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Status of Free Radical and its Scavenging System with Stimulation in *Hevea brasiliensis*

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Tapping panel dryness (TPD) syndrome is expressed initially by prolonged (late) dripping of latex followed by pre-coagulation of latex in the tapping panel itself which culminates into death of latex-bearing cells. The syndrome is found to be due to a cumulative effect of many factors like over-tapping, over dosage of stimulation and sub-optimal agro-climatic conditions. In an attempt to understand the role of free radical and its scavenging systems in occurrence of TPD, studies have been undertaken to estimate free radical (FR) by electron paramagnetic resonance spectrometer with simultaneous assessment of superoxide dismutase (SOD, EC 1.15.1.1), in the laticiferous cellos/latex of five different clones of rubber. The bark of rubber trees when over-exploited with 5% ethephon, showed more free radical accumulation in comparison with the untreated trees; the level of SOD showed less amount in the treated plants. Apparently an increase of free radical and associated decrease of the scavenging system, thus leading to an imbalance in the two, maybe one of the factors for TPD syndrome.

Keywords: Rubber latex, bark, lutoids, free radical, electron paramagnetic resonance, superoxide dismutase, stimulation.

INTRODUCTION

Tapping in *Hevea brasiliensis* is a scientific method of systematic/synchronized wounding (cutting) of a portion of bark, which oozes out the economically important 'latex'. Unlimited cutting of the bark (Eschbach *et al.* 1989), along with other factors like overdosage of stimulant to produce more latex, abnormal changes in environmental factors like low temperature, etc., may bring disorder to the plant, which is broadly termed as tapping panel dryness (TPD). Unambiguous report on clonal variation in occurrence of TPD is still lacking. Ironically, since rubber is a bud-grafted plant, controversy arises regarding the susceptibility/viability/homogeneity of the stock and the scion (Krishnakumar *et al.* 1992), the effect of which may also determine the fate of the plant exposed to TPD. Thus TPD seems to be a syndrome, being a cumulative effect of many factors, yet to be explored.

A true TPD syndrome is physiologically characterized by late/prolonged dripping of latex, followed by pre-coagulation of latex or partial dryness of panel, culminating in cessation/stoppage of flow. The biochemical basis of such a disorder is characterized by bursting of lutoids (Cope *et al.* 1972), a phenomenon associated with a series of biomolecular aspects. It is well established now that it is the loss of membrane permeability of lutoids which exposes the content of lutoids with outer atmosphere and causes coagulation of latex within the plant (Paranjothy *et al.* 1976). One of the causes for loss of membrane permeability is the accumulation of free radical (FR) in the laticiferous cells (Cretin and Bangratz, 1983), generated from stress, viz. wound, cold, etc. Stress-induced increase of FR and lower scavenging system were also reported in many plant systems (Leprince *et al.* 1993, Scandalios 1993, Hendry 1993, Price and Hendry 1991). Presence of NAD(P)H oxidase, which generates toxic-free radicals along with its scavenging system, is reported to be present in the lutoidic tonoplast (Chrestin 1989). Thus it is worthwhile to do a thorough study on estimation of free radical and its scavenging enzyme, superoxide dismutase (SOD).

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MATERIALS AND METHODS

Plant material

Five different clones, viz. RRII-118, RRII-203, RRII-105 RRIM-600 and GT-1, which were under tapping for six years, have been chosen as the experimental material. Plants from each clone were stimulated with 5% ethephon once in a month. Yield of all the plants was recorded for pre-winter and post-winter rest period. Latex from five of these clones was collected, after the 7th application of stimulant in Tris-HCl buffer and transported to Bose Institute, Calcutta for biochemical analysis. The bark of the plants was also transported for electron paramagnetic resonance (EPR) study.

Lutoids were isolated from latex by centrifugation at 23 rpm for 30 min at 0–4°C, lyophilized and kept in the desiccator at –80°C. The bark samples were dried in vacuum till the moisture content came down to 7–10%. The dried bark was powdered and stored in a vacuum desiccator.

Electron paramagnetic resonance study

The lutoids, raw latex and the bark samples were tested for FR estimation. The EPR spectra of all the samples were taken at room temperature using Varian E-112 spectrometer at X-band frequencies (ν = 9.45 GHz) having 100 kHz first modulation according to the method of Nandi *et al.* (1997). The 1,2-diphenyl-2-picryl hydrazyl (DPPH) was used as internal standard (g = 2.0036). However, EPR signal was obtained from the bark only.

