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ARTICLE

Ethephon Application Increases the Rubber Yield from *Hevea brasiliensis* by Altering the Water Relations of the Latex

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Application of ethephon (2-chloro-ethyl phosphonic acid) on the bark of mature trees of natural rubber, *Hevea brasiliensis*, which on hydrolysis releases ethylene resulted in 25% increase in rubber yield. While ethephon application has been a commercial practice in rubber plantations throughout the natural rubber growing countries for the past more than four decades, the exact mechanism by which ethylene increases rubber yield is unknown. In the present study we show that ethephon application lead to improved stability of lutoids which are membrane-bound, vesicle-like subcellular particles present in the latex of *Hevea*. The stability of these particles was directly related to the total volume of latex harvested because their bursting led to the coagulation of rubber particles in the latex, resulting in the plugging of the laticiferous vessels and preventing the free flow of latex.

Ethephon treatment resulted in the loss of semipermeability of the lutoid membrane as evident from the increased concentration of malondialdehyde, a product of lipid peroxidation in the latex collected from the trees treated with ethephon. The loss of semipermeability, likely due to the effect of ethylene led to free movement of ions between the serum present inside and the cytosol present outside the lutoid particles leading to isotonic conditions across the lutoid membrane in the latex of ethephon-treated plants. Because of the loss of osmotic gradient, continued flux of water into the lutoid particles was avoided and thus they remained intact. This led to a delay in the plugging of the latex vessels and hence more latex could be harvested resulting in more rubber yield from the trees treated with ethephon.

Keywords: *Hevea brasiliensis*, ethephon (2-chloro-ethyl phosphonic acid), latex, lutoid, natural rubber, B-serum, C-serum, water potential.

Introduction

Among the nearly 2000 latex-producing plant species, *Hevea brasiliensis* produces latex with the maximum concentration of dry rubber content (John, 1992). Latex is commercially extracted from the bark of *H. brasiliensis* through a process of controlled wounding termed tapping. Rubber yield is controlled by factors related to the physiology of latex flow and its *in situ* regeneration in the bark between successive tapping (Sethuraj, 1981). Stimulation of latex production by external application of ethephon (2-chloro-ethyl phosphonic acid) on the bark is a commercial practice in rubber plantations. Ethephon application prolongs the duration of latex flow and thus increases the total volume of latex during tapping (Ho and Paardekooper, 1965).

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The duration of latex flow in *Hevea* is largely determined by the stability of lutoids present in the latex (Southorn, 1969). Yeang and Hashim (1996) studied the stability of lutoids in the early and late flow latex fractions and observed more damaged lutoids in the late flow fraction and implicated it as the reason for the cessation of latex exudation. Lutoids are membrane-bound vesicles and occupy as much as 15% volume of the latex (Archer *et al.*, 1969; d'Auzac *et al.*, 1995). The serum contained inside these organelles (B-serum) has a high concentration of protons and various cations such as Mg^{2+}, Ca^{2+}, etc., cationic proteins as well as hydrolytic enzymes, e.g. acid phosphatase (d'Auzac, 1989). Lutoids upon bursting release the cations which bring about coagulation of latex after physically interacting with the negatively charged rubber particles which are *cis*-poly isoprenes. Hevein, a low molecular weight protein in the B-serum is also involved in the coagulation of latex (Gidrol *et al.*, 1994).

In spite of a small reduction in the dry rubber content of latex, the large increase in its volume leads to high rubber yield from rubber plants treated with ethephon. Ethephon-induced prolonged flow of latex is due to the lutoids remaining stable (unburst) for a longer period. Although this has been known for a while (Ho and Paardekooper, 1965), the mechanism of ethephon-induced stability of lutoids is unknown. Since ethephon upon hydrolysis on the bark of the tree is known to release ethylene (Audley *et al.*, 1978) which destabilizes cellular organelles and damages membrane structures (Abeles *et al.*, 1992; Myron and Connelly, 1971), it is paradoxical that the membrane-bound lutoids would remain stable for a longer time. Hence the present study was undertaken with the objective of examining the mechanism of ethephon-induced stability of lutoids leading to prolongation of the duration of latex flow.

