ORSTOM-CIRAD / Mahidol-RRIT

REPORT

ON THE RESULTS OBTAINED FROM THE THAI-FRENCH "HEVEA" PROJECT

Research of specific molecular markers to be used in high latex content (yield) diagnosis and early selection of high yielding rubber clones.

UNDER DTEC AGREEMENT

First Period:

September 1993 - December 1995

(November 1995)

ORSTOM-CIRAD / Mahidol-RRIT

REPORT

ON THE RESULTS OBTAINED FROM THE THAI-FRENCH "HEVEA" PROJECT

Research of specific molecular markers to be used in high latex content (yield) diagnosis and early selection of high yielding rubber clones.

UNDER DTEC AGREEMENT

First Period:

September 1993 - December 1995

(November 1995)

REPORT
ON THE RESULTS OBTAINED FROM THE THAI-FRENCH "HEVEA" PROJECT
(November 1995)

Research of specific molecular markers to be used in high latex content (yield) diagnosis and early selection of high yielding rubber clones.

CHAPTER I : INTRODUCTIONS

I: INTRODUCTION:

1) Importance of Rubber in Thailand:

At present, the total rubber planted area, in Thailand, reaches more than 1.8 million hectares (approximately 85% of which being under exploitation) of which more than 85% is in the southern part of the country. Thailand's natural rubber production, unlike that of most rubber producing countries is based nearly 92% on small holders. The average small holding is about 2 hectares. Today, the total population involved in one way or another with rubber is about 5.5 millions, that is to say roughly 10% of the Thai population. This makes the rubber production and industry important economically as well as socially.

While ranking N° 1 in world rubber production, Thailand produced in 1994 about 1.4 million tons of rubber, of the total more than 90% were exported as raw material, bringing rubber to a major rank in Thai export, earning with more than 30,000 millions bahts.

2) Problems linked with rubber production in Thailand and other Asian countries:

a - Rubber yield can be largely improved:

Thailand produced in 1994 about 1.4 million tons of dry rubber from about 1.4 million hectares of rubber plantation in production, that is to say a mean rubber yield of roughly 1 ton/ha. Apart from the social aspects, in the small holding context, this relatively low yield is mainly due to unadapted tapping systems and short harvest time. This rubber yield could almost be doubled through the planting of clones more adapted to those specific conditions especially encountered in Thailand and, over all, through the use of tapping systems more suitable for the existing clones.

b - Manpowers ("tappers") productivity has to be improved:

All rubber producing countries have more and more to face a common problem: an increasing lack of a highly specialized manpower: the tappers. This comes from the fact that tapper-employees are generally underpayed to maintain higher exploitations profitability, and that, with the actual commonly used tapping systems in Asia* each rubber planted rai consumes too much manpower. This could be overcome, and the manpower profitability increased at least 2

fold, through the use of different modern tapping systems, to be adapted to each clone, using decreased tapping frequency (2 tappings/week or even every 4 days) compensated by stimulation of the latex yield with Ethrel (a low cost ethylene chemical releaser which can be cyclically applied at the tapping cut).

* Recommendations in Asia are: tapping every 2 days, but in special conditions (high rubber selling rate,...) trees can even be tapped up to every day, leading, in the long term, to overexploitation.

c - Care has to be taken to avoid any kind of overexploitation (TPD):

Any kind of overexploitation through overtapping or/and overstimulation can lead, in the mean/ long term to a definitive cessation of the latex production, a physiological disorder called the "Tapping Panel Dryness" (TPD), happening when the level of exploitation of the tree exceeds the physiological capability of the tree to regenerate latex. Susceptibility for TPD is a clonal characteristic, hence genetically determined.

d - The diversity of environmental conditions

The diversity of environmental conditions of the rubber planting area and the sensitivity of rubber clones to various stresses is also a major problem. Only early selection of - and even probably, in the next 10 years, the possibility of creating through genetic engineering - suitable new rubber clones, more adapted to the specific conditions encountered in Thailand, could help to solve these problems, especially in the East and North East of Thailand where the Thai government would like to promote rubber plantations in order to fix people in theses areas, providing them with daily incomes from rubber.

