BIOCHEMICAL ASPECTS RELATED TO RUBBER BIOSYNTHESIS AND YIELD IN *HEVEA BRASILIENSIS*

Thesis submitted to
THE UNIVERSITY OF KERALA
for the Degree of
DOCTOR OF PHILOSOPHY
in Biochemistry

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JULY 1992

C E R T I F I C A T E

This is to certify that the thesis entitled **'Biochemical aspects related to rubber biosynthesis and yield in Hevea brasiliensis'** is an authentic record of the research carried out by Smt. Usha Nair, N. under our joint supervision and guidance and that no part of this work has been presented for any other degree.

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DECLARATION

I hereby declare that the thesis entitled **'Biochemical aspects related to rubber biosynthesis and yield in Hevea brasiliensis'** submitted by me for the Degree of Doctor of Philosophy in Biochemistry of the University of Kerala, embodies the results of original research work carried out by me at the Rubber Research Institute of India, under the joint supervision of Dr. P.A. Kurup, Professor and Head (Retd.), Department of Biochemistry, University of Kerala, Trivandrum and Dr. M.R. Sethuraj, Director, Rubber Research Institute of India, Kottayam. I further declare that this thesis has not previously formed the basis for award of any degree.

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A C K N O W L E D G E M E N T

The author places on record her deep indebtedness and grateful thanks to Dr. P.A. Kurup, Professor and Head of the Department of Biochemistry (now Retired), University of Kerala, Trivandrum and to Dr. M.R. Sethuraj, Director, Rubber Research Institute of India, Kottayam, under the joint guidance of whom, the research work for this thesis was carried out.

The author also wishes to express her grateful thanks to Dr. A.O.N. Panikkar, Joint Director (Research & Training), Rubber Research Institute of India, Kottayam for his helpful suggestions in the preparation of the manuscript, and to Dr. K.R. Vijayakumar, Deputy Director (Plant Physiology), Rubber Research Institute of India, Kottayam for providing the necessary facilities to carry out this work.

Her special thanks are due to Dr. Molly Thomas, Kum. S. Sreelatha and Smt. Sheela P Simon, who are the author's colleagues in the Biochemistry Section of the Plant Physiology Division. Thanks are also due to the other members of staff of the Plant Physiology Division and to the Field Staff for the help they have rendered.

She is thankful to Shri. R. Babu for typing the manuscript.

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C O N T E N T S

PREFACE

Productivity of a rubber (Hevea brasiliensis) clone depends on its capability to synthesise latex. In a tree which is under regular tapping, the rate of regeneration of latex is important. In fact the most important factor which determines the physiological limit of rubber yield, is the rate of biosynthesis leading to regeneration of latex before the next tapping. There are reports that HMG-CoA reductase is a key enzyme involved in rubber biosynthesis. However no serious attempt seems to have been made to correlate the activity of this enzyme with rubber yield, probably because in vitro systems to assess the biosynthetic capacity using latex have experimental limitations.

Flow of latex on tapping is another important factor governing rubber yield. It has been suggested that the stability of lutoids is a major factor controlling flow. The role of lutoid B-serum as well as the proteins present in it, including many lutoid enzymes, has been the subject of some investigations. Studies on the relationship between the biochemical composition of the latex or its fractions and flow of latex have also been reported. But most of these investigations have been confined to individual clones in mature trees. The possible correlation between these factors and yield of rubber, with a view for early identification of high yielding clones, does not seem to have been studied.

Early prediction parameters for productivity should be based on comparative studies on a number of clones belonging to different productivity groups. Literature survey does not indicate any well studied communication in this direction. Nor is any report available on biochemical parameters in high yielding and low yielding categories of clones. It is in this context that the following aspects have been studied, in many high and low yielding clones, in an attempt to identify reliable biochemical factors which can be used as parameters for early prediction of high yielding clones:

1. Levels of total lipids, triglycerides, phospholipids, glycolipids and sterols in latex, latex fractions and leaves,
2. Levels of reducing and non-reducing sugars and other carbohydrates in latex and leaves,
3. Concentration of thiols in latex,
4. Concentration of magnesium in latex,
5. Concentration of inorganic phosphorus in latex,
6. Concentration of total proteins in latex and latex components, including lutoid membrane, and electrophoretic pattern of proteins,
7. Activity of HMG-CoA reductase in bark and other parts of the plant and the effect of latex components on the enzyme activity, and
8. Variations in some of these parameters with age of trees.

CHAPTER 1

INTRODUCTION

Although the existence of rubber in plant species has been known to man since several centuries, rubber cultivation did not become an important farming operation until the beginning of the 20th century. Extensive areas are now under cultivation of the Para rubber tree - Hevea brasiliensis - in the different rubber growing countries to recover natural rubber (NR) an indispensable raw material, both in war and in peace.

NR has been reported to occur in over 2000 species (George et al., 1980). The content of NR in these species, however, is not adequate to raise them as an agricultural crop. Even among the eleven species of the genus Hevea, it is only Hevea brasiliensis which contain substantial quantum of rubber. During the war period certain species, like Castilloa elastica, were tried as possible source of NR, but no attempts were made to raise them on plantation scale. Guayule (Parthenium argentatum) is currently receiving attention as a potential commercial source of rubber.

World requirement of NR is almost exclusively met by H. brasiliensis grown over an area of 90,00,000 ha of which India accounts for 4,51,000 ha (Rubber Board, 1992). The tree contains an extensive network of articulated anastomosing laticifers of which those in the bark of the trunk are exploited for latex through a process of controlled wounding termed tapping. Rubber represents 29 to 42 per cent of the weight of the latex exuded by the mature trees in regular tapping. It is present as spherical or pear shaped particles of 0.1 to 0.5 μm diameter surrounded by a phospholipid and protein containing membrane. It is a 1:4 polymer of isoprene and the cis configuration about the double bonds was established by X-ray crystallography. The molecular weight of rubber from fresh latex has been reported to vary from 0.7×10^5 to 40×10^5 in a bimodal distribution. Tanaka (1989) has recently shown that natural rubber has a structure which consists of a terminal group (ω-terminal which is dimethyl allyl, or a derivative of this, attached to two or three isoprenyl units in the ε-configuration, in turn attached to a linear chain of ζ-isoprenyl units terminated (α-terminal) by a ζ-hydroxy-2-methyl-but -2-enol group or a derivative. The structure of natural rubber as suggested by Tanaka is given in Figure 1. It is not proposed to discuss the chemical structure of natural rubber in more detail, since many excellent reviews are available in this respect (Hemming, 1983; Archer and Audley, 1987; Tanaka, 1989).

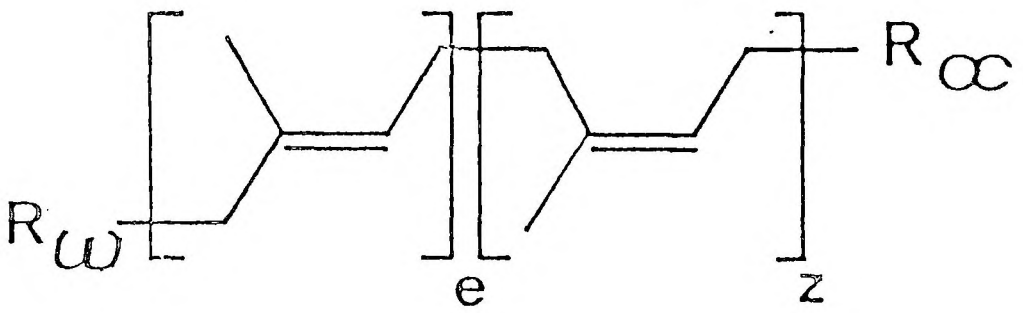

Fig. 1. Structure of Natural Rubber.

Biosynthesis of polyprenols and natural rubber

The polyprenols are the products of the isoprenoid biosynthetic pathway which starts with the production of isopentenyl diphosphate (IDP) from mevalonic acid (MVA) and its isomerisation to dimethyl allyl diphosphate (DMADP). The precursor involved in the biosynthesis of isoprenoids has been shown to be acetyl-CoA. Acetyl-CoA can be generated from pyruvate via its decarboxylation and dehydrogenation, catalysed by pyruvate dehydrogenase complex localised in the mitochondria and the transport of the acetyl-CoA via citrate to the cytoplasm. Alternatively acetyl-CoA can also be produced from acetate by acetyl-CoA synthase. The general out line of the synthesis of isopentenyl diphosphate in the plants is given in Figure 2.

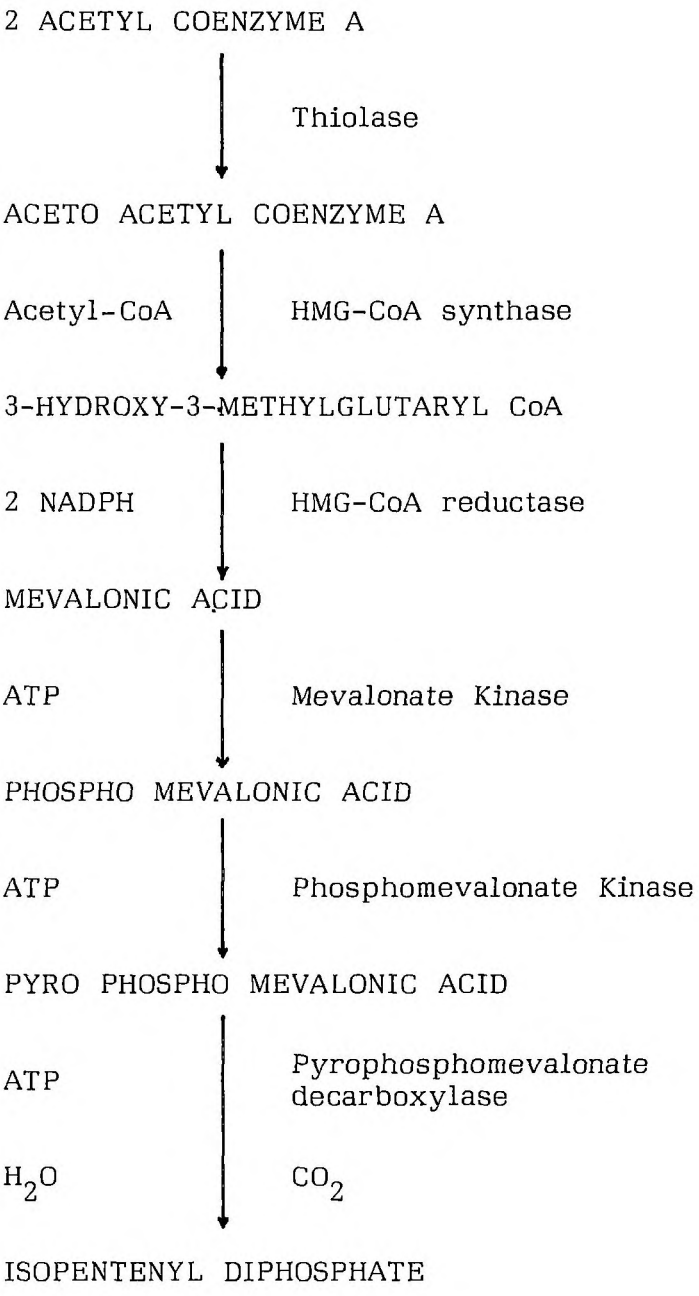

Fig. 2. General outline of IDP biosynthesis in higher plants.

Many of the enzymes involved in this pathway have been identified in many rubber producing plants including Hevea. While thiolase enzyme has been little studied in higher plants, the activity of HMG-CoA synthase has been demonstrated in the latex of Hevea. The enzyme HMG=CoA reductase has been reported in Hevea (Lynen, 1967; Hepper and Audley, 1969; Sipat, 1982). Presence of enzymes involved in the conversion of mevalonic acid to IDP have been reported in Hevea latex and in other plants (Williamson and Kekwick, 1965; Skilleter et al., 1966; Skilleter and Kekwick, 1971). IDP is presumed to be synthesised in the cytosol. Biogenesis of rubber is initiated by the isomerisation of IDP to dimethyl allyl diphosphate (DMADP) catalyzed by IDP isomerase. Activity of this enzyme has been well demonstrated in Hevea latex (Lynen, 1969). The long held view that natural rubber was formed by successive ζ-additions of IDP directly to DMADP assumed that only two enzymes, namely IDP isomerase and rubber transferase, are responsible for the biosynthesis of natural rubber from IDP. It is clear that the formation of natural rubber from IDP requires the isomerisation of IDP to DMADP, then a series of prenyl transfers, the first two or three adding IDP in the ε-configuration to form ε-ε-farnesyl diphosphate (FDP) or $\varepsilon,\varepsilon,\varepsilon$-geranyl geranyl diphosphate (GGDP) and subsequent transfers adding IDP in the ζ-configuration. Apart from the IDP isomerase, atleast one additional enzyme is required to form FDP

or GGDP which are the substrates for the rubber transferase. The latter enzyme mediates sequential ζ-addition of IDP first to the primer molecule and then to the growing chain. In Hevea latex, IDP isomerase appears to be distributed between the serum and the surface of the rubber particles (Barnard, 1965; Archer and Audley, 1987). FDP synthetase mediates the production of FDP from DMADP or geranyl diphosphate (GDP) (Poulter and Rilling, 1981). FDP synthetase activity has been demonstrated in the serum of Hevea latex (Archer et al., 1966; Archer and Audley, 1987) and the soluble fraction of stem of Guayule (Benedict et al., 1983) and leaves (Madhavan and Benedict, 1984). GGDP synthetase, distinct from FDP synthetase which can utilise GDP and DMADP as well as FDP has also been demonstrated in the serum from Hevea latex (Archer and Audley, 1987).

Archer et al. (1966) studied stereo specificities in the formation of ε-ε-FDP and in the extension of the rubber molecule by the ζ-addition of IDP. The formation of FDP in Hevea latex serum exhibited absolute stereo specificity with respect to proton elimination from C-2 of IDP in accordance with the formation of all isoprenoids. The formation of ζ-isoprenyl units in natural rubber was shown to follow alternative stereo specificity. However in a recent study, it has been shown that in the formation of the ζ-configuration sequence in the leaves of 12 taxonomically unrelated plant species, the proton elimination contrary to the

biogenetic rule occurs exclusively (Suga et al., 1986). If this finding is true it would appear that the prenyl transferases involved in the formation of ζ-configuration sequence in this polyprenols are distinct from rubber transferase in Hevea latex. Archer and Cockbain (1969) found the rubber transferase from Hevea latex to be strictly stereo specific yielding exclusively cis-poly isoprene. Lin and Ho (1986) also reported results of purification of rubber transferase from Hevea latex while Madhavan and Benedict (1988) and also Reddy and Das (1988) studied rubber transferase from the leaves of Guayule. The primer required was thought to consist of rubber molecules with an active α-terminal, to function as a prenyl acceptor. The rubber transferase isolated by Archer and Cockbain (1969) from Hevea latex and by Madhavan and Benedict (1984) from Guayule leaf are soluble enzymes of the cytoplasm. Archer et al. (1966) showed complete absence of ζ-prenyl addition activity and exclusively ε-prenyl addition activity in the serum obtained from high speed centrifugation of Hevea latex. It is now known that FDP is a substrate for rubber transferase (Cornish and Backhaus, 1988; Archer and Audley, 1987), and Archer et al. (1966) demonstrated the presence of FDP in their incubate, there is no obvious reason for ζ-prenyl transfer not to have occured. The rubber transferase studied by these workers is in all likelyhood FDP synthetase with GGDP synthetase. In a later study, Madhavan et al. (1989) showed the presence of rubber transferase

activity in washed rubber particles from Guayule, which also had GDP transferase activity. Backhaus and Bess (1986) isolated a polypeptide by electrophoretic stripping of a suspension of Guayule rubber particles. This was a glycoprotein of approximate molecular weight 50,000, integrally bound to the rubber particle membrane. Attempts to solubilise the enzyme to demonstrate rubber transferase activity were unsuccessful. They suggested that this enzyme may be rubber transferase and proposed a model for the action of the enzyme in condensing IDP from the cytoplasm with a growing rubber chain in the interior of the rubber particle. So far there are no reports of the identification of the rubber transferase gene. The factor(s) which govern(s) termination of the rubber chain is also unknown at present.

