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BIOCHEMICAL COMPOSITION OF SOFT BARK TISSUES IN *HEVEA* AFFECTED BY TAPPING PANEL DRYNESS

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Tapping panel dryness (TPD) syndrome is a serious problem affecting the productivity of *Hevea* plantations. There is complete shut down of rubber biosynthesis in the laticiferous cells of fully dry trees. The biochemical composition of the live laticiferous tissues of TPD affected and healthy *Hevea* trees was compared in the present study. The TPD affected laticiferous tissues contained comparatively higher levels of sugars, phenols and soluble proteins than healthy tissues. Also there was significantly higher activity of peroxidase and lower activity of polyphenol oxidase in the affected than in the healthy tissues. The results showed that lack of availability of sucrose was not the cause for TPD. The high peroxidase activity and the accumulation of phenols in the laticiferous tissue indicate possible oxidative stress in the TPD affected bark tissues. It appears that oxidative damage of laticiferous vessels may be responsible for the complete shut down of the rubber biosynthetic machinery, possibly by altering the energy metabolism in fully dry trees.

Key words : Biochemical composition, *Hevea brasiliensis*, Oxidative stress, Tapping panel dryness

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INTRODUCTION

High yielding clones of natural rubber (*Hevea brasiliensis*) are often susceptible to a physiological disorder called tapping panel dryness (TPD). It is hypothesised that this disorder occurs when harvesting of the latex from the trees exceeds the physiological capacity for its regeneration. It is estimated that TPD leads to approximately 15 to 20 per cent decrease in yield (Commere *et al.*, 1989). The common symptoms of TPD include an excessive late dripping of latex with a simultaneous drop in the dry rubber content of the latex in the initial phase. Total inhibition of rubber biosynthesis occurs and no latex is produced towards the final phase.

Investigations to analyse the cause of this disorder were restricted largely to latex biochemistry and general physiology only (Prematillaka *et al.*, 1985; Vijayakumar *et al.*, 1990; Dian *et al.*, 1995). Cytological disorders associated with the development of TPD

were also reported (Gomez, 1990). While there is a general consensus that TPD is a physiological disorder, several studies led to different hypotheses about the origin and development of this disorder. *In situ* coagulation of latex in the laticiferous cells of TPD affected plants has been observed (de Fay and Jacob, 1989). However, it is unclear whether coagulation is a cause or a consequence of this disorder. Certain proteins from the cytosolic compartment of latex were found to be related to TPD and among these, one specific protein (14.5 kDa) was reported to be linked with the coagulation process (Dian *et al.*, 1995). Accumulation of this specific protein is correlated with the disease development.

The present investigations consisted of biochemical analysis of live soft bark tissues, containing the laticifer, of healthy and TPD affected trees of the clone RRII 105 to quantify the variations in some of the major biochemical components related to metabolism in general and rubber biosynthesis in particular.

MATERIALS AND METHODS

An 18 year old rubber plantation with clone RRII 105 (1 ha) from the Central Experiment Station of Rubber Research Institute of India at Chethackal (9°22'N; 76°50'E) was selected for this study. The trees have been under regular tapping, thrice in a week, throughout the year for eleven years. TPD affected and normal trees were identified from such trees.

Using a sharp chisel, bark samples of 2.5 x 2.5 CM were carefully taken from the tap-ping panel of individual trees belonging to normal (n=20) and TPD (n=15) population occurring randomly scattered in the plantation. The samples were collected from the TPD (advanced stage) and normal trees wrapped individually in aluminium foil and transported on ice to the laboratory and stored at –60°C until analysis. From these bark samples, soft bark tissues was carefully removed using a scalpel and individually processed. One gram (fresh weight) of the soft bark was homogenized in liquid nitrogen, extracted with 80% ethanol and used for the estimation of total sugars (Scott and Melvin, 1953), reducing sugars (Nelson, 1944) and phenols (Swain and Hillis, 1959).

Glutathione was extracted from a known amount of the soft bark tissues (5 g) which was powdered in liquid nitrogen and homogenized with 5 per cent (w/v) trichloro acetic acid (TCA). Cellular debris was removed by centrifuging at 10,000 rpm for 20 min. The deproteinised supernatant was extracted with ether and then it was evaporated using a vacuum evaporator. The residue was dissolved in 2.5 per cent TCA and the glutathione content was assayed following the method after Boyne and Ellman (1972).