3) The intrinsic limiting factors and major problems for rubber yield:

Two intrinsic factors are known to limit the latex yield at the level of the latex producing tissues of the rubber tree:

- the duration of the latex flow, which determines the volume of latex collected at each tapping. The duration of the flow is limited by the coagulation of latex leading to the plugging of the extremities of the severed latex vessels;
- the capability of the latex cells to regenerate the exported latex between two tappings;

and a major problem impairing rubber yield,with high economical impact:

- the sensitivity and tolerance of the latex producing tissues to the stresses induced by the exploitation techniques themselves: tapping frequency (traumatic stress/endogenous ethylene) and stimulation intensity (dose/frequency : hormonal stress/exogenous ethylene), leading to the "Tapping Panel Dryness" (TPD) desease.

All these characteristics (phenotypes) have been proven to depend on clones (genotypes), hence to be genetically determined.

4) Towards a latex molecular diagnostic and the future genetic engineering of rubber tree:

Most of these problems (limiting factors) could be minimized or solved by the designing of biochemical and molecular tools for a latex diagnostic allowing :

- the estimation of the physiological status of the latex producing tissues under tapping, leading to recommendations for more adapted tapping systems depending on clones and environmental conditions, for optimum yield and attempting to avoid TPD risks;

- the characterization of parents and hybrids in the rubber breeding programs, as well as for the early selection of their progeny (high yielding/stress tolerant).

In the future, some of these problems might be solved through the techniques of genetic engineering, by modifying the expression of endogenous genes or introducing foreign genes of agronomical interest, specifically in the latex producing tissues. For this, latex cell specific promotors have to be cloned and characterized, in order to design latex specific expression vectors, for transformation rubber tree.

II - OVERALL CONDITIONS OF THE PROJECT DEVELOPMENT (09/93-/95)

A - SETTING UP A LAB FOR THE "HEVEA" PROGRAM.

During the last 3 months of 1993, a lab (50 m2) was set up, to welcome this program, in the building of the Department of Biotechnology, Faculty of Science, Mahidol University, and fully equiped, thanks to the fundings from the French Embassy, the French Institutes (ORSTOM + CIRAD) with a participation of Mahidol University (See annexe I for details). The lab became fully operational at the end of January 1994, with the arrival of the last researchers of the French team and of the whole equipment. In the mean time (November 1993 - January 1994), experiments were initiated in the lab. of Prof Amaret BHUMIRATANA in collaboration with Prof Jarunya NARANGAJAVANA (Department of Biotechnology).
We are thankfull, to Prof. Pornchai MATANGKASOMBUT and Prof. Amaret, respectively the Dean and Vice-Dean of the Faculty of Science, Mahidol University, and to Prof Watanalai PANBANGRED - the Head of the Department of Biotechnology - as well as to every chief of each lab in this Department, to welcome the French team and to let free access to their equipment and facilities, for this project to be performed. We also thank Dr Skorn MONGKOLSULK and Suvit LOPRASET for occasionnal collaboration and let us access to the equipment in CRI.

B - SETTING AND FOLLOWING OF THE FIELD EXPERIMENTS.

For this initial phase of the project, all the field experiments were set up in the Rubber Research Center of Chachoengsao. We are gratefull to Dr. Sanit SAMOTHORN, General Director of the RRIT, and Mr Samuk SONMARK (Director of the RRC Chachoengsao), to let free access to their experimental plantation. We are thankfull to Mr Jirakorn KOSASAIWE and Suchin MOEUN-MEUN as well as to Miss Noparat VICHITCHOLCHAI, to allow us to set up and help us to follow all the field experiments we asked for.

C - THE THAI-FRENCH TEAM.

1) French part :

- Dr. Hervé CHRESTIN (plant cell physiology and molecular biology) arrived in the beginning of september 1993, to set up the lab, order equipments and stocks of chemicals, define in more details the content of the program to be followed, and set up the first experiments in the field (Chachoengsao RRC) as well as in the lab (Dept. of Biotechnology, Mahidol University). Dr; Hervé CHRESTIN became the representative of the ORSTOM French Institute in Thailand but still devoted 80% of his time to the project.

- Dr. Regis LACROTTE (plant physiology and immunology) and Dr Xavier GIDROL (molecular biology) arrived in December 1993 (both of them worked full time in the field and at the bench for the project).

- Dr. Valérie PUJADE-RENAUD (plant cell physiology and molecular biology) joined the team beginning of January 1994 (full time on the project).

Unfortunately, Dr Xavier GIDROL, who was proposed a high responsability position in a Biotech Firm in USA (Colorado), left Thailand in July 1994. Hence only 3 french researchers, instead of 4, have been working on the project between August 1994 and October 1995. Dr GIDROL has recently been replaced in the beginning of November 1995, by Dr. Pascal MONTORO (plant tissue culture and molecular biology/full time on the project).

