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CHANGES IN THE BIOCHEMISTRY OF LEAVES OF TAPPED AND UNTAPPED TREES OF *HEVEA BRASILIENSIS* DURING REFOLIATION, MATURATION AND WINTERING

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Changes in the physiology of leaves were monitored for their entire life time in tapped and untapped trees of *Hevea brasiliensis*. At periodic intervals the biochemical components of the leaves, namely, chlorophyll, soluble proteins, free amino acids (FAA), starch, soluble sugars and malondiadehyde (MDA) and the extent of membrane leakage were measured. Total chlorophyll, soluble protein, starch and sugar progressively increased as the leaves matured, but their levels decreased during senescence and wintering. FAA and MDA contents in the leaf increased during the maturation and remained constant before their concentrations increased drastically during wintering. The total chlorophyll/soluble protein ratio decreased sharply and FAA/soluble protein and MDA/total chlorophyll ratios were found to increase abruptly during wintering. Accumulation of MDA indicates severe peroxidative damage of the membrane systems leading to increased membrane leakage during wintering. Results show that tapping led to higher concentrations of starch and soluble sugars in the leaves possibly due to the increased photosynthesis as a result of increased sink activity (exudation of latex). There was more accumulation of MDA and increased membrane damage suggesting increased stress in the leaves of the tapped trees when compared to the untapped trees.

Key words: Ageing, *Hevea brasiliensis*, Malondialdehyde, Membrane damage, Oxidative stress, Senescence, Tapping, Wintering.

INTRODUCTION

Wintering of deciduous trees is one of the spectacular phenomena in nature and has been a theme of active research by plant biologists for a long time (Biswal and Biswal, (1999). In temperate regions wintering occurs during the fag end of autumn, also known as fall season referring to the leaf fall. From a physiological perspective, wintering is an accelerated process of ageing and senescence triggered by internal factors that are yet to be fully elucidated. The most obvious aspect of wintering is the sudden change in the colour of the leaves from green to a variety of shades depending on the species and the eventual defoliation.

Hevea brasiliensis is a deciduous tree species of tropical origin. Mature trees of this species shed all their leaves between late December and early January, but young plants (1 to 3 year old) are not deciduous in nature. Once the tree enters into the wintering phase in early December, it takes hardly 2 to 3 weeks for the complete change in the leaf colour from green to yellow and for their total defoliation. Within a period of another 2 to 4 weeks the tree gets refoliated with lush green leaves. While some clones of *Hevea* winter partially, certain others winter completely and the clones may be either early or late wintering types (George *et al.*, 1967; Meenattoor *et al.*, 1989; Soman *et al.*, 1995). The exact timing of wintering in *H. brasiliensis* is an important factor that determines the incidence of powdery mildew, a serious leaf disease affecting the young foliage of this crop (Vinod *et al.*, 1996; Edathil *et al.*, 2000). Besides the above observations, there have been few scientific and systematic studies on wintering in this important species. It is not known why the deciduous trait begins to express only after the plants are about four to five years old. The biochemical changes in the leaf during wintering have not been studied in *H. brasiliensis*. Once the trees have attained maturity, they invariably winter almost at the same time every year. Some minor variations have been observed in the timing of wintering between trees in different agroclimatic zones. However, irrespective of the prevailing environmental conditions and management practices such as tapping, fertilizer application etc., mature trees of *H. brasiliensis* go through the wintering process with a precision that is seldom noticed in the biological systems.

A research programme on the wintering phenomenon in tapped and untapped trees of *H. brasiliensis* was initiated and the first set of observations on the changes in the biochemistry of the leaves over a full year period are presented here. Oxidative stress was found to be associated with wintering in *H. brasiliensis*. Data also showed that the tapped trees experienced a slightly higher degree of stress preceding wintering compared to untapped trees.

MATERIALS AND METHODS

The changes in the biochemical composition and membrane leakage in leaves of tapped and untapped trees of *H. brasiliensis* (clone RRIM 600) were monitored for one year (except in January, when no leaves were available) at weekly intervals till leaf maturation and thereafter at monthly intervals. Starting from mid-February, a total of 19 samples were made from the experimental plantation of RRII, Kottayam, which had 16 year old tapped and untapped trees. The field is situated at 76° 36'E and 9° 32' N at an altitude of 73 m above MSL (Thomas *et al.*, 1999) and the soil type is usti kandihumults (NBSSLUP, 1999). Trees of similar age and girth from both the tapped and untapped areas (n=5) were selected. Five leaves each were colleted from three different points from the top of the canopy of selected trees. From each of the 15 leaves, five small discs (10 mm diameter) were cut and all the 75 leaf discs from each tree were pooled to make a composite sample representing that tree for various analyses.

