Latex Production, Diagnosis and Harvest

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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).

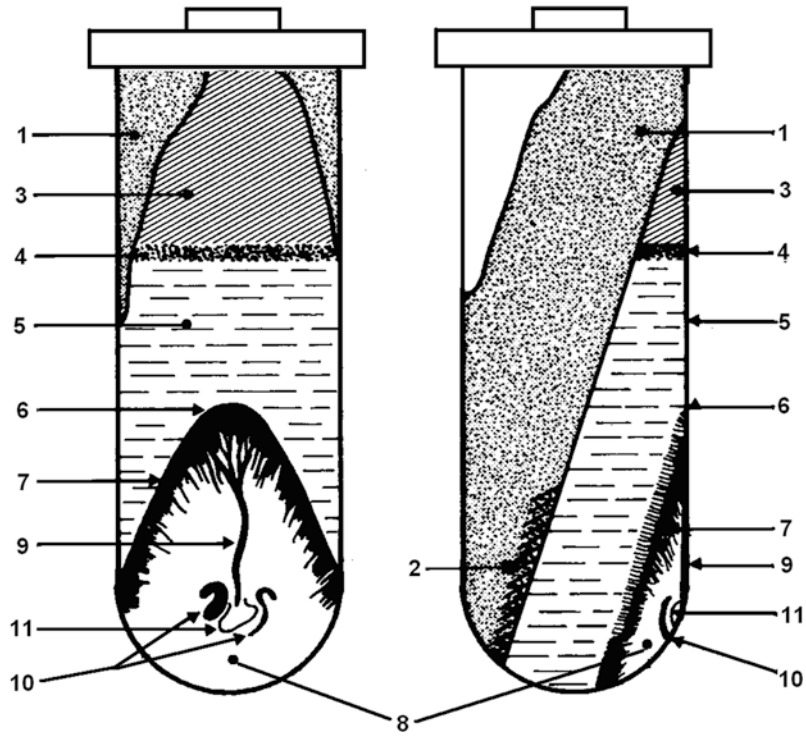

Fig. 5.1 Ultracentrifugation of *Hevea* latex. Fractions 1–3 are white rubber phase. Fraction 4 is the Frey-Wyssling particles. Fraction 5 is the clear serum (C-serum) corresponding to latex cytosol. Fractions 6–11 constitute the 'bottom fraction' of which the fraction 8 is the lutoid fraction