2) The Thai Counterpart:

- In Mahidol University, Dr Jarunya NARANGAJAVANA (plant biotechnology, biochemistry) is the permanent collaborating Thai researcher contributing on this project (DNA methylation/ low molecar weight TPD proteins). Prof. Ginda NIYANAD (immunology / Mahidol University) joined recently our project (contributing on raising antobidies against putative TPD protein markers. We had also occasional collaborations with Dr Skorn MONGKOLSULK's group (Molecular Biology / Mahidol and CRI)(Gene cloning and sequencing).

- in The Rubber Research Center of Chachoengsao (RRIT): Mm Noparat VICHITCHOLCHAI (Agronomist / in charge of the Latex Diagnostic and research on TPD in RRC Chachoengsao) and her assistant, Miss Payao SRISA-AN, are the permanent Thai collaborators on this project, under the supervision of Mr Jirakorn KOSAISAWE (exploitation systems) and Mr Suchin MAEN-MEUN (agronomy). We also had some collaborations with Mr. Terachart VITITCHOLCHAI (agronomy) and Miss Kanikar TEERAWATANASUK (plant breeding).

- Contacts were taken with Dr Watana (Electron microscopy and tissue culture) and occasionnal collaborations with Dr Witsut (Electron microscopy and Latex diagnostic), in the Hat-Yai RRC. These collaborations will be strengthened in the second part of this project.

III - TRAININGS AND STUDENTS

A - SHORT TRAININGS:

- Miss Noparat VICHITCHOLCHAI (RRIT) was given 2 times one week trainings in Mahidol University and Chachoengsao RRC, on the Latex Diagnostic, and a one week training on latex protein extraction and electrophoresis (Mahidol University)
- Miss Kannikar TEERAWATAWASUK (RRIT), was given a one week training in Mahidol University on the Latex Diagnostic.
- Miss Kannikar TEERAWATAWASUK (RRIT), Dr Jarunya NARANGAJAVANA and Dr Sunee KERBUNDIT (Mahidol University), were given a 1 week training on rubber tree tissue culture (embryogenic callus induction) with Dr. Marc-Phillipe CARRON (a specialist from CIRAD who came in Thailand for this purpose in June 1994).
- Mr Witsut was given a 2 days training for the interpretation of the Latex Diagnostic.

B - STUDENTS (See 3rd part of the results):

- Miss Panida KONGSAWADWORAKUL was recruited in May 1994 as a first year MSc student and started her long term lab training in May 1995, on the following subject:"Studies of differential expression of genes potentially involved in the onset of Tapping Panel Dryness (TPD) in *Hevea brasiliensis* ".
- Miss Pritsana CHOMCHAN was trained (part time) as a Bachelor of Science student (06/94-03/95) on the following subject : "Small scale extraction and analysis of DNA and RNA from Rubber Tree (*Hevea brasiliensis*).
- Mr Supat DARA was trained (part time) as a Bachelor of Science student (06/94-03/95) on the following subject:"Direct transfer of DNA constructs into Hevea protoplasts".

CHAPTER II : MATERIAL AND METHODS

A - PLANT MATERIAL / EXPERIMENTAL TRIALS IN THE FIELD.

Because of its widespread use in Asia, and in particular in Thailand, most of the experiments were done with trees of the RRIM 600 clone. Some experiments have been done with the PB 5/51 clone which is more sensitive to TPD. This year, the experiments could only be done in the RRIT Experimental Rubber Research Center (RRC) of Chachoengsao (120 km East of Bangkok / a relatively dry area compared with the traditional rubber area in Southern Thailand/ Tapping is generally stopped during the dryer season: February-April).
Experiments on TPD induction could not be performed in duplicate in the Hat-Yai RRC (South of Thailand), no virgin (or sufficiently young trees with known exploitation history) being available at this time.

1) Experiments for the studies on induction of latex genes expression by ethylene on virgin trees (started in November 1993) :

Ethylene (a very simple gaz molecule!) is a plant hormone, commonly involved in numerous classical physiological events in the plant kingdom, among which: cell senescence, fruit maturation, fruit and leave abscission, inhibition of the elongation but stimulation of the thickenning of roots. Endogenous synthesis of ethylene is also known to occure in reaction to wounding, pathogenes attack and adverse environmental conditions (waterlogging, drought, saline conditions, etc... It is as such considered as the main stress and senescence hormone in plants.

