Research Article

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# Tapping induced changes in respiration rate, biochemical status and ATP content in soft bark tissue of natural rubber plant

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**Abstract**

Various metabolic changes in the bark tissue of rubber trees under tapping were sudied in comparison with untapped trees. Dark respiration including cytochrome-c alternative respiration (AOX) and residual respiration rates werer increased in soft bark tissue of tapped trees. Similar trend in respiration rates was also observed in isolated mitochondria from tapped trees. In an unopened (untapped) tree, the non-phosphorylativ alternative respiration rate was smaller than trees under regular tapping. The day to day metabolic changes in tapping panel area in newly opened trees were studied. The rate of respiration including AOX was gradually increased when tapping progressed. The respiratory substrate, sugar content, suddenly declined in bark tissue of newly opened trees and gradually increased in subsequent tapping days and reached a stable level within two weeks. The ATP contents decreaseed progressively in the soft bark tissue after tapping. On the contrary ATP content steeply increased in the late serum as the tapping days progressed. Tapping induced sink demand and enhanced metabolic activities in the laticiferous tissue were demonstrated by increased levels of carbohydrates and proteins in the soft bark tissue and increased ATP and certain polypeptides inthe range of 62, 38 and 12 k Da size in latex serum. However, bark tissue was undergoing oxidative stress as evident from the increased levels of malondialdehyde (MDA) in tapped trees. The result was discussed in the purview of possible mechanisms involved in unaccountable biomass loss in tapped trees.

*Keywords:* Alternative respiration (AOX), biomas, *Hevea brasiliensis* mitochondira, respiratiion, tapped and untapped trees

## Introduction

Tapping in *Hevea* is a necessary wounding process for the harvest of crop. The key changes in the tapping panel area re increased activities of rubber biosynthetic enzymes (Jacob *e t al.*, 1989) in order to recoup the latex synthesis through activation of respiratory election transport and ATP production (Chrestin *et al.*, 1989; Annamalainathan *et al.*, 2001).

When virgin trees are freshly opened and tapping progresses, the rate of respiration in the bark gradually increases and the tapping process induces a sink demand for sucrose, which is used as a substrate for respiration as well as rubber biosynthesis (Jacob *et al.*, 1989, Annamalainathan *et al.*, 2001). In tapped trees the enhanced bark tissue respiration results in 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. A posititve relationship between latex ATP and latex yield was already reported (Sreelatha *et al.*, 2004).

Tapping stimulates the respiratory rate in the laticiferous tissue. The tapping panel area records higher respiration rate than the untapped area of the tissue. The respiratory activity on the untapped area of the tapped tree is consequently higher than untapped tree (Annamalainathan *et al.*, 1998). Thus tapping, in addition to causing drainage of vital resources through latex also causes loss of photosynthates through increased respiration which can have a bearing on the biomass of the trees. Therefore, a tapped tree loses biomass that is not realized by its rubber yield. The exact reasons for the unaccountable biomass loss in tapped trees are not yet studied throughly.

It has been reported that the cyanide resistant alternative respiratory pathway is induced in tapped trees (Annamalainathan *et al.*, 2001). This is a non phosphorylative pathwya, shares electron from the ubiquinone of electron transport chain in mitochondria and is not coupled to ATP synthesis. The terminal oxidase of this pathway is the alternative oxidase (AOX). The only

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known function for this respiratory pathway is related to the thermogenesis in the anthesis of Arum family (Rask in *et al.*, 1987). The exact role of AOX in plant metabolism remains uncertain. A few evidences are accumulating that it plays a role in strees averting mecahnisms by minimizing the generation of reactive oxygen species (ROS) (Purvis and Shewfelt, 1993; Robson and Vanlerberghe, 2002). Mitocheondrial electron transport chain is a powerful source of free radicals in non-green tissues. The implications of the AOX pathway in tapped trees ar not yet clear.

The present study examined the physiological changes in bark tissues owing to the tapping process and various other factors which are responsible for the tapping induced loss of biomass in rubber trees.

