STUDIES ON TAPPING INDUCED PHYSIOLOGICAL AND BIOCHEMICAL CHANGES IN THE BARK TISSUE OF *HEVEA BRASILIENSIS*

Dissertation submitted in partial
fulfilment of the
requirements for the award of the degree of
MASTER OF PHILOSOPHY
in **Biosciences** of Mahatma Gandhi University

By

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CERTIFICATE

This is to certify that the project work entitled "STUDIES ON TAPPING INDUCED PHYSIOLOGICAL AND BIOCHEMICAL CHANGES IN THE BARK TISSUE OF *HEVEA BRASILIENSIS*" is an authentic record of project work carried by Mr. M. A. Anumod under the supervision and guidance of Dr. K. Annamalainathan, Plant Physiologist, Plant Physiology Division, Rubber Research Institute of India (RRII), Kottayam, for the award of the Degree of Master of Philosophy in Biosciences at School of Biosciences, Mahatma Gandhi University, Kottayam. The work presented in this dissertation has not been submitted for any other degree or diploma earlier.

Priyadarshini Hills,
January 2006

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CERTIFICATE

This is to certify that the project work entitled "STUDIES ON TAPPING INDUCED PHYSIOLOGICAL AND BIOCHEMICAL CHANGES IN THE BARK TISSUE OF *HEVEA BRASILIENSIS*" submitted by Mr. M. A. Anumod for the award of the Degree of Master of Philosophy in Bioscience of Mahatma Gandhi University, Kottayam, was carried out under our supervision and guidance in the Division of Plant Physiology, Rubber Research Institute of India, Rubber Board, Kottayam. It is also certified that this work has not been presented for any other degree or diploma.

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DECLARATION

I hereby declare that the dissertation entitled "STUDIES ON TAPPING INDUCED PSYCHOLOGICAL AND BIOCHEMICAL CHANGES IN THE BARK TISSUE OF *HEVEA BRASILIENSIS*" submitted in partial fulfilment for the award of the degree of Master of Philosophy in Biosciences of the Mahatma Gandhi University, Kottayam, is a bonafide record of dissertation work carried out by me under the supervision and guidance of Dr. K. Annamalainathan, Plant physiologist, Plant Physiology Division, RRII, Kottayam. No part of this dissertation has been previously presented for the award of any degree, diploma, associateship, fellowship or other similar title of any other university or institution.

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ABBREVIATIONS

ADP	-	Adenosine di phosphate amino – methane
AMP	-	Adenosine monophosphate
AOX	-	Alternative respiration
APS	-	Aammonium per sulphate
ATP	-	Adenosine triphosphate
BSA	-	Bovine serum albumin
C	-	celsius
CO_2	-	Carbon dioxide
cyt-c	-	cytochrome c
EDTA	-	Ethylene diamino tetra acetic acid
ETC	-	Electron transport chain
g	-	gram
HEPES	-	N – 2 – hydroxy ethyl piperazine – 2' ethane – sulfonic acid
K	-	Potassium
KCN	-	Potassium cyanide
kDa	-	kilo daltons
kg	-	kilogram
KH_2PO_4	-	Potassium dihydrogen phosphate
KOH	-	Potassium hydroxide
MDA	-	Malondialdehyde
Mg	-	Magnesium
mg	-	milligram
$MgSO_4$	-	Magnesium sulphate
ml	-	millilitre
µm	-	micrometre

mm	-	millimetre
mM	-	millimolar
NaCl	-	Sodium Chloride
NAD	-	Nicotinamide adenine dinucleotide
NADH	-	Reduced form of nicotinamide adenine dinucleotide
NaOH	-	Sodium hydroxide
nm	-	nanometre
O_2	-	Oxygen molecule
OD	-	Optical density
PAGE	-	Poly acrylamide gel electrophoresis
PO_4	-	Phosphate group
RNA	-	Ribonucleic acid
ROS	-	Reactive oxygen species
rpm	-	revolves per minute
SDS	-	Sodium dodecyl sulphate
SH	-	Sulfhydryl group
SHAM	-	Salicyl hydroxamic acid
TBA	-	Thio barbituric acid
TCA	-	Trichloro acetic acid
TEMED	-	$N,N,N^{1}N^{1}$ tetramethyl ethylene diamine
TES	-	N'(tris – hydroxy methyl) methyl – 2 – amino ethane sulfonic acid
Tris	-	Tris hydroxy methyl amino methane
V	-	volt

CONTENTS

INTRODUCTION

INTRODUCTION

Natural rubber harvested from the rubber tree (*Hevea brasiliensis*) emerged as the most versatile industrial raw materials produced from plants during 20th century. The *Hevea* or para rubber tree is the source of 95-98 percent of the natural rubber produced throughout the world (Sekhar, 2004). Currently, *Hevea* is planted in over 20 countries stretching from countries in South East Asia to Africa and some parts of Central and South America, with an estimated cultivated area of more than 9 million hectares.

Natural rubber latex is the cytoplasm of specialized tissues called laticifers, which are embedded in the bark tissues of the tree. Upon tapping laticifers are opened and latex is expelled which contains 30-60% rubber. The main use of natural rubber is in automobile tyres. In addition, natural rubber now finds extensive use in soil stabilization, vibration absorption and road making. It is also used for the manufacture of hoses, foot wear, battery boxes, foam mattresses, balloons, toys and etc.

The physiology of natural rubber tree is widely studied but most of the studies are confined to tapped trees. The changes in physiology and biochemistry of tapping panel compared to an untapped tree is not thoroughly explored. Tapping in *Hevea brasiliensis* is essentially a wounding process for the harvest of the crop. This process is known to enhance the metabolic activities of laticiferous sink tissue in order to

regenerate the components of the latex (Chrestin *et al.*, 1989). Tapping causes loss of carbon through enhanced respiration. Draining of large quantities of carbon resources through the harvesting of latex adversely affects the biomass of the tree. The reduction in biomass due to tapping is only partially accounted for by the removal of rubber through latex. The mechanism of unknown biomass loss (k factor) is yet to be studied thoroughly. A high yielding clone with a trait of low tapping induced biomass loss is desirable in the context of selection for latex-timber clones. In order to find out the metabolic changes in tapped trees and understand the reasons for the biomass loss a study was initiated in tapped and untapped trees of the clone RRII 105 at Rubber Research Institute of India, Kottayam.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Para Rubber

Hevea brasiliensis is the most important commercial source of natural rubber. Latex is obtained from the laticiferous enriched soft bark tissue of the rubber tree by the controlled wounding process known as tapping during which thin shavings of bark are removed. The aim of tapping is to cut open the latex vessels (d' Auzac *et al.*, 1997). Natural rubber, structurally cis-1,4-poly isoprene, is a high molecular polymeric substance with visco-elastic properties. Latex is a varying mixture of water, hydrocarbons, resins, oils, proteins, acids, salts, sugar etc. It contains 25-35% rubber hydrocarbon in small particles suspended in a serum together with 5-6% non-rubber substances. The remaining major component is water (d' Auzac, 1989).

Physiology of Untapped Tree

Tapping is initiated in a rubber tree when it attains 50 cm girth at a height of 150 cm from the bud union. In *Hevea brasiliensis* the trunk girth measurement and the calculated annual girth increment are widely used as parameters of growth, particularly during the period of immaturity (Shorrocks *et al.*, 1965).