Chlorophyll

Five leaf discs were randomly picked from the total of 75 leaf discs from a tree and their fresh weight determined. Chlorophyll was extracted in acetone:DMSO (1:1 v/v) from these leaf discs after overnight incubation and their quantity determined after Arnon (1949).

Soluble proteins

Leaf discs of known fresh weight (approximately 500 mg) were homogenized in liquid nitrogen followed by 50 mM Tris-HCl buffer (pH 7.5). The extract was centrifuged at 20,000 rpm for 20 min at 4°C and the concentration of proteins in the supernatant was determined (Lowry *et al.*, 1951).

Free amino acid, sugar and starch

Leaf discs of known weight from the composite sample were homogenized in liquid nitrogen. The free amino acids and soluble fraction of carbohydrates in the sample were extracted by boiling with 80% (v/v) ethanol for 20 min. The extract was centrifuged at 4,000 rpm for 15 min at room temperature. The pellet was re-extracted by washing with 80% ethanol. The ethanolic extracts were pooled and analysed for free amino acids (Moore and Stein, 1948) and soluble sugars (Scott and Melvin, 1953). The ethanol insoluble residue, which contained starch was oven dried overnight at 50°C and acid hydrolysed with perchloric acid (52%) before determining the starch content after McCready *et al.* (1950).

Malondialdehyde content

Leaf sample (0.05 g) was taken from the composite sample, homogenized in liquid nitrogen and then in 2 ml of potassium phosphate buffer (pH 7.0). The homogenate was centrifuged at 20,000 rpm for 20 min at room temperature. The level of lipid peroxidation in the leaf tissue was measured in terms of malondialdehyde (MDA) content determined by the thiobarbituric acid (TBA) method (Heath and Packer, 1968).

Membrane damage

Immediately after preparing the composite samples, 10 leaf discs were randomly selected and rinsed several times with distilled water. After thorough washing they were incubated in 15 ml distilled water at 10°C for 18 h to allow the diffusion of electrolytes from the discs. The contents were mixed thoroughly and the electrical conductance (initial conductance) was measured at room temperature using a digital conductivity bridge (Global Electronics, India). The leaf samples were then boiled for 30 min to kill the tissues completely. The volume was made up to 15 ml with distilled water and the conductivity was measured once again at room temperature. Electrolyte leakage was calculated by dividing the initial conductivity reading by the conductivity reading after boiling and expressed as percentage (Simonds and Orcutt, 1988).

RESULTS AND DISCUSSION

As in several other species, a gradual reduction in leaf chlorophyll content noticed in *H. brasiliensis* (Fig. 1A) was the first obvious sign of wintering. Based on the changes in the leaf chlorophyll content during the life time of a leaf, four distinct phases could be identified. They are: (i) mid February to mid March (ii) mid March to mid July (iii) mid July to end of November (iv) December.

During the first phase (mid February to mid March), new flushes were produced and the leaf chlorophyll content showed a steady increase. As the young leaves matured, their chlorophyll content increased steadily and reached a maximum by the middle of March when the maturation of leaf was complete. During the second phase (mid March to mid July) the chlorophyll content remained high and more or less constant suggesting that the leaves were physiologically mature and stable (Fig. 1A). During the next phase (mid July to end of November) there was a gradual reduction in the chlorophyll content reflecting the normal pace of ageing in mature leaves. The chlorophyll content decreased at a much faster rate during December (fourth phase) and this corresponded with the active wintering period. By the end of December most of the leaves were shed and those remaining on the trees were almost devoid of any chlorophyll. Defoliation was complete before the middle of January.

The total soluble protein content in the leaves changed more or less in tandem with the changes in leaf chlorophyll content (Fig. 1B) and hence there was a positive correlation between leaf chlorophyll and protein contents both during the wintering phase (Table 1) and the refoliation phases (Table 2).

