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# *Over-exploitation Associated Changes in Free Radicals and its Scavengers in* Hevea brasiliensis

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*Tapping panel dryness (TPD) syndrome, generally considered as a stress-induced physiological disorder, is a consequence of abnormal exploitation in rubber (*Hevea brasiliensis*). Numerous studies in other crops have revealed that stress induces/enhances the formation of free radicals (FR) which gets moped up by its scavenging system. To study the status of free radicals and its scavenging system during occurrence of TPD, normal plants were subjected to frequent tapping and stimulation treatment (10% ethephon), to accelerate the occurrence of the syndrome. A significant negative correlation was observed between FR in the bark and superoxide dismutase (SOD) in the lutoid with tapping. However, latex thiol showed a very low linear correlation with progression of tapping. The study also revealed that the amount of FR in frequently tapped plants (where the wounding was excessive) was less than that of in the stimulated plants, though the damage to the scavenging system (SOD) was higher in the frequently tapped trees. It was also observed that with progression of time, the percentage of TPD was more in the frequently tapped trees than in the stimulated ones. Interestingly however, at the 115th actual tapping, the occurrence of TPD was found to be higher in the stimulated samples than that of in the frequently tapped plants. The control plants showed no TPD syndrome in both cases. This implies that excess generation of active oxygen with defective defense mechanism (in terms of SOD) along with frequency of exploitation, source-sink imbalance* etc. *may determine the yielding potential and the TPD incidence in rubber.*

**Key words:** exploitation; tapping system; stimulation; stress; free radicals; scavengers; tapping panel dryness; superoxidase dismutase

TPD is generally considered as a stress based physiological process induced by tapping. When the level of exploitation of a rubber tree exceeds its physiological capability to generate latex, the tree succumbs to TPD. It is also understood now that abnormal exploitation *viz.* frequent tapping[1] or an overdose of stimulation[2] or an imbalance between these two factors, may be one of the physiological reasons behind this problem. This physiological fatigue is governed by complex interactions between clonal sensitivity and exploitation intensity,

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which is further intensified by fluctuation of environmental factors[3].

Detailed information on the possible factors leading to TPD and its remedial measures are very scanty. The analysis of protein profile in TPD affected rubber trees show over-expression of certain acidic proteins[3] and also the development of heat shock proteins[4]. Little is known regarding how rubber plants react when stress induced FR generation is high. Loss of certain enzymes related to free radical (FR) scavenging system *viz.* superoxide dismutase (SOD) and catalase (CAT) in TPD affected plants, was reported by Chrestin *et al.*[5] It is evident that broad types of stress *viz.* air pollutants, UV radiation, herbicides, drought, cold *etc.* are directly or indirectly related to oxygen toxicity through physiological and biochemical lesions[6]. Such incidences may also occur in rubber trees where various types of stresses *viz.* wounding (tapping cut) and environmental hazards like drought, cold, hailstorms *etc.* are experienced by the plants. Hence, information on generation of FR and its protective measures is nevertheless important in rubber to understand the tolerance of plants to oxidative stress and to reduce cellular damage by adopting alternate exploitation measures. Thus, the present investigation was aimed at studying the concentration of FR [estimated directly by electron paramagnetic resonance (EPR) spectrometer] and the lutoidic SOD as active oxygen scavenging system (AOSS) with over-exploitation.

## MATERIALS AND METHODS

### Plant Material

The study was conducted in rubber (*Hevea brasiliensis*) clone RRIM 600 planted in a randomised block design spaced at normal spacing of 5m × 5m in the farm of Regional Research Station, Rubber Board, Agartala, India. Mature trees, under the half-spiral (half of the stem at 150 cm above the bud-union region at 25° angle of the plant) every alternate day, (denoted as 1/2S d/2) tapping system, were considered for the experiment. Tapping started from April 1996 for a period of two years. In control plants the biochemical analysis was started after seven months of tapping. To accelerate the occurrence of TPD, two sets of 15 trees having uniform girth, were subjected to two different treatments: (a) plants tapped under normal frequency with application of 10% Ethephon applied in the groove of the bark at the tapping region once a month and (b) plants subjected to twice daily tapping (frequent tapping). Both the treatments started from November 1996 till December 1997. Plants under the alternate day normal tapping were taken as control. Samples for biochemical analysis were collected in November and December 1996 and January, March, April, May, September and December 1997. The FR content was measured from the soft bark collected from just below the tapping-cut. For the estimation of thiol, the total serum (supernatant after TCA precipitation of latex protein) was considered. SOD was assayed from lutoids of the latex. Samples were collected from the stimulated plants before the application of stimulant.