## Materials and methods

### Plant material

*Hevea*, clone RRII 105, planted during 1988 in Rubber Research Institute of India was selected as the experimental plant materials. Trees were tapped in the ½ S d/3 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 Shorrock's regression model:

$W+0.002604\ G^{2.393}$ (Shorrocks *et al*, 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). Approximately 5 g tissue was powdered in liquid nitrogen and then homogenized 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 resuspende din 10 ml of wash medium (0.3 M sucrose, 10 mM TES, 1 mM glycine, pH 7.5) and centrifuged at 1100 g for 5 min. The supernatant collected 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 esuspended in wash medium.

### Respiration assay

#### a. Tissue respiration

A thin slice (approximately 0.5 mm uniform thickness) of 150 mg fresh laticifers enriched inner soft bark tissue 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 electrode chamber buffer (*p*H 7.2) contained 10 mM $KH_2PO_4$, 10 mM NaCl, 2 mM $MgSO_4$, 0.1% BSA and 100 mM sucrose.

The cytochrome c (cyt c) and alternative pathways of respiration were measured by adding appropriate inhibitors. The alternative pathway in soft bark tissue was inhibited by incubating the tissue in 3 mM salicyl hydroxamic acid (SHAM) for ten minutes as described by Millenar *et al.* (1998). To inhibit cyt c pathway the tissue was incubated in a range of 50 to 500 µM KCN and at 500 µM of KCN maximum inhibition was found. The respiration was measured after 10 min of pre-incubation with the inhibitors.

#### b. Mitochondrial respiration

For mitochondrial respiratory assay the reaction medium contained : 03 M mannitol, 10 mM TES-KOH *p*H 7.5, 5 mM $KH_2PO_4$, 10 mM NaCl, 2 mM $MgSO_4$ and 0.1% (w/v) bovine serum albumin. All the measurements were carried out at 25ºC. Calibration of the electrode was made by the addition of sodium dithionite to remove all oxygen in the electrode chamber and the oxygen concentration was assumed to be 240 µM per ml of water. The maximum alternative respiration (AOX) or potential AOX activity was measured by the addition of cyt pathway inhubitor (antimycin) followed by addition of AOX inhibtor (salicylic hydroxamic acid). Addition of various substrates (electron donors) and effectors was made to ensure that the respiration may not be limited by substrate supply.

### Estimation of malondialdehyde (MDA)

The method of Health 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 2.5 ml buffer, in a cold mortar and pestle, followed by centrifugation at 8000 g for 20 min. To the supernatant 1 ml of TBA solution (20% (w/v) trichloroacetic acid, 0.01% (w/v) butylated hydroxytoluence) was added. The samples were vortexed and heated to 95ºC for 30 min,

followed by coolmg on ice for 5 min. The samples were then spun at 3000 g for 10 min and absorbances of each sample read at 440 nm, 532 nm and 600 nm, using a spectrophotometer (Shimadzu).

**Estimationof total protein**

Total poretin content of the soft bark tissue was extracted in 50 mM Tris buffer (pH7.4) with 2% 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 suggars and starch**

Total sugars and starch from the soft bark tissue wer extracted in 80 per cent ethanol and estimated by the methods after Scott and Melvin (1953) and MeCready *et al.* (1950), respectiely.

**Extraction and estimation of ATP in latex**

One gram fresh latex was extracted with 2.5% TCA and mad upto 10 ml. The solution was filtered using Whatmann No. 1 filter paper and 2 ml of filtrate was used fro ATP estimation. The samples were neutralized with 0.1 N KOH and made the volume upto 10 ml with 30 mM Hepes buffer (*p*H 7.4).

The ATP content in latex was measured luminoetrically (luminometer-Stratec Electronic GmbH, Brikendfeld, Germany) as described by Amalou *et al.*, (1992) using bioluminescent assay kit (Sigma FL-AA).

**SDS-PAGE analysis of proteins**

Fresh latex was centrifuged at 23,000 g for 45 min and C serum was seperated. Analysis of the proteins from latex C-serum was carried out in 12% SDS-PAGE as described by Laemmli (1970).