During the refoliation and maturation phases, the free amino acid content of the leaves increased reflecting the increase in the total soluble protein and chlorophyll contents (Fig. 1C) and hence a positive correlation existed among them (Table 2). During the wintering period the free amino acid showed a gradual increase (Fig. 1C) possibly due to breakdown of proteins that is known to happen during ageing and senescence (Sultan and Farooq, 1996). Free amino acid content showed a negative correlation with sugar and starch contents during the wintering period (Table 1). This was because with the progress in wintering the starch (Fig. 1F) and sugar (Fig. 1G) contents decreased as expected, but the free amino acids accumulated in the leaves.

The total chlorophyll to soluble proteins ratio remained fairly constant in the young leaves, but increased as the leaves matured (Fig. 1D). However, during wintering, this

Table 1. **Inter-correlation of various biochemical parameters of *H. brasiliensis* leaves (n=18) during the wintering phase**

Chlorophyll	Protein	FAA	Sugar	Starch	MDA	% Leakage
Chlorophyll	+0.95**	-0.74**	+0.85**	+0.89**	-0.95**	-0.96**
Protein		-0.62*	+0.93**	+0.97**	-0.84**	-0.95**
Free AA			-0.50*	-0.57*	+0.74**	+0.72**
Sugar				+0.98**	-0.67**	-0.83**
Starch					-0.73**	-0.88**
MDA						+0.92**
% Leakage						

* Significant at P≤ 0.05 ** Significant at P≤ 0.01

Table 2. **Inter-correlation of various biochemical parameters of *H. brasiliensis* leaves (n=20) during the maturation phase**

Chlorophyll	Protein	FAA	Sugar	Starch	MDA	% Leakage
Chlorophyll	+0.75**	-0.78**	+0.72**	+0.70**	+0.89**	+0.70**
Protein		+0.83**	+0.59**	+0.56*	+0.81**	+0.62**
Free AA			+0.75**	+0.68**	+0.93**	+0.75**
Sugar				+0.88**	+0.83**	+0.75**
Starch					+0.73**	+0.81**
MDA						+0.71**
% Leakage						

* Significant at P≤ 0.05 ** Significant at P≤ 0.01

ratio showed a fast decline indicating that chlorophyll degradation was faster than protein degradation. Reduction in the ratio of chlorophyll to soluble proteins is an indication of senescence (Annamalainathan *et al.*, 1996). Chlorophyll being the site of production of enormous quantities of active oxygen species (AOS) and free radicals (FR) (Asada, 1996) that are produced during senescence, it is only natural that they are extremely vulnerable to degradation. During ageing and senescence, large quantities of AOS and FR are produced in the photosynthesis apparatus, particularly the chlorophylls. This leads to severe oxidative stress resulting in the degradation of several bio molecules including chlorophyll molecules (Grover, 1993). The ratio of free amino acids to soluble proteins (Fig. 1E) also reveals interesting information. This ratio remained more or less constant for most of the time, but showed a drastic rise during the wintering phase suggesting that the accumulation of free amino acids was indeed due to the breakdown of the proteins during senescence.

The starch (Fig. 1F) and soluble sugar (Fig. 2A) contents increased steadily as the young leaves matured and decreased during wintering. It is stated that reduction in starch and sugars in a senescing leaf is due to reduction in chlorophyll. However, there is always an increase in respiration in senescing leaves, which is accompanied by consumption of sugars. Starch and soluble sugar contents showed similar changes with leaf chlorophyll content and therefore there was a positive correlation both during the wintering (Table 1) and the refoliation (Table 2) phases.

MDA content in the leaf increased slightly during the first six weeks and then remained constant until the wintering time. During wintering, it shot up abruptly indicating the severe stress and peroxidative damage of the leaves experienced during that period (Fig. 2B). During this phase, there was a negative correlation between the chlorophyll and MDA contents (Table 1). This

