J. Rubb. Res., **4(4)**, 245–254

Tapping-induced Changes in Respiratory Metabolism, ATP Production and Reactive Oxygen Species Scavenging in Hevea

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Rate of dark respiration was measured in the soft bark tissues of untapped and tapped trees of Hevea brasiliensis. *The respiration rate increased after tapping and decreased when the trees were given tapping rest. Concomitant with increase in respiration, tapped trees also showed increased activity of reactive oxygen species scavenging enzymes such as superoxide dismutase, catalase and peroxidase in the soft bark tissues. Tapping significantly increased the rate of cyanide resistant alternative respiration. The latex (C-serum) of tapped trees contained a high amount of ATP, indicating that the metabolically activated tapped trees produced large amounts of ATP in the serum and the excess ATP molecules were lost through the serum. This excess loss of ATP and enhanced alternative respiration may partially explain the tapping induced loss of shoot biomass which was not reflected in the dry rubber yield.*

Key words: Tapping, respiration, ATP, superoxide dimutase, catalase, peroxidase, bark tissue, *Hevea*

Tapping is essentially a wounding process which results in the drain of large quantities of cellular organelles like lutoids, ribosomes, and metabolic resources such as proteins and sugars, apart from the economically important latex. Between two successive tappings, a tree has to regenerate all these components needed for the biosynthesis of latex. This reconstitution process requires large quantities of carbon intermediaries and energy[1]. In addition to causing the drain of resources, tapping also causes loss of carbon through enhanced respiration which affects the biomass production of the tree[2]. Tapping can amount to a biotic stress in a rubber tree and this elicits a range of physiological and biochemical changes in the trunk.

Photosynthates in the form of sucrose are catabolised in the laticiferous tissue through respiration. This gives rise to a variety of metabolic intermediaries (*eg.* acetate) and releases energy in the form of ATP and nicotinamide adenine dinucleotide (NADH), reduced form, which are then utilised for the synthesis of polyisoprene[3]. In addition, the wounding process of tapping can also increase maintenance respiration[4].

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During the course of respiration in the mitochondria and other metabolic activities in the laticiferous system, production of reactive oxygen species (ROS) is inevitable. The NADH-quinone reductase in the lutoid membrane is a potential source of production of such toxic oxygen species[5]. When the cytochrome pathway is inhibited or restricted in mitochondria, the instability in the reduction state of the ubiquinone pool promotes free radical formation[6]. The ROS scavenging enzymes like superoxide dismutase (SOD), catalase, peroxidase *etc.* play extremely important roles in their safe detoxification[7] in latex and laticiferous system. The presence of these scavenging enzymes in the laticiferous tissue and latex has been shown by Chrestin[8] and Krishnakumar *et al.*[9] A proper functioning of these enzymes is essential to maintain the physiological health of the laticiferous cells, failing which the system may succumb to severe oxidative stress which may be later expressed as tapping panel dryness (TPD) *etc.*[9] Oxidative stress is also known to alter respiratory and energy metabolism[10]. There is practically no information in the literature on the impact of tapping on the interrelationship between dark respiration, ATP production and ROS scavenging activity in tapped and untapped trees. The present study is an attempt to address this interrelationship in tapped and untapped trees of *Hevea*.

MATERIALS AND METHODS

Plant Material

Hevea clone RRII 105, planted during 1988 in the Rubber Research Institute of India was selected as the experimental plant material. Trees were tapped in the 1/2S d/3 system and 15 trees had been left untapped from 1998. Six to eight trees from the tapped and untapped population were randomly selected from a compact area for the present study during 1999–2000. Bark samples were collected from just below the tapping cut in the tapped trees. These were collected before tapping, three hours after tapping and on the subsequent resting days at the same hour. Corresponding samples were collected from the untapped trees.