Site of rubber biosynthesis

It has been suggested that a single ζ-polyisoprene molecule of molecular mass 10^6 in its stable conformation would form a sphere approximately of 0.0075 μm diameter (McIntyre, 1978). The average diameter of the rubber particles in Guayule and Hevea latex (Stavely et al., 1961) is approximately 1 μm. Such particles should contain about 10^6 individual rubber molecules of average molecular mass 10^6. Despite evidence that agglomeration of small rubber particles to form large particles does occur (McIntyre, 1978; Backhaus, 1985) most rubber biosynthesis must occur on the

surface of the existing rubber particles. The outer layer of the rubber particles in vivo consists of phospholipids and protein as a monolayer with the hydrophobic tails of the phospholipid molecules pointing internally (Backhaus and Bess, 1986). The presence in latex of rubber particles of sizes ranging from 0.01 µm to tens of µm (McIntyre, 1978; Backhaus, 1985) indicates that the particles grow from very small units containing a few rubber molecules to those containing millions. According to Archer and Audley (1987) the rubber particles start as an association of GGDP, rubber transferases and possibly other molecules in the form of a miscelle either suspended in the serum or attached to some surface. It is assumed that initially rubber transferase acts at some site in a suitable membrane possibly in the ER on a molecule of FDP or GGDP. The rubber transferase is assumed to form an integral part of the outer layer surrounding the rubber particle and acts as a conduit for the growing chain into this layer. The enzyme will produce by repeated ζ-additions of IDP a tail of increasing length which is inserted in an extended conformation deeply into the surrounding layer. At some critical length the tail adopts a coiled conformation. During this process rubber transferase, nascent rubber molecules and a portion of the outer layer detach to form a separate entity with the rubber chain coiled internally surrounded by a hydrophobic surface formed from phospholipid chains containing the rubber transferase through

which protrudes the actively growing end of the rubber molecule (Paterson Jones et al., 1990).

Role of photosynthesis in rubber biosynthesis

Rubber biosynthesis requires adequate supply of substrates and in addition the rubber transferase which adds IDP, sequentially to the α-terminal of the growing molecule. IDP is derived from acetate via the irreversible decarboxylation of mevalonate (Lynen, 1959). Acetate is derived from the products of photosynthesis. In Guayule photosynthesis continues through out winter. Rubber biosynthesis in this plant peaks in winter when growth is inhibited and decreases to a low level as growth starts and continues (Gilliland and Van Staden, 1986). Photorespiration consumes considerable quantities of carbon fixed by photosynthesis in Guayule in summer but is much less in winter (Kelly et al., 1985). The two major sinks for photosynthesis namely growth and photorespiration are less demanding in winter while photosynthesis continues. The capacity to use photosynthate in synthesising storage carbohydrate is finite and it appears that the production of rubber in Guayule may be a devise to use excess photosynthate when other demands have been effectively satisfied. Rubber therefore may be in all probability a metabolic overspill.

It has been suggested that the production of rubber transferase is stimulated by low night temperature. But there is no

evidence that cold treatment directly stimulates the expression of the gene coding for rubber transferase. Paterson-Jones et al. (1990) suggest that it is the excess IDP itself which causes expression of genes for rubber transferase.

Factors which regulate the flow of latex

As mentioned earlier, rubber yield in Hevea depends not only on the rate of biosynthesis, but also on the flow of latex on tapping. The flow of latex triggered by tapping stops after some time and as early as 1932 Frey-Wyssling attributed this cessation of flow to the coagulation of the latex on the cut. Southorn (1968) used electron microscopy to demonstrate the formation of thick caps of rubber coagulum at the open extremity of latex tubes. Even though many postulates have been put forward to explain the mechanism of plugging including the involvement of bacteria, it soon became evident that the lutoid fraction (Paton, 1953) in the latex plays an important role in the coagulation of the latex. The pioneering work of Southorn and his team (Southorn, 1969) showed that the lutoids can destabilise the negatively charged colloidal suspension of rubber particles. The acidic pH, divalent cations such as magnesium and calcium and the positively charged protein molecules in the lutoid may neutralise the negative charge of the rubber particles. In addition some of the acid hydrolases trapped in the lutoids can attack the protective coating of rubber

particles. The coagulating role of the intra lutoid serum (B-serum) has been demonstrated in a dilute suspension of rubber particles. It was also shown that the main destabilising action of B-serum was not due to its acidic pH. The role of divalent cations - calcium and magnesium - in the lutoids was also discarded since the addition of B-serum and chelating agents like EDTA had no observable effect on the destablisation role of B-serum. The elimination of ions completely from the B-serum by dialysis was further shown to make no difference to its destabilising activity. A positive role for the proteins present in the B-serum most of which are positively charged was suggested when it was found that precipitation of proteins in the B-serum by boiling led to removal of most of the destabilising activity. Further support for the role of the proteins came from experiments using cationic proteins such as cytochrome c which caused immediate flocculation when added to a suspension of rubber particles (Southorn and Yip, 1968). The observation that addition to latex of a cationic surfactant like cetyl pyridinium chloride (CPC) had a powerful destabilising activity on the latex provided additional support and they further suggested that C-serum which contains negatively charged proteins at biological pH opposed the destabilising action of B-serum in vitro when varying proportions of these two serums were put into contact with a dilute suspension of rubber particles. It was also observed that the C-serum which was distinctly protective

in its physiological pH range of 6.5 to 7.3 acquires a destablising effect when the pH was adjusted to 5.5 (Southorn and Yip, 1968).

However, it was shown that the total leakage of B-serum into fresh whole latex was insufficient to convert the latex from a fluid state to coagulum (Southorn and Edwin, 1968). However, latex to which B-serum was added always contained some micro-flocs observable by electron microscopy which consists of an aggregation of rubber particles and damaged lutoids. It was also observed that fresh latex always contained such microflocs (Southorn, 1961; Southorn and Yip, 1968) which may result from the bursting of some lutoids and release of B-serum during and immediately after tapping. The breakage of lutoids within latex vessels inducing the formation of microflocs may play an important role in the stoppage of latex flow (Southorn and Yip, 1968). Usha Nair *et al.* (1978) developed a method to measure the colloidal stability of rubber particles and the flocculating potential of the B-serum *in vitro*. They also studied some of the factors which influence the flocculating potential of B-serum and the colloidal stability of rubber particles and how these factors influence the process of plugging of latex vessel. It was further shown that there is significant clonal variation in B-serum and C-serum activity and bursting index (Usha Nair *et al.*, 1980) and activity of B-serum in bringing about coagulation was found to be positively correlated with cationic proteins in confirmation with the earlier work.

The role of lutoid enzymes in coagulation has also received some attention. The break down of lutoids during or after tapping may liberate some hydrolytic enzymes able to attack the phospholipoprotein films which protect the stability of the rubber particles. The protease (cathepsin) with a very acid optimum pH discovered by Pujarniscle (1968; 1969) in the lutoids may be involved in this process. An NADH quinone reductase originating from the lutoid membrane which is postulated to play a role in bark dryness induced by over exploitation, may also play a role in affecting the stability of the lutoid membrane. The toxic oxygen produced by this enzyme may attack the double bonds of the ethylenic fatty acids in organelles including lutoids resulting in leakage of the organelle components leading to destabilisation of the colloidal suspension.

The efficiency of NADH quinone reductase may depend on the equilibrium between the oxidising and reducing molecules of the latex. This equilibrium depends on the concentration in the latex of certain reducing molecules such as glutathione or ascorbic acid and to the activity of various enzymes such as catalase, superoxide dismutase, peroxidase and phenol oxidase (Chrestin et al., 1984).

The presence of a lutoid phospholipase with an acid optimum pH activated by small amounts of calcium ions may also be able to cause coagulation of latex. But adding phospholipase C or D

to fresh latex showed only a weak coagulant effect. Woo (1976) postulated evidence of the presence of a coagulase in C-serum which was distinct from phospholipase D. But Hanower et al. (1975) failed to reproduce the results reported by Woo. Hanower et al. (1975) searched for a possible role of oxygen in the coagulation of latex in in vitro experiments. But such experiments are difficult to carry out in vivo. Brzozowska-Hanower et al. (1978) claimed that there was good correlation between specific orthodiphenol oxidase activities from five clones and the rate of coagulation of their latex. It has been suggested that the break down of latex organelles which occur at the open extremities of latex tubes allows the release of a particular enzyme (ortho diphenol oxidase) which comes into contact with phenolic substrates, activators like Ca, H_2 and also with atmospheric oxygen. This may lead to the production of polyphenolic or phenol compounds having destabilising effect on the proteins forming the protective surface coating of rubber particles.

These observations thus show that natural coagulation begins by the appearance of microflocs of degraded lutoids and rubber particles. It is clear that lutoids are one of the main elements involved in the stopping of latex flow. The duration of latex flow which is a major factor in rubber production depends on the extent of wholeness (stability) of the lutoids when they flow out of the laticiferous tubes. The stability of the lutoids is measured in terms of the bursting index and the plugging index.

The bursting index (Ribaillier, 1972) measures the ratio of the lutoidic free phosphatase activity to total acid phosphatase activity determined by the bursting of lutoids by a detergent and gives a measure of the degraded lutoids. The plugging index proposed by Milford et al. (1969) is measured by

$$\frac{\text{Initial flow rate}}{\text{Total volume}} \times 100$$

The higher the bursting index or the plugging index shorter the duration of flow and lower the yield.

Attempts are being made at the Rubber Research Institute of India (RRII) and elsewhere to evolve high yielding strains of Hevea brasiliensis by various methods, of which the most important one is hybridization which consists of genetic recombination using artificial pollination of the clones with desirable characteristics and selection of the most promising of their descendants.

Hevea is a perennial tree and has a long vegetative cycle. Exploitation is started when the trees are six to seven years of age and latex is obtained by a traumatizing operation (tapping) which may some times have a harmful effect on the physiological state of the trees. In addition the economic life of the tree lasts for many years. All these features mean that it takes a long time to produce new clones, since information must be collected during

their adult production phase to obtain good knowledge of their behaviour. Therefore perfecting criteria of early selection of new Hevea clones appears indispensable. Selection criteria must take into account the real production potential of the clones and should attempt to forecast their future performance throughout the economic life of the tree. The early attempts to define selection criteria have been reviewed by Bricard and Nicolas (1989).

Methods using early tapping were devised in the 1930's which included the Morris-Mann tapping test on trees about three years old and the 'Testatex' test carried out on one year old trees. Although some positive results were obtained in these studies, they were not able to identify individual seedlings which might give high yielding clones.

Attempts are therefore being made to study the possible differences in some of the biochemical characteristics of the latex, leaf and the bark of some high yielding clones as compared to the low yielding ones. It is possible that some of the biochemical differences may be discernible even at a young age of the plant. Not much detailed investigation seems to have been carried out in this direction. The available reports in this respect are briefly reviewed below.

D'Auzac (1965) attempted to study the relationship between biochemical composition of latex, intensity of some metabolic reactions and the productivity of H. brasiliensis. Latex from two

clones, one high yielding and the other low yielding, was investigated for differences in d.r.c. percentage, biochemical characters including phosphorus, aminoacids, soluble carbohydrates, enzyme activities, O_2 uptake, CO_2 release and nitrogen content in all the fractions. Reactions such as glycolysis, carboxylation and decarboxylation of pyruvate, synthesis of amino acids and the reducing capacity of latex were found to be directly linked with the biogenesis of latex and hence with productivity.

Poliniere and D'Azuac (1966) studied seven clones with respect to physiological, anatomical and certain biochemical characteristics which are useful in the selection of high productivity clones. Wycherley (1969) mentioned the possible utilisation of physiological characters like plugging index (PI) and the activity of certain enzymes. Ho (1976) observed that plugging index in young trees might improve the forecasting of adult yields. Saraswathy Amma and Sethuraj (1975) studied the clonal variations in latex flow characteristics and yield in nine rubber clones and explained variations in yield between the clones in terms of plugging index and initial flow rate.

Milford et al. (1969) studied the importance of latex vessel plugging to yield and clonal behaviour. Subronto et al. (1982) studied the indices of latex flow as parameters of selection in Hevea brasiliensis. Henon et al. (1984) undertook a basic study to define early selection criteria in Hevea which included growth,

yield and physiological and anatomical characters. Yeang Hoong Yeet (1977) studied the protein and enzyme variation in some Hevea cultivars. Serum proteins were separated electrophoretically using poly acrylamide gels. Some clonal differences were observed in the electrophorograms of B-serum and of serum prepared by acetic acid coagulation of latex separated electrophoretically at acid pH.

Coupe (1978) observed that protein biosynthesis of latex may be a factor in Hevea rubber production. He found that the polymerisation of latex ribosomes is in relation with rubber production by Hevea in all circumstances.

Many authors have shown that the internal production factors of trees displayed considerable clonal variation. Eschbach et al. (1983, 1984) showed that in four clones the physiological characteristics of latex could be used to draw a classification of stable clones despite differences of environment and age and also demonstrated that these physiological parameters can be used to form the basis for the diagnosis of the laticiferous system (Jacob et al., 1986; Prevot et al., 1986).

The perfecting of a latex diagnosis has made obvious the advantages of the study of physiological parameters as selection criteria. In addition a number of preliminary studies have shown that they could be used on young trees and should enable early

selection to be carried out (Ditinger et al., 1981; Henon et al., 1981; Henon 1984; Nicolas, 1978; Odier, 1983).

Study of the physiological features of latex led numerous authors to characterise certain biochemical parameters connected with latex production in Hevea. These parameters are:

1. **Total solids content (TSC):-** Viscosity of latex depends on the total solids present. A high TSC may limit yield by hindering flow (Brozozowska et al., 1979; Milford et al., 1969; Buttery and Boatman, 1976). On the other hand a low TSC indicates weak latex regeneration in situ (Eschbach et al., 1984; Prevot et al., 1984).