### Estimation of Free Radicals

The bark samples from each treatment were dried immediately after collection under vacuum desiccators till the moisture content of bark samples became 5%. Dry samples were measured in the presence of liquid nitrogen by EPR using Varian E-112 spectrometer at X-band frequencies ($\upsilon$ = 9.45 GHz) having 800 G field scan, 100 KHz modulation, microwave power 3.5 mW, field set 343 mT, receiver gain

$4\times10^3$, modulation amplitude 2 mT with a scan time of 4 min, according to the method of Das *et al.*[7]. The 1,1,diphenyl-2-picryl hydrazyl (DPPH) was used as the internal standard (g factor = 2.0036) for FR. For a comparative study, the entire field set up mentioned above was set as standard for the instrument. The recordings were replicated thrice for each sample. For estimation of FR from latex or lutoids. the samples were estimated directly in the cuvette. The presence of FR was confirmed by baking the samples[8] at 80°C.

### Assay of Superoxide Dismutase

Lutoids are cytosolic in origin. They were isolated from latex by centrifugation at 23 K r.p.m. for 30 min at 4°C, washed twice in Tris-Cl buffer (pH 7.8) containing sucrose, lyophilised and kept at –80°C under a vacuum desiccator[7]. For gel electrophoretic assay of SOD, the enzyme was extracted from lutoids in 100 mM potassium phosphate buffer containing EDTA, $MgSO_4$, NaCl and β-mercaptoethanol according to the method of Das *et al.*[7] An equal amount (100 μg) of protein[9] was loaded on to each lane of the gel and then electrophoresed in 10% native polyacrylamide gel for 4 h in 100 V at 2°C–4°C. The migration was from cathode to anode. The SOD was localised photometrically using nitro blue tetrazolium as substrate[10]. The achromatic bands were scanned at 632.8 nm in Hetto Densitometer. The gel assay was repeated twice for each sample.

### Assay of Thiol

Thiol was quantified spectrophotometrically by the standard method using di-thionitro benzoic acid (TNB; pH 6.5) as the substrate[11]. The rubber particles were precipitated in trichloro acetic acid (TCA) and the supernatant was taken for total thiol estimation. The reaction mixture was measured at 412 nm.

### Estimation of Tapping Intensity

The actual tapping intensity is calculated according to the standard method of Lukman[12], *i.e.*

$$\text{AI} = \frac{4 \times \text{length of tapping cut} \times \text{actual no. of tapping days}}{\text{Total no. of days in a given period (year)}} \times 100$$

### Frequency of TPD Scoring

TPD incidence was scored after six months of tapping when visible symptoms of damage (*viz.* panel dryness) appeared on the tapping panel. For recording the percentage of TPD incidence, only trees showing the syndrome with an occurrence of more than 50% of the incidence were considered. Such a category was chosen mainly due to fluctuations in the extent of panel dryness at different seasons within a year on individual trees[2]. However, the dryness seemed to be persisting when the occurrence was greater than the 50% value. The scoring of TPD was done at the time of tapping. The percentage of partially TPD affected trees and the extent of tapping panel area turning dry due to TPD syndrome were monitored periodically at two-monthly intervals.

## RESULTS

The EPR response of powdered bark samples at different time intervals showed that there was difference in the FR content (in cm $g^{-1}$ dry bark tissue) between the over-exploited and control plants (*Figure 1*). For all the treatments, plants