The biomass production in an untapped tree is governed by the inherent potential of the tree namely, relative rates of photosynthesis,

partitioning and respiration and environmental variations like micro-climate influenced by canopy architecture, leaf area index and meterological factors, density of planting etc. Some of the assimilates produced in a tree are utilized for rubber biosynthesis in the laticiferous system. When a tree is tapped, a part of latex thus synthesized is extracted (Sethuraj and Raghavendra, 1987).

Untapped trees show lower respiration rates concomitant with lower total protein and sugars content in the bark tissues than a tapped tree (Annamalainathan *et al.*, 1998). The decreased concentration of proteins, starch and sugars suggests a lowered sink demand and metabolic activity in the untapped bark. The sucrose content in latex cytosol of untapped trees generally varies between 30 and 60 mM and exhibits a descending concentration gradient downwards along the trunk. The concentration of sucrose in the latex of untapped trees shows clonal differences, the proportion between the clones differed from those observed in tapped trees (Tupy, 1984).

Physiology of Tapped Tree

Tapping in a rubber tree elicits an array of physiological changes in the trunk. Tapping leads to latex flow, results in regeneration of rubber and latex constituents in the latex vessels. Regeneration phenomenon consists of a set of processes like, migration of latex from the displacement and re-equilibration areas to the drainage area, migration of reserves or

their products from their production or accumulation zones where regeneration of rubber and latex constituents occurs. These different phenomena take place progressively and the reconstitution of latex collected at tapping requires a certain amount of time which depends on the quantity taken from the tree (Tupy, 1984).

The introduction of regular tapping results in a drastic fall of sucrose content in latex, although the production is very low. Later there is a continuing increase of rubber production and also an increase of latex sucrose indicating that the enhanced sucrose transfer the newly induced sink (Tupy, 1984). In the long run, the level of latex sucrose in a commercially tapped tree consistently exhibits a depression with respect to untapped trees. Lower levels of sugar in latex indicate a better conversion of sucrose to rubber under conducive conditions. Accumulation of sugar is indicative of possible under utilization due to prevailing low or high temperature induced inhibition in the metabolic activities (Dey *et al.*, 1995). Compared to an untapped tree tapped tree has higher starch content in the bark tissue. The increased concentration of proteins, starch and sugars shows an enhanced sink demand and metabolic activity in the tapped tree. The total protein content has been reported to be higher in the bark from the tapping panel area than the opposite side of the panel (Annamalainathan *et al.*, 1998)

It needs sufficient time for reconstitution of the cell materials lost during tapping. Sucrose is the initial molecule in isoprenic synthesis. Its utilization is controlled by invertase, whose functioning is connected with latex production (Yeang *et al.,* 1984). Ions such as Mg2+, PO_4 and the SH groups also play a role in the activity of certain key enzymes in the isoprenic metabolism, such as invertase, pyruvate kinsae phosphoenol pyruvate carboxylase etc (Jacob *et al.*, 1983). The intracellular pH is an essential factor in the regulation of metabolism. Alkalinization of the contents of the laticifers causes considerable acceleration of the activity of certain enzymes such as invertase and PEP carboxylase (Chrestin *et al.*, 1985).

Protein synthesis, responsible for the production of enzymes involved in cellular metabolism, plays a large role in the regulation of the latex regeneration (Prevot, *et al.*, 1984). Total protein and sugars contents have been reported to be high in the bark of a tapped tree. Stimulation with ethephon also reported to fasten the regeneration process by activating the laticifers metabolism. It also considerably increases the availability of sugar in latex by the sink effect, alkalinizes the cytosol and activates protein synthesis (Neto *et al.*, 1984).

Tapping and Annual Biomass Increment

The biomass of a tapped tree is substantially lower than that of an untapped tree. The percentage of biomass loss in tapped trees compared to their respective untapped trees is different in different clones. The

relatively higher loss of biomass in the high yielding clones can be attributed to possible increase in their maintenance respiration. Maintenance respiration is generally high in tissues with high metabolic activity. Thus there may be more loss of biomass through respiration in the high yielding clones. The studies conducted in RRII showed that the unaccountable loss of biomass could possibly be due to an increase in the non-phosphorylating alternative respiratory activity (Annamalainathan *et al.*, 1998, 2001). Compared to an untapped tree, a tapped tree recorded more respiration including increased alternative respiration, a key component of the non-phosphorylating electron transport pathway.

The higher the yield, the greater the loss in the girth increment and hence the less the shoot dry weight increment. In general the bark thickness of the tapped trees tend to be higher than that of untapped trees, mainly due to the development of laticifers and phloem parenchyma. The extent of girth depression after tapping varies with the clone. Trees that reach early tappable girth (vigorous trees) tend to yield better than the less vigorous trees (Nugawela *et al.*, 2002).

Partition of Assimilates

Expression of the rubber producing efficiency of *Hevea* trees as the ratio of the weight of dry rubber formed to total dry weight accumulation of the tree during a given period of time is an indication of the partition of

assimilates between the two physiological pathways of growth and rubber formation.

Competition between the growth and rubber forming process is not likely to be based on competition for assimilates. d' Auzac and Pujarniscle (1961) and Chua (1967) had not been able to demonstrate any deficiency of carbohydrate materials in intensively tapped trees. More likely it is a competition for vital growth factors, quantities of which are lost in the serum at tapping, and the effect of such a loss would increase in intensity with rising productivity per unit length of tapping cut. Chua (1967) had observed that large quantity of proteins, RNA and energy-rich phosphate materials are lost in latex serum and possibly lead to a growth depression, since the formation of rubber isoprene is a high energy absorbing process requiring four ATP molecules for every isoprene molecule formed.

During the early years of tapping, yields rise slowly to a plateau, and remaining there for a number of years. In contrast, growth during these latter years is in a slow decline, it being the product of a little changing net assimilation rate and slowly declining leaf area ratio (Templeton, 1968).

Metabolic Changes in Tapping Panel

When virgin trees are freshly opened and tapping progresses, the rate of respiration in the bark gradually increases and this process induces a sink demand for sucrose, which is used as a substrate for respiration as well as rubber biosynthesis (Jacob *et al.,* 1992, Annamalainathan *et al.*, 2001).

The enhanced respiration found in the tapped tree is reflected in the extremely high concentration of ATP in the c-serum of the latex. Regular tapping stimulates latex biosynthesis for which a large quantity of ATP molecules are required. The metabolically active tapped trees with enhanced respiration produced more ATP and at the same time a lot of ATP was lost through serum. Either due to the excessive loss ATP through the latex and or due to the inadequate supply of ADP as a result of poor ATP recycling, the phosphorylating cytochrome pathway (cyt-c pathway) of mitochondrial electron transport chain could not handle all the NADH that was generated and this triggered the non-phosphorylating alternative pathway (Annamalainathan *et al.*, 2001). Stimulating the trees by ethephon application has been shown to increase the ATP content in the cytosol (Coupe *et al.*, 1989). The c-serum of the tapped tree has almost five fold larger ATP concentration than the serum collected from untapped trees. This indicates that regular tapping stimulates latex biosynthesis for which a large quantity of ATP molecules are required.

