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Rubber Biosynthesis in Tapping Panel Dryness Affected Hevea *Trees*

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The activity of rubber transferase (RuT) determined in washed rubber particle (WRP) and whole latex showed a marked increase in the advanced stages of tapping panel dryness (TPD) compared to healthy and early stages of TPD. Prenyl transferase activity measured in the C-serum of latex showed a slight decrease in the early stages, but was substantially large in the advanced stages of TPD. There was a positive correlation between RuT and prenyl transferase activities. The increased activities of RuT and prenyl transferase under in vitro *conditions in the presence of adequate concentration of their substrates suggest the existence of a large number of small rubber particles in a given unit weight of WRP. The mean rubber particle size slightly increased in the early stages of TPD, but was smaller in the advanced stages of TPD. The mean rubber particle size was negatively correlated with RuT activity. It is suspected that both RuT and prenyl transferase remained inactive under* in vivo *conditions possibly due to inadequate supply of their immediate substrates. These findings are discussed in the light of our earlier results that showed enhanced respiration in the TPD affected bark tissues caused by alternate respiration which was known for its low ATP yield.*

Key words: tapping; panel dryness; biosynthesis; prenyl transferase activity; respiration; rubber particle size

It is generally presumed that tapping panel dryness (TPD) is, by and large, a physiological disorder resulting from tapping induced biotic stress to *Hevea* trees[1,2]. In the light of recent reports, involvement of a pathogen in some types of TPD seems to be a possibility, although results are far from being conclusive[3–5]. It is likely that various causes including both physiological and pathological may be responsible for TPD. Very little is known about the mechanism that triggers partial to complete inhibition of synthesis of rubber and latex in the affected tissues[2].

It has been suggested that when the capacity of a tree to regenerate the latex harvested through tapping becomes inadequate, the tree succumbs to TPD[6]. This contention is largely based on the common observation that over-exploitation of the trees either due to frequent tapping and/or chemical stimulation leads to increased incidence of TPD[7] and high yielding clones are more vulnerable than low yielding clones[8]. Our earlier results do not indicate that a deficient supply of carbon source for isoprene synthesis is a limiting factor in TPD affected

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trees, because key intermediates of the isoprene pathway, such as HMG-CoA and mevalonate were found in large concentrations in the affected bark[2]. Therefore excess drainage of photosynthates through latex may not be the primary cause for TPD. The metabolic conversion of mevalonate to isoprene and formation of rubber molecules (*cis*-poly isoprene) is inhibited due to unknown reasons in TPD affected trees.

Once the primary molecules of isopentenyl diphosphate (IPP) and DMAPP are formed from mevalonate, there are three distinct steps *viz.* initiation, elongation and termination in the formation of a rubber particle[9]. The process of initiation of the rubber particle requires an allylic diphosphate molecule which is synthesised by the enzyme *trans*-prenyl transferase existing free in the C-serum of latex[9]. The enzyme rubber transferase (RuT) which is *cis*-prenyl transferase and bound to the rubber molecule is responsible for elongation of the polyisoprene chain by catalysing the polymerisation of *cis*-1,4 polyisoprene units from IPP[10]. Thus, *trans*-prenyl transferase and RuT activities may have an effect on the rubber molecule formation and the rubber particle size. In the present investigation we studied the activities of these two enzymes and related it to the rubber particle size in healthy and TPD affected trees.

MATERIALS AND METHODS

The present study was conducted in a two hectare plantation of 21-year-old *Hevea* (clone RRII 105) at the Central Experiment Station of the Rubber Research Institute of India, Chethackal, Kerala, India. The trees had been under the S/2 d/2 system of tapping for the past 12 years. Incidence of TPD was monitored in these trees on every tapping day for a continuous two-month period before taking the samples for the present study. Based on the percentage of the length of the tapping panel that had gone dry, we identified five distinct TPD categories. They were trees with 10% – 30% (T1), 31% – 50% (T2), 51% – 70% (T3) and 71% – 90% (T4) dry tapping panel. Trees with 0% panel dryness were taken as control. Fresh latex samples were collected from 4–6 trees from each TPD group. The latex samples from the different trees belonging to a given TPD group were pooled together into a composite sample. One volume of this latex sample was mixed with two volumes of an isotonic stabilisation buffer (0.1 M $NaHCO_3$, 50% glycerol, 0.3% (w/v) NaN_3 and 5 mM cysteine) in the field and frozen in liquid nitrogen immediately. These samples were transported on dry ice in sealed containers and stored at –20°C before further processing was carried out at the USDA, Western Regional Laboratory at Albany, California, USA.