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

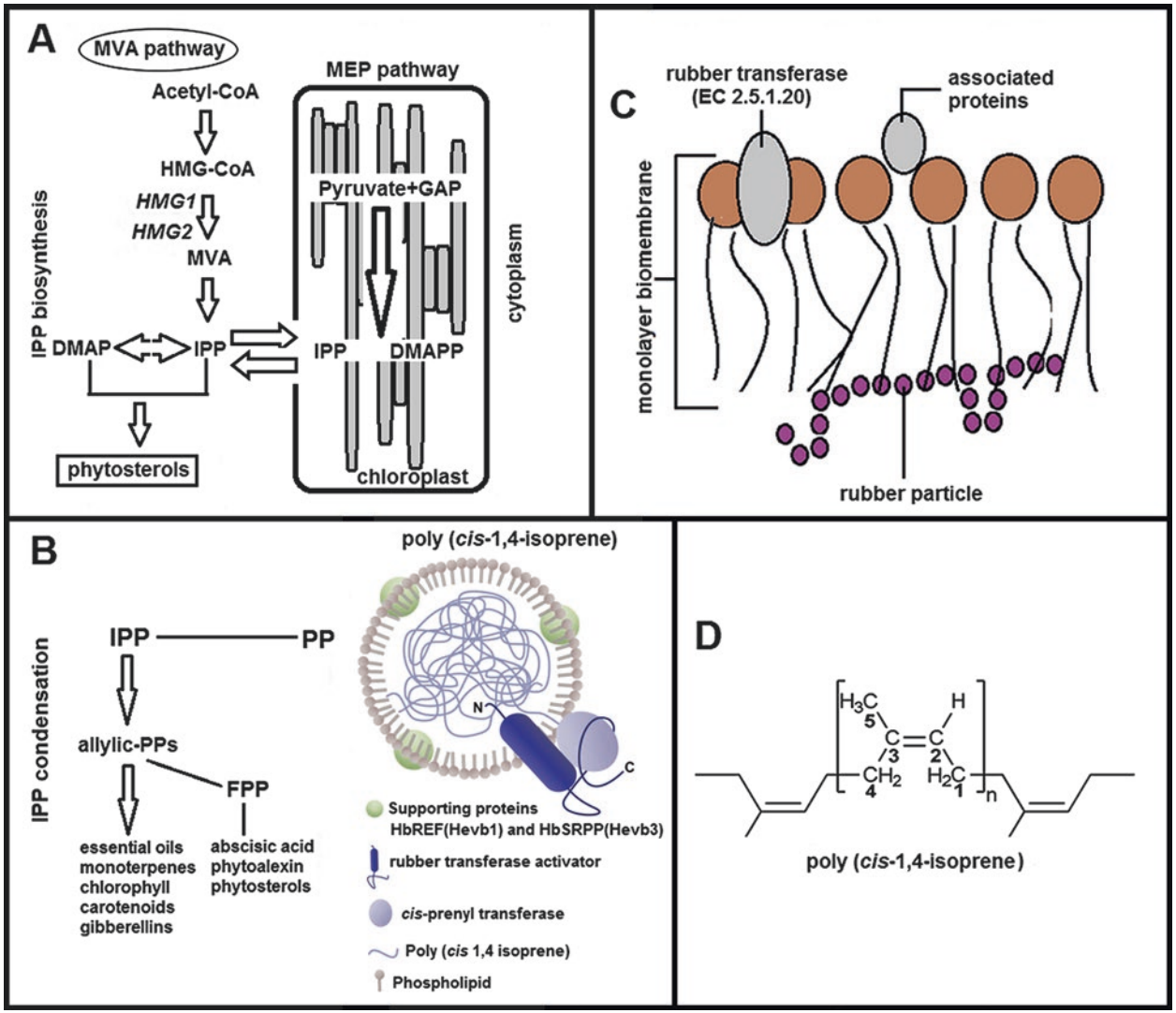

Fig. 5.2 Biosynthesis of rubber particles: (**a**) The monomeric subunit of natural rubber IPP is synthesized by the MVA pathway and the MEP pathway in higher plants from acetyl-CoA or glyceraldehydes-3-phosphate and pyruvate, respectively; (**b**) IPP is used for the synthesis of isoprenoids such as 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)