Tapping stimulates the respiratory rate in the laticiferous tissue (Sethuraj, 1992). The tapping panel area records higher respiration rate than the opposite side of the tapping panel. The respiratory activity on the opposite side of the tapping panel (untapped area of the tapped tree) is consequently higher than untapped tree (Annamalainathan *et al.*, 1998). Thus tapping, in addition to causing drain of vital resources through latex

also causes loss of photosynthates through increased respiration which can have a bearing on the biomass of the trees.

Respiratory Electron Transport Chain

Higher plants have two pathways of mitochondrial electron transport from ubiquinone to oxygen (Umbach and Siedow, 1997). The respiratory chain consists of four main multienzyme complexes and of two smaller sized components (ubiquinone and cytochrome). Two complexes are responsible for NAD-linked substrate (complex-I) and succinate (complex-II) oxidations. Both complexes consist of a flavoprotein associated with several iron-sulfur centers (Beinert and Palmer, 1965). Ubiquinone plays a central role as an obligate intermediate carrier for electrons coming from the substrates and on their way to oxygen. In plant mitochaondria, two accesses to oxygen are offered to the electrons. One is made by complexes III and IV plus cytochrome c. Electron transfer through the cytochrome pathway is coupled to the generation of an electrochemical gradient used to produce ATP. This pathway is sensitive to cyanide and antimycin and hence also known as cyanide sensitive pathway.

The alternative pathway or the cyanide resistant pathway is one of the special features of plant respiration. This pathway, which shares electrons from the ubiquinone pool with cytochrome pathway, is not coupled to ATP synthesis (Wagner and Krab, 1995). So far the only known function of the alternative pathway is related to the thermogenesis during

the anthesis of Arum species (Meeuse, 1975). However, the knowledge about the role of AOX is now being extended. It is thought that the alternative pathway might play a role in preventing the formation of toxic oxygen species when the normal activity of the cytochrome pathway is restricted by any environmental constraints (Purvis and Shewfelt, 1993).

Latex Physiology and Biochemistry

Latex is a specialized form of cytoplasm containing a suspension of rubber and non-rubber particles in an aqueous serum. It can be separated in to four layers, a white upper layer of rubber particles, an orange or yellow layer containing Frey-Wyssling particles, an aqueous serum named c-serum and a bottom fraction containing grayish yellow gelatinous sediments by ultracentrifugation (d 'Auzac and Jacob, 1989). Among these layers, the third one is an aqueous serum named c-serum. The serum contains-aminoacids, proteins, carbohydrates, organic acids, inorganic salts and nucleotide materials.

Natural rubber latex is a rich repository of photosynthates in different forms and several other vital resources such as proteins, minerals etc. C-serum contains nearly half the enzymes detected in the latex. Most of the c-serum proteins are anionic. The proteins are considered to be an alpha-globulin in view of its characteristics. Enzymes of the glycolytic pathway as well as enzymes of rubber biosynthesis are found in serum (Gidrol *et al.*, 1994).

The bottom fraction of latex contains Frey-Wyssling particles, mitochondria and other particulate components of plant cell having density greater than that of serum. Hydrocarbon is 30-45% of latex volume. Hydrocarbons are having the shape of spherical, oval or pear shape. 10-20% of latex is lutoids. They are subcellular membrane bound bodiesranging in size from 2-5μm. The membrane encloses a fluid serum known as lutoid serum or B-serum (Jacob *et al.*, 1989). Sucrose, quebrachitol (methylinositol) and glucose are the major carbohydrates in latex cytosol. It is mainly used for respiration and sugar nucleotides are used for the synthesis of various glycosides in cellular structures (Chrestin *et al.*, 1986).

Total protein content in latex is 1%. Major protein in the serum phase is α-globulin. C-serum contains most of the enzymes. B-serum contains a protein known as heavin. Lutoids from young latex vessels contain a protein deposited in the form of bundle of microfibrils. Two basic proteins are also present in bottom fraction Heveamine A, a cationic protein and a minor one have lysozyme and chitinase activities. These proteins are involved in the mechanism of latex flow (Wickramarachchi, 2001) Main lipids present in latex are fats, waxes, sterols, sterolesters and phospholipids. These lipids play a vital role in the stability and colloidal property of latex (Nair *et al*, 1993).

Most of classic aminoacids are present in latex. Low molecular weight thiols, which are the main reducing agents in latex, include glutathione and cysteine are also present in latex. Ascorbic acid is a very important reducing agent in latex. Malic acid and citric acid make up 90% of organic acids in latex. potassium, magnesium, copper, iron, sodium, calcium and phosphorous form 0.5% of latex (Nair, 2000).

MATERIALS AND METHODS

MATERIALS AND METHODS

Plant Material

Hevea, clone RRII 105, planted during 1988 in Rubber Research Institute of India was selected as the experimental plant material. Trees were tapped in the 1/2S d/2 system and 15 trees were left untapped from 1998 onwards. Ten trees each from the tapped and untapped population were randomly selected from a compact area for the present study during 2005. Bark samples were collected from just below the tapping cut in the tapped trees. Latex was sampled in the morning hours. Corresponding samples were collected from the untapped trees also.

Accounting of Biomass

The shoot biomass of tapped and untapped trees was calculated using the Shorrocks regression model:

$W=0.002604\ G^{\wedge 2.7826}$ (Shorrocks, 1965),

where G is trunk girth (cm) at the height of 150 cm from bud union.

Isolation of Mitochondria

Mitochondria were isolated from bark tissue according to the modified methods of Day *et al.* (1985) and Zhang and Wiskich (1995) from approximately 5g of tissue. Tissue was grind in liquid nitrogen and then in phosphate buffer (pH 7.5). The homogenate was filtered through 4 layers of miracloth and centrifuged for 5 min at 1100 g. The supernatant was centrifuged for 20 min at 18000 g and the pellet resuspended in 10 ml

of wash medium (0.3 M sucrose, 10 mM TES, 1 mM glycine, pH 7.5) and centrifuged for 5 min at 1100 g. The supernatant was centrifuged for 20 min at 18000 g. The mitochondria were found as a tight light yellow-brown band at the bottom of the tube. The final mitochondrial pellet was resuspended in approximately 1 ml of wash medium.

Respiration Assay

Tissue respiration:

A very thin slice (approximately 0.5 mm uniform thickness) of 150 mg fresh laticifers enriched soft bark tissue (just adjacent tissue of cambium) was used for the measurement of dark respiration by using a Clarke type oxygen electrode (Hansatech, UK) as described by Lambers *et al.*,(1983) and modified by Annamalainathan *et al.*,(1998). The assay buffer (pH 7.2) contained 10 mM KH_2PO_4, 10 mM NaCl, 2 mM $MgSO_4$, 0.1 % BSA and 100 mM sucrose.

The cytochrome and alternative pathways of respiration were measured by adding appropriate inhibitors. The alternative pathway was inhibited in soft bark tissue after incubating the tissue in 3 mM salicyl hydroxamic acid (SHAM) for ten minutes as described by Millenaar *et al.*,(1998). Previously the requirement of optimum concentration of SHAM (stock solution in methoxyethanol) for maximum inhibition of alternative pathway was standardised. To inhibit cytochrome pathway the tissue was incubated with a range of KCN from 50 to 500 µmole and at 500 µmole of

KCN maximum inhibition was found. The respiration was measured after 10 min. of pre-incubation with the inhibitors.