Protein extraction and gel electrophoresis

The polyacrylamide gel electrophoresis of SOD from lutoids was done according to the method modified from that of Nandi *et al.* (1997). The enzyme was isolated in 100 mM potassium phosphate buffer (pH 7.8) containing EDTA, $MgSO_4$, NaCl and β-mercaptoethanol. The extract was centrifuged at 20 rpm for 30 min at 0–4°C. The protein fraction of 30 ~ 80% $(NH_4)_2 SO_4$ precipitation was made salt-free and the extract was considered as the enzyme extract.

Total protein was estimated by the method of Bradford (1976). An equal amount (50 µg) of protein was loaded on to each lane of the gel. Electrophoresis was continued for 5 h in 100 V at 2–4°C. The gels were stained according to the method of Beuchamp and Fridovich (1971) using NBT and riboflavin as the substrates.

RESULTS AND DISCUSSION

The average yield in pre-wintering rest (i.e. from Oct. 1995 to Feb. 1st week 1996) and post-wintering rest (i.e. end of March 1996 to July 1996) in all the clones, increased with the application of stimulant (Table 1). However, during pre-wintering rest period, RRII-105 showed significant increase in yield compared with the rest of clones, while in post wintering rest period RRII-203, RRIM-600 and GT-1 showed significant increase in yield, at least in the climatic condition (during Oct. 1995 to July 1996). Ethephon stimulated increase in yield of rubber has been reported by D'Auzac and Ribaillier (1969), George *et al.* (1974) and Coupe and Chrestin (1989). It has also been noticed that with the application of ethephon (ET), while the yield was more in all the clones, the plants were approaching towards the TPD syndrome.

The spectrum of the powdered bark samples (Figure 1) showed a singlet with sixtet. This sixtet arises

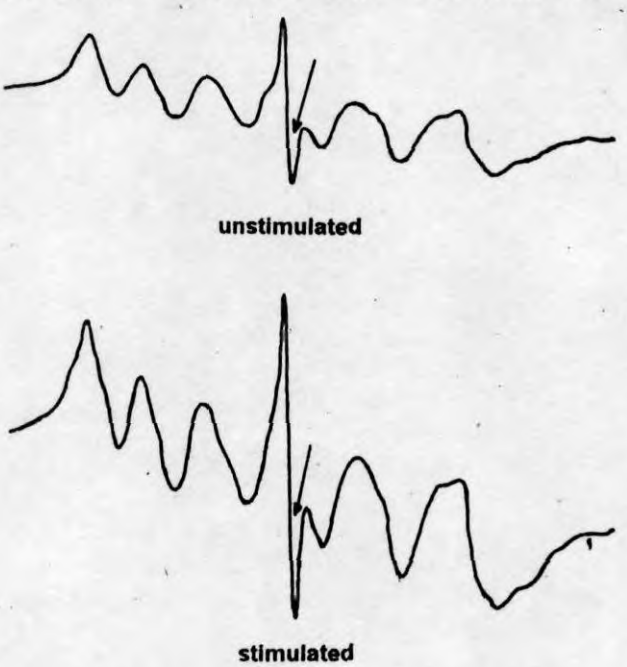

Figure 1. EPR spectra of unstimulated and stimulated (5% ethephon) bark samples in rubber. The height of the peak corresponds to the amount of free radical in cm/mg. (For details of experiment, please see materials and methods.)

Table 1. Average yield (in g) of different clones of rubber plants treated with ethephon (5%, monthly application) during pre-wintering (from 10 Oct. 1995–12 Feb. 1996) and post-wintering rest period (from 22 Mar. 1996 to 7 Jul. 1996). Values are means of 10 plants for each treatment.

Clone	Yield (g per plant ± S.E.)	
	Unstimulated	Stimulated
Pre-wintering rest		
RRII 203	54.3 ± 4	62.3 ± 6
RRII 118	60.0 ± 3	65.4 ± 4
RRIM 600	86.3 ± 4	94.4 ± 5
RRII 105	85.0 ± 7	112.0 ± 10
GTI	45.0 ± 3	52.6 ± 2
Post-wintering rest		
RRII 203	39.6 ± 5	67.0 ± 9
RRII 118	51.1 ± 4	61.0 ± 7
RRIM 600	36.6 ± 6	77.4 ± 10
RRII 105	61.5 ± 8	78.6 ± 5
GTI	39.4 ± 7	52.3 ± 8

Table 2. Free radical content (in cm/mg) from dry-bark of rubber plant treated with or without stimulation (5% ethephon, monthly application). Free radical estimated by EPR spectrometer. The values indicate average of 10 plants in each treatment.