Purification of Washed Rubber Particles

Washed rubber particles (WRP) were prepared from whole latex by suspending in a wash buffer (100 mM Tris-HCl at pH 7.5, 2.5 mM $MgSO_4$, 5 mM DTT) and further by centrifugation/ flotation procedure after Cornish *et al.*[11] with some modification in the centrifugation speed (470 g) and duration (15 min – 20 min). The washed rubber particles collected by this method were pooled and adjusted to 10% glycerol, frozen as droplets in liquid nitrogen and stored in liquid nitrogen until use. The dry rubber content (DRC) was determined in each WRP preparation before doing various assays.

Rubber Transferase Activity

Rubber transferase activity was determined in the latex and WRP by the method of Cornish

and Siler[10]. WRP were diluted with wash buffer (1 mg WRP/25 ml) and incubated for 4 h at 25°C in 2 mM ^{14}C-IPP, 2 mM $MgSO_4$, and 100 mM tris-HCl, pH 7.8. Farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) were used as the initiator molecules[12] in the assay reaction mixture. The radioactivity in the WRP was determined by liquid scintillation spectroscopy and corrected for the background activity determined in the presence of 25 µL 1 M EDTA. The enzyme activity was calculated by determining the IPP incorporation (nmol/g dry rubber). The allylic diphosphate (GGPP) concentrations were changed and the V_{max} of RuT in washed rubber particles was also determined[12].

Prenyl Transferase Activity

The C-serum from the latex samples was collected by centrifuging at 15 000 r.p.m. for 40 min at 4°C. Soluble prenyl transferase activity assay in the C-serum was carried out by incubating the C-serum in a reaction mixture (6 mM ^{14}C-IPP, 50 mM Tris-HCl (pH 7.5), 1 mM $MgSO_4$, 10 mM DTT and 3 mM DMAPP) for 40 min at 25°C[9]. Excess solid NaCl was added to each tube to saturate the samples with some NaCl left undissolved. The samples were then partitioned three times against 750 mL aliquot of n-butanol. The radioactivity in the combined n-butanol fraction was determined by liquid scintillation spectroscopy.

Rubber Particle Size

Rubber particle size was determined by a standard procedure developed in the USDA laboratory[11] using a particle size analyser (Horiba LA-900, Horiba Instruments, USA). The measurement of the rubber particle size was made in both latex and WRP samples by suspending a known volume of each sample in double distilled water. All the samples were sonicated for 2 min within the chamber before analysis to avoid formation of lumps of rubber particles before measuring the particle size.

RESULTS

The frozen WRP prepared from frozen latex samples collected from normal and TPD affected trees showed appreciable RuT activity when assayed at 25°C. The RuT activity showed a progressive increase with increasing concentration of the substrate (GGPP) in the assay medium in both healthy and TPD treatments (*Figure 1 A*). The substrate saturated maximum rate of RuT activity (V_{max}) was more in TPD affected than normal trees (*Figure 1 A*). V_{max} of RuT analysed in the latex from healthy and TPD affected trees showed similar K_m values for IPP (*Figure 1 B*).

V_{max} of RuT expressed on a unit dry rubber basis showed a substantial increase in the latex and WRP samples collected from trees with advanced TPD compared to healthy trees (*Figure 2 A*). In the early stages of TPD V_{max} of RuT activity remained more or less similar in the healthy and TPD treatments (*Figure 2 A*).

The V_{max} of prenyl transferase expressed on the basis of a unit volume of C-serum of latex showed a decreasing trend in the early phases but this was substantially increased in the advanced stages of TPD (*Figure 2 B*). There was a strong positive correlation between the V_{max} of prenyl transferase and RuT (*Figure 2 C*).

The mean particles size in the WRP showed an increasing trend in the early stages of TPD but was remarkably small in the latex collected from trees severely affected by TPD