Respiration assay in isolated mitochondria:

For mitochondrial respiratory assay the reaction medium contained: 0.3 M mannitol, 10 mM TES-KOH pH 7.5, 5 mM KH_2PO_4, 10 mM NaCl, 2 mM $MgSO_4$ and 0.1% (w/v) bovine serum albumin and all measurements were carried out at 25 °C. Calibration of the electrode was carried out by the addition of sodium dithionite to remove all oxygen in the electrode chamber and the oxygen concentration was assumed to be 240 µM. The maximum alternative respiration (AOX) or potential AOX activity was measured by the addition of a cyt-pathway inhibitor (antimycin) followed by addition of AOX inhibitor (salicylic hydroxamic acid). Addition of various substrates (electron donors) and effectors was made to ensure the respiration may not be limited by substrate supply.

Estimation of Malondialdehyde (MDA)

The method of Heath and Packer (1968) was used for the estimation of MDA in bark tissue. Approximately 300 mg of tissue was homogenised in liquid nitrogen and added 2.5ml buffer, in a cold pestle and mortar, followed by centrifugation at 8000 g for 20 min. The supernatant was added with 1 ml of TBA solution (20% (w/v) trichloroacetic acid, 0.01% (w/v) butylated hydroxytoluene). The samples were vortexed and heated to 95 °C for 30 min, followed by cooling on ice for 5 min. The samples were

then spun at 3000 g for 10 min and absorbances of each sample read at 440nm, 532nm and 600nm, using Shimadzu spectrophotometer. The amount of MDA equivalents was calculated from the equation below.

$$\frac{\Delta O.D \times \text{Total}}{1.56 \times 10^5 \times \text{aliquot volume}} \times \frac{1}{\text{Fr.Wt}} = \text{moles/g}$$

Estimation of total Protein

Total protein content of the soft bark tissue was extracted in 50 mM Tris buffer (pH 7.4) with 2 percent SDS. Soluble protein of the bark tissue was extracted with 50 mM Tris buffer (pH 7.4) and centrifuged at 10, 000 rpm for 20 min. The supernatant was taken for soluble protein analysis. Total and soluble proteins were quantified by the method of Lowry *et al.* (1951).

Estimation of Total Sugars

Total sugars from the soft bark tissue were extracted in 80 percent ethanol and estimated by the method described by Scott and Melvin (1953). To a cold mixture of 27ml water and 100ml concentrated sulphuric acid, 100mg anthrone was added. To 0.1ml of tissue extract 3.0 ml cold anthrone reagent was added. Heated the mixture in a boiling water bath for 10 minutes. After cooling the mixture reading was taken at 627nm OD. Standards were also treated in the same way as samples.

Estimation of Starch

The residue of the ethanol extract of tissue was digested by using perchloric acid and the starch content was quantified by the method of McCready *et al* (1950).

Extraction and Estimation of ATP in Latex

About 1gm fresh latex was extracted with 2.5% TCA and made up to 100ml. Filtered the solution using Whatmann No.1 filter paper and 2 ml of filtrate was used for ATP estimation. TCA was removed by successive extractions with cold ether. The residual ether was evaporated. The samples were neutralized with 0.1N NaOH and volume made up to 10ml with 30mM Hepes buffer (pH 7.4).

Principle

The ATP content in latex was measured luminometrically (luminometer- Stratec Electronic GmbH, Brikenfeld, Germany) as described by Fader and Kollar (1984) using a bioluminescent assay kit (Sigma FL-AA). Here ATP is consumed and the light is emitted when firefly luciferase catalyses the oxidation of D-luciferin.

$$\text{ATP + Luciferin} \longrightarrow \text{Adenyl luciferin + Ppi}$$

$$\text{Adenyl luciferin + Oxygen} \longrightarrow \text{Oxyluciferin + AMP + } CO_2 \text{ + light}$$

SDS-PAGE Analysis of Proteins

Analysis of the proteins like total and soluble proteins of soft bark tissue, mitochondria and c-serum was carried out by SDS-PAGE according

to the method of Laemmli (1970). The composition of the various solutions is as follows.

a. Sample buffer (for 10ml)

0.5 M Tris-Hcl, pH 6.8	2.5 ml
Beta-mercaptoethanol	2.5 ml
Glycerol	2.5 ml
1% bromophenol blue	1.25 ml
Distilled water	1.25 ml

b. Separation gel buffer (for 30ml)

Acrylamide (30%)	12 ml
0.5 M Tris-HCl, pH 8.8	7.2 ml
Distilled water	10 ml
10% SDS	0.3 ml
10% APS	0.15 ml
TEMED	10 µl

c. Stacking gel buffer (for 10ml)

Acrylamide (30%)	1.35 ml
0.5 M Tris-HCl, pH 6.8	3.0 ml

Distilled water	5.5 ml
10% SDS	0.1 ml
10% APS	0.05 ml
TEMED	5 µl

d. Acrylamide stock (30%)

Acrylamide	30.0 g
N, N-methylene bisacrylamide	1.6 g

Distilled water added to make up to 100 ml

e. Running buffer

50 mM Tris-Hcl, pH 8.3	3.0 g
Glycine	14.3 g
SDS	1.0 g

Preparation of separating gel:

A linear gel of 1.5 mm thickness was prepared by adding 30% of acrylamide solution followed by 0.5 M Tris-HCl, distilled water, 10% SDS, 10% APS and TEMED.

Preparation of stacking gel:

The stacking solution was layered over the separating gel after inserting a comb and was allowed to polymerize. Protein samples were mixed with equal volume of sample buffer and heated to 100 ^{0}C for 3 min.

After cooling to room temperature the samples were centrifuged at 10, 000 g for 2 min. The supernatant was loaded on the gel and was run at 50 V till the samples cross the stacking layer. Then the voltage was increased to 120 V. Electrophoresis was carried out at 20 ^{0}C.

Staining and Destaining:

The gel after electrophoresis was immersed in staining solution. The stain was prepared by dissolving 500 mg of coomassie brilliant blue (sigma) in 80 ml of methanol, 100 ml of distilled water and 20 ml of glacial acetic acid. The gel was stained for 6 h and destained with 40% methanol and 10% acetic acid mixture for 12 h. The destained gel was preserved in 7% acetic acid solution.

RESULTS

Figure 1. Tapped and untapped trees of *Hevea brasiliensis* (Clone RRII 105) planted in RRII campus, Kottayam.

RESULTS

The tapped and untapped trees of the most popular and high yielding rubber clone RRII 105 was selected as the study material. These trees were analyzed for various metabolic activities and biochemical composition like tissue and mitochondrial respiration, carbohydrate and protein status of laticiferous tissue and ATP content of latex. Additionally studies were carried out to compare the protein profile of soft-bark tissue, mitochondria and latex serum of tapped and untapped trees.

Figure 1 shows the field photograph of tapped and untapped trees. Both the tapped and untapped trees were 17 years old when the present experiments were done. Tapping was initiated during 1998 and one set of trees (around 15) was left untapped. The biomass accumulation of untapped trees was significantly higher than tapped trees (Fig.2).