"Stimulation" with Ethrel (ethylene releaser) is well known to induce a transitory (5-8 tappings) increase in latex yield (+ 50% up to +200% depending on clones). It is a convenient mean to study gene expression in the latex cells, in relation with increased latex production, and therefore is of great help to find out molecular markers of high latex yield.

To study the effects of hormonal stimulation on gene expression in the latex cells, it is important to distinguish between the "direct" effect of ethylene, which can be only observed when working with "virgin" (never tapped) rubber trees, and possible indirect effects of ethylene when working with regularly tapped trees. This indirect effects can be due to:

- interference with endogenous ethylene and other plant hormones probably synthetized in response to tapping (ie: wounding/traumatic stress)
- interference with the regeneration of latex resulting in high metabolic activity).

Kinetics experiments to study the direct effects of ethylene on gene expression in the latex cells were performed as following on homogenous (growth and general aspect) 7 year old virgin trees (RRIM 600 clone):

Six batches of 6 homogenous virgin trees were either stimulated 1h30; 3 ; 6; 12; 24 hours before a first tapping, or not stimulated (control). A 5% Ethrel solution was applied on a 2 cm large, lightly scrapped half spiral band, just beneath the place to be tapped (All the trees were scrapped 24 h before tapping). All the trees were tapped (1/2 spiral) the same day, at the same hour, to avoid any possible influence of micro-environmental and weather conditions, and after every 2 days (d2). The 25 first drops of latex flowing out of the bark were discarded (eventual contaminations such as small bark shavings), the few ml following were collected in a special "fixing buffer" (see methods) and instantaneously deep-frozen in liquid nitrogen, in the field. The samples were stored at minus 70°C and the total RNA were extracted as described in "Methods".

2) Studies on spontaneous TPD (start in January 1994):

a - Five hundred thirty 11-year-old trees (4 th year of tapping) of the RRIM 600 clone were checked for TPD symptoms, according to the classification reported in "Methods". Healthy trees, were also selected as control. After one year experiment on the lower tapping pannel, the tapping pannel was changed (fitrst opening of the higher opposite tapping pannel). The latex was collected either for protein pattern analysis, or for RNA extraction and Northern blot analysis

b - In the same way, trees with TPD symptoms and healthy trees were selected from 12-year old trees (5th year of tapping), clone PB 5/51, and their latex collected for protein pattern analysis or total RNA extraction and Northern blot analysis.

3) Experiments on TPD induction through overexploitation (start in May 1994).

Four hundred, 6,5-year-old virgin (not tapped) trees (clone RRIM 600) were tapped 1/2 spiral (S/2) every 2 days (d2), for 2 months. Five batches of 33 trees were selected considering growth and production. In each batch the 12 most homogeneous trees were selected for studies on latex protein pattern, gene expression (total RNA/Northern blot) and Latex Diagnostic.
Each batch of 33 trees was tapped in 1/2 spiral and submitted to the following treatments (started in July 1994 after the dry season and the 2 month S/2 d2 tapping for tree selection):

Treatment	Tapping Frequency	Ethrel Stimulation Dose	Ethrel Stimulation Frequency	International nomenclature
control				
A	every 2 days	0	0	S/2, d2
(over)stimulation				
D	every 2 days	5%	every month	S/2, d2, ET 5%, m/m
E	every 2 days	5%	every 15 days	S/2, d2, ET 5%, d/15d
F	every 2 days	10%	every month	S/2, d2, ET 10%, m/m
overtapping				
G	every day	0	0	S/2, d1

During the first 7 month of the experimentation, tree per tree latex production was recorded only for one tapping every 2 weeks. Starting from February 1995, the tre/tree latex production was regularly recorded at each tapping as well as their precise monthly cumulated latex production. The latex collections were performed (from the 5 x 12 selected trees) roughly every 2 weeks during the first 7 months, then every 3-4 weeks:

- 7 drops for Latex Diagnostic,
- 1,5 ml for protein pattern analysis,
- 6 ml in 6 ml of the 2 x "fixing buffer" for RNA extraction,

TPD symptoms and the characteristics of the initial latex flow were systematically recorded during the first year of experimentation at each collection of latex, for the 5 x 12 most homogeneous selected trees only, then accasionnally these characteristics were checked also on the 5 whole batches of 28-33 trees under experimentation .