Materials and methods

Plant material

Forty virgin *Hevea* trees (clone RRII 105) with girth ranging from 50 to 60 cm (at a height of 150 cm from the base) were used for the study. These trees had been planted in 1988 in the research farms of the Rubber Research Institute of India situated at 76°36′E and 9°32′N at an altitude of 73 m above MSL. The present studies were conducted between January and August, 1996. Three separate experiments were conducted during this period. Biochemical measurements were done for the first two experiments and water potential measurements were done for the last experiment. Sample size ranged from 8 to 10 trees for each measurement.

Ethephon treatment

The trees were divided into two equal groups of twenty each. Ethephon containing 5% 2-chloro-ethyl phosphonic acid was applied on the bark of one group of plants (@2 ml per tree). Ethephon was applied as a 20 mm wide band using a brush just below the tapping cut, which was previously scraped in order to eliminate the dead tissues. The trees were tapped on alternate days at 7.30 a.m.

The following physiological and biochemical parameters associated with the yield components in *Hevea* were recorded on ethephon-applied trees and compared with the untreated control trees using *t'* test. Sethuraj (1981) described the analysis of yield components in *Hevea*.

Physiological measurements

Dry rubber content (DRC): DRC expressed as a percentage (% DRC) was determined by gravimetric method after acid coagulation and oven drying (80°C for 72 h) of 10 g latex.

Yield: Dry rubber yield (g/tree/tap) was computed by multiplying DRC with the weight of latex harvested in a single tapping from a tree.

Total solid content (TSC): This was determined by drying 1 g latex at 80°C to a constant weight.

Turgor pressure (MPa): Pre-tapping latex vessel turgor (TP) which determines the initial rate of latex flow on tapping was measured using disposable mini-manometers comprising polythene surgical tubing sealed at one end and fitted with a 21 gauge hypodermic syringe needle at the other (Raghavendra *et al.*, 1984). The total length of the manometer was 20 cm. The needle was inserted gently into the bark and latex would be entering the sealed surgical tube. The length of the air column trapped at the sealed end of the surgical tube was measured and the turgor pressure was computed using a calibration curve prepared against known pressures and length of air column in a similar tube. Turgor pressure was measured on the bark at 5 cm below the tapping cut.

Initial flow rate (IFR) per unit length of tapping cut (ml/min/cm): Initial flow rate per unit length of tapping cut was determined by dividing the volume of latex collected in a measuring cylinder during the first 5 min by 5 and length of the tapping cut.

Plugging index (PI): Plugging index (Milford *et al.*, 1969), an index to measure the magnitude of latex vessel plugging during latex flow was determined by measuring the initial flow rate and total volume of latex using the formula, PI = (IFR × 100)/Total volume.

Water potentials of B and C-sera: For separating B and C-sera, fresh latex was centrifuged immediately after collection and before coagulation at 23,000 rpm for 45 min at 4°C. The middle serum (C-serum, representing the cytosol) was removed from the centrifuge tube using a syringe. The fraction at the bottom (lutoid) was removed and subjected to freezing and thawing to rupture the lutoids and liberate the serum contained inside the lutoids (B-serum). This was centrifuged at 20,000 rpm for 30 min to remove the lutoid membrane fragments and the supernatant B-serum was collected. The water potentials of B and C-sera were determined using Dew Point Microvoltmeter (HR 33T, Wescor Inc., Logan, Utah, USA).

Biochemical analysis

For biochemical analysis, latex samples drawn between 5 and 30 min were collected in glass beakers held in ice.