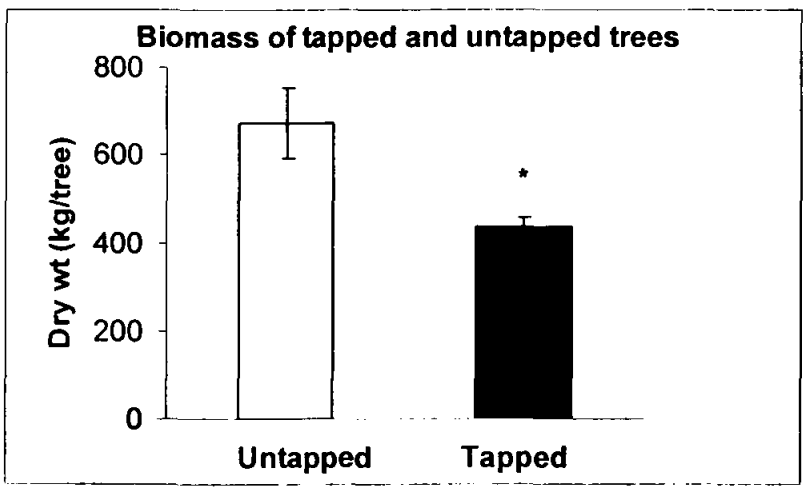

Figure 2. The estimated shoot biomass of untapped (open box) and tapped (closed box) of *Hevea*, clone RRII 105. n = 10,
* indicates significantly different at 5% level.

After seven years of tapping, the trees lost around 34% of biomass as compared to untapped trees. When an untapped tree recorded around 673 kg of dry weight, the tapped tree had only 433 kg. The loss of biomass in a tapped tree was reported to be high even after adding the higher energy value for the rubber yield. Thus there was a missing biomass commonly referred as 'k' factor (Sethuraj, 1992). There would be differential metabolic activities in a tapped tree compared to an untapped tree. Therefore, in order to find out the changes in metabolic activities of tapping panel area the respiratory activities of bark tissues as well as isolated mitochondria were carried out.

Tapping resulted in an enhanced respiratory activity. The soft bark tissue respiration, including cyt-c and alternative oxidase mediated oxygen uptake rates were higher in tapped trees compared to untapped trees (Fig. 3). The residual respiration also was found to be significantly high in tapped trees. It represents the non-respiratory oxygen consumption by other oxidizing enzymes and activities of secondary metabolites.

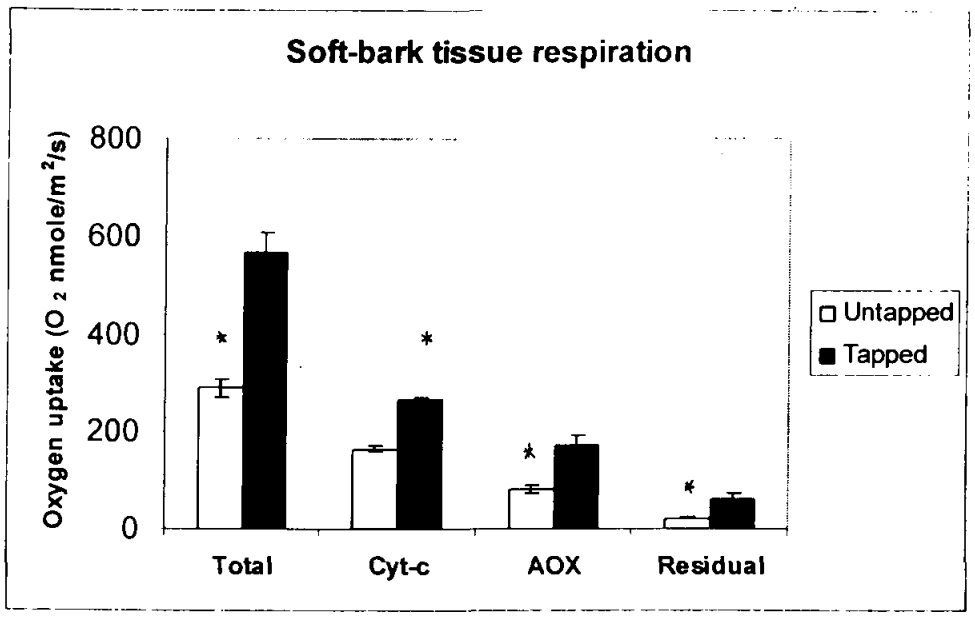

Figure 3. Soft bark tissue respiration in untapped (open box) and tapped (closed box) trees of *Hevea*. n = 6, * indicates significantly different at 5% level.

In order to avoid the interference/errors owing to secondary metabolites the respiratory rates were measured in isolated mitochondria from untapped and tapped tree soft-bark tissue (Fig. 4). NADH was used as a substrate for mitochondrial electron transport chain (ETC) reactions. The NADH dependant total respiration rate was significantly higher in tapped trees. The alternative oxidase mediated oxygen uptake also significantly increased in a higher order due to tapping. The potential or maximum capacity cyt-c and alternative oxidase activities were measured with the addition of ADP in the presence of appropriate inhibitors of electron transport chain and they were found to be higher in a tapped tree than in an untapped tree (Fig. 4). The potential rate of alternative respiration in

mitochondria was recorded in which the cytochrome-c activity was impaired. The capacity is generally defined as the oxygen uptake resistant to the cyt pathway inhibitor and sensitive to the AOX inhibitor.

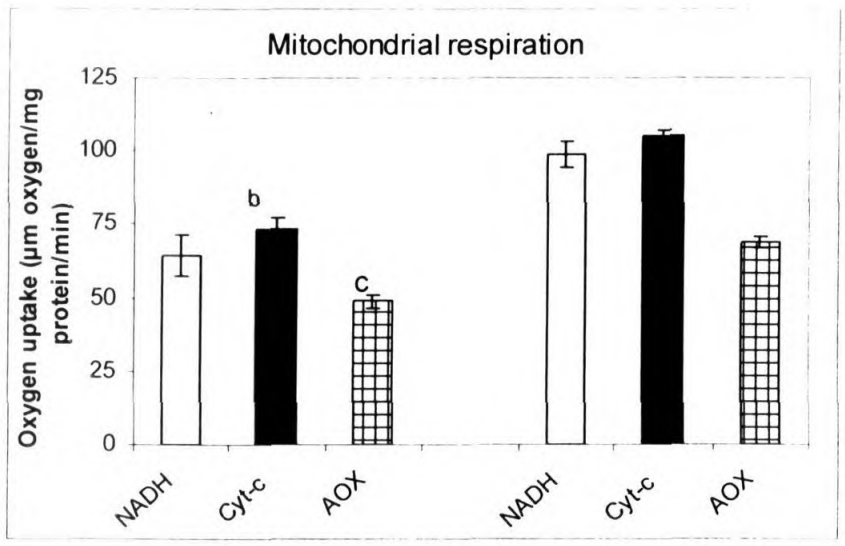

Figure 4. The rate of mitochondrial respiratory O2 uptake (µM O2/mg protein/min). Mitochondria were isolated from soft bark tissue of untapped and tapped trees of *Hevea*. Histograms with different alphabets are significantly different.

ATP content in the latex was higher in tapped trees than the untapped trees (Fig. 5). Tapped trees recorded around 4 fold increase in ATP level. This agrees with the fact that regular tapping stimulates latex biosynthesis for which a large quantity of ATP molecules are required. The latex volume was accounted on the day of bark sampling for respiration and a relationship was established. Respiration rate increased with an increase in latex volume (Fig. 6), indicating requirement of increased metabolic activities for a higher latex biosynthesis.