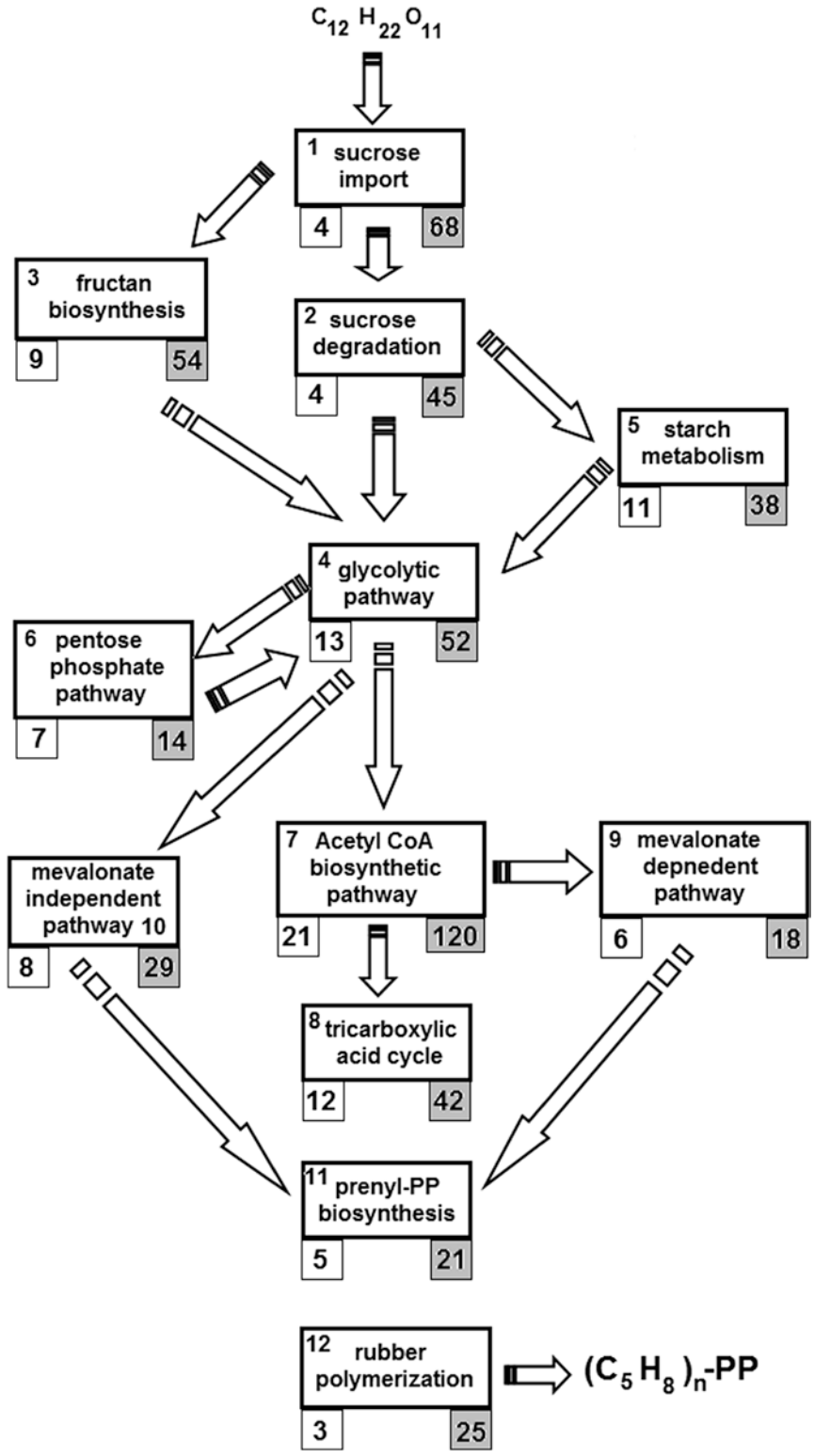

Fig. 5.3 Schematic representation of the metabolic pathway leading to natural rubber biosynthesis. The number of enzymes and associated proteins in each individual pathway is shown in *plain boxes* and the number of orthologs are in *shaded boxes* (after Rahman et al. 2013). http://www.biomedcnetral.com/1471-2164/14/75