Clone	Unstimulated	Stimulated
RRII 203	0.0243	0.038
RRII 118	0.009	0.024
RRIM 600	0.034	0.052
RRII 105	0.020	0.054
GTI	0.046	0.058

from the naturally occurring Mn^{2+} ions of the specimen, with *g* value lying between 1.860 and 2.1602. The *g* value of maximum absorption is 2.00352, suggesting that the radical is quinone in nature (Cadenas 1989, Atherton *et al.* 1993). This is also in comparison with the radical anions of ubiquinone (Nohl and Jordan 1986) and semi-quinone (Knowles *et al.* 1976). Such stable-free radicals were also detected in a wide range of tissues subjected to oxidative injury such as water loss (Hendry *et al.* 1992), drought-affected leaves (Runeckles and Vaartnov 1992), highly irradiated and water-stressed mosses (Seel *et al.* 1991). The EPR response, which is considered to be the most reliable method for FR formation and build-up showed that there is a difference in FR content (in cm/mg dry tissue) of stimulated and

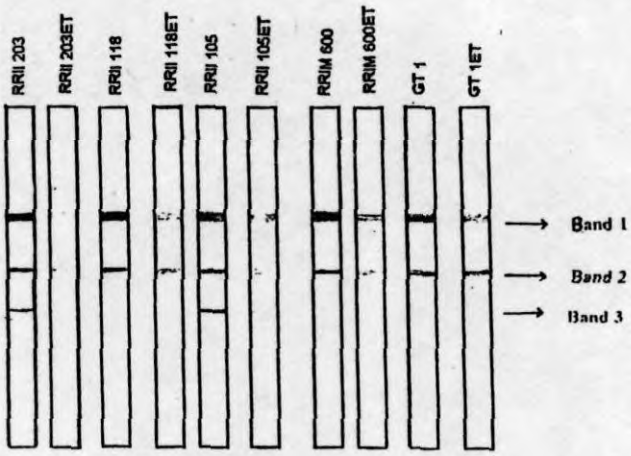

Figure 2. Representative diagrams of SOD bands from lutoids of latex on 10% native polyacrylamide gel. The intensity of black areas corresponds to the concentration of enzyme. Here, ET = 5% ethephon-treated plants; RRII-203, RRII-118, RRII-105, RRIM-600 and GT 1 are names of clones.

unstimulated samples (Table 2). The amount of FR is more in stimulated than that of unstimulated bark sample irrespective of difference in clones. This suggests that over-exploitation of rubber plant, by the application of ethephon, causes higher production and accumulation of free radical. Over-exploitation associated free radical formation was also evidenced when the NAD(P)H-quinone oxide-reductase enzyme was studied colorimetrically in lutoids (D'Auzac *et al.* 1985). This enzyme is active in oxidizing the phenolic and quinonic compounds to produce hydroquinones and semiquinones (Ossipov *et al.* 1995) which are auto-oxidized to superoxide ions, hydroxyl radicals and oxygen singlet (O^{2-}). Chrestin (1989) also reported a stimulation-induced increase of FR-generating lutoidic NAD(P)H oxidase.

The polyacrylamide gel electrophoretic analysis of lutoidic SOD (Figure 2) showed that there was a difference in the banding pattern as well as intensity of bands. The gel showed two common bands and a third faint band which showed polymorphism (either present or absent). The first two isozyme bands in stimulated plants showed less inensity, indicating less concentration of SOD, than that of unstimulated plants irrespective of clones. However, the third band was well discernible only in RRII-203 and RRII-105 in both unstimulated and stimulated samples.

A significant decrease in free radical associated scavenging system with over-exploitation was also shown by D'Auzac *et al.* (1985). It has been reported that the SOD along with catalase was low in stressed plants (Chrestin 1989). The tapping stress-induced increase of free radical and decrease of its associated scavenging mechanism may be one of the causes of TPD syndrome or vice versa in rubber. It is important to distinguish between the cause and effect relationship between TPD and FR accumulation with the associated FR scavenging system. Though it is not very clear, it seems that the FR may be getting generated in the tissue of laticiferous cells and then transported to the lutoids where the SOD along with catalase looks after the FR scavenging mechanism; the efficiency of action of the scaenging system determines the initiation of disaster in rubber yield.

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