Measurement of Respiration

A very thin slice (approximately 0.5 mm uniform thickness) of 200 mg fresh laticifer enriched soft bark tissue (just adjacent to the cambium) was used for the measurement of dark respiration using a Clarke type oxygen electrode (Hansatech, UK) as described by Lambers *et al.*[11] and modified by Annamalainathan *et al.*[2] 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 SHAM (Salicyl hydroxamic acid) for ten minutes as described by Millenaar *et al.*[6] Prior to this the requirement of optimum concentration of SHAM (stock solution in methoxyethanol) for maximum inhibition of alternative pathway was standardised. To inhibit the cytochrome pathway the tissue was incubated with a range of KCN, from 50 µmole to 500 µmole, and maximum inhibition was found at 300 µmole of KCN. The respiration was measured 10 min after pre-incubation with inhibitors.

Assay of ROS Scavenging Enzymes

The activities of reactive oxygen scavenging enzymes, namely superoxide dismutase[12],

catalase[13] and peroxidase[14] were assayed in the soft bark tissue extracts (homogenised in 100 mM PO_4 buffer pH 7.2) and expressed as relative unit activity per mg soluble protein. The soluble protein content in the bark tissue was estimated by the method of Lowry *et al.*[15]

ATP Measurement

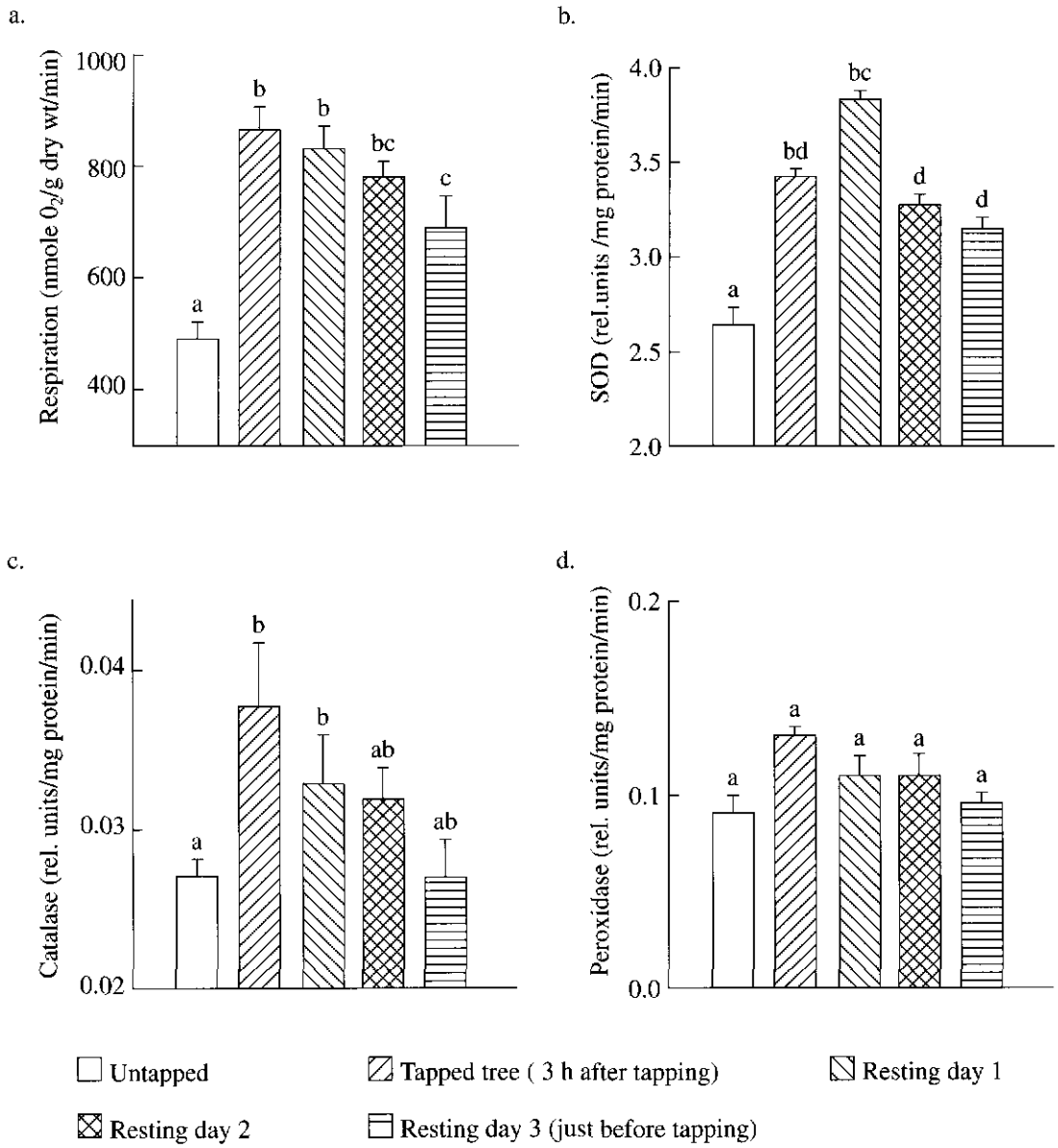
For the measurement of ATP content in the soft bark tissue, one set of bark samples from both the tapped and untapped trees were collected and immediately frozen in liquid nitrogen in the field. These samples were collected from the field in the morning hours (3 h after tapping) along with the samples for respiration. The soft bark tissue was extracted with 20 mM Tris and 2 mM EDTA buffer pH 7.7. Latex samples were collected in ice cold centrifuge tubes and centrifuged at 23 000 g for 45 min in an ultracentrifuge. A sample of C-serum (cytosol) was collected with a syringe from the middle fraction after centrifugation. The ATP content in soft bark tissue and latex C-serum was measured luminometrically (luminometer- Stratec Electronic GmbH, Brikenfeld, Germany) as described by Fader and Kollar[16], using a bioluminescent assay kit (Sigma FL-AA).