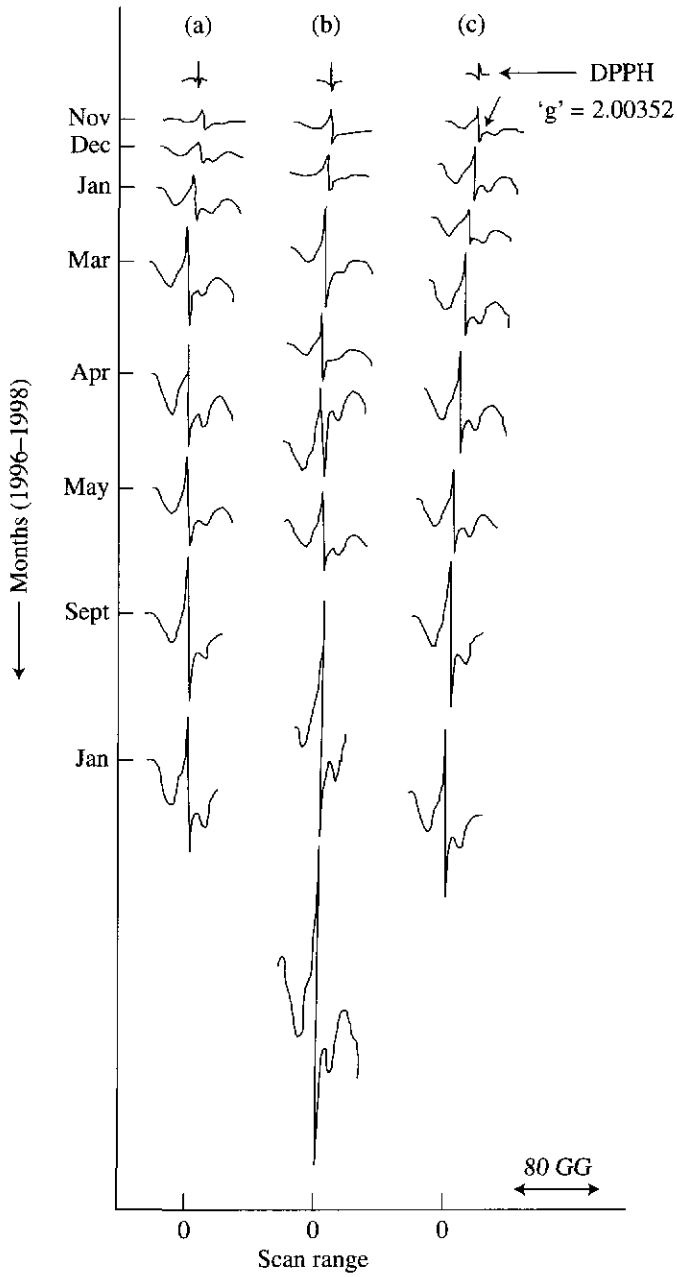



*Figure 1. EPR spectra from soft bark of rubber plants subjected to different TPD accelerated treatments of (a) 1/2S d/2, (b) 1/2S d/2ET (10%) and (c) 1/2S d/0.5 system of tapping. EPR parameters of the spectrum were 800 G field scan, 100 KHz modulation frequency, 3.5 mW microwave power, 343 mT field set, 4 × 103 receiver gain and 2 mT modulation amplitude.*

were considered for the experiment after seven months of tapping in the 1/2S d/2 system. The amount of FR in the samples before imposing the treatments in November 1996 were similar, indicating uniformity of the selected plants. In general, there was a gradual increase in FR content with progression of time, irrespective of the treatments. However, the EPR spectra showed that the FR content was higher in the stimulated samples (*Figure 1b*) compared to that of the frequently tapped set (*Figure 1c*). In the control and frequently tapped trees also, the EPR spectra was similar to at least the first four samplings *i.e.* November, December, January and March (*Figure 1*). It is pertinent to mention here that FR was not detectable from the latex or lutoids by the method adopted for this study.

The isozyme polymorphism of SOD (expressed in scans) in different exploitation systems (*Figure 2*) showed the presence of four isozyme bands seven months after opening of the virgin bark, before imposing the treatments. With progression of tapping after adopting treatments, the intensity of band 3 increased at the initial stage for all the treatments followed by a decline in banding intensity after the 3rd month of tapping. Further, the number of bands also started decreasing and was finally limited to two. However, in both the treatments, the intensity of these two bands gradually declined, compared to that of the control. The extent of decline in banding intensity of the frequently tapped samples was higher compared to that of the stimulated ones.

