RUBBER BIOSYNTHESIS IN TAPPING PANEL DRYNESS AFFECTED *HEVEA* TREES

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Abstract *The activity of rubber transferase (RuT) determined in washed rubber particle (WRP) and whole latex showed a marked increase at 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 of TPD, 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 presence of adequate conversion of their substrates suggest the presence 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.*

Key words: *Hevea brasiliensis;* rubber biosynthesis; particle size; WRP; RuT; TPD; prenyl transferase.

INTRODUCTION

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 (Lacrotte *et al.*, 1995; Krishnakumar *et al.*, 1998a). 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 (Nandris *et al.*, 1991; Zheng *et al.*, 1997; Wu Jilin *et al.*, 1997). At the present juncture, it is likely that various causes including both physiological and pathological may be responsible for TPD. But very little is known about the mechanism that triggers partial to full inhibition of synthesis of rubber and latex in the affected tissues (Krishnakumar *et al.*, 1998a).

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 (Jacob *et al.*, 1994). 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 (Chrestin, 1989) and that high yielding clones are more vulnerable (Sivakumaran and Haridas, 1989). Our earlier results do not indicate that a lack of carbon source for isoprene synthesis was a limiting factor in TPD affected trees because key intermediates of the isoprene pathway such as HMG CoA and mevalonate were found in large concentrations in the affected bark (Krishnakumar *et al.*, 1998a). Therefore, excess drain of photosynthates through latex may not be the primary cause for TPD. The metabolic conversion of mevalonate to isoprene and formation of rubber

particles (cis-poly isoprene) is inhibited due to unknown reasons in TPD affected trees.

Several evidences suggested a relation between TPD and oxidative stress (Christin *et al.*, 1985; Gohet *et al.*, 1997; Krishnakumar *et al.*, 1998a). Oxidative stress has been known to alter the normal metabolic pathways of healthy cells and trigger a series of degenerative processes and may lower the respiratory activity and the ATP status of the tissue (McKersie and Leshem, 1994). Conversion of mevalonate to isoprene is an energy consuming process (Peterson-Jones *et al.*, 1990) and therefore, this must be very sensitive to oxidative stress. This may be the reason for the accumulation of mevalonate in TPD affected tissues (Krishnakumar *et al.*, 1998a).

Once the isoprene molecules are formed from mevelonate, there are three distinct steps *viz.* initiation, elongation and termination in the formation of a rubber particle (Cornish, 1993). The process of initiation of the rubber particle requires an allylic diphosphate molecule which is synthesized by the enzyme trans-prenyl transferase existing free in the C-serum of the latex (Cornish, 1993). The enzyme rubber transferase (RuT) which is cis-prenyl transferase and bound to the rubber particle is responsible for elongation of the polyisoprene chain by catalyzing cis-1, 4 polyisoprene units from isopentenyl diphosphate (IPP) (Cornish and Siler, 1996). Thus, trans-prenly transferase and RuT activities may have an effect on the rubber particle size. In the present investigation we studied the activities of these two enzymes and related 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 years old *Hevea* (clone RRII 105) at the Central Experiment Station of the Rubber Research Institute of India, Chethackal, Kerala, India. The trees were 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 has 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 controls. 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) from the field and frozen in liquid nitrogen immediately. These samples were transported on dry ice in sealed containers and stored at -20°C before the experiments were carried out at the USDA, Western Regional Laboratory at Albany, California, USA.

Purification of Washed Rubber Particles (WRP)

Washed rubber particles 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 according to Cornish *et al.* (1993) with some modifications in the centrifugation speed (470g) and duration (15-20 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 (RuT) Activity

Rubber transferase (RuT) activity was determined in the latex and WRP by the method of Cornish and Siler (1996). Washed rubber particles were diluted with wash buffer (1mg WRP/25ml) and incubated for 4 hrs. at 25°C in 2mM ^{14}C-IPP, 2mM $MgSO_4$, 25mMDTT and 100mM tris-HCl, pH 7.8. Farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) were used as the initiator molecules (Cornish and Siler, 1995) in the assay reaction mixture. The radioactivity in the washed rubber particle was determined by liquid scintillation spectroscopy. The allylic diphosphate (GGPP) concentrations were changed and the V_{max} of RuT in washed rubber particles was also determined (Cornish and Siler, 1995).