RESULTS AND DISCUSSION

The dark respiration rate was recorded just before tapping, 3 h after tapping and on subsequent resting days (day 1, day 2 and on day 3 — just before tapping). When compared to an untapped tree, the respiratory rate in a tapped tree increased after tapping and decreased during the resting days (*Figure 1a*). The longer the resting period, the more the reduction in respiration in tapped trees. However, the rates were always higher in the tapped than the untapped trees at any given sampling time (*Figure 1a*). This increased respiration rate in the tapped trees could be due to both enhanced metabolism for latex biosynthesis as well as wound induced maintenance respiration. This finding is in corroboration with our earlier study in ten different *Hevea* clones[2].

Compared to an untapped tree, a tapped tree showed higher rate of superoxide dismutase, catalase and peroxidase activity in the laticiferous tissue which gradually decreased with resting days (*Figures 1b*, *1c* and *1d*). These enzyme activities indicated enhanced production of ROS in tapped (wounded) tissues. ROS are inevitable by-products of cell metabolism. They trigger the degeneration of cellular components through various reactions such as peroxidation of membrane phospholipids[17]. Normally, any tissue under biotic or abiotic stress would respond to ROS through antioxidants[18]. As explained by Laties[4], in the present study the ROS scavenging and respiratory metabolism also appeared to be similar as in wounding and senescence process. The bark tissue respiration rate showed a significant positive correlation with the SOD ($r = 0.61$, *Figure 2a*), catalase ($r = 0.68$, *Figure 2b*) and peroxidase ($r = 0.4$, *Figure 2c*) activity of the tissue. This indicates that the higher the respiration rate, the greater the free radical production in laticiferous cells.

In order to ascertain the rate of phosphorylating (cytochrome oxidase) and non-phosphorylating (alternative oxidase) pathways, the respiration rate was measured with different inhibitors of these pathways. A tapped tree recorded significantly higher rate of cytochrome and alternative oxidases-mediated O_2 uptake than the untapped tree (*Figure 3*). It is now well documented and accepted that the alternative



(n = 6 ± SE shown); Histograms with same alphabets are not significantly different (at $P \leq 0.05$) and those with different alphabets are significantly different (at $P \leq 0.05$).

Figure 1. Rate of dark respiration (a) and activities of SOD (b), catalase (c) and peroxidase (d) in soft bark tissue of tapped and untapped trees of Hevea brasiliensis.