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

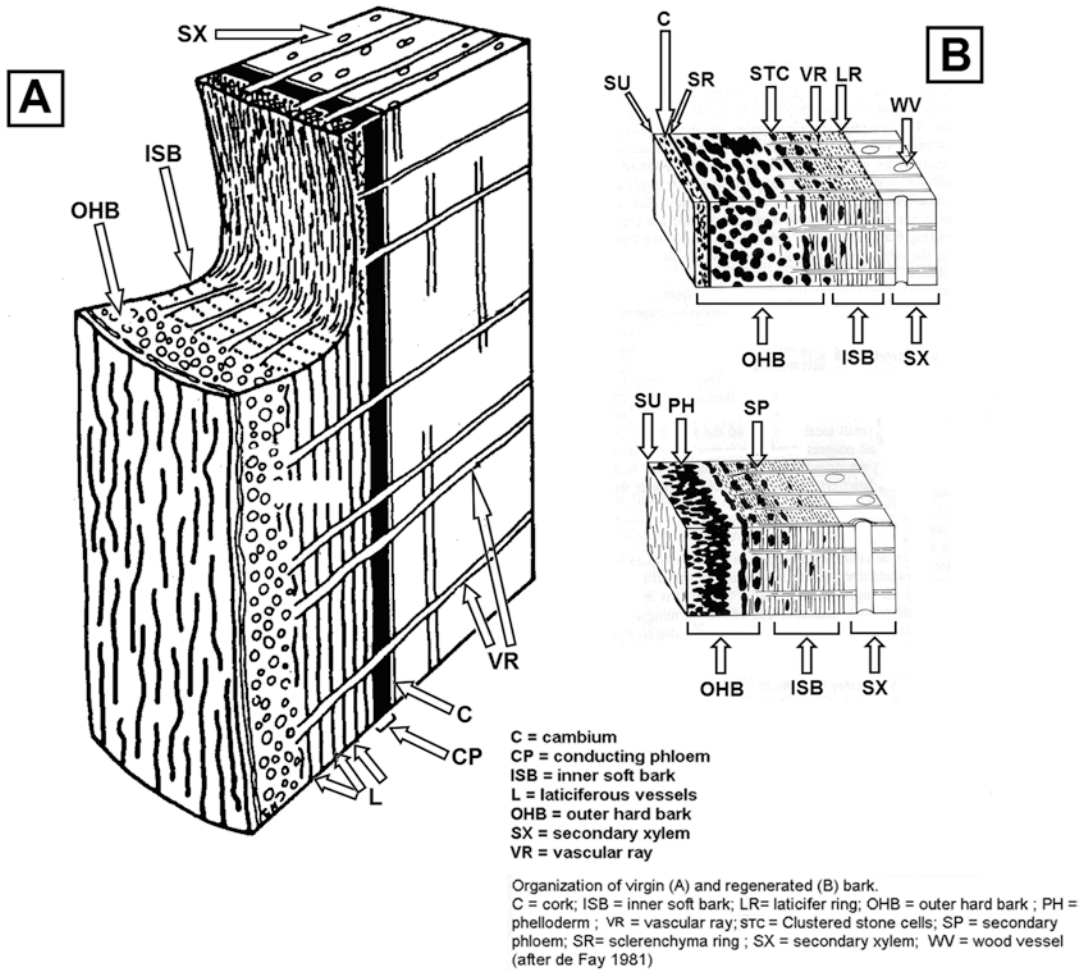

Fig. 5.4 Organization of virgin and regenerated bark. (**a**) Cross section of virgin bark (*C* cambium, *CP* conducting phloem, *ISB* inner soft bark, *L* laticiferous vessels, *OHB* outer hard bark, *SX* secondary xylem, *VR* vascular ray). (**b**) Cross section of regenerated bark (*C* cork, *ISB* 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,

