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Yield Stimulation of the Rubber Tree (RRIM 600) with Ethephon does Not Increase Latex Hev b 6 Protein or mRNA

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Hevea brasiliensis *latex hevein is an allergenic protein designated the allergen name Hev b 6 by the International Union of Immunological Societies. A study was undertaken to determine if latex flow stimulation by ethephon increased Hev b 6 concentration in the latex from mature rubber trees. A two-site immunoenzymatic assay with antibodies specific for hevein revealed no significant differences in Hev b 6 content in control trees and* Hevea *trees stimulated with ethephon. The mRNA transcript levels of hevein determined by northern analysis appeared similar for control and stimulated trees. Using the relative ratio method for Real Time PCR assessment, the steady-state transcript levels of hevein were also similar for control and stimulated trees. It was concluded that latex from mature* Hevea *trees stimulated with ethephon did not result in an increase in hevein mRNA transcripts or in the translated protein. There was therefore no evidence from this study that ethephon stimulation increased allergenicity of latex by increasing Hev b 6 content.*

Key words: allergen; Hev b 6; *Hevea brasiliensis*; Real-Time PCR; ethephon; yield; stimulation; RRIM 600; hevein; latex; allergenicity

Many proteins associated with plant defence have been characterised as being allergenic[1]. Some of these proteins, such as hevein (designated Hev b 6 by the International Union of Immunological Societies) are found in large amounts in *Hevea* latex[2]. The synthesis of several plant defence-related proteins is activated upon exposure to ethylene[3]. In *Phaseolus vulgaris*, for example, Class I chitinases that are the pan allergens responsible for the latex-fruit syndrome have been shown to be induced by treatment with chloroethylphosphonic acid (ethephon), a compound that releases ethylene[4]. This group of proteins have N-terminal sequences similar to that of hevein.

Ethylene-based stimulants have been commonly used to increase the latex yield of rubber trees since the 1960s[5,6]. The content of certain latex proteins, specifically enzymes, are known to change in response to yield enhancement by ethylene-releasing stimulants[7,8]. Although there has been a move towards the use of gaseous ethylene in recent years[9], the most established stimulant of latex flow in the rubber industry is ethephon[10,11]. Broekhaert and co-researchers reported an increase in prohevein RNA transcripts in the latex following treatment of young *Hevea* seedlings with ethephon[12]. In another investigation, treatment of turnip (*Brassica rapa*) with ethephon led to an increase in an

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allergenic (IgE-binding) protein that showed homology to prohevein[13]. Such findings have led researchers to suggest that the contents of some latex allergens could increase as a result of commercial ethephon yield stimulation[14–17]. The increment in these proteins is thought to be a plant defence response by the rubber tree. Since ethephon stimulation, and ethylene-based yield stimulation in general, is a common practice in rubber planting, it is important to investigate how this widespread agronomic practice might affect allergenicity of latex as the source material for a wide variety of manufactured latex goods such as medical gloves.

Although Hev b 6 mRNA has indeed been shown by Broekhaert and co-researchers to be elevated in *Hevea* latex in response to ethephon treatment[12], it should be noted that in the particular study, only young (four-month-old) seedlings were sprayed with a solution of ethephon. Drop samples were then harvested from these seedlings. It was also reported that ethylene induces an over-expression of hevein in latex cells from mature trees, although no precise quantitative measurements, such as those obtained by Real-Time PCR, were made[18]. In the present investigation, therefore, the effect of ethephon applied to mature trees on Hev b 6 protein and mRNA was examined. This includes Hev b 6 protein quantified by immunoassay and more precisely mRNA levels quantified by Real-Time PCR.

MATERIALS AND METHODS

Ethephon Treatment and Latex Collection and Preparation

Ethephon (2.5%) was applied to the tapping cut and on the renewing bark 2 cm above the cut of RRIM 600 trees that were tapped on a half spiral, third daily system. Latex samples from stimulated and control trees were collected before stimulation and five days after stimulation. There were 6 trees that were treated with ethephon to determine the yield stimulation effect, and these were compared to 6 control trees that were untreated. Latex from 3 trees each from the treated and control groups were analysed for the contents of Hev b 6 protein and mRNA in the latex.