2. **Magnesium (Mg) content:-** Magnesium, though present in the cytosol, is concentrated in lutoids (Ribaillier et al., 1971). The effect of this cation is two-fold. Firstly it is needed in the activity of several cytosolic enzymes (D'Auzac, 1975; Jacob, 1970; Jacob et al., 1981; Jacob et al., 1983). Secondly the positive charges of Magnesium released from the lutoids have a destabilising effect on the negative charge of the colloidal suspension formed by latex making it coagulate, thus limiting flow (Ribaillier, 1972; Southorn and Yip, 1968).

3. **Bursting index of lutoids (BI):-** A low BI indicates high stability of latex and hence easy flow and higher yield. Lutoids are rich in positive ions and molecules which when released destabilise latex and cause it to coagulate.

4. Phosphorus:- Phosphorus is an antagonist of Magnesium. Because of its negative charge it also contributes to the colloidal stability of latex as a constituent of membrane phospholipids. In addition it is required for an active metabolism because of its role in phosphorylated compounds and energy processes.

5. Acid phosphatases:- These enzymes are found in the lutoids and are used in the calculation of bursting index of lutoids [ratio of free acid phosphatases released from damaged lutoids to total acid phosphatases (TAP or TP)]

6. Thiols:- The thiols act as protectors of lutoid membrane. They also contribute to the redox balance and activate the key enzymes of the laticiferous system.

7. Sucrose:- Sucrose is the precursor of isoprene synthesis. It is difficult to interpret the role of sucrose content. A high figure indicates good supply to the laticifers or poor utilization leading to weak isoprenic synthesis (Tupy and Primot, 1976; Prevot et al., 1984).

It is possible from these data to define factors favourable for high production.

1. An active metabolism generally associated with high P, RSH, TAP and pH levels.

2. A good sugar supply capacity implying a high sucrose content.

3. Stable latex characterized by low magnesium content and bursting index, high phosphorus and thiol content.

5. Medium total solid content value necessary for good flow.

Bricard and Nicolas (1989) carried out studies to determine the reliability of the above physiological and biochemical parameters and observed that all the above criteria exhibited high reliability. In descending order, total acid phosphatase (TAP) sucrose and magnesium were the most reliable criteria. These were followed by phosphorus, thiol groups, and Total solid content and Bursting index of lutoids was next and finally Plugging index with lowest reliability. Thus pH and lutoid BI, whose importance has been shown as clonal characters and production factors do not appear as being very reliable in their studies.

A triangular relationship was observed in their study between pH, magnesium and the BI of lutoids. The higher the pH of the clones, the lower the magnesium content and BI of lutoids. Phosphorus and thiol groups were positively linked in all cases. A positive correlation between pH and TSC and a negative correlation between thiol group and TSC were also observed. TAP appeared to be independent of other parameters except thiols (positive correlation). Sucrose was also clearly independent of other criteria. These studies were carried out in three phases. The first phase was the immature phase, the second after five years and the

third after eight years. The eight physiological parameters in the first year of production were positively correlated with their juvenile equivalents in all the trials.

Bricard and Nicolas (1989) found that under their experimental conditions only thiol groups and phosphorus was positively linked with production and lutoid bursting index was linked negatively. This was found to be the case both between trees of the same clone and between clones (Odier, 1983). The lack of correlation between sucrose content and production according to them indicated that in carbohydrate catabolism the limiting factor was not the sucrose content but the invertase activity. Clones with high yield relatively possessed low sucrose content because of their high rate of catabolism associated with high invertase activity.

Wititsuwannakul and Sukonrat (1986) studied the temporal variation of HMG-CoA reductase activity in the latex of two clonal types of H. brasiliensis and observed peak activity around sun set. Rubber content in the latex also showed a similar pattern. The results suggested a possible correlation between rubber biosynthesis and the regulation of HMG-CoA reductase activity. The activity of this enzyme may be a limiting factor in rubber biosynthesis (Lynen, 1969). Eventhough by altering exploitation techniques such as interval between tappings, the yield of rubber can be

improved, the limiting factor is the rate of biosynthesis of rubber during regeneration in the interval between successive tappings. The activity of HMG-CoA reductase is a major factor in this context, but very little work seems to have been carried out in this respect so far.

Mudji Lasminingsih (1989) studied the band pattern of proteins of 36 rubber clones using poly acrylamide gel electrophoresis. The results of the experiments showed that the band pattern could be grouped into six patterns. He suggested that the pattern could be used as a genetic maker in the identification of the rubber clones. The pattern was not influenced by the age of the rubber tree and environmental conditions.

Jacob et al. (1986) observed that reduced thiols can play an important role in the mechanism governing lutoid stability and hence latex flow in H. brasiliensis. In addition they had an effect on the metabolism and hence the regeneration of latex between two tappings.

As stability of the lutoids is an important factor in determining latex flow and thereby yield, the lipid composition of the lutoid membrane assumes importance. Very little detailed investigations seems to be carried out on the role of lipids in the stability of the lutoid membrane. The few investigations carried out in this respect appear to be mostly from this Institute.

Sherief and Sethuraj (1978) determined the phospholipids of bottom fraction and neutral lipids of rubber particles in several clonal mother trees of Hevea. They observed that lutoid instability, as indicated by bursting index, was negatively correlated with the phospholipids of the bottom fraction of latex. The neutral lipid content of the rubber particles was found to be positively correlated with the colloidal stability of the latex. Premakumari et al. (1980) studied the seasonal variations in lutoid stability, neutral lipid content of rubber particles and phospholipid content of lutoid fractions in relation to variations in yield in drought susceptible and resistant clones. Drought susceptible clones were found to have lower neutral lipid content of rubber and phospholipid content of lutoids during drought periods. Changes in the plugging index as influenced by drought appeared to be related to variations in neutral lipid content of rubber particles. They observed that lutoid stability was related to variations in phospholipid content of the bottom fraction. Molly Thomas and coworkers (1990) observed that leaf and latex lipid component can possibly be included as early prediction parameters for yield characteristics in Hevea brasiliensis. The content of total lipids, triglycerides, sterols and phospholipids was significantly high in high and medium yielding clones. These authors further observed that many of the physiological and genetic variations in Hevea clones can be related to biochemical differences.

There are some reports on the composition of the lipids in Hevea latex and its sub-fractions. Hasma and Subramaniam (1986) studied the composition of lipids in latex of one clone and observed that total lipids constituted about 1.6 per cent of the latex out of which 54 per cent was due to neutral lipids, 33 per cent glycolipids and 14 per cent phospholipids. The neutral lipids comprised of cartoenoid pigments, free and esterified sterols, free and esterified tocotrienols, free fatty alcohols and their acetates tri, di and mono glycerides and free fatty acids. Tri glycerides alone constituted about 63 per cent of the neutral lipids making them the major component of Hevea brasiliensis lipids. The glycolipids consisted of free and esterified steryl glucosides, di and mono galactosyl di glycerides while the phospholipids contained mainly phosphatidyl ethanolamine, phosphatidyl choline and phosphatidyl inositol.

Bao et al. (1988) studied the composition and distribution of phospholipids in Hevea latex lipids. They detected eight phospholipids in the latex of two clones using one dimensional thin layer chromatography.

Hasma (1987) studied the proteolipids of Hevea latex and found that 40 per cent of the membrane proteins of rubber particles were proteolipids which were closely associated with phospholipids and glycolipids. Marcel et al. (1981) studied the fatty acid composition of the latex of Hevea and characterised a noval

dioxo C-18 fatty acid. Hasma and Subramaniam (1978) also reported the occurrence of a furanoid fatty acid in Hevea brasiliensis latex. Ho et al. (1975) studied the lipids associated with the particles in Hevea latex, while Du Pont et al. (1975) determined the phospholipid composition of the membrane of the lutoids in Hevea brasiliensis latex.

As mentioned earlier, all the investigations on the lipids of the latex or the lutoid membrane were confined to individual clones and no attempt seems to have been made to correlate the lipid components of latex or of the lutoid membrane with yield by studying high yielding and low yielding clones. Not much investigations also seems to have been carried out on the role of the proteins in the mechanism of latex vessel plugging and therefore of yield, except the report by Sherief and Sethuraj (1978) that a high ratio of cationic to anionic proteins in B-serum may enhance the process of plugging. The report of Tata (1980) on the distribution of proteins in the fraction of Hevea latex separated by ultra centrifugation also was confined to individual clones. Tupy et al. (1988) studied the variability of RNA and protein content of rubber cytosol related to initial effects of tapping and hormonal yield stimulation. But these data have not been correlated with the yield of rubber. Kekwick (1988) characterised the proteins of C-serum by two dimensional electrophoresis and also studied the incorporation of S^{35} methionine and (^{3}H) leucine

into proteins and the effect of the treatment of Ethrel on *in vitro* protein synthesis. Other work on the proteins include the band pattern of proteins in some rubber clones by Mudji Lasminingsih (1989), characteristics of amino acid incorporation by isolated polysomes from Hevea brasiliensis by Coupe et al. (1974), the report on the protein and enzyme variation in some Hevea cultivars by Yeang Hoong Yeet et al. (1977) and the various reports on the purification and characterisation of Hevein, a crystalline protein from the B-serum of Hevea latex by Tata (1976). But very few of these reports correlate the protein pattern of the latex or the lutoid membrane with yield.

As discussed previously, one of the major factors determining yield of rubber is the rate of its biosynthesis. Since HMG-CoA reductase is the rate limiting step in many poly prenoid synthesis, the activity of this enzyme may be indicator of the biosynthetic rate. The reports on the activity of this enzyme in Hevea brasiliensis relate mostly to the detection of this enzyme activity in different fractions of latex (Sipat, 1985; Hepper and Audley, 1969). Isa and Sipat (1982) reported the occurrence of a heat stable activator of the enzyme in Hevea brasiliensis latex, while Wititsuwannakul and Sukonrat (1986) studied the diurnal variation of enzyme activity in latex and its relation to rubber content. Their results suggested possible correlation between rubber biosynthesis and the regulation of HMG-CoA reductase activity. These

workers (Sukonrat and Wititsuwannakul, 1988) discussed the possibility of the application of this enzyme parameter for early selection of high yielding rubber clones. Wititsuwannakul et al. (1990) isolated calmodulin from the C-serum fraction of centrifuged fresh latex and reported the activation of HMG-CoA reductase by it. Their data suggested a role of calmodulin in the regulation of HMG-CoA reductase. Thus all the available reports in this respect relate to the detection of the activity of this enzyme in Hevea latex and its sub-fractions, purification of this enzyme and study of its properties.

As is evident from this review, no definite information seems to be available correlating various biochemical factors with the yield of rubber which can be used for early prediction of high yielding clones. In view of this, the following aspects have been studied in many high and low yielding clones in an attempt to find out reliable biochemical factors which can be used for early prediction of high yielding clones:

1. Variations in the content of total lipids, triglycerides, phospholipids, glycolipids and sterols in the latex and its sub-fractions and in the leaves.

2. Variations in the concentration of reducing and non-reducing sugars and other carbohydrates in the latex under various conditions of tapping.

3. Concentration of thiols in the latex.

4. Concentration of magnesium in the latex.

5. Concentration of inorganic phosphorus in the latex.

6. Concentration of total proteins in the latex and its components and in the lutoid membrane and the study of their electrophoretic pattern.

7. Activity of HMG-CoA reductase in the bark and other parts of the plant and the effect of latex components on the activity of the enzyme using partially purified enzyme.

8. Variations in some of the above biochemical parameters with the age of the trees using different clones.

The results of these studies are discussed in this thesis.

CHAPTER 2

MATERIALS AND METHODS

A number of Hevea clones representing two productivity groups were employed for the studies. The clones utilized for the specific studies are given along with each chapter. The methodology adopted for each investigation are given in detail in this chapter.

1. Materials

Clonal populations of Hevea brasiliensis (Willd. ex Adr. de Juss.) Muell. Arg. raised at the Central Experiment Station, Chethackal and at the Experiment Station at the headquarters of the Rubber Research Institute of India were utilized for the studies. The clones chosen, which represented high yielding and low yielding groups, were:-

1. RRII 105
2. PB 235
3. PB 217
4. PB 215
5. GT 1
6. RRIM 600

7. Ch 4
8. Pil B 84
9. Ch 29
10. Tjir 16
11. HP 20
12. RRII 38
13. HP 13

2. Methods

2.1 Collection of latex and separation of fractions

Latex was collected in ice cooled beakers for 30 min after tapping as described by Moir (1959). It was centrifuged at 23,000 rpm in a Sorvall OTD 55B ultracentrifuge to separate the rubber phase, C-serum and bottom fraction (Moir, 1959; Cook and Sekhar, 1953). The rubber fraction was washed by dispersing in double distilled water (2 ml per millilitre latex). This rubber dispersion was used for the extraction of lipids. For the estimation of proteins the rubber dispersion was centrifuged and the supernatent cream was used.

The C-serum was collected by puncturing the centrifuge tubes.

The bottom fraction was washed in 0.4 M Mannitol two to three times to make free from contaminating rubber particles and given a light centrifugation at 5,000 rpm for 15 min. The bottom fraction thus obtained was used for the extraction of lipids.

B-serum was prepared by the procedure of Hsia (1958). The bottom fraction was subjected to repeated freezing and thawing. The clear B-serum was obtained after centrifugation at 40,000 rpm for 45 min.

2.2 Lipids

2.2.1 Extraction,

Total lipids were extracted from rubber cream and bottom fraction according to the procedure described by Bligh and Dyer (1959). A known quantity of rubber cream and bottom fraction were extracted in 2:1 chloroform methanol after keeping for 24 h at room temperature. It was filtered and the residue washed with chloroform methanol (2:1) atleast three times. To the filtrate in a stoppered tube, 0.2 per cent calcium chloride (20 per cent of the total volume of the extract) was added, mixed vigorously and allowed to stand. The aqueous layer was removed with a pasteur pipette. The washed lower layer of chloroform was evaporated to dryness and the residue dissolved in a known volume of chloroform. From this aliquots were used for lipid analysis.

2.2.2 Estimation of total lipids

An aliquot of the lipid extract was taken into a previously weighed 5 ml beaker and evaporated to dryness under nitrogen. The beaker was kept in a vaccum desicator over KOH under reduced

pressure until constant weight was obtained. The difference between the two weights was taken as the weight of total lipids present in an aliquot of lipid extract.

2.2.3 Fractionation of lipids

A known volume of total lipid extract was separated by silicic acid column chromatography according to Hasma and Subramaniam (1986) and succesively eluted with hexane:diethylether (95:5) v/v, hexane:diethyl ether (75:25), diethyl ether, methanol and finally with methanol:acetic acid (90:10).

The hexane:diethyl ether (75:25) eluate was used for the estimation of triglycerides, the diethyl ether eluate for the estimation of sterols and the combined methanol and methanol:acetic acid (90:10) eluates for the estimation of phospholipids and glycolipids.

2.2.4 Estimation of triglycerides

Triglycerides were estimated by the method of Van Handel and Zilversmit (1957).

Reagents

a) Chloroform (AR)

b) Ethanolic KOH - 0.4%.

c) 0.2 N H_2SO_4

d) 0.05 M sodium metaperiodate.

e) 0.5 M sodium arsenite

f) Chromotropic acid - 2 g of chromotropic acid (or 2.24 g sodium salt) was dissolved in 200 ml distilled water. 600 ml of concentrated H_2SO_4 was added slowly to 300 ml of distilled water which was cooled in ice. This cooled and diluted acid was then added to the chromotropic acid solution (0.25 mg/ml).