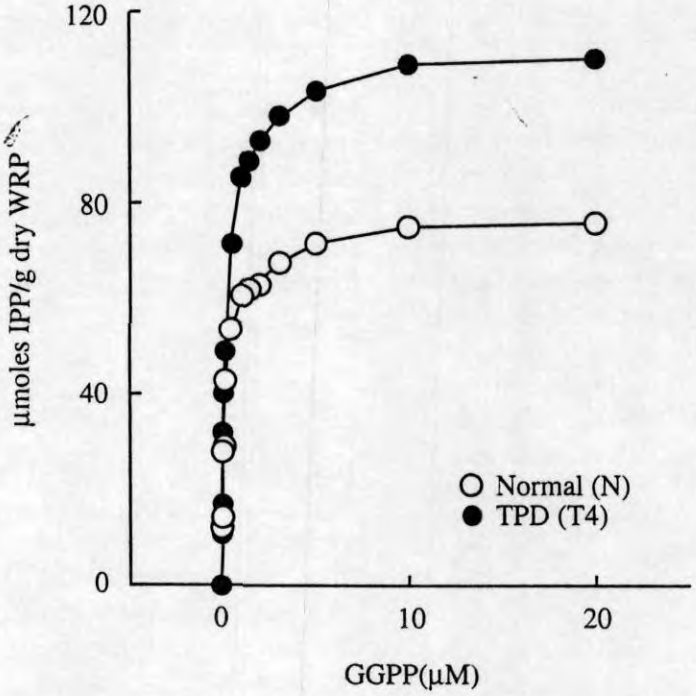

Figure 1 A. The substrate (geranylgeranyl diphosphate) concentration dependent rubber transferase activity in the washed rubber particles (WRP) of normal and tapping panel dryness (TPD) trees.

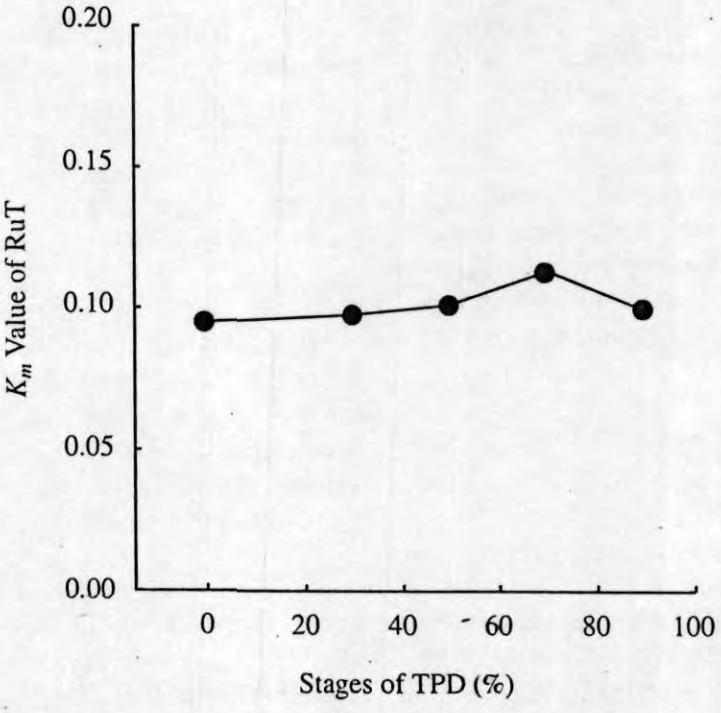

Figure 1 B. K_m value of rubber transferase in the latex harvested from trees at different stages of tapping panel dryness.

(*Figure 3 A*). There was a significant negative correlation between the mean particle size of WRP and V_{max} of RuT measured in WRP (*Figure 3 B*).

DISCUSSION

Contrary to our expectation, there was an increase in the V_{max} activities of RuT and prenyl transferase in the advanced stages of TPD when assayed under optimum conditions *in vitro* (*Figure 2 A* and *B*). The strong positive correlation between the activities of these two enzymes (*Figure 2 C*) is indicative of a stoichiometric equilibrium between the rubber particle initiation and chain elongation processes. Evidently this equilibrium has not been altered in the TPD affected tissues. However, neighbouring laticiferous cells of TPD affected tissues forbid latex synthesis, this may be due to the impermeabilisation of the cell membranes.

The increased activity of RuT (V_{max} expressed on the basis of unit dry weight of WRP) observed in the advanced stages of TPD probably indicates the presence of a large number of smaller rubber molecules, each one with active RuT present on its surface. Therefore, we suspect that due to the poor availability of substrate (IPP), the rubber molecule present in the latex of TPD trees may have a shorter chain length. The chain length is suggested to reflect the relative availability of the substrates[11]. It was noticed that the mean rubber particle size was small in the advanced stages of TPD compared to normal trees (*Figure 3 A*). The smaller particle size, might cause more rubber molecules having more active sites of RuT per unit dry weight of WRP in the TPD affected trees. Since the substrate IPP was in poor supply these sites were not saturated with IPP *in vivo* and therefore when RuT activity was determined with excess IPP *in vitro*, this enzyme showed increased activity in the TPD trees. It may also be noted that the *in vitro* specific activity of RuT was negatively correlated with mean particle size (*Figure 3 B*). Therefore, it is suggested that a high *in vitro* activity of RuT is indicative of the presence of large number of smaller rubber chains in the rubber particles per unit dry weight of WRP in the TPD affected latex.