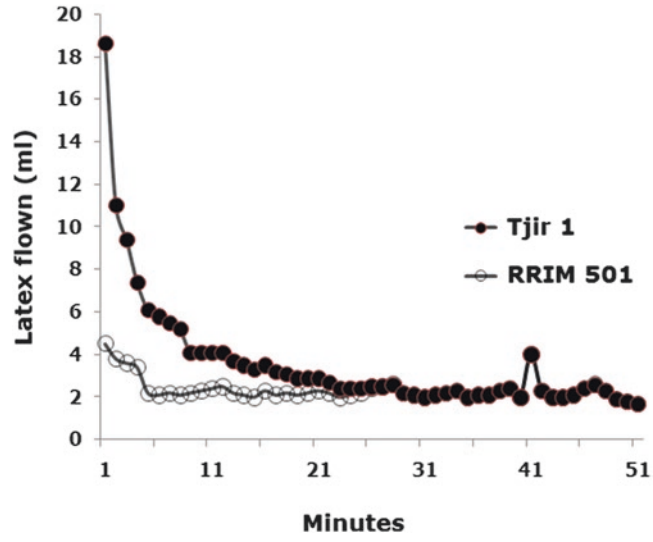

Fig. 5.5 Progression of latex flow of RRIM 501 and Tjir 1

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

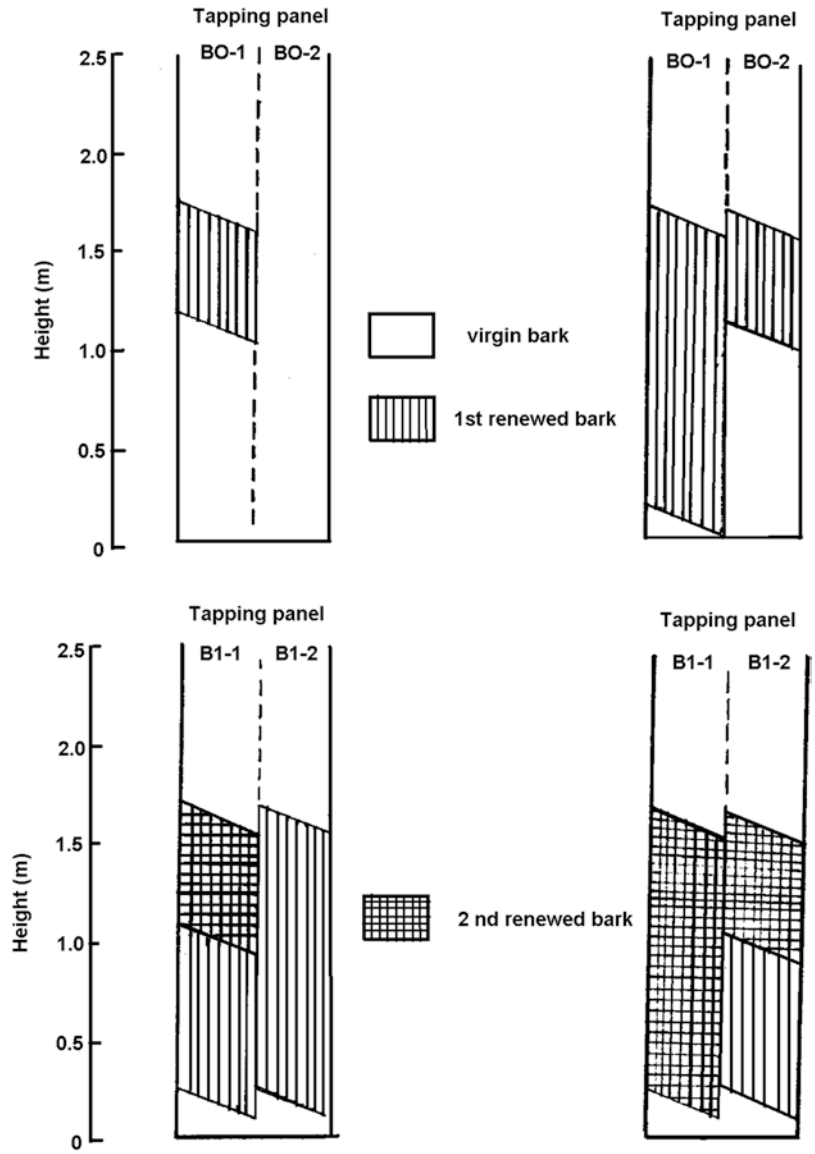

Fig. 5.6 Representation of tapping panels

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).