Upon our request, the 12 most homogeneous trees selected per batch were not "stopped" (continuous tapping) during the last dry season (February-April 1995), in an attempt to induce TPD more efficiently.

B - METHODS

1) Latex collection:

To avoid contamination (bacteria, RNase,...), each tree was equiped with a new (220°C x 4h) sterilized leak, at each tapping. The first 20 drops of latex flowing out of the bark after tapping were discarded, the 7 following ones were collected in 3,5 ml mQ water for Latex Diagnostic (LBD). The following 1,5 ml was collected in 1,5ml Eppendorf tubes and immediately deep-frozen in liquid nitrogen for protein pattern analysis. The following 6 ml were collected, for total RNA extraction, in 15 ml disposable sterile (with screew cap) centrifuge tubes, containing 6 ml of a 2 x alkaline "fixing" buffer (Tris HCl 0.1M, LiCl 0.3M, EDTA 10 mM and 10% SDS, pH 9) under constant carefull manual mixing, and immediately deep-frozen in liquid nitrogen, then stored at -70°C.

2) Evaluation of the "plugging index" of the latex:

The "plugging index" gives an estimation of the ability of the latex to rapidly coagulate, hence to rapidly plugg the severed laticifers, leading to the stopping of the latex flow. The plugging index is the ratio of the mean initial flow rate (ml/min) during the first 5 min after tapping, to the total latex volume collected. In the field, the initial volume of latex collected during the first 5 min of flow, then the total volume collected after tapping, are recorded.

$$PI = \frac{\text{initial volume/5min x 100}}{\text{total volume}}$$

3) Evaluation of the severity of the TPD desease

The severity of the TPD desease for each tree under experiment was checked at each tapping for collection of latex. Six classes of TPD severity, according to the % of dry cut (dry cut length/ total tapping cut length) were defined as follow:

Class	% dry-cut length	symptomes
0	0	Healthy trees / good latex flow all along the tapping-cut
1	< 20 %	Serious difficulty of latex flow / up to 20% scattered dry-cut zones
2	20≤%<40	Completely dry zones on the tapping cut.
3	40≤%<60	" "
4	60≤%<80	" "
5	80≤% ≤100	" " /to completely dry tapping-cut

4) cDNA or gene cloning and gene expression:

a - Extraction of total RNA :

After thawing, most of the rubber was discarded by a 10,000 x g centrifugation for 30 min at 4°C. The recovered "white fraction" was deproteinized through at least 3 phenol/chloroform/ isoamyl alcool (25/24/1, v/v/v) and one chloroform/isoamyl alcool (24/1,v/v) extractions. RNA precipitation was performed overnight in 2M LiCl at 4°C. Additional purification with chloroform/isoamyl alcool was done before overnight ethanolic precipitation (70%) in the presence of 300 mM potatium acetate, pH 5.5, at -20°C. Total RNA were pelleted at 12.000 x g, 30 min, 4°C, then washed with 70% ethanol. The final pellet was resuspended in 50 µl DEPC sterile mQ water for spectrophotometric quantification at 260 nm, then stored at -70°C. For the long term , total RNA were stored in 70% ethanol at -70°C.

b - Northern Blot Analysis (Gene expression).

After 3 min denaturation with formaldehyde/formamide/Tris buffer at 65°C, total RNA (10 µg) were subjected to denaturating 1,2 % agarose/formaldehyde gel electrophoresis (Sambrook *et al.* 1989), then transfered onto a nylon membrane (Hybond N, Amersham) according to the manufacturer's recommandations.
The various probes used were full- or partial-length cDNA or PCR-cloned gene fragments, radiolabelled with α^{32}P-dCTP using the Megaprime DNA labelling system from Amersham. Hybridization was performed overnight in an hybridization oven at 65 °C with homologous probes, or between 45°C and 55°C with heterologous probes, depending on the suspected sequence homology, in 5 x SSC solution (from a 20 x SSC stock solution: 3M NaCl, 300 mM trisodium-citrate, pH 7), 10 x Denhardt's reagent (from a 50 x stock solution: 1% ficoll, 1% PVP, 1% BSA), 7% SDS, 20 mM sodium phosphate buffer, pH 7.2, and 100 µg deproteinized fragmented denaturated salmon sperm DNA.
Final washes were generally performed at 65°C in 0.5 or 0.1 SSC and 0.5% SDS for homologous probes, and in 1x SSC, and 0.5% SDS for heterologous probes at the same temperature as for hybridization.