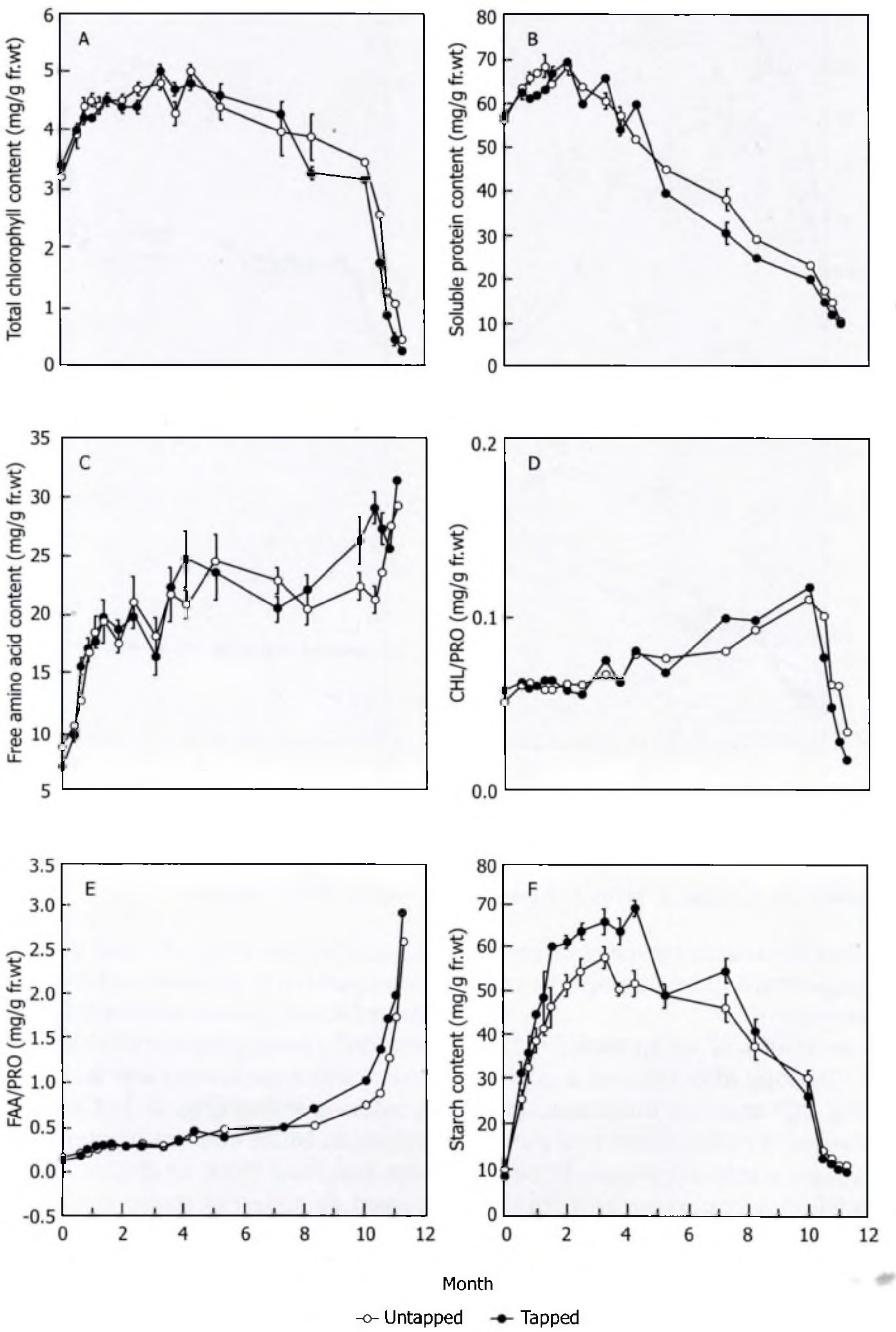

Fig. 1. Changes in the biochemical composition of leaves of tapped (-•-) and untapped (-o-) leaves of *H. brasiliensis*; A. Total chlorophyll; B. Soluble protein; C. Free amino acid (FAA); D. Chl/Pro ratio; E. FAA/Pro ratio; F. Starch