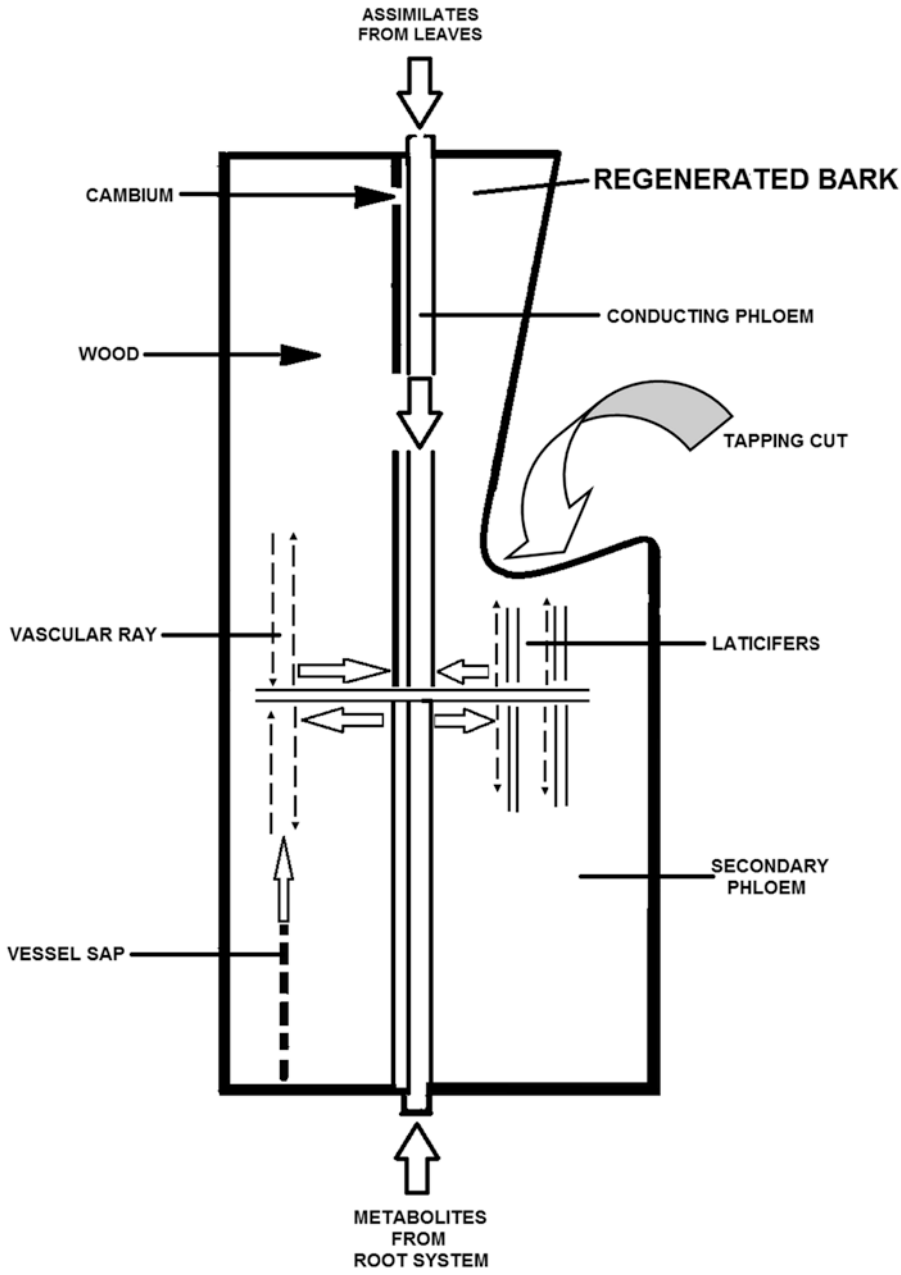

Fig. 5.7 Diagrammatic representation of tapping panel and its functions

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

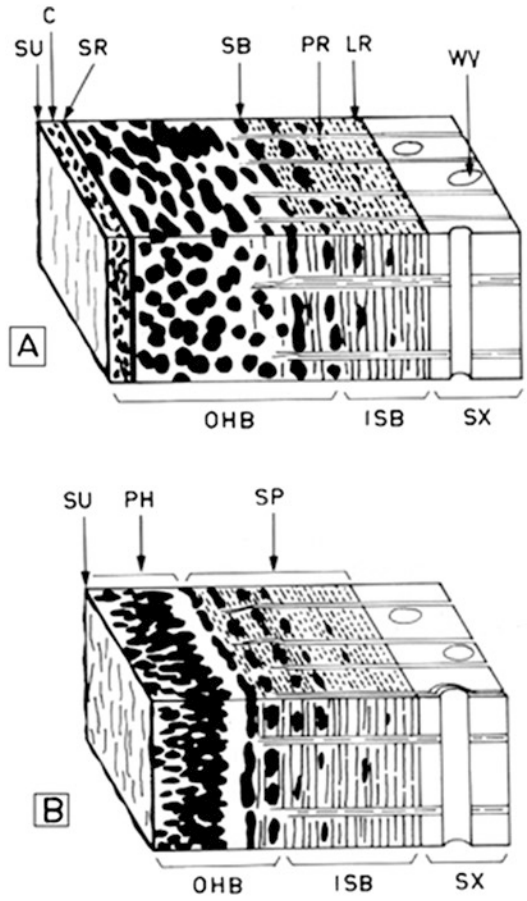

Fig. 5.8 (**a**) Virgin bark (**b**) regenerated bark. *C* cork, *ISB* inner soft bark, *LR* laticifer ring, *OHB* outer hard bark, *PH* phelloderm, *PR* vascular ray, *SB* clustered stone cells, *SP* secondary phloem, *SR* sclerenchyma ring, *SX* secondary xylem, *WV* wood vessel