c - Construction of a latex cDNA library

Poly(A+) RNA were isolated by passing latex total RNA on oligo-dT cellulose columns according to the method described by Aviv et Leder (1972).
cDNA was synthesized from 5 µg poly(A+) RNA extracted from latex, using the Amersham "cDNA Synthesis System Plus". Oligo-dT primers were used to perform the first strand cDNA synthesis.
The cloning of the cDNA was performed using the Amersham "cDNA Cloning System-Lambda gt11", except that the vector used was Lambda Zap II (Stratagene). The double-stranded cDNA was blunt-ended, ligated with dephosphorylated EcoRI adaptors, then purified on Sephadex G25 in order to eliminate the unligated adaptors. The cDNA-ligated adaptors were phosphorylated before ligation in equimolar proportion with the Lambda Zap II vector linearized with EcoRI and dephosphorylated. Phage were packaged using the Stratagene "Gigapack II Gold Packaging extract" and plated in the host strain XL1 blue.

d- Extraction of *Hevea* genomic DNA .

Genomic DNA was extracted from young leaves (clone RRIM 600). Ten grams of liquid nitrogen-frozen leaves (cleaned with steril mQ water upon collection) were ground to a fine powder in a mortar, then transferred in centrifuged tubes and suspended in 3 ml/g of extraction buffer (100 mM Tris.HCl, 100 mM EDTA, 250 mM NaCl, 100 µg/ml proteinase K, pH 8). The suspension was brought to 1% sarkosyl (with 10% stock solution), and incubated for 2 hours at 55°C. The lysate was fuged 10 min at 5,000 x g at 4°C, to pellet debris.
DNA precipitation was achieved by adding 0.6 vol isopropanol, on ice. The precipitated DNA was pelleted at 7.500 x g, at 4°C, and dissolved in 9 ml TE buffer. Solid CsCl (9.7 g) was added and gently mixed until complete dissolution. After 30 min incubation on ice, the lysate was centrifuged 10 min at 7,500 g, 4°C, the supernatant collected and 0.5 ml of 10 mg/ml ethidium bromide was added. After a second 30 min incubation on ice, the RNA precipitate was pelleted by centrifugation 7,500 x g, 4°C, 10 min. The supernatant was transferred in 2 ultracentrifuged tubes. After overnight centrifugation at 60.000 rpm (20°C), the DNA band was sucked off with a syringe. Ethydium bromide was removed by repeated extractions with isopropanol previously equilibrated over a CsCl saturated aqueous phase. The DNA was precipitated by 2 vol water and 6 vol ethanol for 1 h at -20°C, and pelleted at 7,500 x g, 10 min at 4°C. The pellet was resuspended in TE buffer and reprecipitated by adding 1/10 vol of 3 M sodium acetate and 2 vol ethanol, 1 hour at - 20 °C. After centrifugation the pellet was briefly air- dried and resuspended in TE buffer.

e - Construction of a latex genomic DNA library

The genomic DNA (RRIM 600 clone) was extracted as described above and aliquots (50 µg) were digested with EcoR 1 until getting genomic DNA fragments ≤ 10 kb.The genomic fragment from the various aliquots were pooled and after sucrose density fractionation, only the fragments 3,5 ≤ kb ≤ 7 were selected and pooled for insertion in the vector. These genomic fragments were inserted in λ Zap using the Zap Express/Ecor 1/ CIAP-Treated vector Kit from Stratagene. This *Hevea* genomic library was constructed and amplified according to the manufacturer recommandations.

f - Screening of libraries and cloning

The cDNA or genomic libraries were screened with radiolabelled inserts (probes). The hybridizations were performed as described for Northern blotting.

The phagemid Bluscript containing the clones of interest were excised from the Lambda Zap vector using the helper phage R408 (Stratagene) and subsequently multiplied in XL1 blue cells. The length of the inserts was either checked by PCR, using the T3 and T7 primers and the Taq DNA polymerase from Promega (conditions for amplification were 1 min at 94°C, 2 min at 50°C and 3 min at 72°C, for 20 cycles) starting from 5 ml of an overnight bacterial culture, or through digestion of the plasmids prepared by Miniprep (Quiagen kit), with the appropriate restriction enzymes.