The hexane:diethyl ether (75:25) eluate was concentrated to a known volume. Aliquots of 1 ml were pipetted out into three tubes. 1 ml of working standard of glycerol was pipetted out into each of the three tubes. The solvent was evaporated at 60-70°C. 0.5 ml of ethanolic KOH was then added to two out of the three tubes (saponified sample) and 0.5 ml of ethanol was added to the third tube (unsaponified sample). The tubes were closed and kept at 60-70°C for 15 min. 0.5 ml of 0.2 N H_2SO_4 was added to each tube and the tubes were then placed in a gently boiling water bath for 15 min to remove alcohol. They were then cooled to room temperature. 0.1 ml of sodium metaperiodate was added to each tube and kept for 10 min. 0.1 ml of sodium arsenite solution was then added. A yellow colour of iodine appeared and vanished within a few minutes. 5 ml of chromotropic acid was added to each tube and mixed. The tubes were closed and then heated in a boiling water bath for 30 min. They were then cooled

and the absorbance read at 570 nm. The triglycerides were quantified by multiplying the amount of glycerol with its conversion factor 10.4 (Hasma, 1984).

2.2.5 *Estimation of sterols*

Sterols were estimated by the method of Abell (1952).

Reagents

a) Ethanolic KOH. 6 ml of 33 per cent KOH in water was added to 94 ml of absolute ethanol.

b) Petroleum ether (AR) (60-80°C).

c) Colour reagent. 20 ml of acetic anhydride was chilled in ice. 1 ml of concentrated H_2SO_4 was added to this with mixing. It was again chilled for 10 min and 10 ml of glacial acetic acid added and allowed to attain room temperature.

The diethyl ether eluate was made upto a known volume. Aliquots were evaporated to dryness and 5 ml of ethanolic KOH was added and shaken well. It was then warmed in a water bath at 37 to 40°C for 55 min. After cooling to room temperature 10 ml of petroleum ether (60-80°C) was added and mixed. 5 ml of water was added to this and shaken vigorously for one minute. It was centrifuged at a low speed for 5 min. 4 ml of petroleum ether layer was pipetted out into a test tube and evaporated to dryness at 60°C. A standard (β sitosterol) was also treated in the same

manner. 6 ml of colour reagent was added to each tube and kept at 25°C after thorough shaking. 6 ml of colour reagent was taken as the blank. After 30-35 min the optical density was read at 620 nm.

2.2.6 Estimation of Phospholipids

Phospholipids were estimated by the method of Zilversmit and Davis (1950).

Reagents

a) 5 N H_2SO_4

b) 2.5 per cent ammonium molybdate

c) ANSA. 0.2 g of 1-amino-2-naphthol-4-sulfonic acid was mixed with 1.2 g of sodium bisulphite and 1.2 g of sodium sulphite. 0.25 g of this mixture was dissolved in 10 ml of water.

Methanol and methanol:acetic acid eluate was made up to a known volume. An aliquot was pipetted into a kjeldahl flask and evaporated to dryness. 1 ml of 5 N H_2SO_4 was added and digested in a digestion rack till it became light brown. It was then cooled to room temperature. One or two drops of 2 N HNO_3 was added and digested again till it became colourless. The kjeldahl flask was cooled. 1 ml of water added and heated in

a boiling water bath for 10 min. 1 ml of 2.5 per cent ammonium molybdate and 0.1 ml ANSA were added to this. The volume was then made upto 10 ml with distilled water and the absorbance measured at 660 nm within 10 min. The amount of phospholipids was determined by multiplying the amount of phosphorus with the conversion factor 25.9 (Hasma, 1984).

2.2.7 Estimation of glycolipids

Glycolipid was estimated as sugar using the method of Dubois et al. (1956) as modified by Roughan and Batt (1968). A known aliquot of the methanol and methanol acetic acid eluate was evaporated to dryness. 1 ml of water and 1 ml of H_2SO_4 were added and kept in a water bath at 80°C for hydrolysis. The lipid layer was removed using diethyl ether. 1 ml of water and 1 ml of 5 per cent aqueous phenol were added to the aqueous layer and vortexed. This was followed by the rapid addition of 4 ml of concentrated H_2SO_4 (AR) to ensure maximum heating of the mixture. The contents of the tubes were thoroughly mixed and allowed to stand at room temperature for 15 min. The intensity of colour was read at 490 nm using galactose as standard. The amount of glycolipids was determined by multiplying the amount of sugar in the methanol eluate with the conversion factor 4.7 (Hasma, 1984).

2.3 Extraction and estimation of carbohydrates, sugars and free aminoacids in latex

2.3.1 Extraction

10 ml of latex was extracted with 80 per cent ethanol in a boiling water bath for 10 to 15 min. The extract was made up to a definite volume (25 ml).

2.3.2 Estimation of Reducing Sugars

Reducing sugars were estimated by the method of Nelson (1944).

Reagents

a) Copper reagent A

25 g anhydrous Na_2CO_3, 25 g Rochelle salt, 20 g $NaHCO_3$ and 200 g anhydrous Na_2SO_4 were dissolved in ca. 800 ml H_2O and diluted to one litre.

b) Copper reagent B

15 per cent $CuSO_4 \cdot 5\ H_2O$ containing 1-2 drops of concentrated H_2SO_4 per litre.

Copper solution was prepared on the day of use by mixing copper reagent A and B in the proportion 25:1.

c) Arsenomolybdate solution

25 g ammonium molybdate was dissolved in 450 ml of water and 21 ml of concentrated H_2SO_4 added with mixing. 3 g of $Na_2HAsO_4.7H_2O$ dissolved in 25 ml of water was then added. The mixed solution were kept at 37°C for 24-28 h.

To 1 ml of the alcoholic extract, 1 ml of water and 1 ml of copper reagent were added and the solution heated in a boiling water bath for 20 min. After cooling, 1 ml of areseno molybdate solution was added, and kept for 15 min for colour development. The samples were made upto 25 ml with water and the optical density measured at 520 nm.

2.3.3 Estimation of sucrose

Sucrose was estimated as reducing sugar produced by hydrolysis. 1 ml of the alcoholic extract was evaporated to dryness. 1 ml of H_2O and 1 ml of 1 N H_2SO_4 were added and the samples were hydrolysed for 30 min by keeping in a water bath at 60°C. After cooling, 1 ml of 1 N NaOH was added. The reducing sugars in the samples were estimated before and after acid hydrolysis and the difference was taken as the measure of sucrose.

2.3.4 Estimation of total soluble sugars

Total soluble sugars was estimated by the method of Scott and Melvin (1953).

Reagents

2 g anthrone was dissolved in a litre of concentrated H_2SO_4.

1 ml of the alcoholic extract was evaporated to dryness in a water bath. 1 ml of H_2O was added and the tubes were kept in an ice bath. 4 ml of anthrone reagent was added and the tubes were kept in ice bath for 10 min. The tubes were shaken well, heated in a boiling water bath for 10 min, and cooled. The optical density was measured at 620 nm..

2.3.5 Estimation of cyclitols

Quebrachitol, l- and m-inositols were estimated by the modified method of Bealing (1969) as described by Low (1978).

Suitable aliquot of the alcohol extract was evaporated to dryness and mixed with a drop of concentrated HCl. It was then heated for 10 min in a boiling water bath, cooled and centrifuged. The clear supernatent was adjusted to pH 6.5 with NaOH. It was then boiled with 10 ml of 0.3 N barium hydroxide for 15 min. 1 ml of 5 per cent zinc sulphate was then added. The mixture was centrifuged and the supernatent was made upto a volume of 10 ml with water and used for inositol estimation by periodate oxidation adopted from Agranoff et al. (1958). Aliquots of 0.2 to 0.4 ml were incubated with 2.5 ml sodium acetate (1 M), 0.3 ml sodium

periodate (0.01 M) and water to a final volume of 3.8 ml. After 2 h at 65°C, the absorbance was measured at 260 nm against a water blank. The amount of total inositol present was estimated against a standard curve prepared using myo-inositol.

2.4.5 Estimation of starch in leaves

Leaflets were oven dried at 80°C. The powdered samples were extracted with 80 per cent boiling ethanol and centrifuged. The residue was again extracted with 80 per cent ethanol. The supernatent was used for the estimation of reducing and non-reducing sugars.

The residue was used for the extraction and estimation of starch by the method of McCready et al. (1950). The residue was solubilised with 52 per cent perchloric acid for 30 min. After filtration the samples were made upto 100 ml with distilled water. To 1 ml of the extract, 4 ml of water and 10 ml of freshly prepared anthrone reagent (0.2 per cent) were added in cold. The tubes were heated for 7.5 min at 100°C in a water bath, cooled and the colour intensity measured at 630 nm. Standard curves were prepared with known amounts of glucose and the starch content was calculated by multiplying the glucose equivalent present in the sample with 0.9.

2.5 Determination of total phosphorus

Total phosphorus was estimated by the method of Tunnicliffe (1955). 0.1 g of the dried latex sample was taken in a porcelain crucible. 2 ml of 10 per cent alcoholic magnesium nitrate was added and heated in a water bath until the alcohol has evaporated. A blank was also run by taking 2 ml of magnesium nitrate solution. The crucibles were heated to 500°C for 30 min. After cooling, 5 ml of distilled water and 0.5 ml of concentrated H_2SO_4 were added and heated for three hours in a water bath keeping the crucible covered with a watch glass. After cooling, the solution was transferred to a 50 ml standard flask. To 5 ml of the made up solution, was added 1 ml of sulfomolybdic acid and 5 ml freshly prepared ammonium sulphate. The volume was made upto 25 ml and optical density read between 5 and 10 min at 660 nm.

2.6 Extraction and estimation of proteins in latex

2.6.1 Rubber cream

Proteins were extracted from rubber cream according to the method of Dennis and Light (1989). The rubber cream was dispersed in distilled water and centrifuged under refrigerated conditions at 18000 rpm for 30 min. A known weight of the washed rubber fraction was dispersed in 2 per cent sodium dodecyl sulphate and kept in the cold overnight. The dispersion was centrifuged

at 12000 rpm for 30 min. The protein in the clear solution was precipitated by adding cold acetone. The precipitate was dissolved in 0.1 N NaOH after centrifugation. The protein was estimated by the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.6.2 Bottom fraction

Lutoid membrane for protein estimation was prepared according to Xavier Gidrol et al. (1988). Ice cooled latex was centrifuged at 23000 rpm for 45 min. The supernatent serum and rubber fractions were discarded. Crude lutoid fraction was washed three times with distilled water. The sediment obtained by centrifugation of the lutoid supernatent at 35000 g for 10 min at 4°C was used as the membrane fraction.

For the solublisation and extraction of proteins the method of Howard Evans (1979) was adopted.

200 mg of lutoid fraction was solublised by the addition of 2 ml of buffer containing a final concentration of 2 per cent SDS, 3 per cent β mercaptoethanol, 10 per cent glycerol, and 0.1 ml 1 M urea. The solution was heated for 3-5' at 100°C and centrifuged at 15000 rpm for 30 min. To the supernatent, 2 ml 2.5 per cent TCA was added and the precipitate dissolved in 1 ml of 0.1 N NaOH. An aliquot was used for protein estimation.

2.6.3 C-serum and B-serum

To suitable aliquots of C-serum and B-serum, 5 ml of 2.5 per cent TCA was added. The precipitate was dissolved in 1 ml of 0.1 N NaOH and aliquots used for protein estimation.

2.6.4 Latex

Nitrogen content of the total solid film prepared after the removal of the non-protein N by dialysis against water was determined by the micro kjeldahl method and the proteins estimated by multiplying the percentage N by 6.25.

2.7 Electrophoresis

SDS-PAGE was performed according to Laemmli (1970). The gels were run at 4°C with a current of 20 mA per gel. Staining was carried out with Coomassie blue R-250.

2.8 Determination of free amino acids

Free amino acids were determined by the method of Moore and Stein (1948).

Reagents

a) Ninhydrin solution. 0.2 g of reagent grade Stannous chloride was dissolved in 125 ml of citrate buffer. This solution was added to 5 g of ninhydrin dissolved in 125 ml of methyl cellosolve.

b) 0.2 M citrate buffer pH 5. 10.5 g of reagent grade citric acid monohydrate was dissolved in 100 ml of 1N NaOH and diluted to 250 ml.

c) Diluant solvent. Equal volumes of water and reagent grade n-propanol were mixed.

0.5 ml of the alcoholic extract was taken and evaporated to dryness. 1 ml of ninhydrin reagent was added, mixed and heated for 20 min in a boiling water bath. 5 ml of diluant was added to each tube and the contents were mixed. The optical density was read at 570 nm after 15 min.

2.9 Extraction and estimation of thiols, inorganic phosphorus and magnesium in latex

About 1 g of latex was extracted with 2.5 per cent TCA and made upto a known volume. Aliquots were used for the estimation of thiols, inorganic phosphorus and magnesium.

2.9.1 Determination of thiol groups

Thiols were measured by the method of Boyne and Ellman (1972).

Reagents

a) 0.5 M Tris solution.

b) 10 M DTNB solution.

In a 20 ml volumetric flask, 79.4 mg DTNB (5,5'dithio-bis-2-nitrobenzoic acid) and 140.3 mg EDTA were taken and dissolved in water. The pH was adjusted at 6.5 with 0.5 M Tris and made up to volume.

Reagent was stored in the refrigerator.

To 2 ml of the extract, 0.1 ml DTNB solution and 2 ml Tris were added and the O.D was measured at 412 nm within 30 min. Reduced glutathion was used as the standard.

2.9.2 Determination of inorganic phosphorus

Inorganic phosphorus was determined by the method of Taussky and Shorr (1953).

Reagents

Sulpho-molybdenum solution - 10 per cent.

In a 1000 ml volumetric flask, 700 ml distilled water, 278 ml 95-97 per cent sulphuric acid and 100 g ammonium molybdate were added. The volume was made upto 1000 ml and stored in the cold.

At the time of analysis, 5 g of ferrous sulphate and 10 ml of sulfomoybdic solution were mixed in a 100 ml volumetric flask and the volume made up to 100 ml with distilled

water. 0.2 ml of extract was pipetted and the volume made up to 2 ml with 2.5 per cent TCA. 2 ml of sulphomolybdic acid reagent was then added and the absorbance measured at 740 nm using potassium dihydrogen orthophosphate as standard.

2.9.3 Determination of magnesium

Magnesium was estimated by atomic absorption spectrophotometry using the method RRIM (1973).

To 0.1 ml of the extract was added 1 ml of strontium chloride and the volume made up to 25 ml. The concentration of magnesium was obtained using atomic absorption spectrophotometer model No. GBC 902.

2.10 Determination of HMG-CoA reductase activity

HMG-CoA reductase activity of the bark was estimated as described by Rao and Ramakrishnan (1975).

Reagents

a) Saline arsentate.