The latex samples were treated with 0.1% Triton X-100® (1 part latex: 4 parts 0.125% Triton X-100®) which disrupted the lutoids and allowed the release of the lutoid proteins. The serum phase (a mixture of B-serum and C-serum) was recovered after high speed centrifugation of the treated latex[19]. Other than latex collected directly from the tree for analysis where the volume was recorded, the remaining exuded latex was coagulated into a cup-lump that was then air-dried. The final volume of tapped latex was estimated from the weight of the cup lump and the total solid content of the latex. The latter was determined by weighing a small sample of latex before and after drying in the oven.

Hev b 6 Protein Assay

In the assay that employed an ELISA format, monoclonal antibodies against Hev b 6.02 were used as capture and detection antibodies with the latter conjugated to horseradish peroxidase. Following appropriate dilution, the serum samples were assayed for the latex allergen Hev b 6 by a two-site immunoenzymetric assay that employs hevein (Hev b 6.02) as the calibration standard following the manufacturer's instrutions. A commercial immunoassay, the *FIT* kit for Hev b 6 (FIT Biotech, Tampere, Finland) was used following the manufacturer's instructions. As the immunoassay detection does not discriminate between Hev b 6.01 (prohevein, the precursor to hevein) and Hev b 6.02 (hevein), the results in this study are presented as 'Hev b 6'.

Statiscal Analysis

Statistical differences between treatments were determined by analyses of variance.

RNA Isolation

To investigate the extent that hevein mRNA is induced by ethephon treatment, latex was collected from three control and three stimulated trees into an equal volume of RNA extraction buffer (0.1 M Tris. HCl, 0.3 M LiCl, 10 mM EDTA, 10% SDS, pH 9.5) in ice. Total RNA from latex was isolated according to the method described by Kush and co-researchers[20]. The total RNA concentrations was determined spectrophotometrically at 260 nm. RNA integrity was verified using a 1.7% agarose-formaldehyde gel.

Primer Design

Primers for hevein cDNA sequence[12] and 18s rRNA of *Hevea*[21] as internal control were designed using DNASIS Max Software (Hitachi Software Engineering Co.) from nucleotide sequences obtained from GenBank (*Table 1*). The upstream hevein PCR primer corresponds to the region from base 675 to base 697 (5'-tcaagcaataagcaacaacaagg-3'); the downstream hevein PCR primer corresponds to the region from base 852 to base 872 (5'-gaaggaggaagggaaaaccaa-3'). The upstream 18s rRNA PCR primer corresponds to the region from base 254 to base 273 (5'-atgataactcgacggatcgc-3'); the downstream 18s rRNA PCR primer corresponds to the region from base 403 to base 422 (5'-cttggatctggtagccgttt-3'). All primers were synthesized at First Base Laboratories Sdn. Bhd., Malaysia.

Reverse Transcription

In order to quantify hevein mRNA transcript levels in latex of control and stimulated trees, the first strand cDNA was synthesized using 2.0 µg of total RNA with gene specific primers using RevertAid™ M-MuLV Reverse Transcriptase (Fermentas Life Sciences) according to the manufacturer's instructions (Fermentas Life Sciences).

qPCR

Real Time Polymerase Chain Reaction (qPCR) and analysis were achieved using a Line-Gene Fluorogenic Quantitative PCR Detection System (Bio Flux Corporation). SYBR Green I was used in the qPCR system as an intercalating dye[22].

The qPCR reactions for 18s rRNA consisted of 10 µL of a mixture containing 1 × PCR buffer, 0.5 mM $MgCl_2$, 0.2 mM dNTP, 0.3 µM of forward and reverse primers (18s rRNA),

TABLE 1. CHARACTERISTICS OF PRIMERS USED IN qPCR

Gene	GenBank Acc. no.	Forward primer[a]	Reverse Primer[a]	Amplicon size (bp)
Hevein	M36986	675-697	852-872	198
18s rRNA	AY435212	254-273	403-422	169

[a]Co-ordinates according to GenBank (http://www.ncbi.nih.gov).

1 × SYBR Green I dye, 1 µL of transcribed cDNA and 1.0 U of Taq polymerase. The resulting retrotranscribed cDNA was PCR amplified with the following conditions: denaturation at 94ºC for 2 min; 50 cycles of denaturation at 94ºC for 15 s, annealing at 60ºC for 15 s and extension at 72ºC for 15 s.

The qPCR reactions for hevein consisted of 10 µL of a mixture containing 1 × PCR, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 µM of forward and reverse primers (hevein), 1 × SYBR Green I dye, 1 µL of transcribed cDNA and 1.0 U of Taq polymerase. The resulting retrotranscribed cDNA was PCR amplified with the following conditions: denaturation at 94ºC for 3 min; 35 cycles of denaturation at 94ºC for 30 s, annealing at 50ºC for 15 s and extension at 72ºC for 30 s.