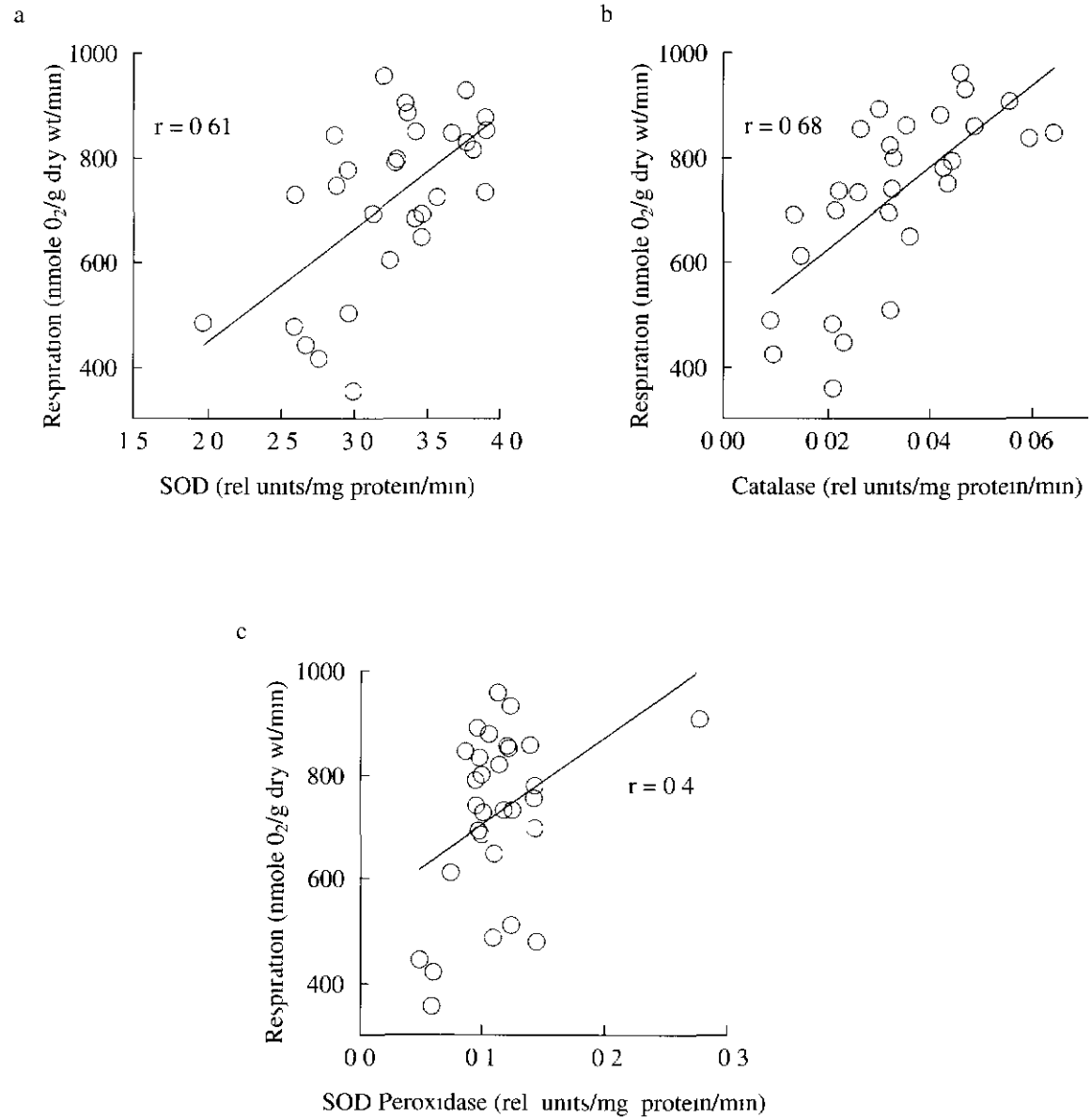
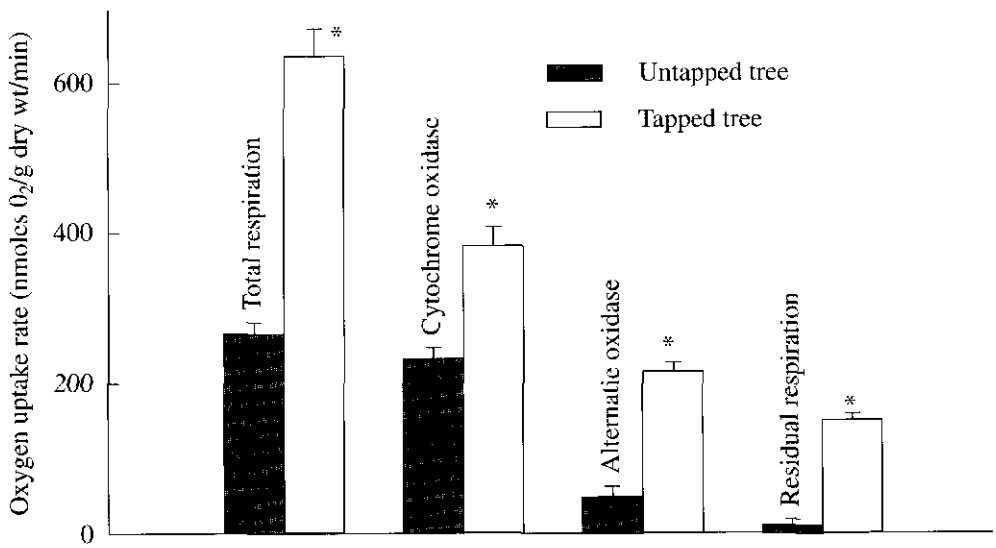