Proline was analysed by extracting the soft bark tissue in sulfosalicylic acid (3%) and reacted with acid ninhydrin reagent (Bates *et al.*, 1973). The method developed by Heath and Packer (1968) with modifications was used for the determination of malondialdehyde, a product of lipid peroxidation. Soft bark tissue (0.5 g) was homogenized with 0.1M Tris-HCl buffer (pH 7.4) and equal volume TCA (20%, w/v). An aliquot of the supernatant was heated for 20 min. in a boiling water bath with equal volume of TCA solution (0.75% w/v in 0.1 M HCl) and then cooled quickly in an ice bath. The absorbance at 532 nm was read and the value for nonspecific absorption at 600 nm was subtracted. The concentration of malondialdehyde was

calculated using its extinction coefficient of 155 per nM per cm.

Soluble proteins in the bark tissues were extracted by homogenizing soft bark tissue (1 g) with 0.1 M phosphate buffer (pH 6.5) containing 1mM EDTA, 1m M PMSF and 1mM DTT. PVPP (0.5 g) was added to the homogenizing mixture and the homogenate was kept at 4°C for 1 hour. The supernatant was collected by centrifuging at 15,000 g for 30 min. The proteins in the supernatant were precipitated overnight using cold acetone (final con. 80%) at 4°C. The precipitated proteins were collected by centrifuging at 10,000 rpm for 30 min. After removing the acetone content by keeping the precipitate in a vacuum evaporator for 30 min, the proteins were redissolved in 0.1 M phosphate buffer (pH 6.5). The protein content was estimated by the method of Bradford (1976) using BSA as standard.

To analyse the peroxidase and polyphenol oxidase enzyme activity, 1g of soft bark tissue was homogenized in 0.1 M phosphate buffer (pH 6.5). The supernatant collected after centrifuging at 23,000 rpm for 30 min. was used for the determination of the peroxidase (Addy and Goodman, 1972) and polyphenol oxidase (Karr and Mishra, 1976) activities. The proteins in the enzyme extracts were precipitated in 10 per cent TCA and the precipitate was redissolved in 0.1 N NaOH after washing with 80 per cent ethanol and the protein content was determined after Lowry *et al.* (1951). The enzyme activity was calculated and expressed in units of activity per min. per mg protein.

The hydroxymethyl glutaryl coenzyme A (HMG-CoA) and mevalonate contents were measured as described by Nair *et al.* (1990). The powdered soft bark tissues (3g) were homogenized in 1 per cent saline arsenate and diluted with an equal volume of perchloric acid. The extract was centrifuged at 3,000 rpm for 15 min. The supernatant was treated with 2 M freshly prepared hydroxylamine in water to analyse the HMG-CoA level. The hydroxylamine reagent was mixed with an equal volume of 4.5 M sodium hydroxide solution to estimate the mevalonate content. Ferric chloride reagent was added and incubated at room temperature for 10 min and the absorbance read at 540 nm against a blank.

The values recorded in the biochemical analyses from healthy and TPD affected *Hevea* bark samples were compared using independent t-test (Panse and Sukhatme, 1995).

RESULTS AND DISCUSSION

It has been shown that sucrose is the major sugar present in the *Hevea* laticiferous tissue, which is the primary precursor for rubber synthesis (Jacob and Prevot, 1992). Vijayakumar *et al.* (1990) related higher levels of sugars in the latex of TPD affected plants with fast induction of this syndrome through intensive exploitation. A recent study has shown that trees that show TPD tendencies contained higher levels of sucrose in their latex (Thomas *et al.*, 1998). Inadequate supply of carbohydrates for the synthesis of rubber being the primary cause of TPD is, thereofre, unlikely.

TPD affected bark contained a significantly high content of protein compared to healthy bark (Fig. 2a). Increased levels of nitrogen, indicating a high content of protein, was observed in the latex of TPD affected trees (Paranjothy *et al.*, 1975).