The correlation between bark FR content (in cm $gm^{-1}$ dry bark tissue) with duration of time (in months) also showed an overall increase of FR in all the treatments studied (*Figure 3a*). The 'r' values in the control, stimulated and frequently tapped samples were 0.88, 0.82 and 0.91, respectively. In the control, the reduction in FR content was 47% while for the stimulated and frequently tapped samples it was 62% and 52%, respectively. The correlation of the lutoidic SOD concentration (in ng 100 $\mu g^{-1}$ total protein) with time (months) also revealed the fact that with progression of tapping, the amount of scavenging enzyme decreased (*Figure 3b*). The 'r' values for control, stimulated and frequently tapped plants were found to be –0.77, –0.73 and –0.82, respectively. The SOD concentration from November 1996 to December 1997 in control, stimulated and frequently tapped plants were 8.6%, 24% and 43%, respectively. However, a very low 'r' value was observed between thiol content and the progression of time (*Figure 3c*), irrespective of treatments.

A comparative study on bark FR and lutoidic SOD concentration (*Figure 4*) showed that there was a significant negative correlation between these two factors in all the treatments. The 'r' values for the control, stimulated and frequently tapped plants were found to be –0.68, –0.74 and –0.84, respectively.

The data on trend of TPD showed that with time, the incidence of TPD syndrome also increased in the over-exploited samples (*Figure 5*). However, in the control, even after 24 months of tapping, occurrence of TPD (scored as >50% occurrence of the symptom) was found to be negligible. Among the two different TPD-imposed treatments, the frequently tapped plants developed more TPD than that of the stimulated plants, the 'r' values being 0.98 and 0.97 for stimulated and frequently tapped trees respectively, indicating a strong positive correlation for occurrence of TPD with time after 14 months of imposition of treatments.

The status of yield and related biochemical changes at an actual tapping intensity[6] of 115 in