1 g of sodium arsenate/litre of physiological saline.

b) Dilute perchloric acid - 50 ml/litre.

c) Hydroxylamine hydrochloride reagent. 138.98 g/litre.

d) Hydroxylamine hydrochloride reagent for mevalonate.

Equal volumes of hydroxylamine hydrochloride reagent and water were mixed freshly before use.

e) Hydroxylamine hydrochloride reagent for HMG-CoA.

Equal volumes of hydroxylamine hydrochloride reagent and sodium hydroxide solution (180 g/litre) were mixed freshly before use.

f) Ferric chloride reagent.

5.2 g of trichloroacetic acid (TCA) and 10 g ferric chloride oride were dissolved in 50 ml of 0.65 N hydrochloric acid and diluted to 100 ml with the latter.

Soft tissues of the bark were homogenised in one per cent saline arsenate and diluted with an equal volume of perchloric acid. The extract was centrifuged at 3000 rpm for 15 min. One ml of the supernatent was treated with 0.5 ml of 2 M freshly prepared hydroxylamine in water. In the case of HMG-CoA, hydroxylamine reagent mixed with an equal volume of NaOH solution was used. The solutions were mixed and kept for 5 min and 1.5 ml of ferric chloride was added. After 10 min the OD was read at 540 nm against a similarly treated blank.

2.11 Determination of Plugging Index, Bursting Index and d.r.c.

2.11.1 Plugging index as proposed by Milford *et al.* (1969) was determined by measuring initial flow rate and total volume of latex obtained and using the formula

$$\frac{\text{Initial flow rate}}{\text{Total volume}} \times 100$$

Initial flow rate was determined by dividing the volume of latex collected during the first 5 min by 5.

2.11.2 Bursting index was measured by the method of Ribaillier (1968), the formula being

$$BI = \frac{\text{activity of liberated acid phosphatase}}{\text{activity of total acid phosphatase}} \times 100$$

The liberated phosphatase activity was determined by incubating a known volume of fresh latex with P-nitrophenyl phosphate in 0.4 M mannitol at pH 5 for 10 min at 27±2°C and thereafter estimating the liberated P-nitrophenol colorimetrically. The total phosphatase activity was determined in a similar manner with the modification that 0.1 per cent teepol B-300 was included to rupture the lutoids completely.

2.11.3 Dry rubber content

Determination of dry rubber content (d.r.c.) was carried out according to IS 5430-1981.

2.12 Statistical analysis

Statistical analysis was carried as described in Panse and Sukhatme (1957).

CHAPTER 3

VARIATION IN THE CONCENTRATION OF LIPIDS IN THE LATEX AND LEAVES OF HIGH YIELDING AND LOW YIELDING CLONES

Evolving desirable genotypes through breeding is time consuming in Hevea brasiliensis because of the long breeding cycle, around thirty two years. It would be advantageous to evaluate yield potential of genotypes at young stages of growth and correlate the same with productivity at mature stage. Biochemical parameters are useful tools in this respect. Among the various biochemical characters lipid composition assumes importance in view of the role of the lutoids in controlling the flow of latex and of the fact that lipid composition of the lutoid membrane plays a role in the stability of lutoids.

Jacob et al. (1976) suggested that phosphatidic acid of the lutoid membrane might contribute towards lutoid stability and that high phosphatidic acid content of the lutoid membrane might confer a highly electronegative charge which must be particularly useful in maintaining the colloidal stability of latex. Ho et al.

(1976) found that neutral lipid content of rubber particle is also one of the factors governing plugging index. Sherief and Sethuraj (1978) reported that the content of phospholipids in the lutoids and neutral lipids in the rubber particles are associated with differences in plugging indices. The levels of these lipids were found to be negatively correlated with plugging indices. However, as discussed in the introduction, no systematic study on the concentration of various lipids in the latex or its sub-fractions or in any part of the tree in high yielding and low yielding clone seems to have been carried out with a view to use them as criteria for early prediction.

For the purpose of early prediction of yield characteristics, it is difficult to separate lutoid particles and rubber particles from latex samples obtained from young Hevea plants, while it is easy to obtain leaves. In view of this, a comparative study has been made on the concentration of various lipids in the latex and its sub-fractions and leaves of some selected high and low yielding clones in the young and mature stages to ascertain whether any of the lipid parameters in any fraction of the latex or leaves can be used for early prediction of the yield. The results obtained are discussed in this chapter.

Materials and Methods

The clones selected for the study of lipid composition of

the latex or its sub-fractions were two high yielding clones viz. RRII 105, PB 235 and two low yielding clones, Pil B 84 and Ch 4. Both were grown at the Central Experiment Station. For the study of lipids of the whole latex and leaves in young plants, the high yielding clones were RRII 105, RRIM 600 and GT 1 and low yielders RRII 38 and HP 20. These plants were located in the farm at Rubber Research Institute of India, Kottayam. The procedures for the determination of dry rubber content (d.r.c.), Plugging index (PI), Bursting index (BI) and Initial flow rate (Ifr) are given in Chapter 2. The concentration of total lipids, triglycerides, phospholipids, sterols, and glycolipids were determined in the latex, latex fractions and also in the leaves as described in Chapter 2. Procedure for collection of latex, separation of latex into different sub-fractions, as well as collection of leaves are also given in Chapter 2. Six trees of each of the clones were selected for the study from a completely randomised planting. For each clone the trees were selected based on their yield performance/vigour and only those with values around the mean were included.

Results

A. Yield

Dry rubber yield, PI, BI, d.r.c., Ifr and total volume of latex of the clones used in the study are given in Table 1a and 1b.

Table 1a. Dry rubber yield, latex volume, dry rubber content, initial flow rate, plugging index and bursting index of four Hevea clones grown in CES of RRII, Chethackal at the second year of tapping (mean of 6 trees).

Clones	Yield of rubber g tree^{-1} tap^{-1}	Latex volume ml tree^{-1} tap^{-1}	d.r.c. %	I.f.r. ml min^{-1} tree^{-1}	P.I.	B.I.
Ch 4	25.29	71.48	35.29	2.36	5.54	36.41
Pil B 84	18.05	54.77	35.08	2.00	7.04	30.20
RRII 105	97.75	271.28	39.98	7.84	3.53	21.20
PB 235	104.41	279.55	41.45	4.80	2.78	16.12
CD (0.05)	10.05	17.35	2.15	0.667	1.23	4.89

Table 1b. Dry rubber yield, latex volume, d.r.c. and plugging index of five clones in the RRII farm at 36 months.

Clones	Yield of rubber g tree^{-1} tap^{-1}	Latex volume ml tree^{-1} tap^{-1}	d.r.c.	P.I.
RRII 105	7.50	14.20	24.35	4.16
RRIM 600	3.70	13.20	20.80	4.26
GT 1	3.40	8.30	24.40	4.12
HP 20	1.24	7.40	27.00	6.12
RRII 38	1.02	6.80	27.40	5.18
CD (0.05)	1.17	5.18	3.50	0.95

The rubber yield and d.r.c. were significantly higher in the high yielding clones when compared to those in the low yielders, in trees in the second year of tapping. The PI and BI on the other hand were significantly lower in the high yielders in this group. The initial flow rate as well as the total volume of latex per tree per tap was significantly higher in the high yielders in the second year of tapping when compared to the low yielders. In the younger trees (third year of growth) the yield of rubber was significantly higher in the high yielders but the d.r.c. was not significantly different while the PI was significantly lower in the high yielders.

B. Lipids

B1 Concentration of total lipids in latex and latex fractions in the second year of tapping.

These results relate to two high yielding clones RRII 105 and PB 235 and two low yielding clones Ch 4 and Pil B 84.

a) Latex

Concentration of total lipids in whole latex of the high yielding and low yielding clones are given in Table 2. There was no significant difference in total lipids in the whole latex of high yielding and low yielding clones.

Table 2. Total lipids in the latex in high and low yielding clones at the second year of tapping.

Clones	Total lipids (mg g^{-1} dry weight)
Low Yielding Clones	
Ch 4	36.23
Pil B 84	41.54
High Yielding Clones	
RRII 105	35.17
PB 235	39.80
CD (0.05)	N.S.

Table 3. Total lipids in the rubber cream in high and low yielding clones at the second year of tapping.

Clones	Total lipids (mg g^{-1} dry weight)
Low Yielding Clones	
Ch 4	36.08
Pil B 84	39.30
High Yielding Clones	
RRII 105	41.23
PB 235	57.33
CD (0.05)	5.54

b) Rubber cream

The results are given in Table 3. There was significant difference in total lipid content of the rubber cream between the two low yielders and only one of the high yielding clone, namely PB 235. However there was no significant difference between the two low yielders and the other high yielder namely RRII 105. The total lipid content of the cream in the high yielder PB 235 was significantly higher than that of the other high yielder RRII 105.

c) Bottom fraction

The results are given in Table 4. The concentration of total lipids in bottom fraction was significantly more in the high yielders when compared to one of the low yielders namely Pil B 84. On the other hand the value in the other low yielder was not significantly different when compared to that in the high yielders.

B2 Concentration of total lipids in latex of young plants (42 months)

These studies were carried out in three high yielding clones RRII 105, RRIM 600 and GT 1 and two low yielding clones RRII 38 and HP 20. The results are given in Table 5. The concentration of total lipids in latex was significantly higher in all three high yielders when compared to the corresponding values in the low yielders.

Table 4. Total lipids in the bottom fraction in high and low yielding clones at the second year of tapping.

Clones	Total lipids (mg g^{-1} dry weight)
Low Yielding Clones	
Ch 4	144.9
Pil B 84	118.7
High Yielding Clones	
RRII 105	146.7
PB 235	147.48
CD (0.05)	17.49

Table 5. Total lipids in the latex in high and low yielding clones at 42 months.

Clones	Total lipids (mg g^{-1} dry weight)
High Yielding Clones	
RRII 105	60.13
RRIM 600	58.28
GT 1	60.26
Low Yielding Clones	
RRII 38	53.26
HP 20	51.62
CD (0.05)	1.18

B3 Concentration of total lipids in leaves of young plants (24 months)

The data relate to three high yielding clones (RRII 105, RRIM 600 and GT 1) and two low yielders (RRII 38 and HP 20). The results are given in Table 6. The concentration of total lipids in the leaves was significantly lower in the low yielding clones when compared to that in the high yielding clones.

C. Triglycerides

C1 Concentration of triglycerides in latex and latex fractions in the second year of tapping

The data relate to two high yielding clones viz., RRII 105 and PB 235 and two low yielding clones Ch 4 and Pil B 84.

a) Latex

The results are given in Table 7. The concentration of triglycerides in one of the high yielding clones PB 235 was significantly higher when compared to both the low yielders while that in the other high yielding clone RRII 105 was not significantly different.

b) Rubber cream

The results are given in Table 8. The concentration of triglycerides in the rubber cream in one of the high yielding clone PB 235 was significantly higher when compared to both the low

Table 6. Total lipids in the leaves in high and low yielding clones at 24 months.

Clones	Total lipids (mg g^{-1} dry weight)
High Yielding Clones	
RRII 105	118.82
RRIM 600	106.08
GT 1	94.80
Low Yielding Clones	
RRII 38	89.69
HP 20	89.98
CD (0.05)	3.24

Table 7. Triglycerides in latex in high and low yielding clones at the second year of tapping.

Clones	Tryglycerides (mg g^{-1} dry weight)
Low Yielding Clones	
Ch 4	3.37
Pil B 84	3.50
High Yielding Clones	
RRII 105	2.62
PB 235	11.18
CD (0.05)	4.06

Table 8. Triglycerides in the rubber cream in high and low yielding clones at the second year of tapping.

Clones	Triglycerides (mg g^{-1} dry weight)
Low Yielding Clones	
Ch 4	5.38
Pil B 84	5.77
High Yielding Clones	
RRII 105	6.02
PB 235	20.58
CD (0.05)	2.13

Table 9. Triglycerides in the bottom fraction in high and low yielding clones at the second year of tapping.

Clones	Triglycerides (mg g^{-1} dry weight)
Low Yielding Clones	
Ch 4	21.60
Pil B 84	24.45
High Yielding Clones	
RRII 105	33.69
PB 235	32.57
CD (0.05)	6.43

yielders. However there was no significant difference in the other high yielding clone RRII 105.

c) Bottom fraction

The results are given in Table 9. The concentration of triglycerides in the bottom fraction was significantly higher in both the high yielders when compared to the low yielders.

C2 Concentration of triglycerides in latex of young plants (42 months)

The data relate to three high yielders RRII 105, RRIM 600 and GT 1 and two low yielders RRII 38 and HP 20. The results are given in Table 10. The concentration of triglycerides in whole latex in 42 month old plants was significantly higher in all the three high yielders when compared to the low yielding clones.

C3 Concentration of triglycerides in leaves of young plants (24 months)

The data relate to three high yielders RRII 105, RRIM 600 and GT 1 and two low yielders RRII 38 and HP 20. The results are given in Table 11. The concentration of triglycerides in the leaves was significantly more in all the three high yielding clones when compared to the low yielders.

Table 10. Triglycerides in the latex of high and low yielding clones at 42 months.

Clones	Triglycerides (mg g^{-1} dry weight)
High Yielding Clones	
RRII 105	8.39
RRIM 600	7.95
GT 1	8.06
Low Yielding Clones	
RRII 38	7.05
HP 20	6.49
CD (0.05)	0.217

Table 11. Triglycerides in the leaves in high and low yielding clones at 24 months.

Clones	Triglycerides (mg g^{-1} dry weight)
High Yielding Clones	
RRII 105	10.88
RRIM 600	9.50
GT 1	10.85
Low Yielding Clones	
RRII 38	8.13
HP 20	7.69
CD (0.05)	0.49

D. Phospholipids

D1 Concentration of phospholipids in the latex and its sub-fractions in the second year of tapping

The data relate to two high yielding clones RRII 105 and PB 235 and to two low yielders Ch 4 and Pil B 84.

a) Latex

The results are given in Table 12. There was no significant difference in the phospholipids of whole latex in high yielding and low yielding clones.

b) Rubber cream

The results are given in Table 13. There appears to be no significant difference in the phospholipids in the rubber cream of the high yielding clones when compared to the low yielding clones.

c) Bottom fraction

The results are given in Table 14. The bottom fraction of both the high yielders had significantly more phospholipids when compared to that in the low yielders.

D2 Concentration of phospholipids in latex of young plants (42 months)

The results which relate to the three high yielding clones

Table 12. Phospholipids in the latex in high and low yielding clones at the second year of tapping.

Clones	Phospholipids ($mg\ g^{-1}$ dry weight)
Low Yielding Clones	
Ch 4	7.96
Pil B 84	7.40
High Yielding Clones	
RRII 105	7.80
PB 235	8.30
CD (0.05)	N.S.

Table 13. Phospholipids in the rubber cream in high and low yielding clones at the second year of tapping.

Clones	Phospholipids ($mg\ g^{-1}$ dry weight)
Low Yielding Clones	
Ch 4	2.12
Pil B 84	2.17
High Yielding Clones	
RRII 105	2.05
PB 235	2.02
CD (0.05)	N.S.

Table 14. Phospholipids in the bottom fraction in high and low yielding clones at the second year of tapping.