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

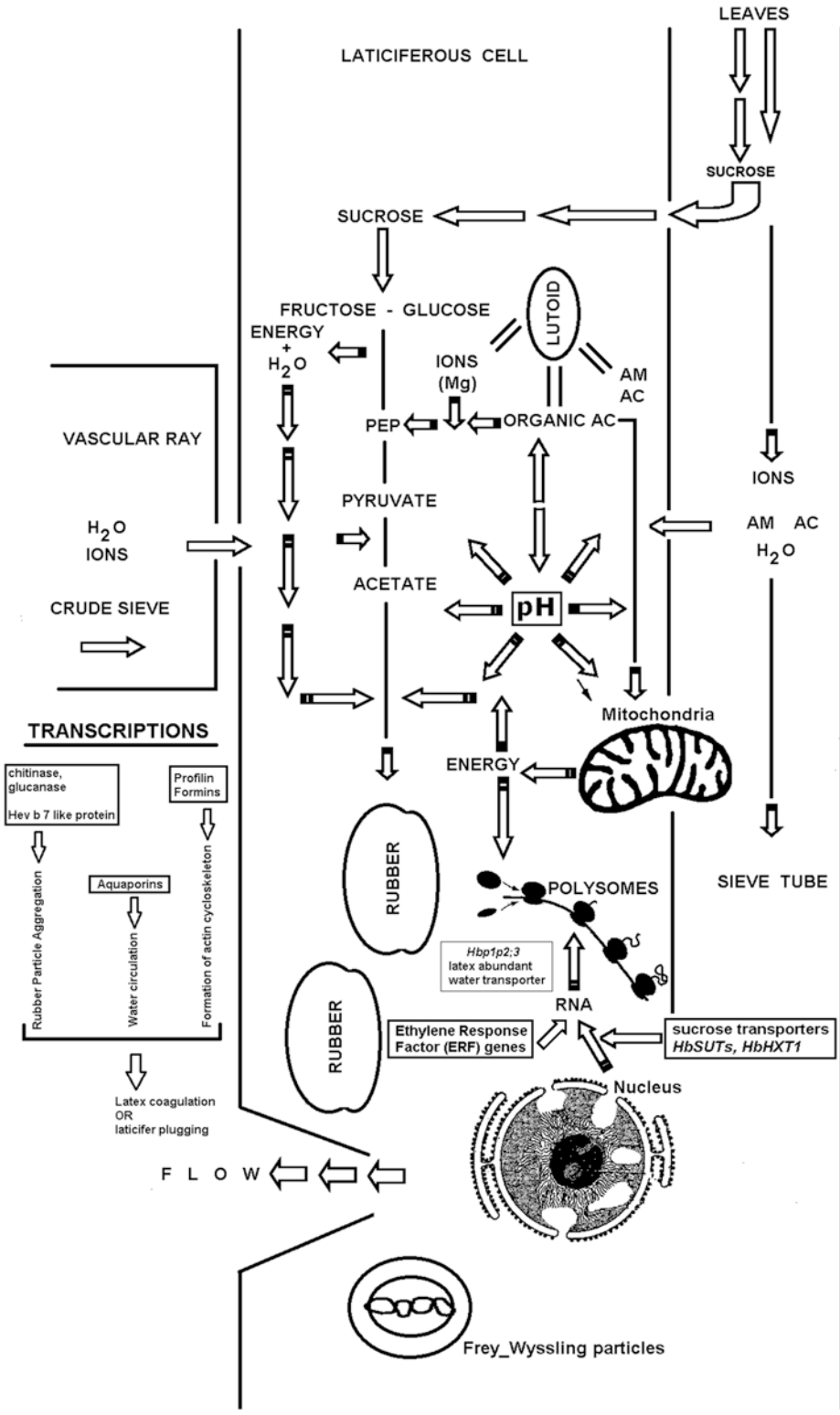

Fig. 5.11 Diagrammatic representation of the probable events after ethylene stimulation

(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.