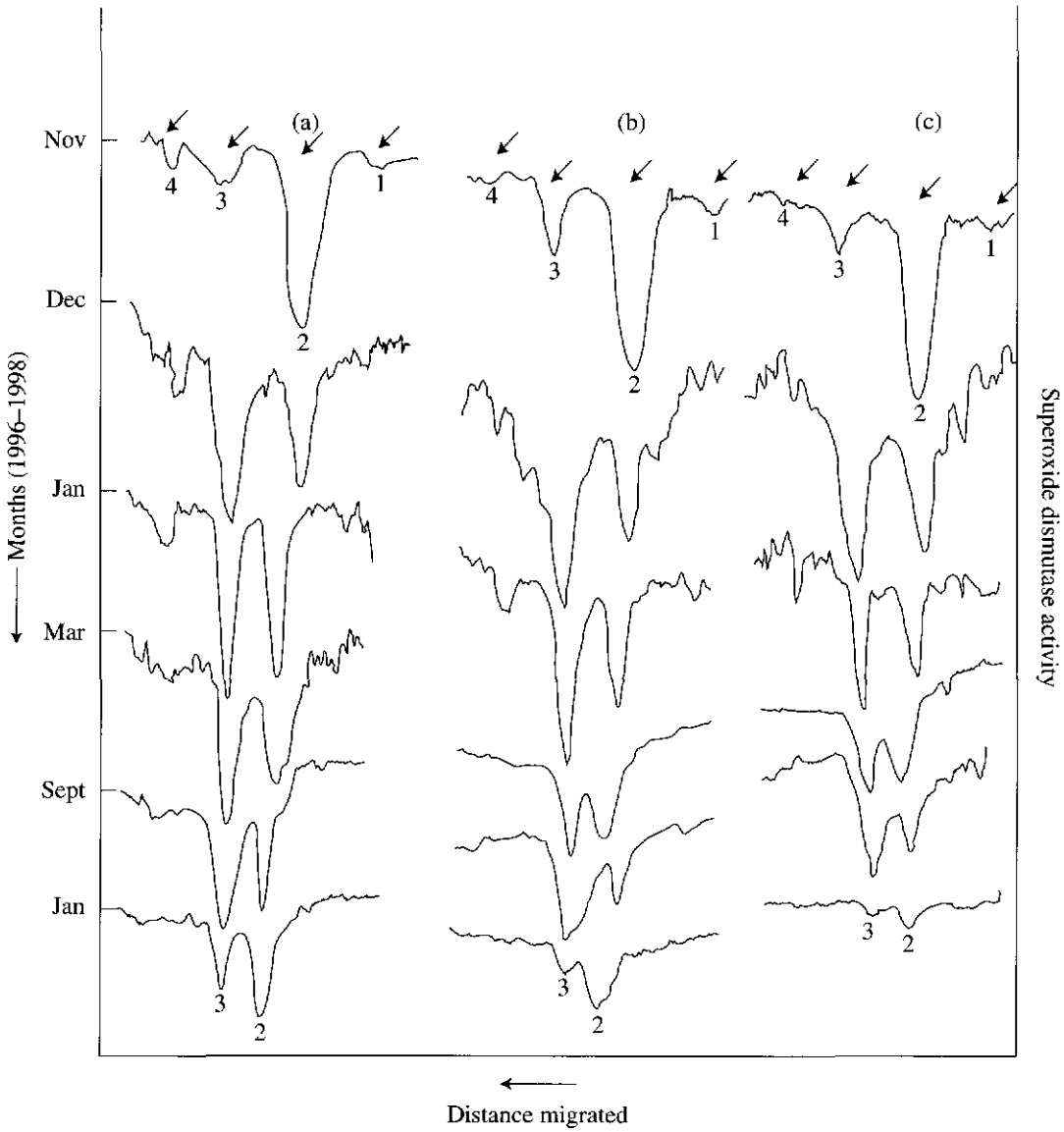



*Figure 2. Densitometric scans (at 632.8 nm) of superoxide dismutase from lutoids of rubber plants subjected to TPD accelerated treatments of (a) 1/2S d/2, (b) 1/2S d/2ET (10%) and (c) 1/2S d/0.5 system of tapping. The distance migrated in each gel was 100 mm. The arrow indicates the peak corresponding to the number of SOD band.*