g - Southern blotting

Genomic DNA was extracted from young leaves as previously described (see genomic library). Samples of 0.5 - 0,8 (RRIM 600) mg DNA, digested with various restriction enzymes (EcoR1, Xho 1, BamHI, HindIII, Sac I, Xba I...), were separated on a 1% agarose gel, transfered to nylon membrane (Hybond N+, Amersham) under alkaline conditions, and hybridized as previously described for Northern blotting with the radioactive labelled probes. Washes were performed at 65°C in up to 0.5 x SSC and 0.5%SDS.

h - Cloning through PCR

We could obtain some clones using either homologous primers designed from rubber tree cDNA or published gene sequences, or heterologous primers designed from consensus sequences deduced from heterologous genes or cDNA, and either genomic DNA or our cDNA library as template. PCR were run using a Peltier cooler "Minicycler Model PTC 150" PCR machine, from MJ Research Inc. After agarose electrophoresis, the PCR DNA bands of expected size were extracted either by freezing/thawing and centrifugation, or by electroelution using the "sicpack" electro-eluter from Hoefer.

The DNA fragments were ligated in the pMosBlue Ti vector (Amersham), according to the manufacturer's recommandations, and clones introduced into *EColi* (DH5α).

i - Sequencing

Dideoxy sequencing was performed either using the T7 Sequencing kit (Pharmacia) and radioactive dCTP, or by automatic sequencing (PerkinElmer), using the PerkinElmer cold sequencing kit, in the Dr SKORN's Lab, in the Chulaborn Research Institute (CRI). Either the GCG software (Devereux *et al.*, 1984), or the software (CRI) was used for sequence analysis.

4) Protein analysis.

a - Protein extraction:

After thawing on ice, the 1,5 ml latex samples were centrifuged (12.000 x g; 1h , 4°C).

- The rubber particles fraction (white supernantant) was either collected for analysis or discarded.
- The clear intermediary fraction (cytosol) was sucked off and recentrifuged (same conditions)
- The bottom fraction (organels: essentially the vacuoles = lutoids) was either collected for further analysis or discarded.
- The membrane proteins from the rubber particles or from the latex organels (vacuoles) were obtained after 3 washings of these crude fractions with 1 ml of 100 mM Tris , 10 mM EDTA, pH 8.0. The organels were lysed by 3 successive freezing and thawing. The membrane proteins were extracted either from the washed lysed organels or from the rubber particle membranes by boiling 3 minutes in 50 mM Tris; 10 mM EDTA; 0.5% SDS; 0.2% Triton; pH 8.0.

b - Protein measurement and electrophoresis:

Protein concentrations were measured according to Bradford (Anal. Biochem, (1976), 72, 248)

The 1-D and 2-D SDS-PAGE (IEF pH 3-10 or 5-7) electrophoresis were run as decribed respectivelly by Laemmli (Nature, 277 (1970) 680-685) and O'Farrel *et al.* (Cell,, 12, (1977), 133-1142), loading 20 µg of protein for small gels and up to 200 µg for large gels.

The proteins were visualized either by Brillant Blue Staining (Sigma method) or by silver nitrate staining (Bio-Rad) (Helmut *et al.*: Electrophoresis: 8, (1987), 93-99 /).

c - Western blotting.

The proteins from latex (cytosol or rubber particles membrane) were submitted to 1D-SDS-PAGE electrophoresis (12,5%) and electro-transferred onto a PVF Immobilon membrane. Western blot analysis were performed using specific polyclonal antibodies (rabbit) raised against the purified proteins from latex and the Vecastain (anti-rabbit) kit for staining.

6) Ultrafiltration.

In order to get rid of low molecular weight proteins such as hevein (MW ≈ 9.5 kD) or to concentrate other proteins, clear latex cytosol or vacuolar sap (obtained by sonication of the latex pellet, 30 sec, 4°C, then centrifugation 30 min, 12.000 x g, 4°C) was submitted to ultrafiltration, eventually followed by 3 subsequent washes with equal volumes of 20 mM phosphate buffer pH 6, either under nitrogen pression (7 bars) in an Amicon cell, using Amicon PM 10 or PM 30 membrane, or by ultra-filtration centrifugation using ultrafiltrating 12 kDa cut-off microcentrifuge systems (4,000 x g). The concentrated "washed proteins" were diluted to the same initial volume with the same phosphate buffer as used for washing.