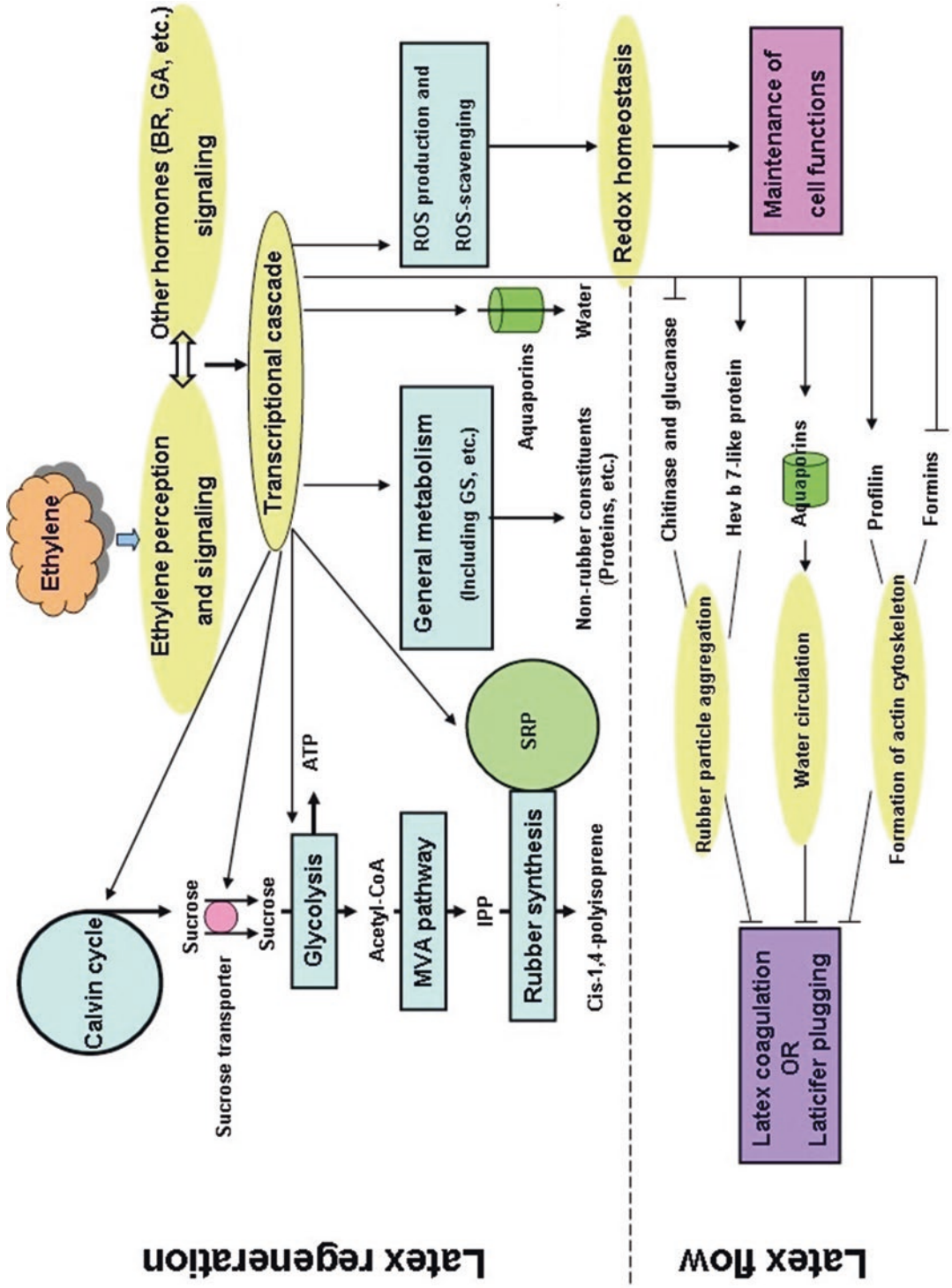

Fig. 5.12 General scheme of ethylene-induced biochemical pathways in latex cells. Factors in shaded boxes are activated and inhibited by ethephon or ethylene, respectively. Factors are: *Chi* (chitinase), *Glu* (glucanase), *GS* (glutamine synthetase), *HEV* (hevein), *HMG* (3-hydroxy-3-methylglutaryl-coenzyme 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.