Clones	Phospholipids (mg g^{-1} dry weight)
Low Yielding Clones	
Ch 4	4.51
Pil B 84	3.69
High Yielding Clones	
RRII 105	7.60
PB 235	6.10
CD (0.05)	1.27

Table 15. Phospholipids in the latex in high and low yielding clones at 42 months.

Clones	Phospholipids (mg g^{-1} dry weight)
High Yielding Clones	
RRII 105	16.69
RRIM 600	18.40
GT 1	16.14
Low Yielding Clones	
RRII 38	15.14
HP 20	15.42
CD (0.05)	0.480

RRII 105, RRIM 600 and GT 1 and the two low yielders are given in Table 15. The phospholipid content of the whole latex in young plant was significantly higher in the high yielders when compared to that of the low yielders.

D3 Concentration of phospholipids in the leaves of young plants (24 months)

The results which relate to the high yielders RRII 105, RRIM 600 and GT 1 and the low yielders RRII 38 and HP 20 are given in Table 16. The leaves of the high yielding clones contained significantly more phospholipids when compared to that in the low yielders.

E. Sterols

E1 Concentration of sterols in the latex and latex fractions in the second year of tapping

The data relate to the two high yielding clones RRII 105 and PB 235 and the two low yielding clones Ch 4 and Pil B 84.

a) Latex

The results are given in Table 17. There was no significant difference in the concentration of sterols in the whole latex in high yielding and low yielding clones.

b) Rubber cream

Results are given in Table 18. The rubber cream from

Table 16. Phospholipids in the leaves in high and low yielding clones at 24 months.

Clones	Phospholipids ($mg\ g^{-1}$ dry weight)
High Yielding Clones	
RRII 105	6.08
RRIM 600	6.07
GT 1	6.79
Low Yielding Clones	
RRII 38	5.12
HP 20	4.66
CD (0.05)	0.37

Table 17. Sterols in the latex in high and low yielding clones at the second year of tapping.

Clones	Sterols ($mg\ g^{-1}$ dry weight)
Low Yielding Clones	
Ch 4	4.88
Pil B 84	5.80
High Yielding Clones	
RRII 105	5.64
PB 235	6.52
CD (0.05)	N.S.

only one of the high yielders, PB 235, had significantly more sterols when compared to that from the low yielders while the sterol concentration in the other high yielder was not significantly different.

c) *Bottom fraction*

The results are given in Table 19. The concentration of sterols in the bottom fraction in one of the low yielders, Pil B 84, was significantly lower when compared to that of the other high yielders while in the case of the other low yielder there was no significant difference.

E2 Concentration of sterols in latex of young plants (42 months)

The results which relate to the three high yielders RRII 105, RRIM 600 and GT 1 and the two low yielders RRII 38 and HP 20 are given in Table 20. The concentration of sterols in whole latex was significantly lower in the low yielders when compared to that in the high yielders.

E3 Concentration of sterols in the leaves of young plants (24 months)

The results which relate to the clones RRII 105, RRIM 600, GT 1 (high yielders) and RRII 38 and HP 20 (low yielders) are given in Table 21. The concentration of sterols was significantly more in all the high yielders when compared to the low yielders.

Table 18. Sterols in the rubber cream in high and low yielding clones at the second year of tapping.

Clones	Sterols (mg g^{-1} dry weight)
Low Yielding Clones	
Ch 4	3.74
Pil B 84	3.87
High Yielding Clones	
RRII 105	3.82
PB 235	4.90
CD (0.05)	0.20

Table 19. Sterols in the bottom fraction in high and low yielding clones at the second year of tapping.

Clones	Sterols (mg g^{-1} dry weight)
Low Yielding Clones	
Ch 4	31.78
Pil B 84	26.47
High Yielding Clones	
RRII 105	36.15
PB 235	34.75
CD (0.05)	6.43

Table 20. Sterols in the latex in high and low yielding clones at 42 months.

Clones	Sterols (mg g^{-1} dry weight)
High Yielding Clones	
RRII 105	8.97
RRIM 600	9.15
GT 1	9.79
Low Yielding Clones	
RRII 38	7.43
HP 20	6.28
CD (0.05)	0.415

Table 21. Sterols in the leaves in high and low yielding clones at 24 months.

Clones	Sterols (mg g^{-1} dry weight)
High Yielding Clones	
RRII 105	21.75
RRIM 600	19.97
GT 1	18.63
Low Yielding Clones	
RRII 38	17.84
HP 20	15.46
CD (0.05)	0.66

F. Glycolypids

F1 Concentration of glycolipids in the whole latex and its sub-fractions in the second year of tapping

The data relates to the two high yielders RRII 105 and PB 235 and the two low yielders Ch 4 and Pil B 84.

a) Latex

The results are given in Table 22. There was no significant difference in the concentration of glycolipids in the whole latex in high yielding and low yielding clones.

b) Rubber cream

The results are given in Table 23. There was no significant difference in the concentration of glycolipids in the high yielding and low yielding clones.

c) Bottom fraction

The results are given in Table 24. Only one of the high yielders (PB 235) showed significantly higher value when compared to the low yielder Ch 4. In the other case there was no significant difference.

F2 Concentration of glycolipids in the leaves of young plants (24 months)

The results which relate to the three high yielding clones

Table 22. Glycolipids in the latex in high and low yielding clones at the second year of tapping.

Clones	Glycolipids (mg g^{-1} dry weight)
Low Yielding Clones	
Ch 4	23.50
Pil B 84	15.60
High Yielding Clones	
RRII 105	17.28
PB 235	21.62
CD (0.05)	N.S.

Table 23. Glycolipids in the rubber cream in high and low yielding clones at the second year of tapping.

Clones	Glycolipids (mg g^{-1} dry weight)
Low Yielding Clones	
Ch 4	6.84
Pil B 84	6.99
High Yielding Clones	
RRII 105	7.14
PB 235	7.47
CD (0.05)	N.S.

Table 24. Glycolipids in the bottom fraction in high and low yielding clones at the second year of tapping.

Clones	Glycolipids (mg g^{-1} dry weight)
Low Yielding Clones	
Ch 4	20.20
Pil B 84	25.72
High Yielding Clones	
RRII 105	19.60
PB 235	31.25
CD (0.05)	7.54

Table 25. Glycolipids in the leaves in high and low yielding clones at 24 months.

Clones	Glycolipids (mg g^{-1} dry weight)
High Yielding Clones	
RRII 105	70.86
RRIM 600	72.42
GT 1	63.90
Low Yielding Clones	
RRII 38	62.70
HP 20	62.55
CD (0.05)	2.69

Table 26. Relation between triglyceride content in rubber cream, d.r.c. and plugging index.

Clones	Triglyceride (mg g^{-1} dry weight)	d.r.c.	Plugging index
Tjir 16	5.56	30.7	3.57
Ch 29	5.56	32.5	4.35
PB 215	9.16	44.6	2.90
PB 217	20.80	39.0	3.00
GT 1	7.06	40.1	2.83

Triglyceride x PI. r = -0.30 (NS)

Triglyceride x d.r.c. r = 0.50 (NS)

Table 27. Relation between phospholipids and bursting index.

Clone	Phospholipids (mg g^{-1} dry weight)	Bursting index
Tjir 16	0.575	43.39
Ch 29	3.03	33.90
PB 215	8.51	12.55
PB 217	12.07	11.65
GT 1	8.49	9.64
CD (0.01)	3.38	8.00

Phospholipids x BI. r = -0.769.

RRII 105, RRIM 600 and GT 1 and the two low yielding clones RRII 38 and HP 20 are given in Table 25. Two of the high yielders, namely RRII 105 and RRIM 600, showed significantly higher concentration of glycolipids in the leaves when compared to that in the leaves of both the low yielders. On the other hand, the concentration of glycolipids in clone GT 1 was not significantly different.

Discussion

Due to practical difficulties, mainly non-availability of trees of the clones, belonging to different age groups like 24 months, 42 months and second year of tapping, it was not possible to use the same clones for study of lipid profile in the different ages of the tree though this would have been ideal. However the same clones were used at 42 months and 24 months of age. When the lipid profile of the latex and its fractions in the second year of tapping ie., when the trees are nine years old, was examined in relation to rubber yield, certain differences are noticeable. Whereas the total lipids in the whole latex shows no significant difference in the high yielders and low yielders, that in rubber cream and bottom fraction shows a variable pattern in the high yielders and low yielders. But if the latex is collected at an earlier age (42 months), the high yielding clones showed higher values.

Regarding the composition of lipids in latex and its fractions a definite pattern could be seen only in the case of triglycerides and phospholipids in the bottom fraction. The high yielding clones showed a higher concentration of triglycerides and phospholipids in the bottom fraction when compared to that in the low yielders while the whole latex and rubber cream showed a variable pattern. The concentration of sterols and glycolipids showed no significant difference or only a variable pattern in the whole latex or its sub-fractions. The whole latex from young plants (42 months old) also showed a higher concentration of total lipids, triglycerides, phospholipids and sterols in the high yielders when compared to the low yielders. Thus in the mature tree, the bottom fraction of the latex contains higher concentration of phospholipids and triglycerides in the high yielding clones. In this connection, Sherief and Sethuraj (1978) have reported a negative correlation between the triglyceride content of the rubber cream of latex and plugging index. No such correlation is observed in the present study. They however, did not study the triglyceride content of the bottom fraction in relation to plugging. That no correlation exists between the triglyceride content of rubber cream and PI or d.r.c. was also confirmed by studying more clones belonging to high yielding and low yielding categories (Table 26). A negative correlation was found between the phospholipid content of bottom fraction and bursting index. This observation is in agreement with that

of Sherief and Sethuraj (1978). This correlation was further confirmed by studying more high and low yielding clones (Table 27).

As mentioned earlier, it is desirable to identify biochemical parameters in the tree at an early age which could be used for early prediction of yield. Collection of sufficient latex from young tree to separate the bottom fraction by ultracentrifugation may present practical difficulties. But it is easy to collect a few ml of latex from the young tree without any harm to the tree. The higher concentration of total lipids, triglycerides, phospholipids and sterols in the whole latex in the high yielders in the younger age of 42 months may be a possible criteria for use for early prediction.

The lipid profile of the latex from still younger trees would have been more desirable, but collection of latex at this age is difficult. Therefore attention was turned to other parts of the tree which can be collected with ease even at a tender age. In this respect the leaves assume importance. The leaves can be collected from young plants in sufficient quantity without any damage to the tree. The results of the present study on the lipid profile of the leaves collected from high yielding and low yielding clones at the age of two years reveal a promising pattern. The concentration of total lipids, sterols, triglycerides and phospholipids in the leaves is significantly more in the high yielding clones when compared to the low yielding ones. The trees used

in this connection were of the same age and under the same conditions of photosynthesis and soil so as to preclude variations on these accounts. No previous data appear to be available in this respect. The lipids in the leaves are derived from photosynthetic activity. The higher concentration of lipids in the leaves may indicate higher photosynthetic activity. In this connection Samsudin et al. (1987) have found a positive correlation between photosynthetic rate and yield in Hevea brasiliensis over five years. Thus the present study indicates that the concentration of total lipids, triglycerides, phospholipids and sterols in the leaves may be used to identify high yielding clones at the age of two years of the tree. Of course this has to be confirmed by using larger number of high yielding and low yielding clones.

As discussed above, the bottom fraction of the high yielding clones have more phospholipids and triglycerides when compared to the low yielders. Since the lutoids form most of the bottom fraction it means that the lutoids in the high yielding clones have more phospholipids and triglycerides. As discussed in the introduction the stability of the lutoid is an important factor governing latex flow and thereby yield of rubber. The high yielders have significantly lower bursting index compared to low yielders indicating greater lutoid stability in the former. The results now obtained indicate that the greater stability of lutoids in the high yielding clones may be associated with higher content of phospho-

lipids and triglycerides in the membrane. (The dry bottom fraction contains mostly the lutoid membrane). It is significant that in addition to phospholipids which is normally recognised as a membrane component, appreciable amounts of triglycerides are also present in the lutoid membrane. In this connection Ho et al. (1976) also reported appreciable amounts of triglycerides in the lutoid fraction. The role of phospholipids and triglycerides in the membrane in determining the stability of the lutoid particles is not clearly understood.

At the physiological pH, the rubber particles, C-serum and bottom fraction are all negatively charged and the mutually repellant action of the negatively charged particle may be involved in maintaining the colloidal nature of latex thereby preventing its coagulation. The phospholipids by their negative charge may play a role in this. The higher phospholipid content of the lutoid fraction in the high yielders may indicate higher negative charge thereby increasing the stability of the latex. Coagulation of latex involves among other things release of enzymes as well as positively charged proteins from the lutoids. But how exactly the higher concentration of phospholipids or their orientation in the lutoid membrane affects the release of these is not at present clearly understood.

CHAPTER 4

VARIATION IN THE CONCENTRATION OF TOTAL SUGARS, REDUCING SUGARS, NON-REDUCING SUGARS, CYCLITOLS AND TOTAL PHOSPHORUS IN YOUNG AND MATURE STAGES

Sucrose is the most predominant sugar in Hevea latex while quebrachitol (1-O-methyl-l-inositol) is the most abundant carbon compound in Hevea latex after rubber. Low (1978) studied the distribution and concentration of sugar and total cyclitols in high, medium and low plugging clones of Hevea. The high plugging clone (Tjir 1) was shown to contain significantly higher total cyclitol than the low plugging clone (RRIM 501) while the sucrose content of the high plugging clone was significantly lower than that in the low plugging clone. Eschbach et al. (1984) on the other hand showed that there is no correlation between the sucrose content of latex and yield. A high sugar content in latex may signify good supply to the laticiferous cells associated with good utilisation in the metabolism of these cells. At the same time, a high sugar

content in latex could also signify low metabolic utilisation of the sugar leading finally to low productivity. The latter appears to be the case in trees affected by bark dryness. Thus no definite information is available on the relation between sugar content of the latex and yield of rubber.

It has been suggested that stable latex is characterised by high phosphorus, among others. Here again, no definite information is available on the relation between phosphorus content and yield which could be obtained only by a study of high yielding and low yielding clones. In view of this, a systematic study has been undertaken on the concentration of these biochemical constituents in the latex in some of the high yielding and low yielding clones at young and mature age of the tree in order to find out whether their concentration can be used to predict high yielders.

Materials and Methods

The high yielding clones used in this study were RRII 105, PB 235, GT 1, RRIM 600 and the low yielding clones RRII 38, HP 20, Ch 4 and Pil B 84. The yield characteristics of these clones are given in Chapter 3. The methods for the determination of the concentration of total sugars, reducing sugars, non-reducing sugars, cyclitols and total phosphorus are given in Chapter 2.

Results

A. Concentration of total sugars, reducing sugars and non-reducing sugars in the latex of trees

a) At the age of 36 months

The results are given in Table 28. There was considerable variations in the concentration of total sugars and non-reducing sugars in the latex in the different clones while that of the reducing sugars was more or less similar. No relation thus appears to exist between the concentration of these components and yield of rubber.

b) At the fourth year of tapping

Results are given in Table 29. As in the case of young trees, no relation was found to exist between the concentration of these components and yield of rubber in the mature tree.