Figure 2 Relationship between dark respiration of the soft bark tissue and its SOD (a), catalase (b) and peroxidase (c) activities

oxidase-mediated electron transport chain after ubiquinone in mitochondria is non-phosphorylating and also not coupled with generation of a membrane potential and electron motive force[19,20]. The high rate of alternative oxidase recorded in the tapped tree could be explained by (i) wound induced diversion of electrons to alternative oxidase leading to more heat generation as observed in many cases with abiotic stresses[21,22], (ii) increased production of NADH due to stimulation of metabolism and by tapping and subsequent diversion of electrons *via* the alternative pathway as an 'overflow' mechanism to oxidise the excess NADH[11] and (iii) stabilisation of the ubiquinone pool through enhanced alternative pathway thereby preventing more ROS formation[22]. The stimulation in alternative pathway in a metabolically active bark tissue such as the tapped bark also indicates that the cytochrome pathway was inadequate to handle all the reductant (NADH). It is also likely that the turnover of ATP (through various energy consuming metabolism, particularly latex synthesis) is a limiting factor and the reductant was preferentially oxidised through the non-phosphorylating alternative pathway because of inadequate availability of adenosine diphosphate (ADP).

After simultaneously treating with both the inhibitors, KCN and SHAM, a considerable amount of oxygen uptake (residual respiration) was recorded in the tissues. The residual respiration rate was very meagre in untapped trees