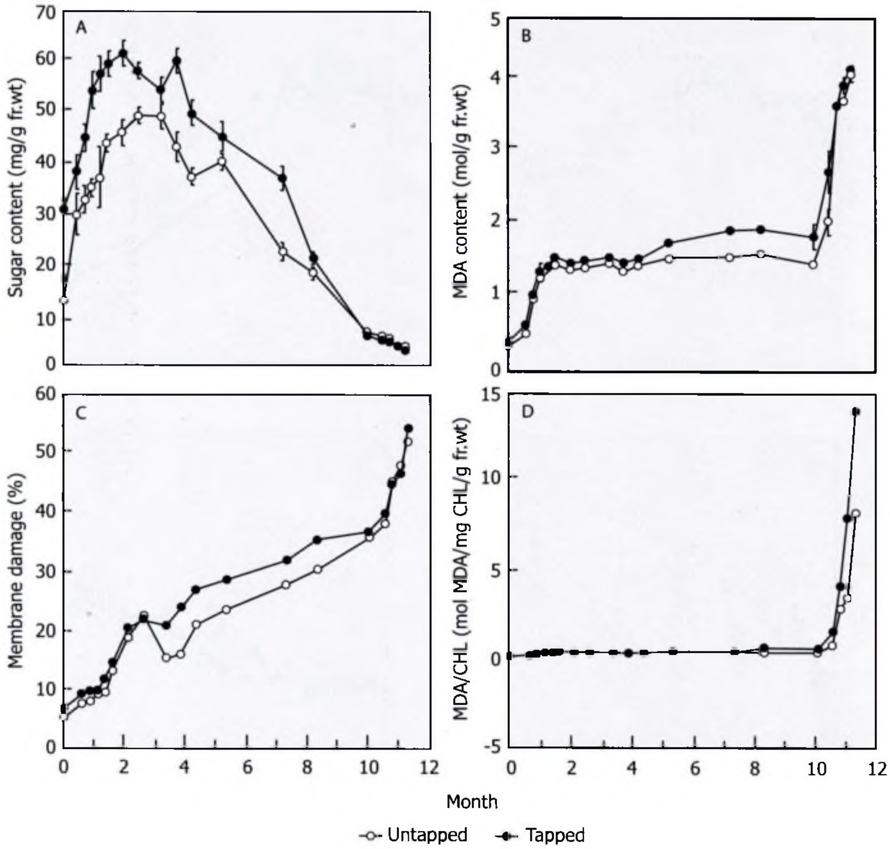

Fig. 2. Changes in the biochemical composition of leaves of tapped (-●-) and untapped (-○-) leaves of *H. brasiliensis*; A. Sugar; B. MDA; C. Membrane damage; D. MDA/Chl ratio

indicates that the wintering leaves were experiencing peroxidative damage to their membrane system.

Concomitant with an increase in MDA, membrane leakage also showed a parallel increase (Fig. 2C) and thus there was a positive correlation between these two parameters during the wintering phase. It may be noted that MDA accumulates as a result of peroxidative damage (Rodriguez *et al.*, 2000) which also damages the membrane system (Dhindsa *et al.*, 1981) as evidenced by the increase in the membrane leakage in wintering leaves.

The MDA content increased along with an increase in the chlorophyll content during the refoliation phase. The MDA content also increased (Fig. 2B) and therefore they were positively correlated (Table 2). During the refoliation phase, membrane leakage also showed a corresponding increase. However, the values were always less than those of the wintering leaves (Fig. 2C). Usually, any increase in MDA content or membrane leakage resulting from oxidative stress is perceived as a sign of stress induced damage triggering ageing and senescence (Kunert and Ederer, 1985). However, it may be noted that production of AOS and FR are inevitable consequences of aerobic lie and that they often have some positive role also to play in the growth and development of the organism (Slain, 1988). For instance, as a cell matures, the rate of cross linking in cellulose present in the cell wall which determines its strength, is increased by the catalytic action triggered by FR. Therefore, the small increase in the MDA content noticed in the maturing leaf could be viewed as a normal aspect of development. The cell succumbs to FR and AOS when their production goes out of control and the various mechanisms involved in their safe and effective removal from the system become inadequate, as must have been the case during the wintering phase. Loss of chlorophyll is one of the consequences of severe oxidative stress (Shimazaki and Sakaki, 1980).

The ratio of MDA to chlorophyll content (Fig. 2D) reveals an interesting pattern. Except during the wintering phase when this ratio shot up, it remained more or less constant throughout the year. Between the tapped and untapped trees there were some subtle differences in the biochemical composition of the leaves. For instance, the starch ($p<0.003$) and soluble sugar ($p<0.003$) contents were substantially more in the leaves of tapped trees almost throughout the year except during the active wintering period (last 5 sampling dates). This may be because tapping stimulated more photosynthesis due to the increased sink activity (Annamalainathan *et al.* 1998). The MDA content ($p<0.01$) and membrane leakage ($p<0.01$) showed an increasing tendency in the fully mature leaves of tapped trees compared to untapped trees. However, during the wintering time these parameters were more or less similar both in untapped and tapped trees. These results suggest that oxidative stress preceded wintering and the leaves of the tapped trees were under more stress than the untapped trees.

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