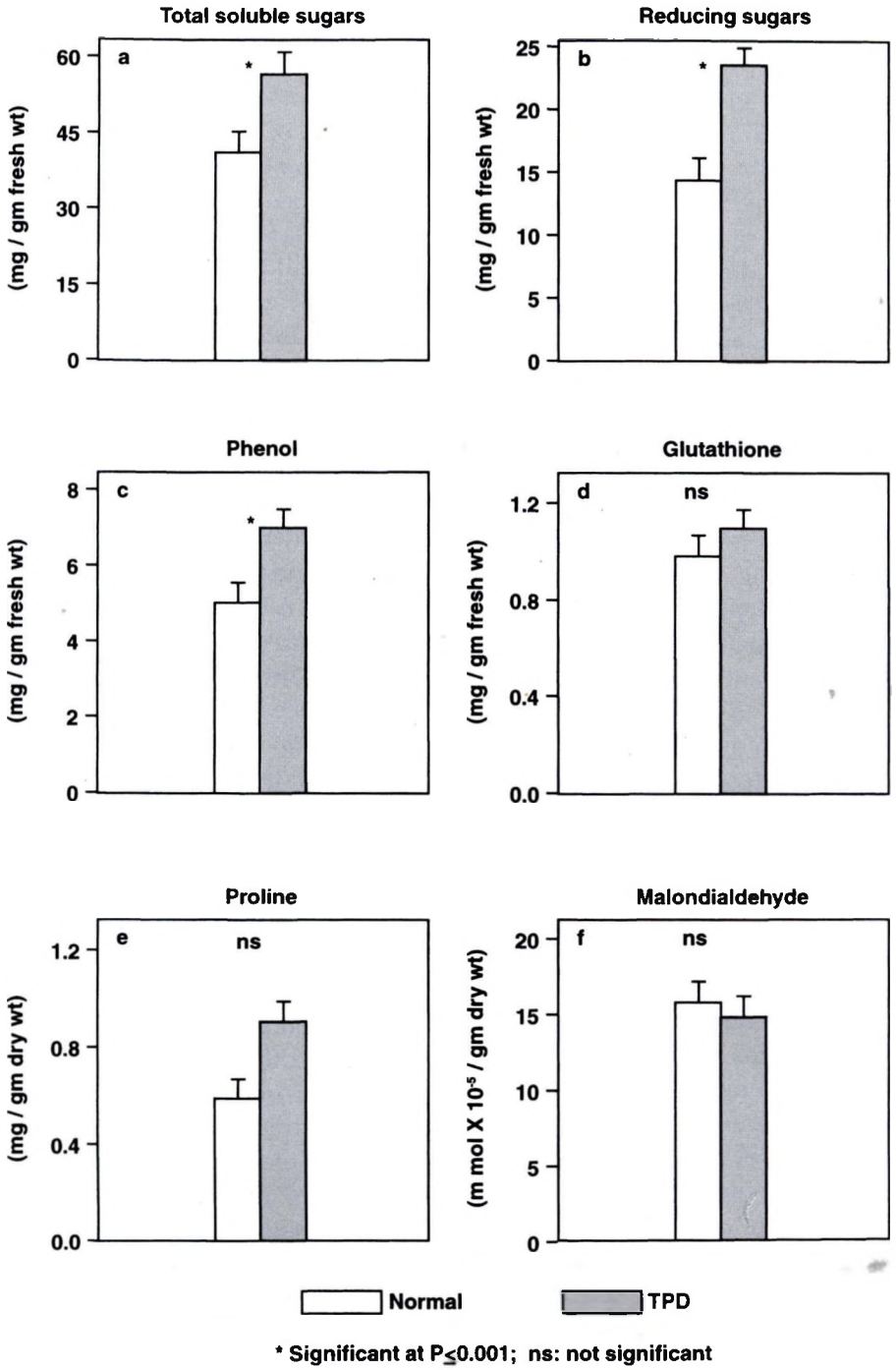

Fig.1. Concentration of total soluble sugars (a), reducing sugars (b), phenol (c), glutathione (d), proline (e) and extent of lipid peroxidation (f) in healthy and TPD affected bark tissues (n=15-20±SE); ** Significant (t-test at $P \leq 0.001$); ns : not significant

Accumulation of a few species of low molecular weight proteins has been reported in the latex of TPD affected trees (Nataraja *et al.*, 1997) and some of these may be involved in the *in situ* coagulation of latex (Dian *et al.*, 1995).

There was significantly less activity of polyphenol oxidase in the bark of TPD affected than healthy plants (Fig. 2c), which led to an accumulation of phenols in the former (Fig. 1c). This indicates an alteration in the regulation of secondary metabolites due to TPD. Phenolic compounds are known to accumulate in plants experiencing biotic stress caused by pests and diseases (Gupta *et al.*, 1995). Stress related compounds such as glutathione (Fig. 1d), proline (Fig. 1e) and melondialdehyde (Fig. 1f) indicating stress induced peroxidative damage of lipids were not significantly altered in TPD affected compared to healthy bark tissues.