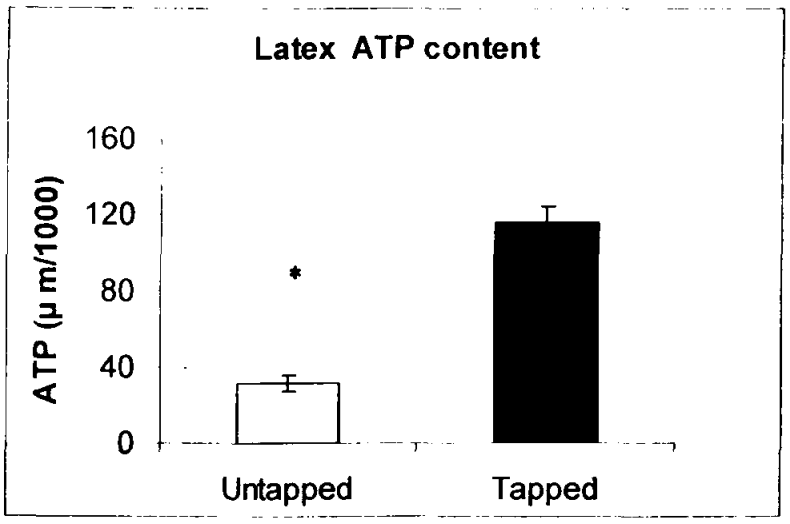

Figure 5. ATP content of latex in untapped (open box) and tapped trees (closed box) of *Hevea*. * indicates significant at 5% level.

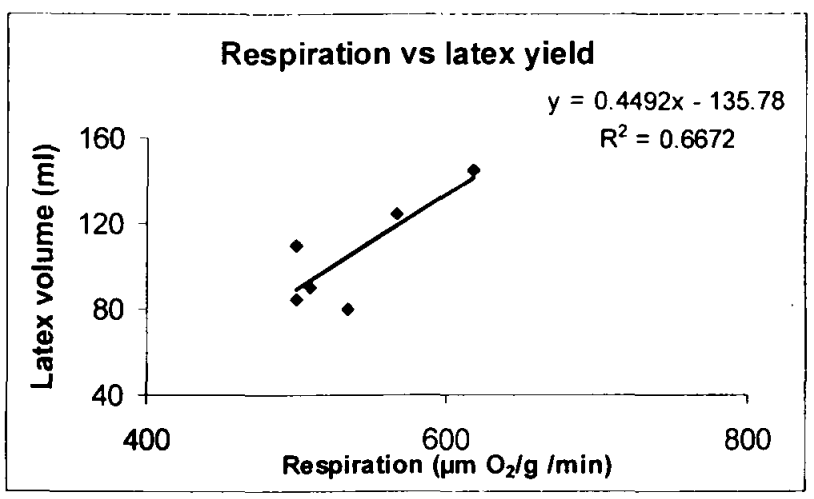

Figure 6. Relationship between respiration rate and latex yield (ml) in tapped trees of *Hevea*.

The quantity of total sugars in the tapped trees did not have any significant difference from the untapped trees. However, sugar content was slightly higher in soft bark tissue of tapped trees (Fig.7). Tapped trees had significantly higher starch content than untapped trees. Starch is accumulated in tissues and converted to simple sugars for the biosynthesis of isoprene in laticiferous tissue. Tapped trees showed high metabolic rate

concomitant with high carbohydrates content. The increased concentration of carbohydrates suggests an enhanced sink demand and metabolic activity in the tapped bark compared to the untapped bark. Tapped trees recorded significantly higher level of protein content than untapped trees in the soft bark tissue (Fig. 8).

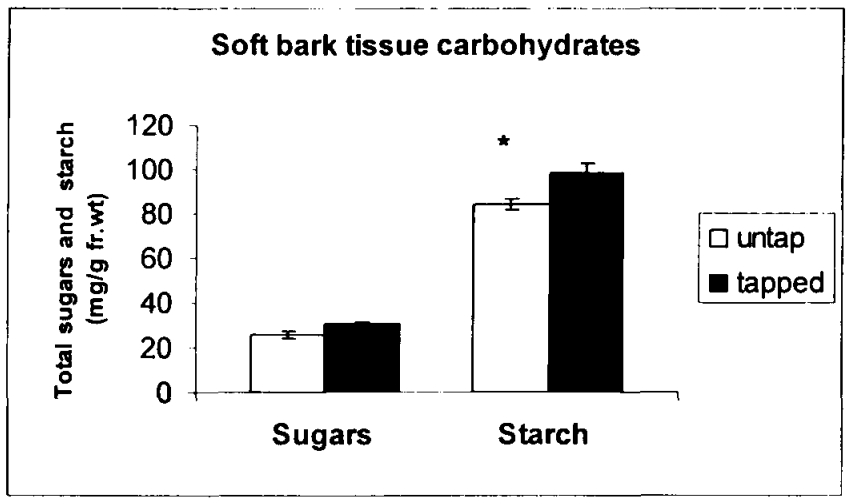

Figure 7. Sugars and starch content in soft bark tissue of untapped(open box) and tapped (closed box) trees of *Hevea*. * indicates significant difference at 5% level.

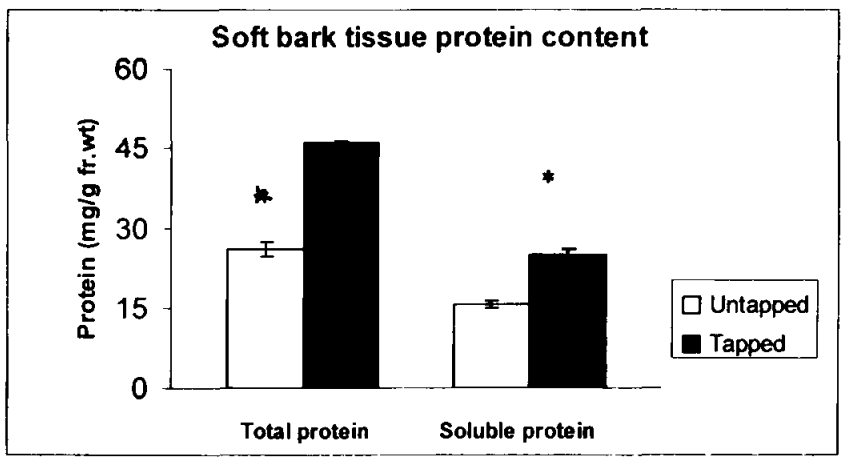

Figure 8. Total and soluble protein content in soft bark tissue of untapped(open box) and tapped (closed box) trees of *Hevea*. * indicates significant difference at 5% level.

Compared to untapped trees tapped trees had more malondialdehyde (MDA) content (Fig.9). MDA is a product of lipid peroxidation. Tapping mediated wounding inflicted mechanical stress in tapped trees. This stress might have lead to the production of ROS species. These ROS ultimately cause lipid peroxidation of bi-layer membranes of the mitochondria and other cell organelle.