Bursting index (BI): Bursting index is a measure of lutoid instability and was calculated from the formula, BI = (activity of liberated phosphatase × 100)/ activity of total phosphatase (Ribaillier, 1968). The liberated phosphatase activity (i.e. the activity of phosphatase leaked from the lumen of lutoids into the cytosol before their rupture) was determined by incubating a known volume of fresh latex with *p*-nitro

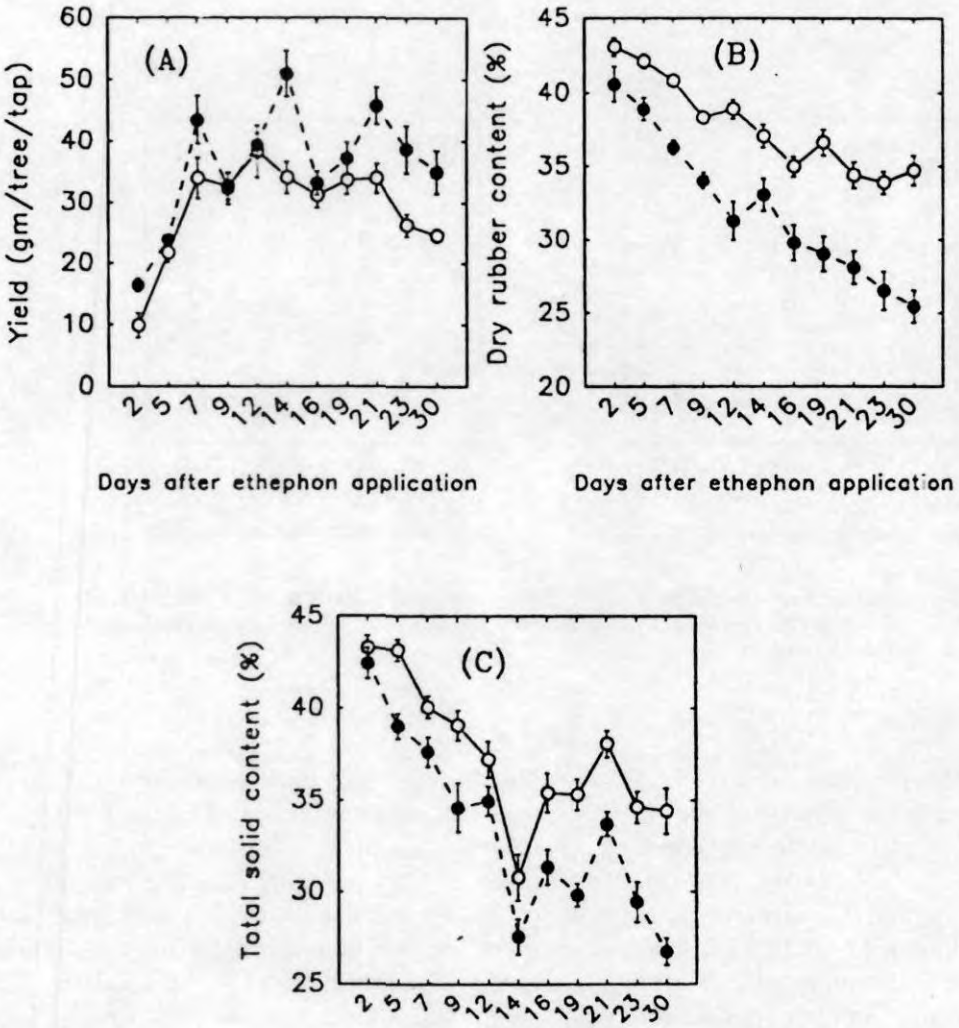

Figure 1. Dry rubber yield (A), dry rubber content (B) and total solid content (C) of control (O) and ethephon (●) applied plants on different tapping days. (Each point is an average of 10 trees ± SE bars shown.)