Standard Curve

To determine the PCR efficiency of hevein gene amplification, a standard curve for hevein was generated using first strand cDNA (derived from total RNA from a treated tree) at different dilutions (0.1, 0.5, 1.0, 5.0 and 10 µg) and the threshold values (C_t) was determined. The C_t was subsequently used to generate a linear regression line.

Normalisation of the Real-Time PCR analysis of hevein gene expression was carried out by comparing the control (untreated) and treated sample to an internal control, house-keeping gene (HKG). The suitability of the 18s rRNA as the HKG was verified by comparing the C_t value for control tree and a treated tree (ethephon treated) using first strand cDNA at different dilutions (10 µg, 12.5 µg and 15.0 µg). The C_t value was determined at the log-linear phase of the PCR cycle. Since there is no alteration found in 18s rRNA expression between the control and treated sample, therefore, the 18s rRNA was chosen to be the internal control for normalisation.

Melt Analysis

Since SYBR green I was used in qPCR for detection of DNA, this would allow both specific and non-specific PCR fragments formed to be measured[23]. PCR products were melted at 75ºC to 95ºC to generate a melting curve to confirm the presence of desired amplicons.

Relative C_t Method

PCR reactions were performed whereby amplification of the hevein gene was compared to that of a HKG in treated and control trees. The C_t value of hevein were normalised with the C_t value of 18s rRNA in both treated and control samples. The normalised values from each group were then averaged to calculate the relative expression level by dividing the average normalised value of treated samples to the average normalised value of the control samples.

Northern Analysis

For Northern analysis, 10 µg of total RNA was separated in 1.4% agarose gel containing 6% formaldehyde and blotted onto Biotrans(+)™ Nylon membrane. The blot was prehybridised using High SDS buffer (7% SDS, 50% formamide, 5 × SSC, 2% blocking reagent, 50 mM sodium-phospahte, pH 7.0 and 0.1% sodium-laurylsarcosine, Roche Diagnostics GmbH, Germany) at 50ºC for 2 h and hybridised using High SDS buffer with the cDNA for hevein (198 bp) and 18s rRNA (169 bp) labelled by the random primer with DIG-High Prime (Roche Diagnostics GmbH, Germany). The membranes were washed twice initially for 15 min in 2 × SSC, containing 0.1% SDS at room temperature followed by another two washes in 0.5 × SSC, containing 0.1% SDS for 15 min at 68ºC. Detection of the

DIG-labelled probes on membranes was performed using the chemiluminescent alkaline substrate (CSPD) according to manufacturer's instruction (Roche Diagnostics GmbH, Germany).

RESULTS AND DISCUSSION

Hev b 6 Content in the Latex

In *Hevea brasiliensis*, the prohevein gene controls the synthesis of the 19 kDa prohevein (Hev b 6.01) latex protein which, in post-translation, breaks down into two sub-units, *viz*. the 4.7 kDa Hev b 6.02 (hevein) representing the N-terminus of prohevein, and a 13 kDa C-terminus (Hev b 6.02)[12,24]. The predominant molecular form of Hev b 6 present in latex is hevein[24]. The major IgE-specific epitopes (that are involved in the allergic response) reside on the hevein moiety of the protein[25,26]. The two-site immunoenzymetric assay used in the present study employed antibodies specific for hevein. Since prohevein incorporates the entire hevein domain, the immunoassay for Hev b 6 detected both Hev b 6.01 and Hev b 6.02 which are the molecular forms of Hev b 6 that are relevant to allergy.

One day following treatment of the trees with ethephon, the volume of latex obtained from tapping increased significantly (*Figure 1A*). At the same time, the latex rubber content – reflected in the total solids – decreased as expected, showing that the treated trees were responding well to ethephon stimulation (*Figure 1B*). Hev b 6 protein contents in the latex five days after ethephon stimulation were compared between three untreated and three ethephon-treated trees (mean latex volumes 224 and 607 mL per tree per tapping, respectively). No significant difference was observed in Hev b 6 protein content (*Figure 2*). Hence, ethephon yield stimulation did not increase Hev b 6 protein content in the latex despite an increase latex yield.