Prenyl Transferase Activity

The C-serum from the latex samples was collected by centrifuging at 15,000 rpm for 40 min. at 40°C. Soluble prenyl transferase activity assay in the C-serum was carried out by incubating the C-serum in a reaction mixture (6mM ^{14}C-IPP, 50mM Tris-HCl (pH 7.5), 1mM $MgSO_4$, 10 mM DTT and 3mM DMAPP) for 40 min at 25°C (Cornish, 1993). 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 750ml 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 after a standard procedure developed in the USDA laboratory (Cornish *et al.*, 1993) 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 two min. within the chamber to avoid formation of lumps of rubber particles before measuring the particles size.

RESULTS

The frozen washed rubber particles (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 (Fig.1A). The substrate saturated maximum rate of RuT activity (V_{max}) was more in TPD affected than normal trees (Fig.1A). V_{max} of RuT analysed in the latex from healthy and TPD affected trees showed similar K_m values for IPP (Fig.1B).

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 (Fig. 2A). At the early stages of TPD, V_{max} of RuT activity remained more or less similar in the healthy and TPD treatments (Fig. 2A).

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 (Fig. 2B). There was a strong positive correlation between the V_{max} of prenyl transferase and RuT (Fig. 2C).

The mean particle size in the WRP showed an increasing trend at the early stages of TPD but was remarkably small in the latex collected from trees severely affected by TPD (Fig. 3A). There was a significant negative correlation between the mean particle size of WRP and V_{max} of RuT measured in WRP (Fig.3B).

DISCUSSION

Contrary to our expectation, there was an increase in the activities of RuT and prenyl transferase (V_{max}) at the advanced stages of TPD when assayed under optimum condition *vitro* (Fig. 2 A&B). The strong positive correlation between the activities of these two enzymes (Fig. 2C) 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.

The increased activity of RuT (V_{max} expressed on the basis of unit dry weight of WRP) observed at the advanced stages of TPD probably indicates the presence of large number of smaller rubber particles, each one with active RuT present on it. We suspect that due to poor availability of the substrate (IPP), this enzyme remained relatively inactive under *in vivo* conditions and thus the mean rubber particle size was small at the advanced stages of TPD compared to normal trees (Fig.3A). Chain length is suggested to reflect the relative availability of the substrates (Cornish *et al.*, 1993). Because of the small size, there will be more number of rubber particles and hence more active sites of RuT per unit dry weight of WRP in the TPD affected trees, and this explains the large *in vitro* RuT activity in these trees in presence of excess IPP in the assay medium. It may also be noted that the *in vitro* specific activity of RuT was negatively correlated with mean particle size (Fig.3B). Therefore, it is suggested a high *vitro* activity of RuT is indicative of the presence of large number of smaller size 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 (Fig. 2C). 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 unavailability of substrates such as IPP, GGPP, DMAPP, *etc.*

Our earlier studies have shown that the biochemical composition of soft bark tissues collected from healthy and TPD affected trees had substantial accumulation of carbon precursors and intermediates for the synthesis of rubber particles such as HMG CoA and mevalonate in TPD affected bark tissues (Krishnakumar *et al.*, 1998a; see also Table1). It would appear that there was some inhibition in the metabolic conversion of mevalonate into isoprene units in the TPD affected trees. This conversion requires abundant supply of ATP which has to be derived from respiration of the bark tissues (Jacob and Prevot, 1992). 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*.

Respiratory rates were significantly higher in TPD affected than normal bark tissues (Table.1; Krishnakumar *et al*., 1998b). Whether this had also resulted in a high ATP status in the TPD affected bark was not known. TPD affected tissues were probably experiencing oxidative stress leading to increased peroxidative damages (Table 1; Krishnakumar *et al*., 1998a). 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 (Wen and Liang, 1993). The possibility of increased diversion of carbohydrates through the alternate pathway can not be ruled out in the TPD affected bark tissues.

The results of the present study show that mean rubber particle size was significantly small at the advanced TPD stages, although both RuT and trans-prenyl transferase enzymes could be fully activated *in vitro* in presence of adequate supply of their intermediate substrates. While intermediates of isoprene path such as HMG CoA and mevalonate were found at greater concentrations in the TPD affected than healthy bark tissues, it is suggested that poor conversion of mevalonate to IPP possibly due to poor supply of ATP may be the cause for small particle size in the former. The role of increased respiration vis-a-vis alternate path way and ATP status in the TPD affected bark tissues is being investigated.