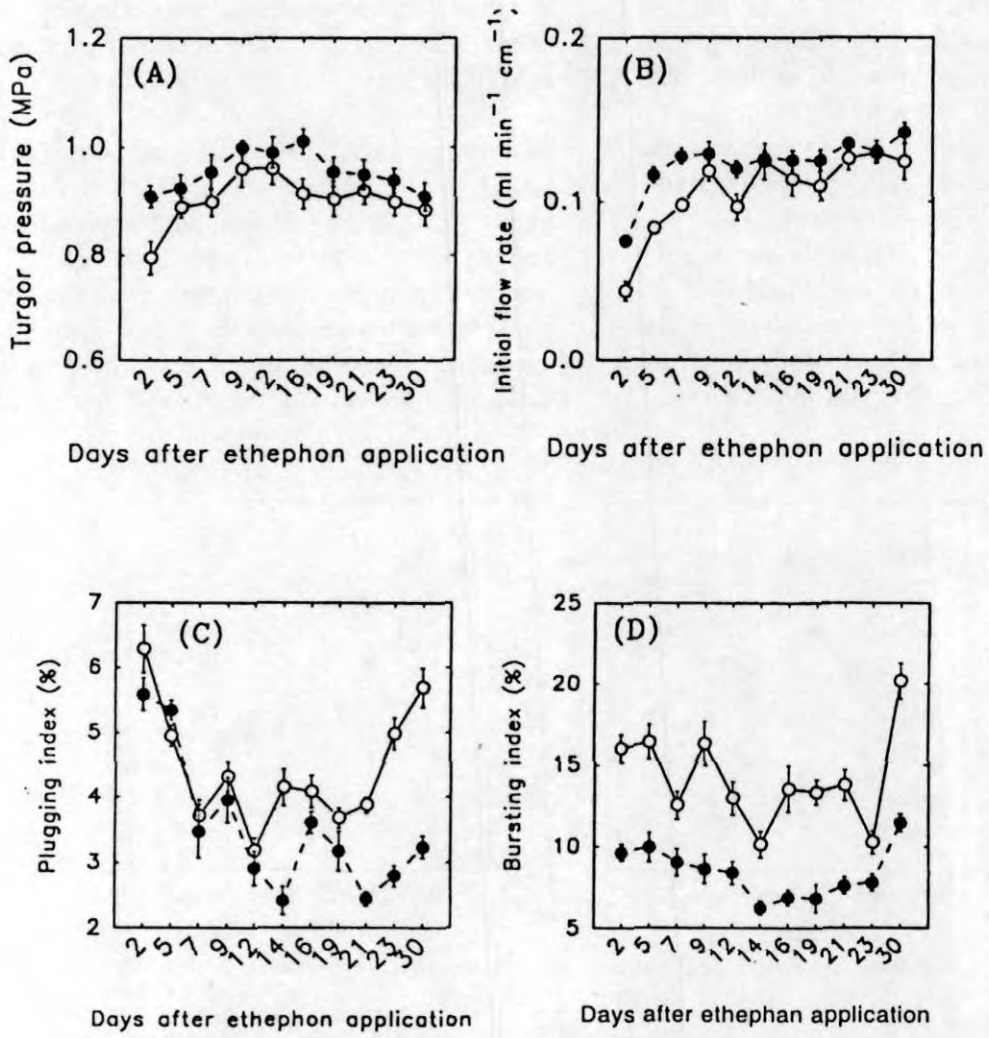

Figure 2. Turgor pressure (A), initial flow rate (B), plugging index (C) and bursting index (D) of control (○) and ethephon (●)-applied plants on different tapping days. (Each point is an average of 10 trees. ± SE bars shown.)

henyl phosphate in 0.4 M mannitol at pH 5 for 0 min at 27 ± 2°C and thereafter estimating the lib-rated *p*-nitro phenol calorimetrically using *p*-nitro henol as the standard (Ribaillier, 1968). The total hosphatase activity (i.e. the activity of phosphatase ter the rupture of the lutoids) was determined in a milar manner with the modification that 0.5% triton -100 was used to rupture the lutoids completely to lease all the phosphatase from lutoid lumen. A rge bursting index means that the liberated phos-hatase activity in relation to the total phosphatase tivity was more, indicating that there was more akage of phosphatase from the lutoids (i.e. lutoids ere less stable).