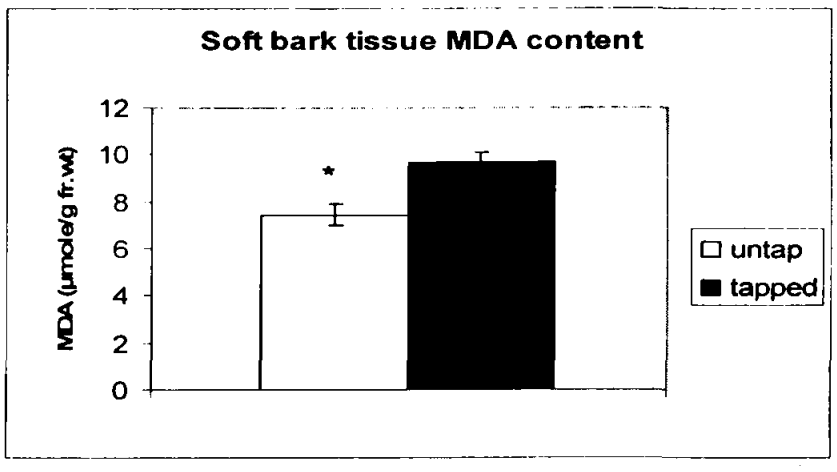

Figure 9. Malondialdehyde content in soft bark tissue of untapped (open box) and tapped (closed box) trees of *Hevea*. * indicates significant difference at 5% level.

The SDS-PAGE profile of mitochondria shows an enhanced level of protein in the approximate molecular weight range of 70-72 kDa in the tapped trees (Fig 10). Tapped trees also recorded higher expression of a 82 kDa protein in the soluble protein fraction as well as in the total protein profile whereas the level of expression of same protein was small in the untapped trees (Fig 11). The c-serum of tapped trees showed differences in many proteins with enhanced levels in the molecular weight range of 12, 18, 38-40 and 62 kDa (Fig. 12).

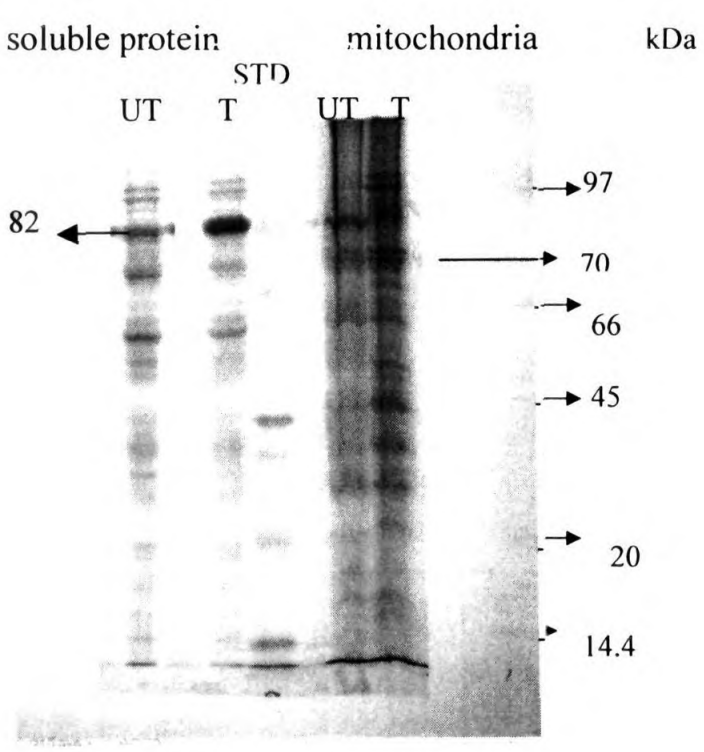

Figure 10. SDS-PAGE profile of soluble protein and mitochondrial protein of soft bark tissue of untapped(UT) and tapped (T) trees of *Hevea*. Molecular weight standards and changes in protein bands are indicated.

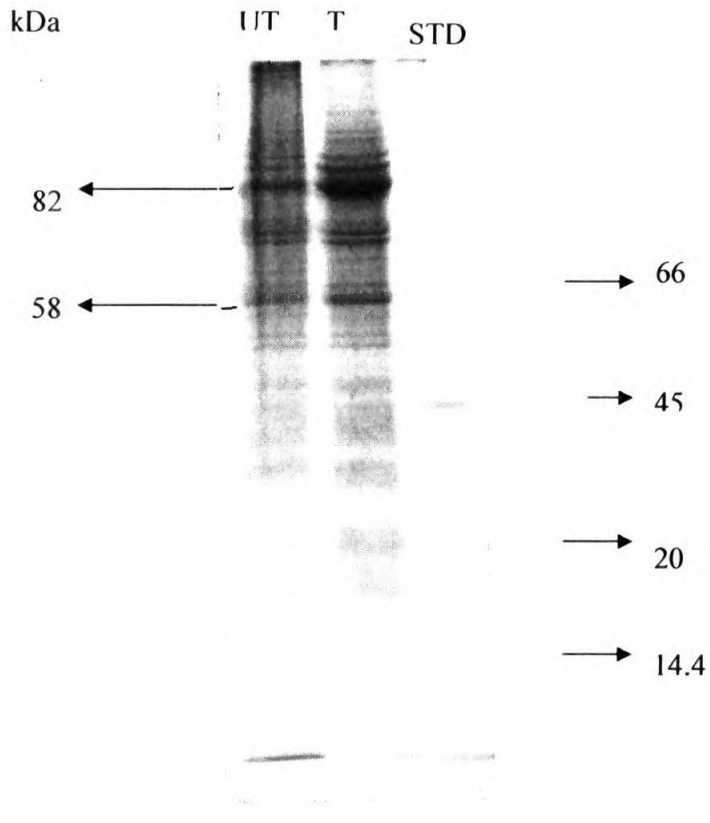

Figure 11. SDS-PAGE profile of total proteins extracted from soft bark tissue of untapped (UT) and tapped (T) trees of *Hevea*. Molecular weight standards (STD) and changes in protein bands are indicated.

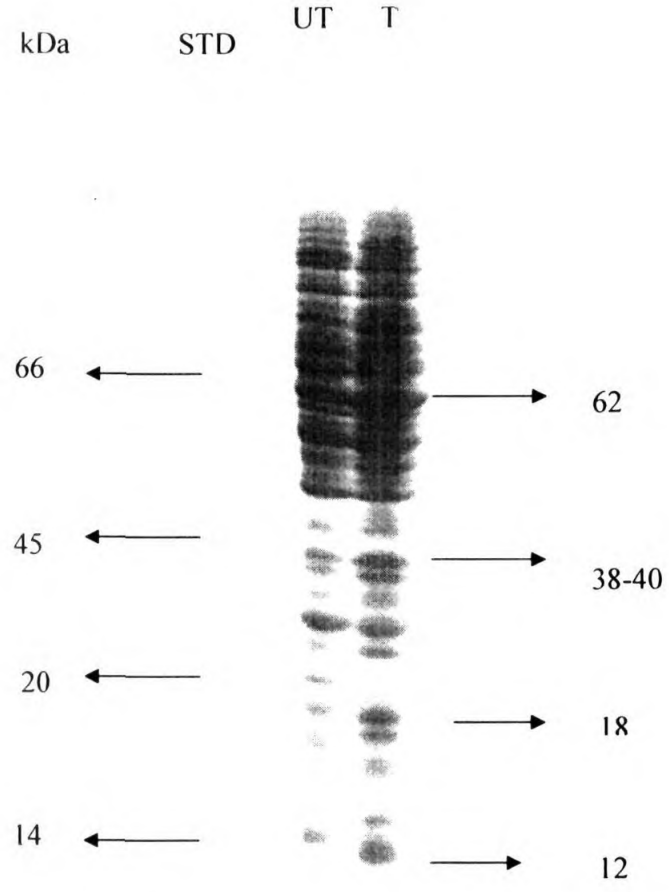

Figure 12. SDS-PAGE profile of latex c-serum proteins collected from untapped (UT) and tapped trees (T) of *Hevea*. Molecular weight standards (STD) and changes in proteins are indicated.