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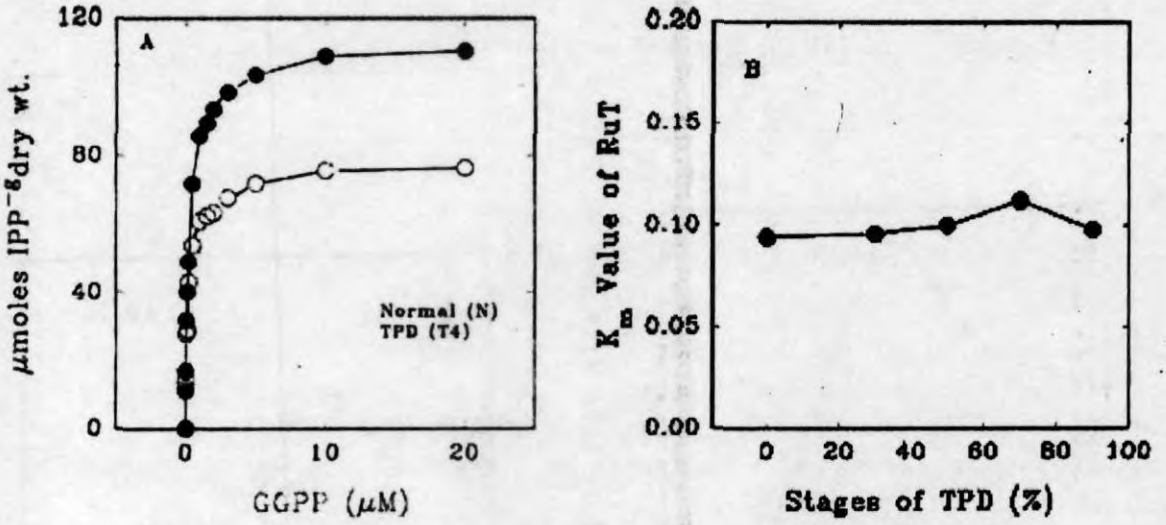