Northern Analysis

Northern blot analysis showed that hevein was expressed in the total latex RNA of control and stimulated trees, however, the steady-state levels of hevein mRNA appeared to be the same for control and stimulated trees (based on the intensity of the signals, *Figure 3*). The intensity of the 18s rRNA signals in total latex RNA of untreated and stimulated trees indicated that the levels of loading of total RNA for untreated and stimulated trees were similar.

qPCR Analysis

Specificity of qPCR in amplifying the desired products (hevein and 18s rRNA) in treated and control trees was determined by gel electrophoresis (*Figures 4* and *5*) and additionally with melting curve analysis. The derived melting temperature of qPCR product was specific for hevein at 83ºC (*Figure 6a*) and for 18s at 87ºC (*Figure 6b*). The gel electrophoresis results were in agreement with melting curve whereby only a single prominent band expected amplicon size of 198 bp for hevein and 169 bp for 18s rRNA was obtained.

For reliable quantification of the hevein mRNA transcript levels in latex of control and stimulated trees, the amplification efficiency of the housekeeping gene (18s rRNA) was tested to serve as an internal reference. The 18s rRNA expressed at roughly the same level for the control and stimulated trees (*Figure 7*). The average normalised value of control and treated trees was 1.8 and 1.7, respectively (*Table 2*). The relative expression level of hevein in treated trees compared to control trees was 0.93. Therefore the steady-state levels of hevein mRNA appeared to be the same for control and treated trees.

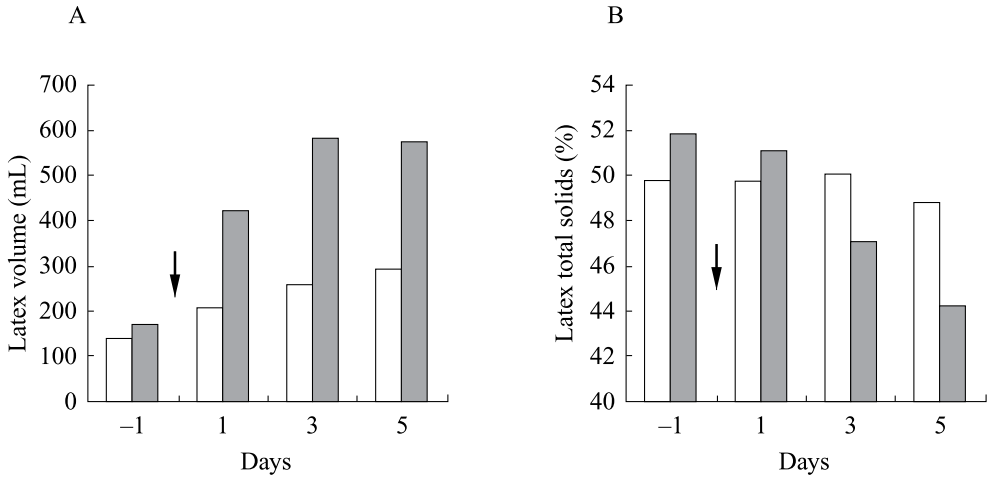

Figure 1. Volume (A) and total solids (B) of tapped latex following ethephon stimulation on Day 0 (arrow). Values are the means of six trees per treatment. Shaded bars are of ethephon-treated trees while unshaded bars are of controls. In (A), there was no statistical difference between the means of untreated and treated trees on Day 0 (pre-treatment). From Day 1 to Day 5, latex yields from the two groups of trees were significantly different ($P<0.05$) on each tapping day. In (B), the difference in total solids content between latex from treated and control trees was statistically different ($P<0.05$) on the 5th day.

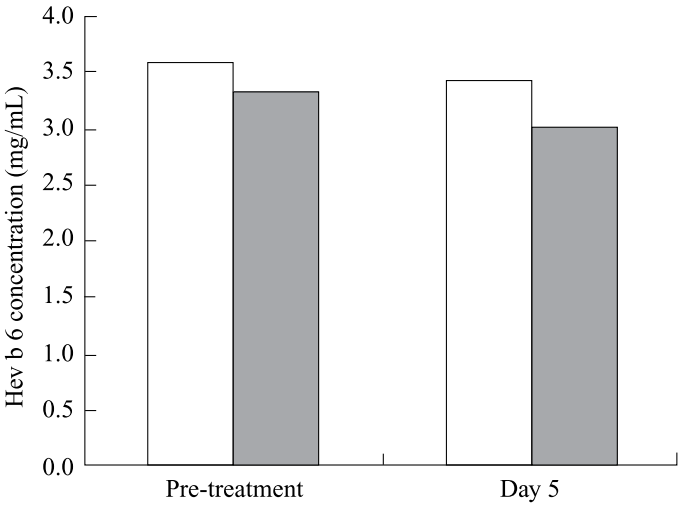

Figure 2. Concentration of Hev b 6 (mg/mL latex serum) in the latex of 3 control trees (unshaded bars) and 3 trees treated with ethephon (shaded bars). Values are the means of three trees per treatment. The means were not significantly different before or after treatment.