The bark of TPD affected trees had significantly higher peroxidase activity than the healthy trees (Fig. 2b). Peroxidase enzyme is known to promote a variety of biological reactions including biosynthesis and degradation of growth regulators (Grambow and Langenbeek-Schwich, 1983). Peroxidase enzyme system enhances the biosynthesis of ethylene (Machackova and Zmrhal, 1981; Abbas, 1997). Higher activity of peroxidase was negatively correlated with cytokinin in the tissues of TPD affected trees (Krishnakumar *et al.*, 1998). While ethylene tilts the metabolic equilibrium from anabolic to catabolic (Wang *et al.*, 1990) leading to senescence, cytokinins have antisenescence effects in plants through prevention of free radical production as well as their scavenging activities (Leshem, 1984).

Increased activity of peroxidase in the TPD affected bark may be an indication of increased production of active oxygen species such as superoxide radicals (O^-_2). Chrestin (1985) reported an abnormal rise in the production of toxic oxygen species in TPD affected plants. Overproduction of such active oxygen species and inability of the tissue to safely remove them from the system would lead to oxidative stress to the cellular constituents including mitochondria. Inhibition of the mitochondrial activity could lead to a possible accumulation of carbohydrates as observed in the present investigation (Fig. 1a&b). This leads to decreased availability of ATP for the conversion of sucrose into rubber which is an extremely high energy consuming process (Jacob and Prevot, 1992). It may be noted that in the rubber biosynthetic pathway a large amount of energy is required particularly in the down stream reactions after the formation of mevalonate. The relatively large accumulation of HMG- CoA (Fig. 2d) and mevalonate (Fig. 2e) in the TPD affected bark also suggests that the availability of carbon skeleton for cis-polyisoprene synthesis was not a limiting factor, but the metabolic conversion of sucrose into rubber was impaired.

In conclusion, the results of the present investigation suggest that the bark tissues of the TPD affected plants are metabolically damaged possibly due to action by free radicals, which inhibited the metabolic conversion of sucrose into rubber. Production of free radicals and active oxygen species damages membrane systems including lutoids. This may be the reason that plants in the later stages of TPD produce latex containing unstable lutoid particles. This leads to early bursting of lutoids resulting in premature *in situ* coagulation of latex on the panel (Chrestin, 1985).