**Results and Discussion**

Tapped and untapped trees of the most popular and high yielding rubber clone, RRII 105, were analyzed for various metabolic activities and biochemical composition. Tapping was initiated in the tapped trees during 1998 and same aged trees (15 numbers) were left untapped. Both the tapped and untapped trees were 17 years old when the experiments were carried out. The shoot biomass accumulation of untapped trees was significantly higher than tapped (Table 1). After seven years of tapping, the trees lost around 34% of biomass as comapred to untapped trees. When an untapped tree recorded around 673 kg of dry shoot weight, the tapped tree had only 435 kg. The loss of biomass in a tapped tree was reported tobe 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). Compared to untapped trees, there would be differential metabolic activities in tapped trees. Therefore, in order to find out the changes in metabolic activities of tapping panel area the respiratory activities in bark tissue as well as isolated mitochondria were studied.

Table 1. The estimated shoot biomass, latex ATP content, soft bark tissue carbohydrates and proteins in untapped and tapped trees of *Hevea brasiliensis*

| Trees | Shoot biomass (kg/tree) | Latex ATP content (M/kg) | Soft bark tissue carbohydrates | | Soft tissue proteins | |
|---|---|---|---|---|---|---|
| | | | Total sugars (mg/g) | Starch (mg/g) | Total protein (mg/g) | Soluble protein (mg/g) |
| Untapped | 673 ± 77 | 32 ± 4 | 26 ± 1.5 | 83 ± 2.5 | 26 ± 1.8 | 16 ± 1.3 |
| Tapped | 435* ± 23 | 115* ± 8.9 | 30.5 ± 1.2 | 98.5* ± 3.7 | 46 ± 2.1 | 25* ± 1.7 |

*indicates tapped trees significantly different from untapped trees at 5% level

Oxygen consumption is a preferred measurement to study the respiratory efficiency of tissue. Tapping resulted in an enhanced respiratory activity in the bark tissue. The soft bark tissue respiration, including cyt-c and alternative oxidase (AOX) mediated oxygen uptake rates were higher in tapped trees compared to untapped trees (Fig. 1.) The residual respiration also was significantly increased in tapped trees. It represents the non-respiratory oxygen consumption by other oxidizing enzymes and activities of secondary metabolites.

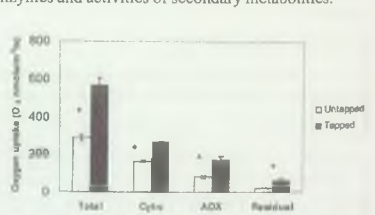


Fig. 1. Soft bark tissue respiration in untapepd and tapped trees of *Hevea.* (n : 6, * indicates significant difference between untapepd and tapped trees at 5% level.)

In order to avoid the interference/errors owing to secondary metabolities the respiratory rates were measured in isolated mitochondira from the soft bark tissue of untapped and tapped tree soft-bark tissue (Fig. 2). NADH was used as a substratre 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

rate was signficnatnly increased due to tapping. The potential or maximum capacity of cyt-c and altenative oxidase activities were measured with the addition of ADP in the presence of appropriate inhibitors of electron trnasport chain and they were found to be higher in tapped trees than in untapped tress (Fig. 2). The potential rate of alternative respiration in mitochondria was recorded in which the cyt-c activity was impaired. The capacity is generallydefined as the oxygen uptake resistant to the cyt-c pathway inhibtor and sensitive to the AOX inhibitor.

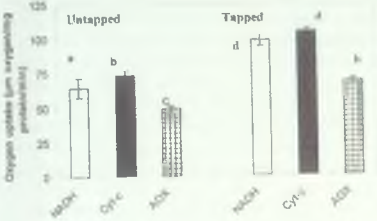


Fig. 2. The rate of mitochondrial respiratory $O_2$ uptake (uM $O_2$/mg protein/min.). (Mitochondria were isolated from soft bark tissue of untapped and tapped trees of *Hevea*. Histograms with different alphabets are significantly different.)

ATP content in the latex was higher in tapped trees than the untapped trees (Table 1). It is a fact that regular tapping stimulates latex biosynthesis for which a large quantity of ATP molecules are required and hence the tapped trees showed around 3.5 fold increase in latex ATP level. Respriation rate increased with an increase m latex volume (data not shown), indicating requirement of increased metabolic activities for a higher latex biosynthesis.

The quantity of toal sugars in the tapped trees did not differ significantly from those of untapped trees. However, sugar content was slightly higher in soft bark tissue of tapped trees (Table 1). Tapped trees had significantly higher starch content than untapped trees. Starch is accumulated in tissues and converted to sugars for the biosynthesis of rubber in laticiferous tissue. Tapped trees showed increased metabolic rate coenomitant 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.