An increase in the activity of prenyl transferase (V_{max} expressed per unit volume of C-serum of the latex) also indicated that this enzyme was not inhibited, but probably this was down regulated *in vivo* in tune with the activity of RuT as indicated by the strong positive association between the activities of the two enzymes (*Figure 2 C*). Thus, it seems that rubber particle initiation and elongation processes were adversely affected in the TPD affected trees compared to the normal trees possibly due to the unavailability of substrates such as IPP, GGPP, DMAPP *etc.* due to the impaired ATP status in the laticiferous tissues. This decreased ATP status in the latex of TPD affected *Hevea* trees was reported earlier[13].

Several evidence suggested a relation between TPD and oxidative stress[2,14,15]. Oxidative stress has been known to alter the normal metabolic pathways in a healthy cell by triggering a series of degenerative processes and may lower the respiratory activity and the ATP status of the tissue[16]. Conversion of mevalonate to isoprene is an energy consuming process[17] and therefore, this must be very sensitive to oxidative stress.

Our earlier studies have shown that the soft bark tissues had substantial accumulation of carbon precursors and intermediates such as HMG-CoA and mevalonate for the synthesis of rubber particles in TPD affected trees[2]. It would appear that there was some inhibition in the metabolic conversion of mevalonate into

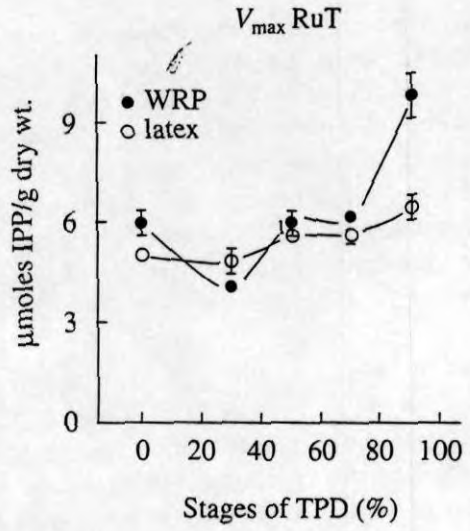

Figure 2 A. V_{max} of rubber transferase (RuT) in the latex and washed rubber particles (WRP) of trees at different stages of tapping panel dryness (TPD).

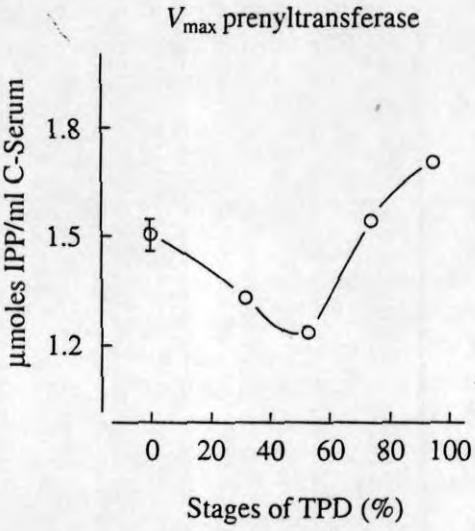

Figure 2 B. V_{max} of prenyltransferase in the C-serum of latex harvested from trees at different stages of tapping panel dryness.

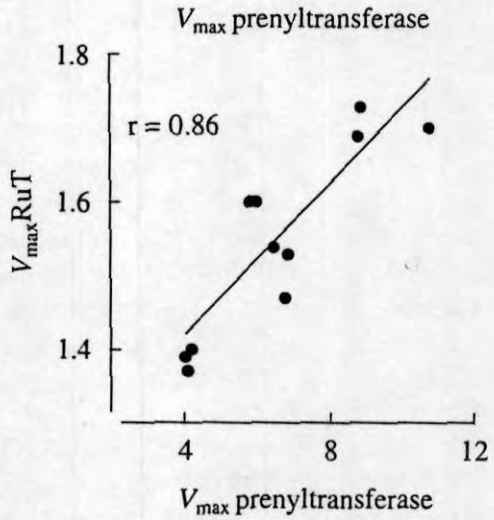

Figure 2 C. Correlation between V_{max} rubber transferase and V_{max} prenyltransferase in tapping panel dryness trees.