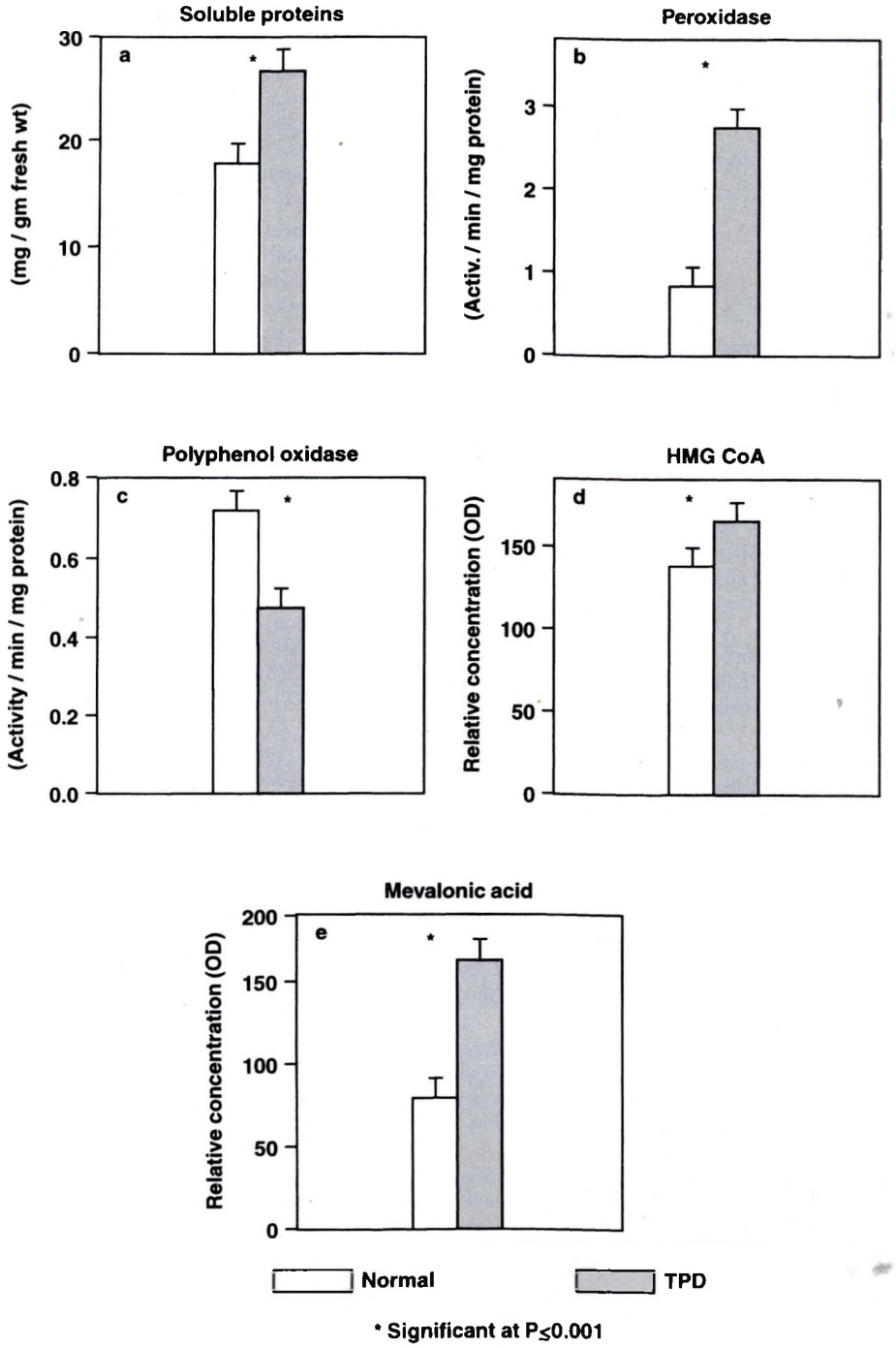

Fig.2. Concentration of soluble proteins (a), activity of peroxidase (b), polyphenol oxidase (c), relative concentrations of HMG-CoA (d) and mevalonic acid (e) in healthy and TPD affected bark tissues (n=15-20±SE); ** Significant (t-test at $P \leq 0.001$); ns : not significant

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REFERENCES

Abbas, S. (1997). Ethrel induced senescence and lipid peroxidation by peroxidase/H_2O_2/phenolic system in primary leaves of *Phaseolus mungo* L. *Plant Physiology and Biochemistry,* **24** : 35-38.

Addy, S.K. and Goodman, R.N. (1972). Polyphenol oxidase and peroxidase activity in apple leaves inoculated with a virulent and an avirulent strain for *Erwinia amylovora. Indian Phytopathology,* **25** : 575-579.

Bates, L.S., Waldren, R.P. and Teare, I.D. (1973). Rapid determination of free proline for water stress studies. *Plant and Soil,* **39** : 205-207.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry,* **72** : 248-254.

Boyne, A.F. and Ellman, G.L. (1972). A methodology for analysis of tissue sulfhydryl compounds. *Analytical Biochemistry,* **46** : 639-653.

Chrestin, H. (1985). Etudes des systemes generateurs dioxygene toxique et des mecanismes detoxifiants. *Reunion Encoche Sechu du* 1985, Abidjan, Cote d'Ivoire, pp. 30-32.

Commere, J., Eschbach, J.M. and Serres, E. (1989). Tapping panel dryness in Cote d'Ivoire. *IRRDB Workshop on Tree Dryness,* 1989, Penang, Malaysia, pp. 83-98.

de Fay, E. and Jacob, J.L. (1989). The bark dryness disease (Brown bast) of *Hevea.* In : *Physiology of Rubber Tree Latex* (Eds. J. d'Auzac, J.L. Jacob and H. Chrestin). C.R.C. Press, Boca Raton, pp. 407-409.

Dian, K., Sangare, A. and Diopoh, J.K. (1995). Evidence for specific variation of protein pattern during tapping panel dryness condition development in *Hevea brasiliensis. Plant Science,* **105** : 207-216.

Grambow, H.J. and Langenbeek-Schwich. (1983). The relationship between oxidase activity, peroxidase activity, hydrogen peroxidase and phenolic compounds in the degradation of indol-3-acetic acid *in vitro. Planta,* **157** : 131-137.

Gomez, J.B. (1990). Lutoids of *Hevea brasiliensis* : Morphological consideration. *Journal of Natural Rubber Research,* **5** : 231-240.

Gupta, S.K., Gupta, P.P. and Kaushik, C.D. (1995). Changes in leaf peroxidase, polyphenol oxidase, catalase and total phenols due to *Alternaria* leaf blight in *Brassica* species. *Indian Journal of Mycology and Plant Pathology,* **25** : 175-180.