Figure 3. Northern blot analysis of the expression for hevein and 18s rRNA in 10 µg of total latex RNA of control (C) and stimulated trees (S). C: control; S: Stimulated with ethephon.

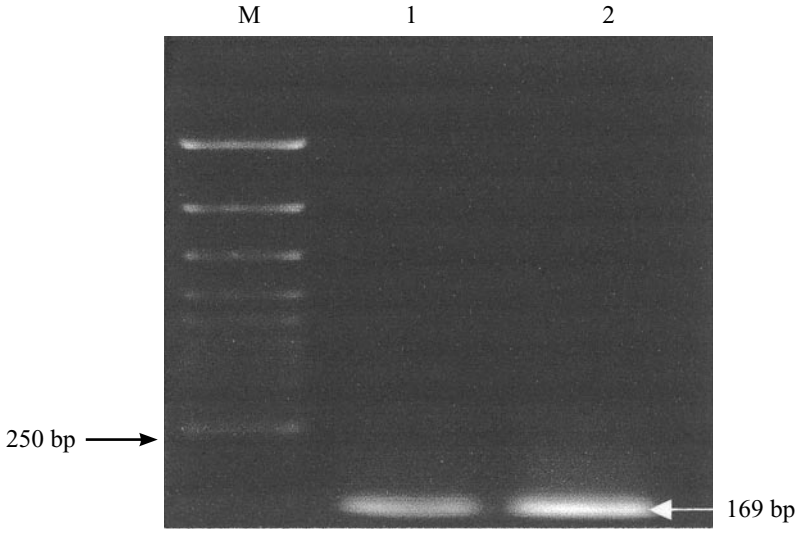

Figure 4. Gel analysis of the 18s rRNA amplified fragment from total RNA of latex from control (1) and stimulated trees (2) using 18s primers. The expected amplicon size was 169 bp. The molecular marker (M) is 1 kb ladder (Fermentas, USA).

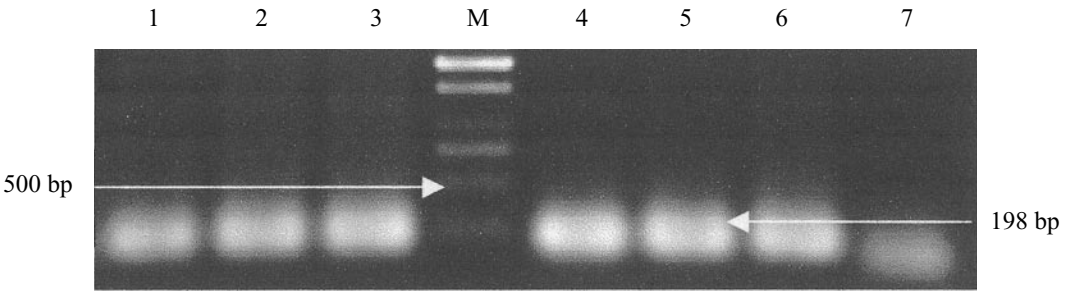

Figure 5. Gel analysis of the hevein amplicons of latex from control (lane 1, 2 and 3) and treated trees (lane 4, 5 and 6). The expected amplicon size was 198 bp. Lane 7 is a negative control. The molecular marker (M) is 1 kb ladder (Fermentas, USA).