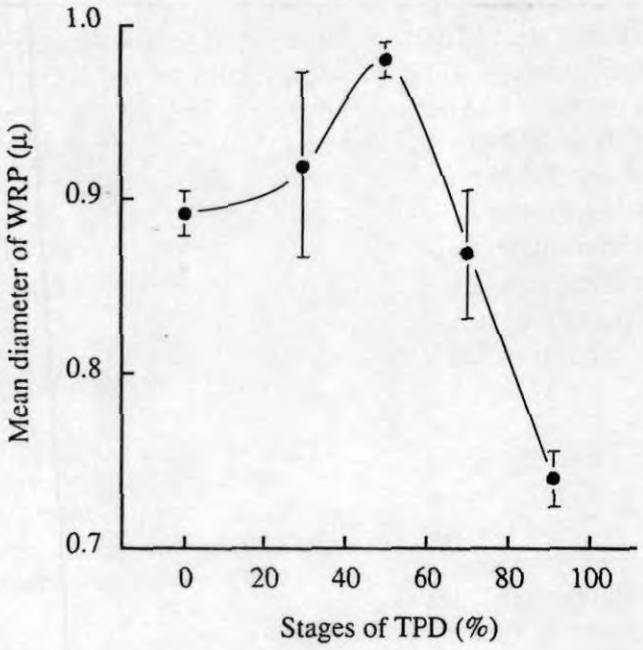

Figure 3 A. The mean diameter of washed rubber particles (WRP) harvested from trees at different stages of tapping panel dryness (TPD).

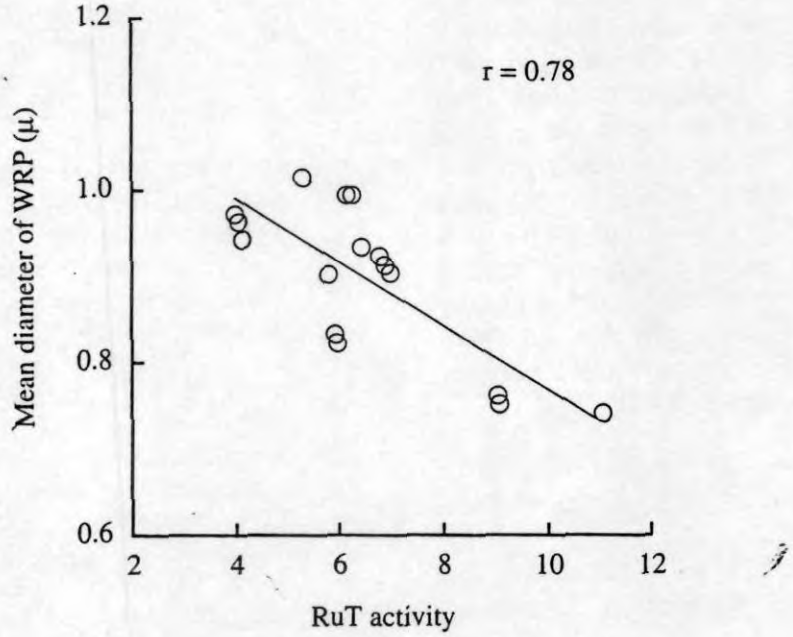

Figure 3 B. Correlation between mean diameter of washed rubber particles and rubber transferase (RuT) activity at different stages of tapping panel dryness.

isoprene units in the TPD affected trees. This conversion requires an abundant supply of ATP which has to be derived from respiration of the bark tissues[18]. Inadequate supply of ATP due to impaired respiratory metabolism may be the reason for the accumulation of mevalonate in TPD affected tissues[2]. Reduced availability of substrates rendered RuT and prenyltransferase enzymes inactive *in vivo*. These enzymes could be activated with adequate supply of their immediate substrates *in vitro*.

Earlier studies showed that the respiratory rates were significantly higher in TPD affected than in normal bark tissues[13]. TPD affected tissues were probably experiencing oxidative stress leading to increased peroxidative damages[2]. It has been reported that respiratory rates could go up under such stress conditions as a result of enhanced alternate pathway without concomitant increase in the ATP status of the tissues[19]. Generally, the alternate respiration (cyanide insensitive pathway) yields only low ATP levels than that of the normal cytochnome respiration[20].

The results of the present study show that the mean rubber particle size was significantly small in the advanced TPD stages. Both RuT and *trans*-prenyl transferase enzymes from TPD trees could be fully activated *in vitro* in the presence of adequate supply of their intermediate substrates. While intermediates of isoprene pathway such as HMG-CoA and mevalonate were found in greater concentrations in the TPD affected than healthy bark tissues[2], it is suggested that there was poor conversion of mevalonate to IPP possibly due to inadequate supply of ATP.

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