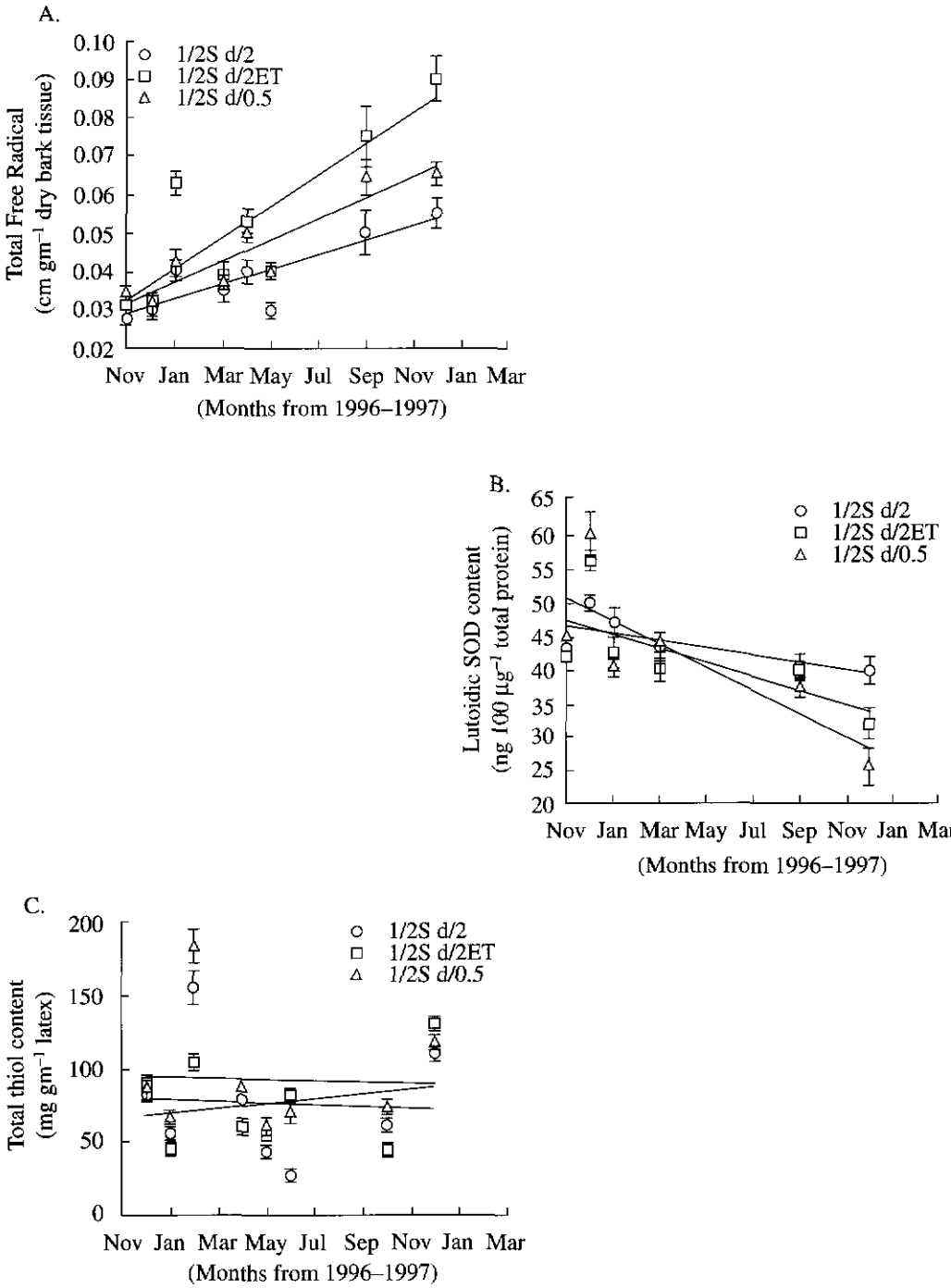
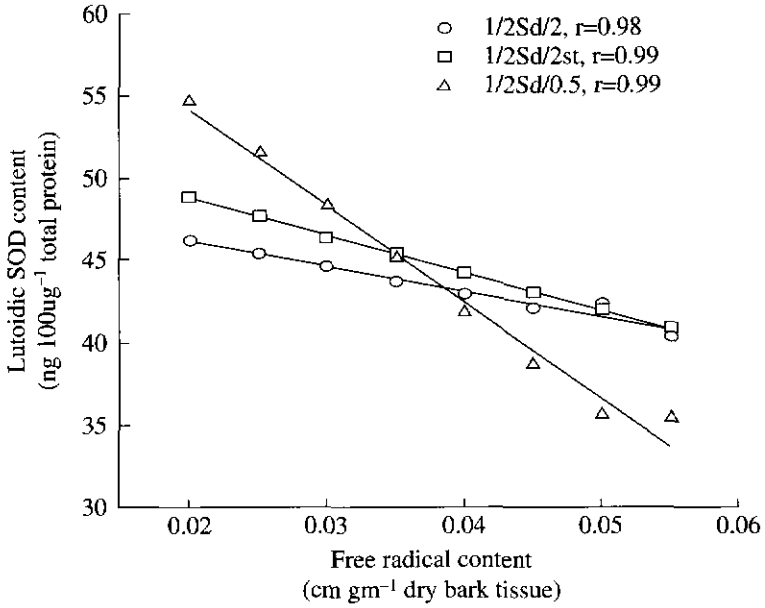
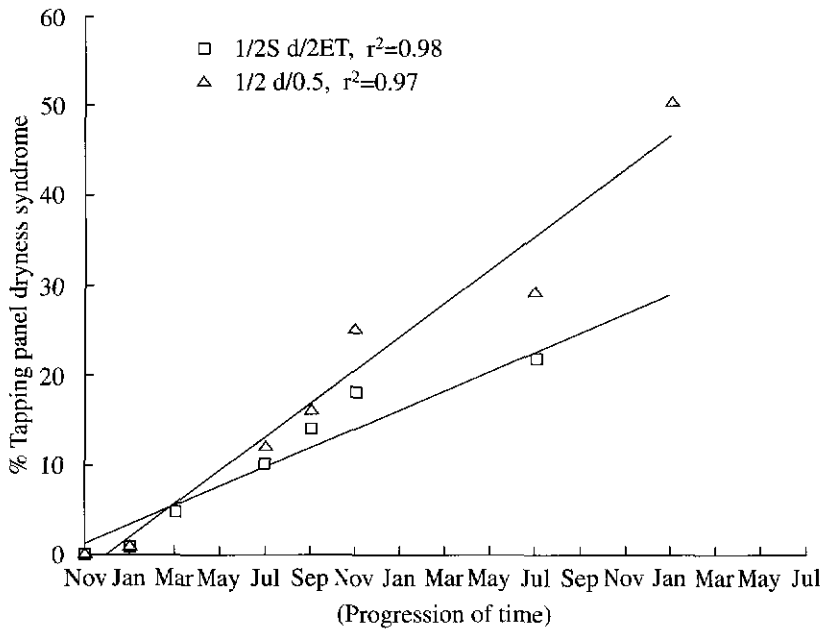



*Figure 3. Trend of free radicals and its scavengers with time.*




The estimated 'r' values derived from 15 plants in each treatment.

