Gnanaharan and Dhamodaran 1993) that gives dry salt retention of 5 kg/m^3 and 12 mm penetration (Gnanaharan 1996). To circumvent fungal attack, sodium pentachlorophenate (NaPeP) at 0.5–1.0% is seen effective (Gnanaharan 1983; Jose et al. 1989).

Pressure treatment is more popular. There are two types, viz. vacuum pressure method (Bethel process) and oscillating pressure method (OPM). In the former, impregnation of preservatives into the wood is done by creating vacuum and pressure. However, partially dried timber ensures maximum penetration and retention of preservatives. The preservatives used are copper-chrome-arsenic (CCA) (copper sulphate, potassium or sodium dichromate and arsenic pentoxide), copper-chrome-boric (CCB) (copper sulphate, sodium or potassium dichromate) and boric acid and borax (Hong and Liew 1989; Gnanaharan and Dhamodaran 1993). The oscillating pressure method (OPM) is more complex that requires automated plant equipment for introducing 10–15 cycles of vacuum and pressure for 10 min in a total time of 2 h. This is popular in Malaysia (Dahlan et al. 1994).

14.2.2 Production and Consumption

Of late, selection for timber has become a very important objective. An estimation from RRIM shows that a hectare of rubber plantation can yield around 190 m^3 of rubber wood, and 2.7 million m^3 of *Hevea* wood would be available from Malaysia (Arshad et al. 1995). Approximately 741 million m^3 of wood must be available from 8,927,000 hectares worldwide. The demand is expected to increase fast, and the RRIM has been making earnest efforts in deriving latex-timber clones (Othman et al. 1995). Lately, there is some interest generated among the scientists to evolve rubber as a factory producing useful chemicals especially life-saving drugs (Yeang et al. 2002). In the future, new requirements linked with environmental concerns such as reforestation or carbon sequestration might appear but implication for breeding is not clear for now (see Chap. 10). As far as rubber wood is concerned, the processing technique needs to be standardized following the environment of the country in question, since the infestation of pests differs with the environment.

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