B. Concentration of cyclitols

a) At the age of 36 months

Results are given in Table 30. Though the concentration of cyclitols in the latex was more in two of the high yielding clones (RRII 105 and RRIM 600) when compared to that of the low yielders, between the high yielding clone GT 1 and the low yielding clone RRII 38, there was no significant variation.

Table 28. Total sugars, reducing sugars and non-reducing sugars (mg/100 g wet weight) in the latex of high and low yielding clones at 36 months.

Clones	Total sugars	Reducing sugars	Non-reducing sugars
High Yielding Clones			
RRII 105	487	94.94	390
RRIM 600	381	93.85	280
GT 1	501	101.63	400
Low Yielding Clones			
RRII 38	800	86.80	710
HP 20	344	76.55	260
CD (0.05)	110	20.30	60

Table 29. Total sugars, reducing sugars and non-reducing sugars (mg/100 g wet weight) in the latex of high and low yielding clones at the fourth year of tapping

Clone	Total sugars	Reducing sugars	Non-reducing sugars
Low Yielding Clones			
Ch 4	324	97	226
Pil B 84	353	97	256
High Yielding Clones			
RRII 105	429	118	310
PB 235	347	91	254
CD (0.05)	N.S	N.S	N.S

Table 30. Cyclitols in latex in high and low yielding clones at 36 months.

Clones	Cyclitols (mg g^{-1} dry weight)
Yigh Yielding Clones	
RRII 105	2.956
RRIM 600	2.584
GT 1	2.185
Low Yielding Clones	
RRII 38	2.106
HP 20	1.771
CD (0.05)	0.156

Table 31. Cyclitols in latex in high and low yielding clones at the fourth year of tapping.

Clones	Cyclitols (mg g^{-1} dry weight)
Low Yielding Clones	
Ch 4	3.54
Pil B 84	3.07
High Yielding Clones	
RRII 105	4.46
PB 235	3.34
CD (0.05)	0.53

b) At the fourth year of tapping

The results are given in Table 31. The high yielders generally showed a higher concentration of cyclitols in latex. But in the case of the low yielder Pil B 84 and high yielder PB 235 there was no significant difference.

C. Concentration of total phosphorus

a) At the age of 36 months

The results are given in Table 32. The concentration of total phosphorus in the latex appears to be more in the high yielding clone when compared to the low yielding clone, except in the case of GT 1. The concentration is significantly more in this when compared to only one of the low yielders HP 20.

b) At the fourth year of tapping

Results are given in Table 33. The concentration of total phosphorus was significantly more in both the high yielders when compared to one of the low yielders Ch 4. But when compared to the other low yielder Pil B 84, there was no significant difference.

D. Concentration of starch, non-reducing and reducing sugars in the leaves at the age of 42 months

The data relates to three high yielding clones (RRII 105,

Table 32. Total phosphorus in latex in high and low yielding clones at 36 months.

Clones	Phosphorus (% dry weight)
High Yielding Clones	
RRII 105	0.249
RRIM 600	0.238
GT 1	0.224
Low Yielding Clones	
RRII 38	0.215
HP 20	0.203
CD (0.05)	0.014

Table 33. Total phosphorus in latex in high and low yielding clones at the fourth year of tapping.

Clones	Phosphorus (% dry weight)
Low Yielding Clones	
Ch 4	0.143
Pil B 84	0.227
High Yielding Clones	
RRII 105	0.222
PB 235	0.219
CD (0.05)	0.038

Table 34. Concentration of starch and sugars in the leaves, at 36 months, in high and low yielding clones.

Clones	Starch (mg g^{-1} dry weight)	Non-reducing sugars (mg g^{-1} dry weight)	Reducing sugars (mg g^{-1} dry weight)
Yigh Yielding Clones			
RRII 105	69.0	47.5	52.4
RRIM 600	82.1	71.1	51.7
GT 1	78.8	72.8	40.4
Low Yielding Clones			
RRII 38	78.2	83.2	54.6
HP 20	67.4	68.8	56.9
CD (0.05)	N.S	N.S	N.S

RRIM 600 and GT 1) and two low yielding clones (RRII 38 and HP 20). The results are given in Table 34. There was no significant difference in the concentration of starch, non-reducing sugars and reducing sugars in the leaves of high yielding clones when compared to those in the leaves of the low yielding clones.

Discussion

The results obtained in the present study on total sugars, reducing sugars and non-reducing sugars do not indicate any difference in high yielding and low yielding clones. This is not in agreement with the results reported for sugars and cyclitol by Low (1978), who found that low yielders had higher total cyclitols and lower sugars in the latex when compared to the high yielders. The concentration of cyclitols however in some of the high yielders included in the present study was more when compared to the low yielders although no definite trend was discernible. The results now obtained on the sugars appear to be in agreement with those reported by Eschbach et al. (1984). In this connection, the higher concentration of cyclitols in the latex of many high yielders now obtained, indicate some relationship with higher osmotic concentration seen in the latex in high yielders (Satheesan et al., 1982). Cyclitols are reported to be the major osmotic component in the latex (Sheldrake, 1973)

Even though a high concentration of total phosphorus in the

latex is generally believed to be associated with yield, no clear cut evidences are obtained in the present study. However, generally a higher concentration of total phosphorus was noticed in many high yielding clones.

No significant difference in the concentration of starch, non-reducing and reducing sugars was noticeable in the leaves as well. This is the case irrespective of the age of the tree. Thus none of these parameters can be made use of for early prediction of yield.

CHAPTER 5

VARIATION IN THE CONCENTRATION OF PROTEIN AND FREE AMINO ACIDS IN LATEX AND LATEX FRACTIONS OF HIGH YIELDERS AND LOW YIELDERS

The previous work carried out in this aspect relate to the study of the proteins in latex and its fractions in individual clones. Tata (1980) studied the protein content in 18 latex samples, rubber fraction, C-serum and bottom fraction but did not make any attempt to correlate these factors with yield. No detailed investigation seems to have been carried out to find out whether there is any qualitative or quantitative difference in proteins in the latex and its fractions in low yielders and high yielders which can be used to identify high yielders. In view of this, investigations have been carried out on the proteins in latex and its fractions and free amino acids in the latex in some high yielding and low yielding clones. The results are discussed in this chapter.

Materials and Methods

The clones used in this study were Ch 4, Pil B 84, RRII 38 and HP 20 (low yielders) and RRII 105, PB 235, GT 1 and RRIM 600

(high yielders). Procedures used for the estimation of total protein and estimation of free amino acids are given in Chapter 2.

I. Concentration of total proteins in latex and its fractions at the third year of tapping

Results are given in Table 35. The concentration of total proteins in latex was lower in the high yielders when compared to the low yielders even though the difference was not statistically significant. Similar results are also obtained in the case of total proteins of the rubber cream (Table 36). The concentration of total proteins in the C-serum recorded higher values in the high yielders when compared to the low yielders. But here again the difference was not statistically significant (Table 37). The concentration of total proteins in the B-serum and lutoid membrane was significantly more in the high yielders when compared to that in the low yielders (Tables38 and 39).

II. Concentration of free amino acids in latex

a) Young plants (36 months)

Results are given in Table 40. The concentration of free amino acids was significantly more in the latex of high yielders when compared to the low yielders.

Table 35. Total proteins in the latex in high and low yielding clones at the third year of tapping.

Clones	Protein ($g\ 100\ g^{-1}$ dry weight)
Low Yielding Clones	
Ch 4	2.10
Pil B 84	2.09
High Yielding Clones	
RRII 105	1.32
PB 235	1.53
CD (0.05)	0.56

Table 36. Protein in rubber cream in high and low yielding clones at the third year of tapping.

Clones	Protein ($g\ 100\ g^{-1}$ dry weight)
Low Yielding Clones	
Ch 4	3.08
Pil B 84	3.08
High Yielding Clones	
RRII 105	2.00
PB 235	2.23
CD (0.05)	0.87

Table 37. Proteins in C-serum in high and low yielding clones at the third year of tapping.

Clones	Protein ($mg\ ml^{-1}$)
Low Yielding Clones	
Ch 4	9.89
Pil B 84	9.88
High Yielding Clones	
RRII 105	10.15
PB 235	10.92
CD (0.05)	0.331

Table 38. Proteins in B-serum in high and low yielding clones at the third year of tapping.

Clones	Protein ($mg\ ml^{-1}$)
Low Yielding Clones	
Ch 4	11.46
Pil B 84	11.86
High Yielding Clones	
RRII 105	14.10
PB 235	13.77
CD (0.05)	0.362

Table 39. Proteins in the lutoid membrane in high and low yielding clones at the third year of tapping.

Clones	Protein ($mg\ g^{-1}$)
Low Yielding Clones	
Ch 4	9.68
Pil B 84	10.30
High Yielding Clones	
RRII 105	18.18
PB 235	16.20
CD (0.05)	3.98

TABLE 40. Amino acids in latex in high and low yielding clones at 36 months.

Clones	Amino acid ($mg\ 100\ g^{-1}$ wet weight)
High Yielding Clones	
RRII 105	299.17
RRIM 600	266.49
GT 1	276.16
Low Yielding Clones	
RRII 38	152.80
HP 20	222.46
CD (0.05)	13.26

Table 41. Amino acids in the latex in high and low yielding clones at the third year of tapping.

Clones	Amino acid (mg 100 g^{-1} wet weight)
Low Yielding Clones	
Ch 4	235.5
Pil B 84	210.2
High Yielding Clones	
RRII 105	278.5
PB 235	281.9
CD (0.05)	40.3

SDS-PAGE Pattern of C-serum of four Hevea clones

A,B - Ch 4; C,D - Pil B 84; E,F - RRII 105; G,H - PB 235

I - Marker (Bovine Albumin)

b) Mature plants (9 years)

The results are given in Table 41. As is the case of young trees, the concentration of free amino acids was significantly more in the high yielding clones, when compared to that in the low yielders.

A preliminary attempt was made to study the electrophoretic pattern of C-serum in both high yielding and low yielding clones. The pattern, however, did not reveal any differences, although some of the bands were prominent in the case of high yielders.

Discussion

The results now obtained reveal significant difference in the total proteins of the B-serum and lutoid membrane and free amino acids in the latex in the high yielding and low yielding clones. The concentration of total proteins in the B-serum and lutoid membrane is significantly higher in the high yielding clones when compared to the low yielding clones. Similarly the concentration of free amino acids in the latex was more in the high yielding clones.

Thus the higher concentration of total proteins in the B-serum and in the lutoid membrane and that of free amino acids in the latex in the high yielders could be considered as criteria for

identifying high yielders. If this pattern is observed in the latex from younger trees it would be possible to utilise these parameters for early prediction of productivity. The electrophoretic pattern of the C-serum in the high yielders and low yielders appears to be similar. But the intensity of some of the bands appear to be more in the high yielders. Detailed work on this aspect is required particularly on the proteins of latex from younger plants before their reliability as criteria for early prediction can be established.

A higher concentration of proteins in the lutoid membrane may possibly contribute to the stability of the lutoids which, as discussed previously, is one of the major factors determining rubber yield. Both latex flow and plugging index are influenced by stability of the lutoids, a higher stability facilitating more flow of latex and thereby more yield of rubber. The higher concentration of proteins in the lutoids in the high yielders may also possibly be involved in greater stability of the lutoids. In this connection it has been shown (Chapter 3) that the concentration of phospholipids is significantly more in the lutoid membrane in the high yielding clones. The higher concentration of phospholipids may also be involved in the greater stability of lutoids.

The higher concentration of free amino acids in the latex of high yielding clones may indicate either lesser utilisation of

those amino acids in protein biosynthesis or increased protease activity. The higher concentration of proteins in the latex in the low yielder may indicate more utilisation of amino acids for protein synthesis. But the low yielders have lower concentrations of proteins in the lutoid membrane and B-serum.

CHAPTER 6

VARIATION IN THE CONCENTRATION OF THIOLS, INORGANIC PHOSPHORUS AND MAGNESIUM IN LATEX OF HIGH YIELDERS AND LOW YIELDERS

Eventhough reduced thiols are believed to play an important role in the mechanism governing lutoid stability, no systematic investigation seems to have been carried out on the relations between free thiol content and yield, by comparing high yielding and low yielding clones. However, it is generally believed that free thiols may have beneficial effect on yield. High thiol content in latex is reported to be associated with stable latex and several authors have demonstrated that there is direct correlation between thiol concentration and production (Eschbach et al., 1984; Prevot et al., 1984; Jacob et al., 1986).

Eventhough inorganic phosphorus and magnesium content in latex have been investigated in many clones, no systematic study seems to have been carried out on the extent of presence of these substances in high yielding and low yielding clones and also to find out whether they can be used for early predictions except for the work by Henon et al. (1984). Magnesium is an indispensable

activator of numerous enzymes in latex while it is also an inhibitor of some such as invertase and acid phosphatase. Subronto (1978) demonstrated the existence of an inverse correlation between Mg^{++} content and production. But Eschbach et al. (1984) observed a positive correlation between magnesium and production. Thus the data with relation to Mg^{++} and yield appers to be contradictory. Regarding inorganic phosphorus d'Auzac (1964), Eschbach et al. (1984) and Subronto (1978) reported direct correlation between Pi content of the latex and production of certain clones. The data available with respect to Pi also appear to be inadequate.

Materials and Methods

The high yielding clones used in this study were RRII 105, PB 235, PB 217, PB 215 and GT 1. The low yielding clones were Ch 4, Pil B 84, Ch 29 and Tjir 16. The yield characteristics and other data regarding clones RRII 105, PB 235, Ch 4 and Pil B 84 are given in Chapter 3. The details in respect to the other clones are summarised in Table 1c.

Procedures used for the estimation of thiols, inorganic phosphorus and magnesium are furnished in Chapter 2.

Results

a) Concentration of thilols in the latex in the fifth year of tapping

The content of thiols in the latex are given in Table 42.

Table 1c. Total volume, plugging index and d.r.c. of five Hevea clones at the sixth year of tapping.

Clone	Volume $tree^{-1}$ tap^{-1}	Plugging index	d.r.c.
Tjir 16	122.16	3.57	30.7
Ch 29	86.16	4.35	32.5
PB 215	292.00	2.90	44.6
PB 217	314.00	3.00	39.0
GT 1	204.00	2.83	40.1

Table 42. Thiols in latex in high and low yielding clones at the fifth year of tapping.

Clones	Thiols (mM wet weight)
Low Yielding Clones	
Ch 4	0.46
Pil B 84	0.49
Tjir 16	0.65
Ch 29	0.46
High Yielding Clones	
PB 215	0.63
PB 235	0.35
PB 217	0.71
RRII 105	0.41
GT 1	0.68
CD (0.05)	0.164

Table 43. Inorganic phosphorus in latex of high and low yielding clones at the fifth year of tapping.

Clones	Pi (mM wet weight)
Low Yielding Clones	
Ch 4	13.6
PIl B 84	6.39
Tjir 16	9.98
Ch 29	12.43
High Yielding Clones	
PB 215	13.42
PB 235	16.69
PB 217	16.48
RRII 105	17.28
GT 1	11.91
CD (0.05)	3.87

Table 44. Magnesium in latex in high and low yielding clones at the fifth year of tapping.