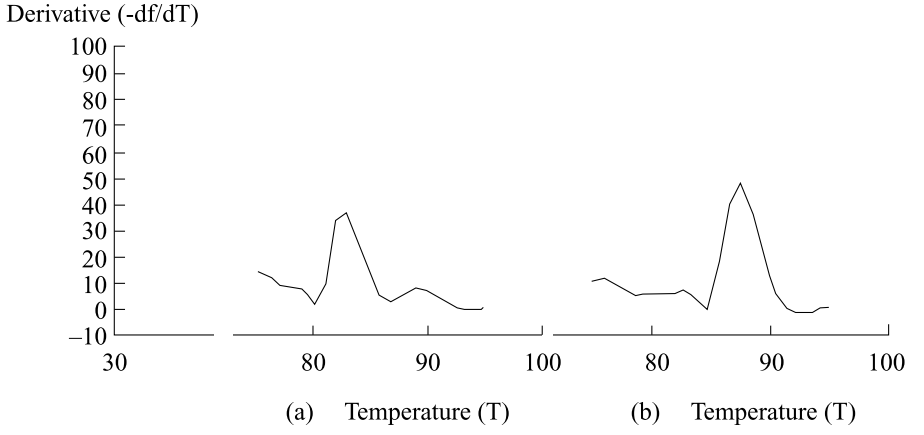

Figure 6. Melting curve of hevein transcript primed using hevein internal primers. (a): A single melting peak Tm = ~83.0ºC, was obtained for both control and treated trees. Melting curve of 18s rRNA transcript primed using 18s rRNA internal primers. (b): A single melting peak Tm = 86.5ºC – 87.5ºC, was obtained for both control and treated trees.

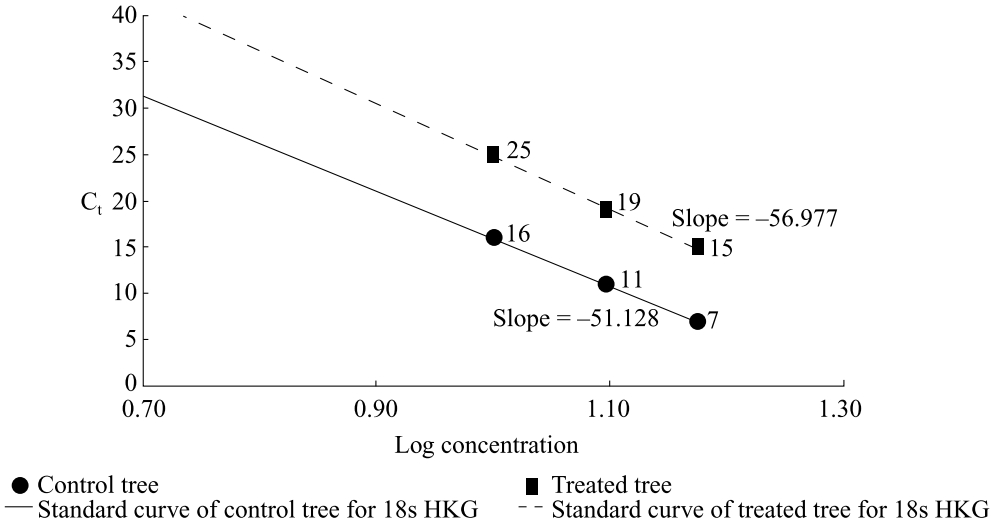

Figure 7. Standard curves of 18s rRNA for control and treated trees is shown, respectively. The 18s rRNA was used as a normaliser gene in this study as the rates of change in expression level remain similar for both control (untreated) and stimulated trees.

TABLE 2. COMPARATIVE C_t VALUES FOR HEVEIN IN CONTROL AND TREATED TREES AFTER NORMALISATION WITH 18s rRNA

Samples	C_t value		Normalised value	Average normalised value
	Hevein	18s rRNA	(C_t Hev/C_t 18s)	
Control tree 1	10.17	5.38	1.89	
Control tree 2	10.96	5.55	1.97	1.84
Control tree 3	9.18	5.58	1.65	
Treated tree 1	10.32	6.14	1.68	
Treated tree 2	10.78	5.53	1.95	1.71
Treated tree 3	8.49	5.79	1.49	

CONCLUSION

Ethephon stimulation did not alter Hev b 6 protein content in Hevea latex of RRIM 600. The absence of an effect on hevein was confirmed in Northern blot and Real Time PCR analyses which showed that the steady-state levels of Hev b 6 mRNA appeared to be the same for untreated and stimulated trees. Taken together, these results refute the presumption of an increase in Hev b 6 in *Hevea* latex as a result of ethephon yield stimulation. The published results on four-month-old seedlings maintained in a growth chamber did not therefore apply to mature trees that were commercially exploited. There

was therefore no evidence that ethephon stimulation increased the allergenicity of latex by increasing Hev b 6 content.

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