Figure 1. The RuT Acitivity of latex from both healthy and TPD trees

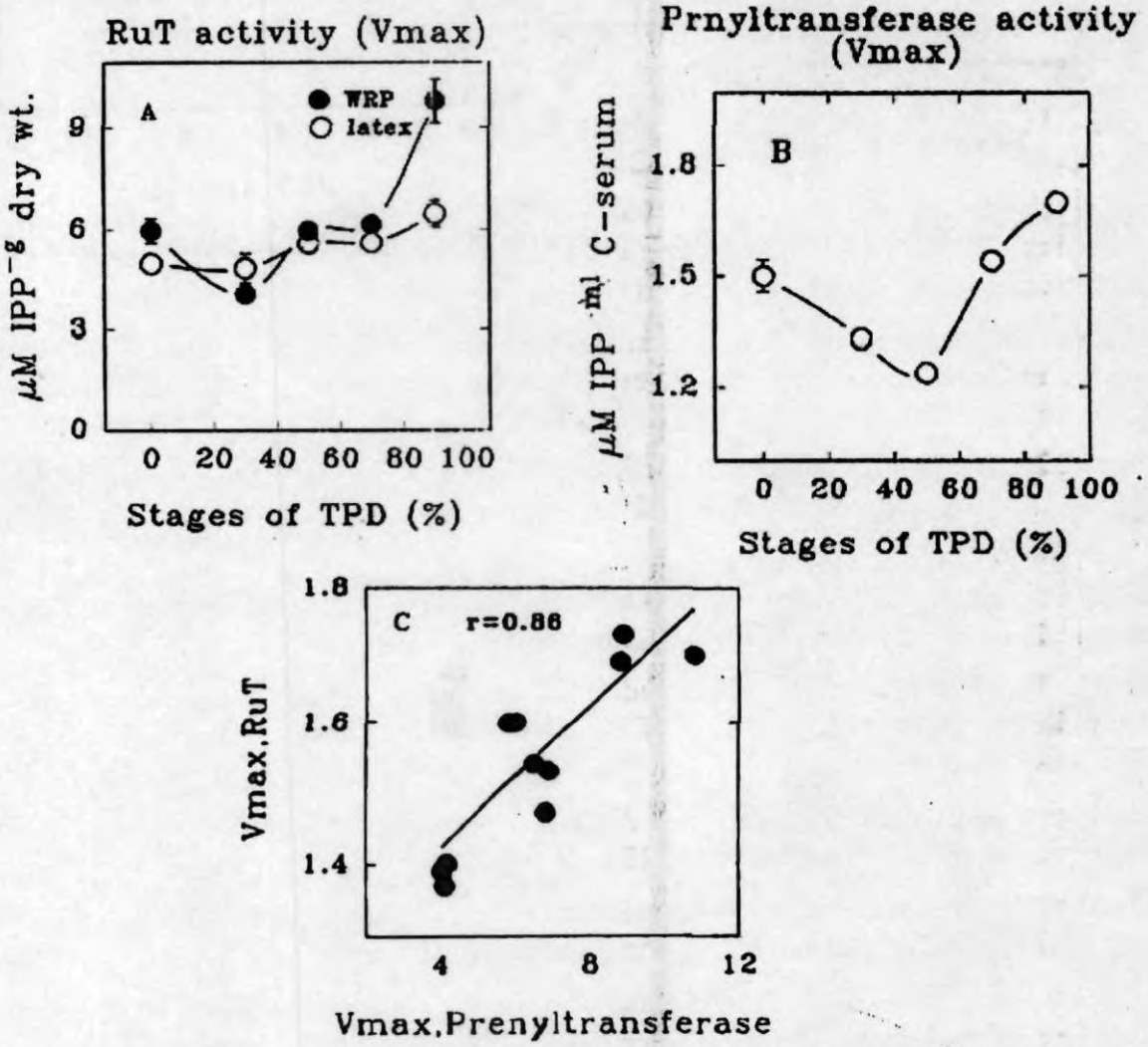

Figure 2. Correlation between the V_{max} of prenyl transferase and RuT.

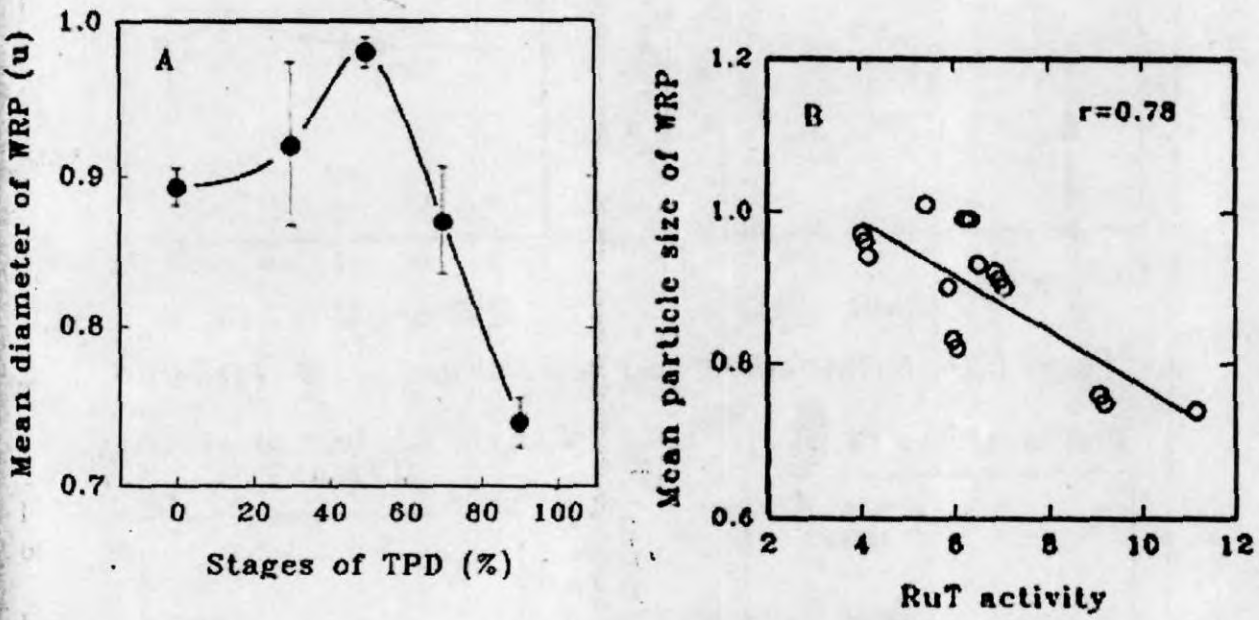

Figure 3. Correlation between the mean particle size of WRP and the V_{max} of RuT