Clones	Magnesium (mM wet weight)
Low Yielding Clones	
Ch 4	23.99
Pil B 84	23.40
Tjir 16	36.94
Ch 29	34.20
High Yielding Clones	
PB 215	30.98
PB 235	14.71
PB 217	20.75
RRII 105	45.79
GT 1	18.78
CD (0.05)	15.00

No definite pattern in the concentration of thiols was seen in respect of the clones studied. Both low and high concentrations were observed in the high yielding clones as well as in the low yielders.

b) Concentration of inorganic phosphorus in latex in the fifth year of tapping

The results are given in Table 43. Except in one case, the concentrations of inorganic phosphorus was higher in high yielding clones when compared to low yielders. In one of the low yielding clones also the inorganic phosphorus concentration recorded a higher value.

c) Concentration of magnesium in latex in the fifth year of tapping

The results are given in Table 44. Wide variations in the concentration of magnesium was noted in individual clones both in the high yielding and low yielding groups. The variation was both similar in low yielders and high yielders.

Discussion

The results obtained with thiols are generally not in agreement with previous reports, which indicate direct correlation between thiol concentration and yield. The results with magnesium are also not inagreement with those reported by Subronto (1978) and

by Eschbach et al. (1984). The former reported an inverse correlation with Mg^{++} and yield while the latter workers reported a positive correlation. The results obtained with Pi appear to be in agreement with those reported by d'Auzac (1965), Eschbach et al. (1984) and Subronto (1978).

The data now obtained relate to trees under the fifth year of tapping (12 years old). The concentration of thiols, Pi and Mg^{++} in the latex of younger trees has to be studied in detail to find out the relation between their concentration and yield.

The results from the present study indicate that neither the content of thiols nor that of magnesium in latex show a definite pattern in the high yielders and low yielders. As such it is not possible to relate a higher or lower concentration with yield. However in the majority of the high yielders studied the concentration of Pi in the latex appears to be higher when compared to the low yielders although results to the contrary are also obtained in one or two cases. A large number of clones have to be studied to establish relationship, if any, between Pi content of the latex and rubber yield.

CHAPTER 7

ACTIVITY OF HMG-CoA REDUCTASE IN THE BARK IN HIGH YIELDING AND LOW YIELDING CLONES

The activity of HMG-CoA reductase, which catalyzes the rate limiting step in rubber biosynthesis, could be an indicator of rubber biosynthesis and consequently of yield of rubber as discussed in the introduction. Most of the work on this enzyme in Hevea brasiliensis relates to the detection of enzyme activity in different fractions of latex. No attempt seems to have been made to correlate the activity of the enzyme to the yield of rubber by studying high yielding and low yielding clones except for a report from Sukonrat and Wititsuwannakul (1988) who discussed the possibility of application of this parameter for early selection of high yielders. In view of this, an attempt has been made to study the activity of this enzyme in different parts of the tree using some high yielding and low yielding clones. Limited work has also been carried out to detect possible presence of inhibitors and/or stimulators of this enzyme activity in the latex using partially purified enzyme. The results are discussed in this chapter.

Materials and Methods

Five clones were used in this study. Two of them, RRII 105 and PB 235, are high yielders. Ch 4, Pil B 84 and HP 13 represented the low yielders. The yield characteristics of these clones are given in Table 1a and Table 45. Bark samples used for the study were collected from trees which were in the second year of tapping.

The bark samples were taken in polythene bags, covered by ice, on the day of tapping, 30 to 60 minutes after complete cessation of latex flow. Physiologically mature leaves and petioles were also collected in polythene bags and kept in ice. Latex was collected in ice cooled containers. The different parts collected were used immediately for the determination of the activity of HMG-CoA reductase. Tissues were homogenised in one per cent saline aresenate. Latex was diluted with 1 per cent saline arsenate. The procedure used for the determination of HMG-CoA reductase is given in Chapter 2. The ratio of HMG-CoA to mevalonate is taken as a measure of enzyme activity existing in the trees, a low ratio indicating high activity and a high ratio low activity.

Partial purification of the enzyme from the bark

Partial purification of the enzyme was carried out by following the procedure of Reddy and Das (1986). About 5 g of soft

bark was homogenised in a medium containing 100 mM potassium phosphate buffer (pH 7.0), 70 mM NaCl, 30 mM EDTA and 10 mM DTT. The suspension was centrifuged at 50,000 g max for 30 min after sonication for 15 seconds. To the supernatent solid $(NH_4)_2SO_4$ was added to a saturation of 40 per cent. The protein precipitating at this saturation was collected by centrifugation at 5,000 rpm for 20 min. It was dissolved in minimum volume of phosphate buffer and dialyzed against the same buffer, till free of $(NH_4)_2SO_4$. This partially purified enzyme was used for the study of possible inhibitor/activator in the latex. Protein in the solution was determined by the method of Lowry et al. (1951).

Enzyme assay

The micro assay method described by Shapiro et al. (1974) was used. The details are as follows:

The reaction system contained in a total volume of 100 ul approximately 0.2 mg enzyme protein in phosphate buffer, 0.38 mg NADPH and 0.15 mg DTT in phosphate buffer, 10 ul of (RS-(3-^{14}C) HMG-CoA 120 n moles (56 mci/mmol). Incubation was carried out at 30°C for 30 min. Reaction was terminated by adding 30 ul of 10 N HCl and 20 ul of 5 per cent (RS)-mevalonolactone to the system. The mixture was allowed to stand for 30 min for complete lactonization of the incubation product mevalonic acid. The precipitated protein was removed by centrifugation. The supernatent

was subjected to TLC, using benzene : acetone (1:1 v/v) as the solvent system for the separation of mevalonate. Iodine vapour was used for locating. The procedure used was according to that of Sipat (1985). The zone of mevalonolactone was scraped into a scintillation vial. 10 ml, of cocktail of toluene containing 0.5 per cent (w/v). 2,5 diphenyloxazole was added. Radioactivity was measured in a Liquid Scintillation Counter.

For studying the effect of rubber phase and C-serum, 10 µl of the rubber dispersion and C-serum was added to the incubation medium. 10 µl of water was added to the control.

Results

1. Activity of HMG-CoA reductase in different parts of the tree in high yielders and low yielders

The results which relate to RRII 105, PB 235, Pil B 84, Ch 4 and HP 13 in the mature trees are given in Tables 46 a, b & c. As can be seen, very low activity is seen in the latex. leaves and petioles did not show any variation between high and low yielders. On the other hand, the bark contained significant amount of activity. In view of this bark was chosen for determining the variations in enzyme activity in high yielders and low yielders.

Table 45. Total volume PI and d.r.c. of clone HP 13 at the second year of tapping.

Clone	Volume ($tree^{-1}$ tap^{-1})	d.r.c.	PI
HP 13	94.5	31.0	3.54

Table 46a. HMG-CoA reductase activity in latex in high and low yielding clones.

Clones	HMG-CoA reductase activity (HMG-CoA/mevalonate)
Low Yielding Clones	
Ch 4	2.94
Pil B 84	3.68
High Yielding Clones	
RRII 105	3.70
PB 235	2.92

Table 46b. HMG-CoA reductase activity in leaves.

Clones	HMG-CoA reductase activity (HMG-CoA/mevalonate)
Low Yielding Clone	
HP 13	1.79
High Yielding Clone	
RRII 105	1.67

Table 46c. HMG-CoA redcutase activity in petioles.

Clones	HMG-CoA reductase activity (HMG-CoA/mevalonate)
Low Yielding Clone	
HP 13	1.2
High Yielding Clone	
RRII 105	1.4

2. Variation in the activity of HMG-CoA reductase in the bark in high yielding and low yielding clones

The results which relate to high yielders RRII 105 and PB 235 and low yielders Ch 4 and Pil B 84 (in the second year of tapping) are given in Table 47. As can be seen, both the hign yielding clones showed significantly higher enzyme activity in the bark when compared to low yielding clones.

3. Effect of latex fractions on the activity of partially purified enzyme from the bark

The bark of RRII 105 and HP 13 were used for this study.

(i) Activity of the partially purified enzyme

Results are given in Table 48. No significant difference in the activity of the purified enzyme was observed in high yielding and low yielding clones when compared on protein basis.

(ii) Effect of C-serum and rubber phase on the activity of the partially purified enzyme in the case of high yielder RRII 105 and low yielder HP 13

Results are given in Table 49. The latex from the high yielder was used in the case of the partially purified enzyme from the bark of the high yielder. Similarly the latex from the low yielder was used for the bark from the low yielder.

Table 47. HMG-CoA reductase activity in the bark in high and low yielding clones.

Clone	HMG-CoA reductase activity (HMG-CoA/mevalonate)
Low Yielding Clones	
Ch 4	2.26
Pil B 84	2.70
High Yielding Clones	
RRII 105	1.55
PB 235	1.52
CD (0.05)	0.174

Table 48. Activity of partially purified enzyme from bark extract of high and low yielding clones.

clone	Radiactivity recovered as ^{14}C mevalonate (cpm mg^{-1} protein)
Clone RRII 105	37,176
Clone HP 13	33,800

't' value : Not significant.

Table 49 Effect of C-serum and rubber phase on the activity of the partially purified enzyme in high and low yielding clones.

Clones	Radiactivity recovered as ^{14}C mevalonate (cpm mg^{-1} protein)
High Yielding Clones	
Bark extract	43,765
Bark extract + C-serum	94,338
Bark extract + R Dispersion	43.957
Low Yielding Clones	
Bark extract	49,740
Bark extract + C-serum	78,470
Bark extract + R Dispersion	50,185

As can be seen, the rubber phase in both the high yielder and low yielder did not have any significant effect on the activity of the enzyme. On the other hand, the addition of C-serum significantly stimulated the enzyme activity in both cases. The stimulation was significantly more in the case of the high yielding clones.

Discussion

The method used for determining the enzyme activity in the bark in the high yielding and low yielding clones determines the ratio of HMG-CoA to mevalonate, the conversion of former to latter being brought about by the enzyme. This method thus gives a true measure of the actual activity exerted by the enzyme in the tree at the time of removal of the bark. When this method was followed, the high yielding clones showed significantly lower ratio's of HMG-CoA/mevalonate, indicating that more of the former has been converted to the latter. Thus higher enzyme activity exists in in vivo conditions in high yielding clones when compared to the low yielding clones. The higher activity obtained may be due to a higher concentration of enzyme present, higher concentration of possible activators of enzyme activity present or lower concentration of possible inhibitors of enzyme activity present.

When the enzyme is partially purified, the possible activator and/or inhibitor of enzyme activity present may be removed and the activity measured in *in vitro* systems with optimum conditions of substrate, pH etc. is only a reflection of the concentration of the enzyme. When assayed under these conditions, the enzyme activity was similar in both high yielding and low yielding clones, indicating that the concentration of the enzyme is more or less similar in both these groups of clones. This may, in other words, indicate that there is no difference in the biosynthetic ability as far as the enzyme is concerned.

The observation that the rubber phase of the latex has no effect on the activity of the enzyme in both the clones while the C-serum exerts a stimulating effect, is an indication of the presence of activator or activators of enzyme in the C-serum. The extent of this activation is significantly higher in high yielding clones. This observation, along with the observation that the enzyme activity *in vivo* is more in high yielding clones, signifies that the higher activity observed in the high yielding clones is not likely to be due to any difference in the biosynthetic rate of the enzyme but due to difference in the concentration of activator/activators present. The high yielding clones possibly contain high concentration of stimulators in the C-serum. In this connection, Wititsuwannakul (1990) have reported that *Hevea*

calmodulin is able to activate HMG-CoA reductase. It is possible that this may be the substance, or one of the substances, acting as activator for enzyme activity.

The activity of HMG-CoA reductase in the bark and measured by the ratio of HMG-CoA to mevalonate may be used to score high yielders and low yielders. For early prediction, however, it is necessary to determine the enzyme activity in the bark of the young plants.

SUMMARY OF IMPORTANT FINDINGS

1 Variations in lipids concentration

a) Studies on the composition of lipids in latex and its fractions revealed that the high yielding clones showed a higher concentration of triglycerides and phospholipids in the bottom fraction when compared to that in the low yielding clones.

b) Triglycerides in the rubber cream showed no correlation with plugging index and d.r.c., whereas the phospholipids of the bottom fraction showed significant negative correlation with bursting index.

c) The concentration of total lipids, triglycerides, phospholipids and sterols in the leaves and whole latex was higher in the high yielders when studied at the younger age of 24 to 42 months. These may be used reliably to identify high yielding clones.

2 Variations in the concentration of sugars, cyclitols and phosphorus

a) No relation was apparent between the concentration of total sugars and non-reducing sugars in the latex and rubber yield in young as well as mature trees.

b) The high yielders generally showed a higher concentration of cyclitols and total phosphorus in latex.

c) No significant difference in the concentration of starch and non-reducing and reducing sugars was noticed in leaves of high yielding clones compared to that in the low yielding clones.

None of these parameters could therefore be used for early prediction of yield.

3 Variations in the concentration of proteins and free amino acids

a) The concentration of total proteins in the B-serum and lutoid membrane and that of free amino acids in the latex was significantly higher in the high yielding clones. The higher concentration of proteins in the lutoid membrane may possibly contribute to the stability of the lutoid which is one of the major factors determining rubber yield. The higher concentration of proteins in the B-serum and in the lutoid membrane and that of free amino acids in the latex in the high yielders could be used as one of the criteria for identifying high yielders.

b) In the lutoid membrane in the high yielders higher concentration of proteins and phospholipids was noted which may possibly be involved in imparting greater stability of lutoids facilitating more latex flow and thereby more rubber yield.

4 Variations in the concentration of thiols, inorganic phosphorus and magnesium

a) The results obtained indicate that neither the content of thiols nor that of magnesium in latex had a definite pattern in high yielders and low yielders making it not possible to correlate this parameter with yield.

b) However in the majority of the high yielders studied, the concentration of Pi in the latex was higher when compared to that in the low yielders. This character could therefore be used along with other parameters.

5 Activity of HMG-CoA reductase

a) The method used for determining the activity of HMG-CoA reductase in the bark in the high yielding and low yielding clones (ie. determining the ratio of HMG-CoA to mevalonate) gave a true measure of the actual activity exerted by the enzyme in the tree at the time of removal of the bark.

b) Higher enzyme activity existed in in vivo conditions in high yielding clones when compared to low yielding one. However when the enzyme was partially purified, and the possible activators and/or inhibitors of enzyme activity present removed, the enzyme activity was similar in high yielding and low yielding clones indicating that the concentration of the enzyme is similar in clones belonging to both the groups.

c) The addition of respective C-serum significantly stimulated the activity of the enzyme in in vitro studies in both high yielders and low yielders. This stimulation was significantly more in the case of high yielders. The results indicate that the higher activity of the enzyme observed in vivo in high yielders may be due to higher concentration of stimulator/s of the enzyme action present in the C-serum.

d) Further work involving a set of clones at different stages of growth will be rewarding now that a quick method of determining HMG-CoA reductase activity has been standardised.

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LIST OF SCIENTIFIC PAPERS

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