*Figure 4. Comparative study of free radicals and SOD.*




The estimated 'r' values derived from 15 plants in each treatment.

*Figure 5. Trend of tapping panel dryness with progression of time.*

each treatment is shown in *Table 1*. In stimulated plants, the yield (in gm $tap^{-1}$ $tree^{-1}$) was higher at this stage in comparison to control; for the frequently tapped plants, it was found to be lower. It was also observed that in comparison to the control (1/2S d/2), while the frequently tapped system was having lower amount of FR and higher concentration for SOD, the stimulated plants were showing higher amount of FR and lower SOD concentration. However, in the stimulated samples, the amount of total thiol was high compared to that of the other treatments. The TPD (scored as occurrence of >50% TPD symptom) was seen to be higher at this stage than that of the frequently tapped trees; TPD was found to be negligible in the control.

## DISCUSSION

The EPR signal of the FR (*Figure 1*) when compared with the internal standard (DPPH), showed that it was a single line spectrum with the width of ≈ 4.8 mT and a 'g' value of 2.00352 that seems to be of quinone derivative[13]. Such a spectrum is also comparable with that of quinone[8], semi-quinone[14] and ubiquinone[15] radicals. In bark, the signals were comparable to the internal standard *i.e.* DPPH. Increase in the first four signals in all the treatments (*Figure 1*) may be linked to the stabilisation of the bark and the latex cell regeneration metabolism in response to stress. Since the control was also experiencing wounding stress (along with the climatic fluctuation), increase in quinolic-FR was observed with progression of time. Formation of free radicals by NAD(P)H-quinone reductase was also studied by d'Auzac *et al.*[16] The EPR response of the stimulation treatment showed a significant increase in FR content over the control and frequently tapped plants (*Figure 3a*). This was more pronounced when the plants approached the occurrence of TPD during the later stages of tapping.

An initial increase in the intensity of bands was observed with the commencement of treatments, subsequently followed by a decline in activity (*Figure 3*). The activity is expressed in terms of band intensity and number of bands. It is relevant to mention here that the SOD extracted from the C-serum and lutoids (B-serum) showed a similar banding pattern. The trend of SOD concentration with that of the tapping intensity revealed a significant negative correlation indicating that the AOSS was less active in TPD affected plants (*Figure 3b*). Plants possess inherent enzymatic and non-enzymatic antioxidant defense mechanisms[17]. The initial increase in activity of SOD may be due to this inherent protective measure by the cells from the attack of active oxygen, which was also not very high in all the treatments.

There was no linear fit between thiol and progression of time for all the treatments *viz.* control, stimulated and frequently tapped samples (*Figure 3c*). A reduction in thiol content was also reported in the stimulated plant[18] as well as in the tapping induced TPD affected plants[19]. The low-linear correlation of thiol with time may be due to acceleration of the TPD syndrome in the virgin bark of the rubber plant or may be a limitation of the method used. It is interesting to note here that though the thiol contents in different tapping systems were widely different, an increasing trend was observed in all the treatments after January (*Figure 3c*). It is reported that the environmental parameters have a critical role in damaging the oxidative defense mechanism[20], which may affect the scavenging mechanism of thiol. The increase of thiol content after January indicates that it became more responsive when plants entered into the defoliation

phase, after experiencing the low temperature stress It seems that an elaborate regulatory mechanism is involved in this enzymatic and non-enzymatic anti-oxidant defense mechanism, imbalance in the pathway may not be responsive to defense system To get an indepth information of the defense mechanism of thiol, detailed study on changes in different forms of thiol is worth investigating