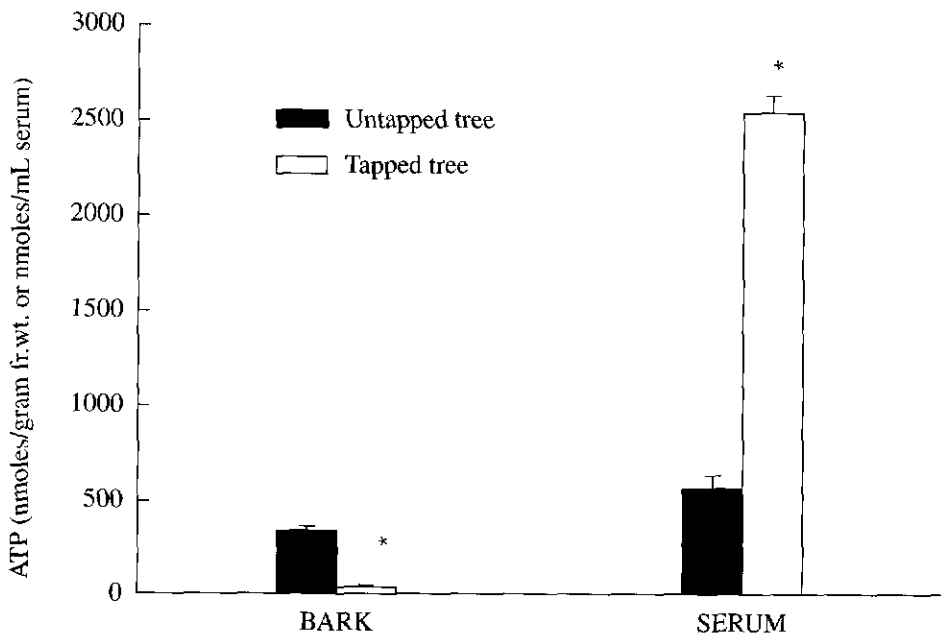


(n = 6 ± SE shown): Single* indicates statistically different (at $P \leq 0.05$)

Figure 3. Rate of respiration in soft bark tissues of tapped and untapped trees without any inhibitors (total respiration), with SHAM (to determine cytochrome oxidase mediated respiration), KCN (to determine alternative oxidase mediated respiration) and with combination of both SHAM and KCN (to determine residual respiration).

and significantly high in tapped trees (*Figure 3*). The residual respiration normally recorded in a tissue represents the non-respiratory oxygen consumption by other oxidising enzymes like lipoxygenase, peroxidases, polyphenol oxidase *etc.*[23] It appears that these enzymes were also stimulated through tapping due to wound induced secondary metabolic process.

The enhanced respiration found in the tapped tree was related to the extremely high concentration of ATP in the C-serum of the latex. The C-serum of the tapped tree had almost five-fold larger ATP concentration than the serum collected from untapped trees (*Figure 4*). This agrees very well with the fact that regular tapping stimulates latex biosynthesis for which a large quantity of ATP molecules are required. Regular tapping is known to accelerate latex synthesis machinery needing to increase metabolic flux[1]. Our results show that the metabolically active tapped trees with enhanced respiration produced more ATP and at the same time a lot of ATP was lost through serum. It is likely that either due to the excessive loss of ATP through the latex and or due to the inadequate supply of ADP as a result of poor ATP recycling, the phosphorylating cytochrome pathway of mitochondrial electron transport chain could not handle all the NADH that was generated and this triggered the nonphosphoryl ating alternative pathway in the tapped trees (*Figure 3*). Stimulating the trees by ethephon application has been shown to increase the ATP content in the latex cytosol[24].



(n = 6 ± SE shown); Single* indicates statistically different (at $P \leq 0.05$).

Figure 4. ATP content of soft bark tissue and C-serum of latex in tapped (open box) and untappd (closed box) trees of Hevea.

Compared to the serum, the bark tissue contained lower concentration of ATP in both tapped and untapped trees (*Figure 4*). We are unsure why the ATP content was less in the bark of a tapped tree. It appears that there was enhanced flux of ATP from the mitochondria into the cytosol and thus most of the ATP from the bark was lost through the latex. This may explain why there was less ATP in the bark of tapped trees. It may be noted that these bark samples were collected 3 h after tapping by which time ATP drain through latex must have reached a maximum, leaving very small amounts of ATP in the bark.

CONCLUSION

The present study shows for the first time how tapping stimulated respiration and ATP production in the *Hevea* bark. The tapping induced stimulation in alternative pathway might be an indication that the cytochrome pathway has been inadequate to handle all the reduction product in the laticiferous tissue due to the tapping induced stimulation in metabolism. Alternatively it is also possible that the ATP-ADP turnover was a limiting factor so that there was increased diversion of electrons for the non-phosphorylating alternative pathway. The loss of large quantities of ATP through latex serum also could amount to a considerable degree of biotic stress on prolonged tapping. Tapping also seems to have activated the ROS scavenging metabolism; failure of which in due course of time, could result in severe oxidative stress. The non-phosphorylating alternative respiration and the loss of resources including ATP and diversion of additional energy for ROS scavenging are also factors that might explain how a tapped tree is loosing its biomass, that is not accounted for either by rubber production or its standing biomass[2,25].

ACKNOWLEDGEMENT

The authors thank Visalakshyammal, C.C. Joseph and P. Babuji for their assistance in the laboratory and field.

Date of receipt: July 2001
Date of acceptance: November 2001

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