DISCUSSION

DISCUSSION

Tapping in natural rubber tree is a necessary wounding process for the harvest of the latex. The harvested latex contains around 30-40% dry rubber content. The remaining portion of the latex comprises water, serum proteins, sugars and lot of minerals like K, PO_4 and etc. Between two successive tapping days the tree has to recoup the latex. Thus the laticiferous sink activity is significantly increased in a tapped tree compared to an untapped tree. The tree loses a lot of resources other than the energy rich polyisoprenes through latex. Latex is a rich repository of photosynthates. In tapped trees there is partition of assimilates between the two physiological pathways of growth and rubber formation. However, the percentage of allocation for these two processes is a clonal character (Templeton, 1968). Such a partitioning process is not warranted in an untapped tree except for a maintenance factor with small amount of latex in their latex vessels. Therefore the biomass of a tapped tree is significantly lesser than that of an untapped tree of similar age. Tapping additionally causes loss of photosynthates through increased respiration which can also have a bearing on the biomass of the trees.

In the present study the loss of biomass after seven years of tapping was around 34%. It has already been reported that RRII 105 has the highest

percentage of biomass loss in a study in which 10 clones were included (Annamalainathan *et al.*, 1998). There was a direct positive relationship existing between the yield and shoot biomass loss (Sethuraj, 1992).

Tapped trees recorded higher cyt-c activity for the enhanced metabolism including rubber biosynthesis. For maintaining higher biosynthesis it has been reported that maintenance respiration was generally high in tissues with high metabolic activity (Szaniawski, 1981). The enhanced respiration found in the tapped tree was related to the extremely high concentration of ATP. The results also showed that lot of ATP was lost through the serum and that may have a bearing on the biomass in a tapped tree.

The increased rate of alternative respiration (AOX) and its implication in the metabolic status are studied in a few plants. In the present study the tissue and mitochondria isolated from bark tissues adjacent to the tapping panel area recorded 1.5 to 2 fold increase in AOX activity compared to the untapped tree. This pathway is non-phosphorylating and operation of proportionately higher rate of AOX is required to maintain the same energy status as for the cyt-pathway.

The increased rate of AOX recorded in the tapped tree could be explained by wound induced diversion of electons to AOX, eventually more heat generation (Ribas-Carbo *et al.*, 2000). Alternative pathway

branches from the classical cytochrome pathway at the ubiquinone site, thereby bypassing two out of the total three phosphorylating sites. Therefore 65% energy of the electron remains uncoupled (Lambers, 1980; Moore and Siedow, 1991). The energy thus dissipated is as heat. As reported by Laties (1982) wounding also induce increased mitochondrial respiration resulting in heat production.

It is known that tapping induces hyper metabolic rate resulting in the production of high level of NADH and likely that the excess electrons from the substrate is being directed through AOX pathway as an 'overflow' mechanism to oxidise the excess NADH. Thereby possible stabilization of the ubiqinone pool and preventing ROS generation could have taken place in mitochondria (Ribas-Carbo *et al.*, 2000). The alternative pathway plays the important role of by passing the electrons transport when the normal activity of the cyt-pathway is restricted or impaired by any inhibitors or other cellular parameters which are not conducive (Wagner, 1995; Yip and Vanlerberghe, 2001). Further it has been implicated that cyanide resistant and SHAM sensitive alternative respiration is associated with thermogenesis in the flowers of inflorescence of Araceae, Annonaceae and Aristolochiaceae (Raskin *et al.*, 1987).

There was a positive relationship existing between the total sugars, starch and protein content of soft bark tissue and rate of respiration. The

level of carbohydrates determines the rate of respiration and control energy status of the tissue (Krishnakumar *et al.*, 1998). Rate of respiration was positively correlated with the concentration of sugars, starch and protein content in *Hevea* bark tissue (Annamalainathan *et al.*, 1998). The gel profile also showed increased level of certain proteins like the 82 and 70 k Da peptides in the soft bark and mitochondria of tapped trees. This might be due to the over expression of certain enzymes due to tapping and enhanced sink demand. Serum also showed enhanced amounts of certain proteins indicating that induction of rubber biosynthetic enzymes and other proteins related to flow of latex. Due to high metabolic activity, tapped trees may contain certain enzymes which may be present in an insignificant amount in untapped trees.

During the course of enhanced respiration in the mitochondria and other metabolic activities in laticiferous system, production of ROS is inevitable. The NADH-quinone reductase in the lutoid membrane is a potential source of such toxic O_2 in latex containing tissues, eventually trigger lipid peroxidation of membranes. In the present study it was found that the soft bark tissue of tapped trees recorded increased MDA content. Under normal condition a set of antioxidant enzymes are active and detoxify the free radicals. However, when the capacity of defense mechanism inadequate to scavenge the radicals the tissue succumbs to the oxidative stress result in peroxidation of lipids and proteins (Asada, 1992).

Annamalainathan *et al.*, (2001) have reported increased activities of ROS scavenging enzymes in soft bark tissues of tapped trees.

The present study shows how tapping stimulated enhanced respiration, particularly the alternative respiration in *Hevea* bark tissue. Tapping iunduced alternative respiration indicate diversion of excess electrons through non-phosphorylative ETC, possibly due to metabolic stimulation and resultant increased production of NADH. Tapping also stimulates the ATP turn over. The excess ATP molecules are drawn in latex. Tapping induced wounding process also could result in oxidative stress ultimately peroxidation of membranes. These are all factors that might explain how a tapped tree is loosing its biomass, that is not accounted. Tapping also stimulates the sink demand and biosynthesis of rubber as evidenced from the increased level of certain enzymes in bark and c-serum of tapped trees.

SUMMARY

SUMMARY

The objective of the present study is to analyze the physiological and biochemical changes in bark tissue of tapped trees in comparison with the similar aged untapped trees of *Hevea brasiliensis*. The trees were analyzed for various metabolic activities and biochemical compositions like tissue and mitochondria respiration, carbohydrate and protein status of laticiferous tissue and ATP content of latex. Additionally a few experiments were carried out to compare the protein profiles of soft bark tissue, mitochondria and the latex serum of tapped and untapped trees.

The biomass accumulation of untapped trees was significantly higher than tapped trees. Compared to untapped trees the tapped trees recorded increased rate of tissue and mitochondrial respiration and carbohydrate and protein contents. Tapping also stimulated more ATP production that was reflected in latex. The lipid peroxidation rate was higher in tapped bark tissue than untapped trees. The SDS-PAGE protein profiles of mitochondria and c-serum of latex revealed increased expression of certain soluble proteins in tapped trees.

The present study explains how tapping stimulated the respiration and other sink activities in *Hevea* bark tissue. Tapping particularly stimulated the alternative respiration which is non-phosphorylative waste

process. Tapping also stimulated higher ATP turn over and the excess ATP molecules were lost in latex serum. The tapping mediated wounding process resulted in setting of oxidative stress in bark tissue. These are all factors explain how a tapped tree is loosing its biomass that is not accounted for by the dry rubber yield.

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