Tapped trees recorded significantly higher level of protein content than untapped trees in the soft bark tissue (Table 1). The SDS-PAGE profile of the latex 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 k Da compared to the serum from untapped trees (Fig. 3). The increased leel of protein bands in tapped trees indicated higher metabolic flux with enhanced protein trun over including probable increase in rubber biosynthetic enzymes also.

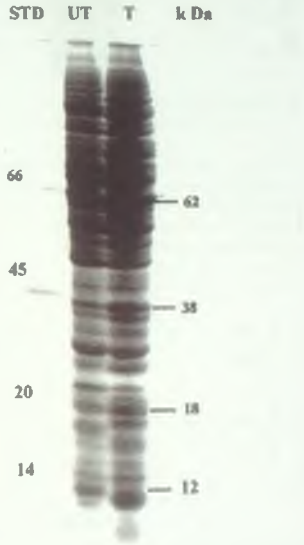


Fig 3. SDS-PAGE profile of latex C-serum proteins collected from untapped (UT) and tapped trees (T) of *Hevea*. (Molecular weight standards (STD) are marked in the left side and changes in major proteins size are indicated in the right side.)

Compared to untapped trees, tapped trees had more malondialdehyde (MDA) content, a product of membrane lipid peroxidation (Fig. 4). Tapping mediated wounding inflicted mechanical stress in tapped trees. This stress mediated production of ROS might have caused lipid peroxidation of bi-layer membranes of the mitochondria and other cell organcelles resulting in MDA accumulation.

The day to day respiration rate, sugar content in bark tissue and ATP content in the bark tissue and latex C-serum were analyized in a set of newly opened trees. The respiratory rate was gradually increased in laticidferous enriched bark tissue. Simultaneously the

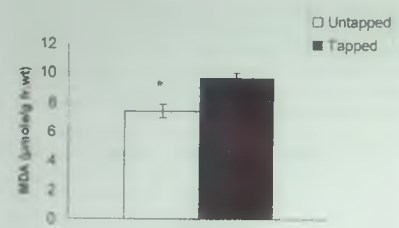


Fig. 4. Malondialdehyde content in soft bark tissue of untapped and tapped trees of *Hevea*. (* indicates significant difference at 5% level.)

alternative respiration was also increased when the tapping progressed (Fig. 5a). The sugar content suddenly declined ina newly opened trees, however, started increase from third tapping onwards and atained stable level from seventh tapping day onwards (Fig. 5b). This observation very well explained the sink demand and subsequent supply of metabolites from the photosynthetic source. The ATP content of bark tissue was gradually reduced and on the contrary it was steeply increased in C-serum (Fig. 5c & d). This experiment clearly demonstrated how the metabolic activities were modulated/activated in trees under tapping compared to an untapped tree.

Tapping in natural rubber tree is an unavoidable wounding process for the crop harvesting. The harvested latex contains around 30-40% dry rubber content and the remaining portion of the latex comprises water, serum proteins, sugars and othe mineral nutreitns like K, $PO_4$ etc (d' Auzac and Jacob 1989). In tapped trees the tree has to regenerate the latex betwen the tapping days. The laticiferous sink activity is significantly increased in tapped trees compared to untapped trees. Due to crop harvesting the tree is losing a lot of resources other than the energy rich polyisoprenes. Latex is a rich repository of photosynthates. There is partitioning of photosynthates