Determination of lipid peroxidation: Lipid peroxidation was measured in terms of the level of malondialdehyde, which is a product and a routinely used index of lipid peroxidation. The method as described by Heath and Packer (1968) with modifications was used for the determination of malondialdehyde in lutoid membrane and C-serum. About 0.5 g lutoid membrane (lutoid from which B-serum was removed) was homogenized with 2 ml 0.1 M Tris-HCl buffer pH 7.4 and then mixed with an equal volume of trichloroacetic acid (20% w/v). An aliquot of the supernatant was heated for 20 min in a boiling water bath with an equal volume of thiobarbituric acid solution (0.75% w/v in 0.1 M HCl) and then quickly

cooled in an ice bath. The absorbance at 532 nm was read and the value for non-specific absorption at 600 nm was substracted. The concentration of malondialdehyde was calculated using its extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer, 1968). For the determination of malondialdehyde in C-serum, 1 ml of C-serum was mixed with 1 ml of Tris-HCl buffer, pH 7.4 and then combined with 2 ml of trichloroacetic acid (20% w/v). An aliquot of the supernatant was used for the estimation of malondialdehyde, as described for lutoid membrane.

Results

Rubber yield was high and dry rubber and total solid contents were low in the ethephon-treated plants compared to the control plants on all tapping dates (Figures 1A, B and C). Turgor pressure and the initial flow rate of latex (Figures 2A and B) showed an increase whereas the plugging index and bursting index (Figures 2C and D) showed marked decrease in the ethephon-treated plants compared to control plants. From the data given in Figures 1 and 2, we computed the overall means of these above parameters for the entire experimental period. A significant increase in yield (25%, $P < 3.2 \times 10^{-5}$) was observed in the ethephon treated compared to the control plants (Figure 1A) for the entire experimental period. Similarly significant changes in turgor pressure (4% increase, $P < 1.7 \times 10^{-4}$, Figure 2A), initial flow rate (10% increase, $P < 2.1 \times 10^{-4}$, Figure 2B), plugging index (18% decrease, $P < 3.4 \times 10^{-4}$, Figure 2C) and bursting index (25% decrease, $P < 0.01$, Figure 2D) were also noticed in the ethephon-applied trees for the whole duration of the experiment. The dry rubber content was decreased by 11% ($P < 1.1 \times 10^{-6}$, Figure 1B) and the total solid content was decreased by 9% ($P < 4.2 \times 10^{6}$, Figure 1C) in the ethephon-applied trees during the course of the experiment. Significant positive correlation was observed between turgor pressure and initial volume ($r = 0.62$, $P < 0.01$) (Figure 3A) and between turgor pressure and total volume ($r = 0.64$, $P < 0.01$) (Figure 3B).

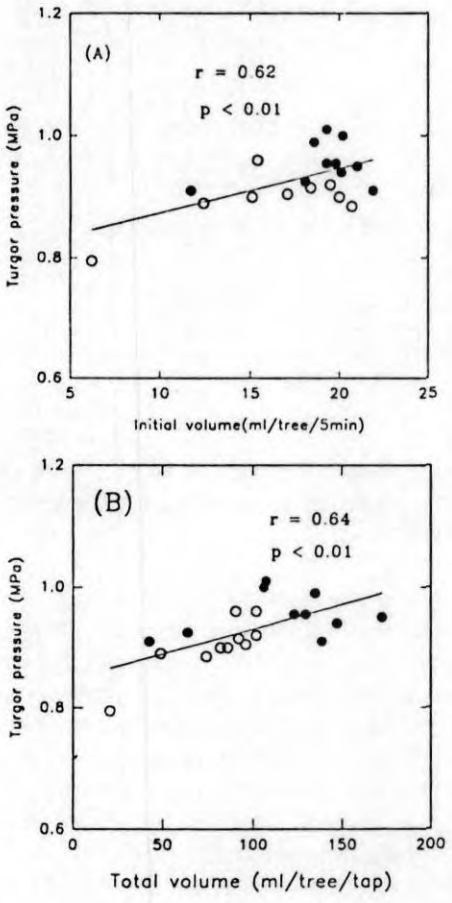

Figure 3. Correlation between turgor pressure and initial volume (A) and correlation between turgor pressure and total volume (B). O, control; ●, Ethephon-applied.