Heath, R.L. and Packer, L. (1968). Photoperoxidation in isolated chloroplast. 1. Kinetics and striometry of fatty acid peroxidation. *Archives in Biochemistry and Biophysics,* **125** : 189-198.

Jacob, J.L. and Prevot, J.C. (1992). Metabolism of the laticiferous system and its biochemical regulation. In : *Natural Rubber : Biology, Cultivation and Technology* (Eds. M.R. Sethuraj and N.M. Mathew). Elsevier, Amsterdam, pp. 116-136.

Karr, M. and Mishra, D. (1976). Catalase, peroxidase and polyphenoloxidase activities during rice leaf senescence. *Plant Physiology,* **57** : 315.

Krishnakumar, R., Sreelatha, S., Gopalakrishnan, J., Jacob, J. and Sethuraj, M.R. (1998). Increased peroxidase activity is related to the decreased cytokinin content in TPD affected *Hevea* trees. In : *Developments in Plantation Crops Research* (Eds. N.M. Mathew and C. Kuruvilla Jacob). Allied Publishers Limited, New Delhi, pp. 142-146.

Leshem, Y.Y. (1984). Interaction of cytokinins with lipid associated oxygen free radicals during senescence : A prospective mode of cytokinin action. *Canadian Journal of Botany,* **62**:2943-2949.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin-Ciocalteu reagent. *Journal of Biological Chemistry,* **193** : 265.

Machackova, I. and Zmrhal, Z. (1981). Is peroxidase involved in ethylene biosynthesis ? *Physiologia Planterum,* **53** : 479-482.

Nair, U.N., Thomas M., Sreelatha, S., Simon S.P., Vijayakumar, K.R. and George, P.J. (1990). Clonal variations in the activity of 3-hydroxy-3-methyl glutaryl-CoA reductase in bark of *Hevea brasiliensis. Indian Journal of Natural Rubber Research,* **3** (1) : 40-42.

Nataraja, K.N., Krishnakumar, R., Jacob, J. and Sethuraj, M.R. (1997). Analysis of the protein profiles of healthy and TPD affected *Hevea brasiliensis* bark tissues. In : *IRRDB Workshop on Tapping Panel Dryness in Hevea brasiliensis,* 1997, Hainan, China, pp. 17-20.

Nelson, N. (1994). A photometric adaptation of the Somogyi method for the determination of glucose. *Journal of Biological Chemistry,* **153**:375.

Panse, V.G. and Sukhatme, P.V. (1995). Statistical method for agricultural workers. ICAR, New Delhi, pp. 53-56.

Paranjothy, K., Gomez, J.B. and Yeang, H.Y. (1975). Physiological aspects of brown bast development. *Proceedings of International Rubber Conference*, (1975), Kuala Lumpur, Malaysia, pp. 181-202.

Prematillaka, S.P., Yapa, P.S.A. and Bamunuaechi, A. (1985). Serum pattern in healthy and brown bast affected trees of *Hevea*. *Journal of the Rubber Research Institute of Sri Lanka*, **64** : 7-12.

Scott, T.A. and Melvin, E.H. (1953). Determination of dextran with anthrone. *Analytical Chemistry*, **25** : 1656.

Swain, T. and Hillis, W.E. (1959). The phenolic constituents of *Pranus domestica*. The quantitative analysis of phenolic constituents. *Journal of Science, Food and Agriculture*, **10** : 63-68.

Thomas, M., Sreelatha, S., Simon, S.P., Nair, N.U., Thomas, K.U., Jacob, J. and Sethuraj, M.R. (1998). Availability photosynthates and occurrence of tapping panel dryness syndrome in *Hevea brasiliensis*. In : *Developments in Plantation Crops Research* (Eds. N.M. Mathew and C. Kuruvilla Jacob), Allied Publishers Limited, New Delhi, pp. 139-141.

Vijayakumar, K.R., Sulochanamma, S., Thomas, M., Sreelatha, S., Simon, S.P. and Sethuraj, M.R. (1990). The effect of intensive tapping on induction of tapping panel dryness and associated biochemical changes in two clones of *Hevea*. *Proceedings of IRRDB Symposium*, 1990, Kunming, China, pp.103-109.

Wang, S.W., Wang, C. and Wellburn, A.R. (1990). Role of ethylene under stress condition. In : *Stress Response in Plants Adaptation and Acclimatation as Mechanisms* (Eds. R.G. Alscher and J.R. Cumming). Wiley Liss, New York, pp. 147-173.