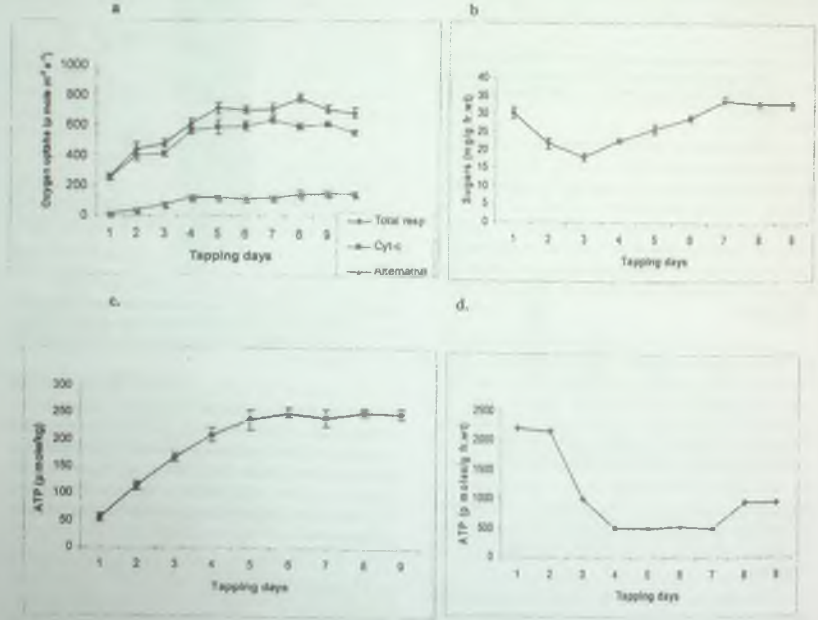


Fig. 5. The changes in respiratory rate (a), soft bark tissue sugars (b) and ATP contents in latex serum (c) and ATP content in soft bark tissue (d) as the tapping is progressing in a newly opened tree

for latex production and biomas allocation in a tapped tree (Templeton 1968). However, the percentage of allocation for these two processes is a clonal character. Therefore, the biomass of a tapped trees is significantly lower than an untapped tree of the same age. There existed a direct positive relationship between the yield and shoot biomass loss (Annamalainathan *et al.*, 1998). In the present study the loss of biomass in the seven years old tree was around 34.5%. It has already been reported that RRII 105 lost the highest percentage of biomass compared to other *Hevea* clones studied (Annamalainathan *et al.*, 1998).

There was a positive correlation existing between the total sugars and starch content of soft bark tissue. The level of carbohydrates determines the rate of respiration and energy status of the tissue. Rate of respiration was positively correlated with the concentration of sugars and starch in *Hevea* bark tissue (Low and Gomez, 1984, Annamalainathan *et al.*, 1998). The unaccountable biomass loss in tapped trees could possibly be explained in many ways (Sethuraj 1992). The increased alternative respiration in the bark tissues as well as in isolated mitochondria indicated more diversion of electrons through the non-phosphorylative respiratory pathway. If alternative pathway opertaes in an increased order the net ATP molecules are lesser in the cytosol of rubber trees (Krishnakumar *et al.*, 2001). However, activity of cyt-c also increased in the tapped trees explains the higher energy demand for the rubber biosynthetic pathway in the laticiferous tissue.

The AOX in higher plants has been generally considered as a wasteful metbolic process, but many studies have shown that it has certain vital physiological signficance. It is considered to be protective pathway in mitochondria akin to photorespiration in chlorplast. Alternative respiration bypasses two out of the three phosphorylating sites in mitochondrial electron transport and hence this may be an unsuitable trait for the overall energy metabolism of the cell. However, AOX may have a protective role during environmental and and biotic stresses as reported in many studies. Various findings indicate that the functional role of AOX is tissue and organ specific. If the expression of AOX is eliminated by antisense techique, the plants succmb to drought or other abiotic factors mediated oxidative stress (Ribas Carbo *et al.*, 2005; Annamalainathan *et al.*, 2006). Thus like photorespiration, alternative respiration also seems to be a necessary evil in order to regulate cellular metabolism. Needless to say the productio of biomass requires the input of carbohydrates, partly to generate ATP and NAD(P) H for biosynthetic reactions and partly to provide the carbon skeletons (Penning de Vires *et al.*, 1974).

The rate of respiration depends on three major energy-requiring process *i.e.* growth, maintenance and ion transport. Once biomass is produced, energy must be spent for the general repair and maintenance of the system. The estimated cost of maintaining plant biomass ranges from 20 to 60% of photosynthates produced per day in woody species (Ryan *et al.*, 1994) and a major part of the maintenance respiration is associated with protein turn over and maintaining ion gradients across membranes, approximately 0.26 g glucose is being spent for the turn over of one gram of protein (De Viser *et al.*, 1992). It is expected that ina tapped tree the rateof protein turn over is increased as evidenced from the increased protein level in C-serum. Therefore, the tapped tree is losing more biomass than the amount accountable for by its yield.

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