In control plants, the water potential of B-serum was significantly less than the C-serum ($P < 0.01$) whereas C and B-sera water potentials did not vary significantly in the ethephon-treated plants (Figures 4A and B). The water potential gradient between C and B-sera (i.e. C-serum water potential minus B-serum water potential) was significantly higher in control than ethephon-treated plants ($P < 0.003$) (Figure 4C). There was a positive correlation ($r = 0.72$, $P < 0.01$) between the water potential gradient (between C and B-sera) and the bursting index of lutoids (Figure 4D).

The concentration of malondialdehyde in the C-serum and in the lutoid membrane showed a significant increase with ethephon application (Figures 5A and B). The overall mean concentration of malondialdehyde in the C-serum (computed from all the measurements) was 59% more ($P < 1.48 \times 10^{-6}$) in the ethephon treated than control trees. When measured in the lutoid membranes this was 64% more ($P < 2.54 \times 10^{-4}$) in the ethephon treated than control trees.

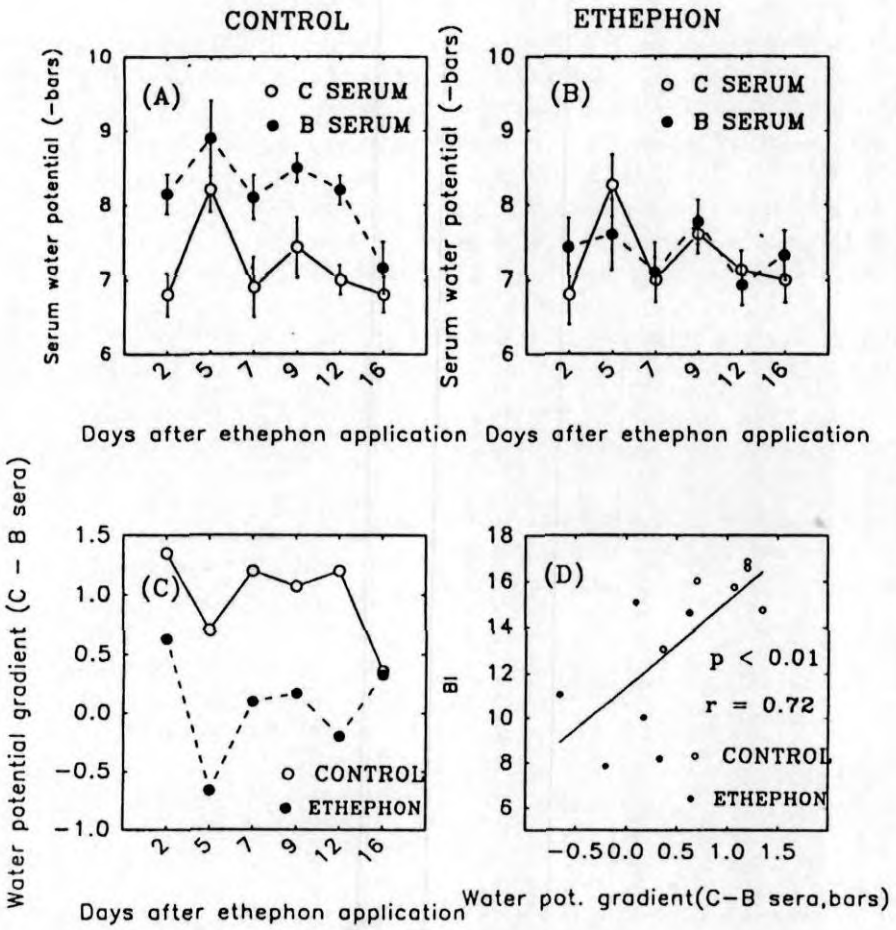

Figure 4. Water potentials of B- and C-sera of control (A) and ethephon-applied plants (B), water potential gradient between B- and C-sera (i.e. C-serum water potential minus B-serum water potential) of control and ethephon-applied plants (C) and the correlation between water potential gradient and bursting index, BI (D).