A significant negative correlation was observed between lutoidic SOD and bark FR (*Figure 4*) In the control, stimulated and frequently tapped sets, about 45%, 55% and 70% of the variances could be explained for the inverse relation between SOD and FR with a confidence level of 95% The FR that was generated in the bark cells may affect the lutoidic membranes in the laticiferous cells of the bark tissue The initial increase in SOD in all the treatments may be linked with the stabilisation of the lutoids—failure of which leads to coagulation of latex due to rupture of the lutoidic membrane It is also reported that a high amount of FR along with a low amount of AOSS *viz* thiol, SOD and catalase (CAT) may be one of the consequences of TPD in rubber[18] Thus, a study based on the relationship between lutoidic FR and SOD, and peroxidase would be worth investigating Moreover, a balance between these two factors is a prerequisite for the relative stability of the cell membrane It is interesting to note that when the FR was increasing in the soft bark cells, the SOD in the lutoids was also responding accordingly and was thus balancing the stability of lutoids

The assessment of TPD in all the treatments (*Figure 5*) showed that during the experimental period, when the control plants were not affected by TPD with progression of time, the over-exploited plants were found to be highly affected with TPD syndrome ($r^2$=0 98) Further, with time, the frequently tapped plants showed more TPD than that of the stimulated plants The data on FR and SOD showed that though the amount of FR in frequently tapped trees were less in comparison to that of the stimulated trees, the associated SOD was also very low

The comparative study on yield with FR, thiol, SOD and TPD, at the 115th actual tapping (*Table 1*), showed that in case of intensive tapping, though the plants were yielding less and FR was low, with higher amount of SOD, a fair amount of TPD percentage[5 7] was seen With a similar value of FR and thiol content and a slightly lower concentration of SOD however, the control plants yielded more with no visible symptoms of TPD (scored as stated earlier) Further, the 10% ethephon treated samples with higher FR and thiol content and lower SOD yielded more and showed high TPD incidence The data also revealed that at the end of the experiment (*i e* after 14 months of imposing treatments) when the control and stimulated plants experienced an AI of 115, the frequently tapped plants had an AI of 465 The TPD incidence in frequently tapped plants may be due to the short duration between two successive tappings Adequate amount of time interval is thus necessary for regeneration of the latex metabolic system after necessary repair In the case of control plants, as the time period between two successive tappings was more or less adequate for the system (without any yield stimulation), the plants were not showing visible TPD symptoms In the stimulated plants, although the time period between two tappings was adequate, the excessive dose of stimulant triggered the occurrence of TPD

But in terms of AI, the stimulated plants were found to be more prone to TPD (*Table 1*) In the frequently tapped plants, the significant

TABLE 1. YIELD AND ITS BIOCHEMICAL COMPONENTS AT THE ACTUAL 115TH TAPPING OF *HEVEA* PLANTS UNDER OVER-EXPLOITATION TREATMENTS

| Components | Control (1/2S d2) | Tapping with 10% stimulant (1/2S d/2) | Frequent tapping (1/2S d/0.5) |
|---|---|---|---|
| Yield (gm $tap^{-1}$ $tree^{-1}$) | 13.96 | 17.99 | 6.85 |
| Free Radical (cm $gm^{-1}$ dry bark tissue) | 0.06 | 0.08 | 0.04 |
| Thiol (mg $gm^{-1}$ latex) | 78.53 | 93.10 | 76.77 |
| SOD (ng 100 $\mu g^{-1}$ total protein) | 39.46 | 33.30 | 47.00 |
| TPD% (scored as >50% incidence) | 0 | 26.79 | 7.60 |

increase in TPD started after May, the time when the tapping cut approached the bud-union region (40 cm–50 cm from the initial cut). At the end of the study, the bark consumption for 1/2S d/0.5 was about 130 cm–140 cm. In the control and stimulated plants, when the plants experienced an AI of 115 (at the end of the experiment), the bark consumption was 40 cm – 50 cm below the origin of the tapping cut. However, due to a higher dosage of stimulation (10% ET), an imbalance between the causes of TPD and its repair might have occurred in the 1/2S d/2 tapping system that led to more TPD.

The susceptibility of TPD towards the bud-union region was also reported by Keuchenius[21]. Tupy[22] also reported the depressive effect of high tapping frequency with assimilates (*i.e.* sucrose) of latex. Moreover, the more the incision *i.e.* tapping cut, the lower the chance of regeneration of latex by means of the assimilates produced by photosynthesis and stored in the trunk and bark[23]. It is clear now that by maintaining a delicate balance between the rates of FR generation and its removal through controlled tapping intensity, stimulation dosage and also considering the position of tapping panel, it may be possible to get a reasonably good yield from rubber plants without passing through the phase of TPD syndrome which involves an elaborate regulatory mechanism.

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