Discussion

The increase in rubber yield due to ethephon treatment was in spite of a significant reduction in the dry rubber content of the latex (Figure 1B), suggesting that the increase in yield in ethephon-treated plants was due to increase in the total volume of the latex harvested. A significant increase in turgor pressure (Figure 2A) and initial flow rate (Figure 2B) (favouring more flow of latex with force) and a decrease in the plugging index (Figure 2C) and bursting index (Figure 2D) (delaying the coagulation of latex on the panel and termination of the dripping of latex) led to increased volume of latex in ethephon-treated trees compared to control plants. As observed in previous studies (Abraham *et al.*, 1968; 1971), the ethephon-induced yield increase progressively disappeared with time.

The central question that we have tried to address in the present study is the mechanism of ethephon-induced stability of lutoid particle. Ethephon when applied to the trees, produces ethylene (Audley *et al.*, 1978) which damages the membrane systems of the cell (Abeles *et al.*, 1992). In the present experiments, we have found that there was increased peroxidative damage as evident from the increased accumulation of malondialdehyde in the latex of the ethephon-applied trees (Figures 5A and B). The extent of lipid peroxidation indicates the severity of stress per-

ceived by the membrane systems. Lipid peroxidation which begins with the formation of a lipid-free radical, results in the formation of lipid hydroperoxides with malondialdehyde as one of the breakdown products (Dhindsa *et al.*, 1981). In spite of the increased damage to the membrane the lutoids remained stable as evident from the reduced bursting index in the ethephon-applied trees (Figure 2D).

It appears that the reduced bursting of the lutoids which prolonged flow of latex was due to altered water relations in the B and C-sera of the latex of the ethephon-treated plants. There was a steeper water potential gradient between the C-serum (high water potential) and B-serum (low water potential) in control than ethephon-treated plants (Figure 4C). The C and B-sera of ethephon-treated plants were in near isotonic situation when compared to the untreated control plants. This suggests that there was free movement of osmotically active ions across the lutoid membrane resulting from the peroxidative damage and subsequent loss in the semipermeability of the lutoid membrane in the ethephon-treated plants. Our observations on malondialdehyde (Figures 5A and B) supports this suggestion. This loss in the integrity of the lutoid membrane in ethephon-treated trees (where ethylene is released through the hydrolysis of ethephon) is in agreement with the well-known effect of ethylene on membrane systems (Abeles *et al.*, 1992). Ethylene damages the functional integrity of membrane-bound organelles making the membrane permeable to ions due to loss of its semipermeability (Abeles *et al.*, 1992).

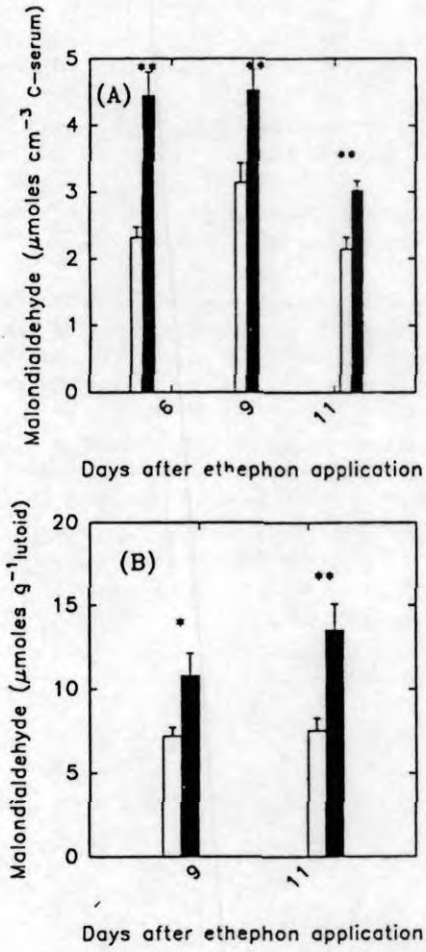

Figure 5. Concentration of malondialdehyde in the C-serum (A) and lutoid membrane (B) of control (open bars) and ethephon-applied (solid bars) plants on different tapping days. Each point is an average of eight trees. (** indicates t' test significant at $P \leq 0.01$, * indicates a significance at $P \leq 0.05$. ± SE shown). □, control; ■, Ethephon-applied.

The near isotonic condition existing between B and C-sera in the ethephon-treated plants means that there is less net osmotic flux of water from the cytosol into the lumen of the lutoids. Therefore, the lutoids will not enlarge and burst in the ethephon-treated trees as fast as they would in the control trees. Thus the lutoids remain stable (i.e. unburst) for a longer time preventing the fast coagulation of latex on the panel resulting in the delayed plugging of the latex vessels (Figure 2C). This is the reason for the prolonged flow of latex leading to increased volume of latex, and thus more yield, in ethephon-treated trees.

The situation was just the opposite in the control trees. The steep water potential gradient existing between the C and B-sera in the control plants (Figure 4C) indicates that there is accumulation of osmotically active ions in the lutoid lumen, indicating the continued maintenance of the semipermeability of the lutoid membranes. Because of the maintenance of a steep water potential gradient, there will be more entry of water into the lumen of the lutoid, leading to the enlargement and eventual bursting of the lutoids resulting in faster coagulation of latex on the tapping panel and termination of latex flow in the control trees as evident from their high plugging index (Figure 2C).

The above interpretation is further supported by the significant positive correlation observed between the

water potential gradient (between C and B-sera) and the bursting index of lutoids (Figure 4D). As the water potential gradient between the cytosol (C-serum) and lutoid lumen (B-serum) increased there was increased bursting of lutoids. This indicates that the bursting of the lutoids is controlled by the osmotic entry of water from the cytosol into the lutoid lumen as determined by the water potential gradient. Our results show that ethephon prevented the bursting and thus improved the stability of lutoids by altering the water relations of the latex by affecting the permeability of the lutoid membrane through its peroxidative damage. Increased stability of lutoids lead to delayed plugging of the laticiferous vessels. This caused prolonged flow of latex and thus increased the rubber yield from ethephon-applied trees.

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gated. Irrespective of the concentration of the total protein in the leaf extracts there were specific bands present or missing in the bud grafted plants propagated from a single mother plant. This suggests that low concentration of the protein was not responsible for the missing bands. Specific bands were missing even when the total protein concentration was high. Similarly, there were visible differences in the banding patterns of enzymes between the stock and scion bark tissues. But there was no variation in the isozyme profiles of different leaf samples from a single bud grafted plant. The results indicated that the enzyme polymorphism observed in the homogeneous scion may be due to the differences in the gene expression due to some unknown effects of the heterozygous rootstocks.

IV Poster 6

DISSIMILARITIES IN THE GENETICS BETWEEN THE ROOTSTOCK AND SCION AND THEIR RELATIONSHIP WITH THE OCCURRENCE OF TAPPING PANEL DRYNESS SYNDROME IN *HEVEA*

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Tapping panel dryness (TPD) syndrome is a physiological disorder characterised by partial to complete inhibition of latex production and thus affecting the productivity of *Hevea*. In the present study the hypothesis that a greater genetic distance between the rootstock and scion may interfere with the physiology of the latter and eventually express subtle symptoms of delayed incompatibility response including TPD. This was addressed by subjecting the DNA from the rootstock and scion portions of two healthy and three fully TPD affected trees of *Hevea* (clone GT1 as the scion) to RAPD analysis and computing the genetic distance between the heterozygous rootstocks and the scion. The RAPD profiles of the scion tissues were identical because they all came from the same clone, GT1. But the RAPD profiles of the rootstocks were different confirming their genetic heterozygosity. The indications from the present preliminary study is that the genetic distance between the rootstock and scion tissues was higher in the TPD affected than healthy trees.

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