CHAPTER III - RESULTS AND DISCUSSION

As stated in the introduction, there are **2 major intrinsic yield limiting factors** at the level of the latex producing tissues:

- the latex flow limitation by **coagulation** processes at the tapping cut,
- the capacity of **regeneration** of the latex between tappings,

and a major problem impairing rubber yield,with high economical impact:

- the onset of **Tapping Panel Dryness** (TPD) desease caused by overexploitation and most probably also by other stresses.

Moreover, **"stimulation"** with Ethrel (ethylene releaser) is well known to induce a transitory increase in latex yield. It is therefore a convenient mean to study gene expression in the latex cells, in relation with increased latex production, and is of great help to find out molecular markers of high latex yield.

These are the reasons why our researches were foccussed on the latex yield limiting factors and stimulation, in an attempt to characterize **some major genes, the expression** of which may be involved in the control of major limiting factors (coagulation, regeneration and the onset of TPD) and in the response to yield stimulation with Ethrel. These results will be presented in a 1st part of this report.

In a second part, we will briefly report the main results obtained by the M. Sc and B. Sc. students who has been working or are still working on the molecular Biology of rubber in this project

In a third part of this chapter, we will present our preliminary results obtained on the cloning of latex cells specific promotors which will be usefull to design specific vectors for the future genetic engineering of rubber tree.

In a fourth part we will present some of the results on the Latex Diagnostic (LD) obtained in the RRC Chachoengsao (with Mrs Noparat's group) related to overexploitation of rubber.

Endly, this report will enclose a general discussion and conclusions, with prospects for a two years extension of this project (01/96 - 12/97)

RESULTS : 1st PART

THE MAIN RESULTS OBTAINED ON MOLECULAR MARKERS LINKED TO THE LATEX YIELD LIMITING FACTORS

I - COAGULATION OF LATEX AND THE LATEX FLOW

We had previously (IMCB-Singapore) started to characterize the molecular basis of latex coagulation (Gidrol et *al.,1994*), which is a major limiting factor of rubber production:
Hevein, a ≈ 9.5 kDa (2 binding sites) lectin-like protein, one of the most predominant proteins in the latex of rubber tree, is compartmentalized in the vacuolar structures (lutoids) of the latex, where it represents ≈70% of the soluble proteins. As a lectin, Hevein has been shown to bind chitin and to be probably involved in rubber tree defence against fungi invasion.
We have shown that, when released in the cytosol, upon bursting of the lutoids (vacuolar compartment), hevein induces latex coagulation by bringing together the rubber particles (RPs). We could show that the hevein-RPs bridging is mediated by N-Acetyl-D-glucosamine, and involves a 22 kDa receptor glycoprotein localized on the surface of the RPs (Gidrol et al. 1994).

1) cDNA and gene fragments cloning:

a - Hevein :

The Hevein cDNA had been cloned and sequenced (Broekaert *et al.*, 1990). We obtained (in collaboration with IMCB-Singapore) our own hevein clone through PCR (1 min DNA denaturation at 95°C, 45 sec annealing at 65°C, 1 min polymerization at 74°C / 30 cycles) using two 24 mers primers, designed from the published hevein sequence (5' and 3' extremities of the published sequence) and our own cDNA library as template. The insert obtained had the expected length (≈ 1 kb) and was cloned in the pMosBlue Ti vector (Amersham) in *EColi* (DH5α). The verification of the sequence of the ≈ 350 first bases of each 5' and 3' ends of the insert did show that it was a hevein clone .

b - The hevein-receptor :

We had previously (in IMCB-Singapore) cloned part (≈ 400 bp from the 3'end) of the rubber particle bound hevein receptor, through immunoscreening of an expression latex cDNA library. Using this first partial cDNA clone as a probe, we could clone a longer insert (≈ 750 bp) through the screening of our cDNA library. But the Northern blot analysis, as well as the sequence of the insert showed that it was not a full length cDNA, which is expected to be ≈ 1100 bp long. This new longer cDNA clone shares 98% homology for the 300 bp at its 3' end, with the previous shorter clone, but doesn't include the start codon and the expected full open reading frame (≥ 600 bp for a 22 kDa protein). The difficulties for obtaining the full length cDNA clone of the receptor protein most probably result from GC highly rich sequences in the poly(A+) RNA (sequence 2: Annexe 2), which may give rise to the formation of secondary superstructures within the poly(A+) RNA, impairing the functionning of the reverse transcriptase during the synthesis of the full length corresponding 1st strand cDNA.

Action of exogenous chitinases and of lutoids extracts on the glycosylated status of the hevein receptor

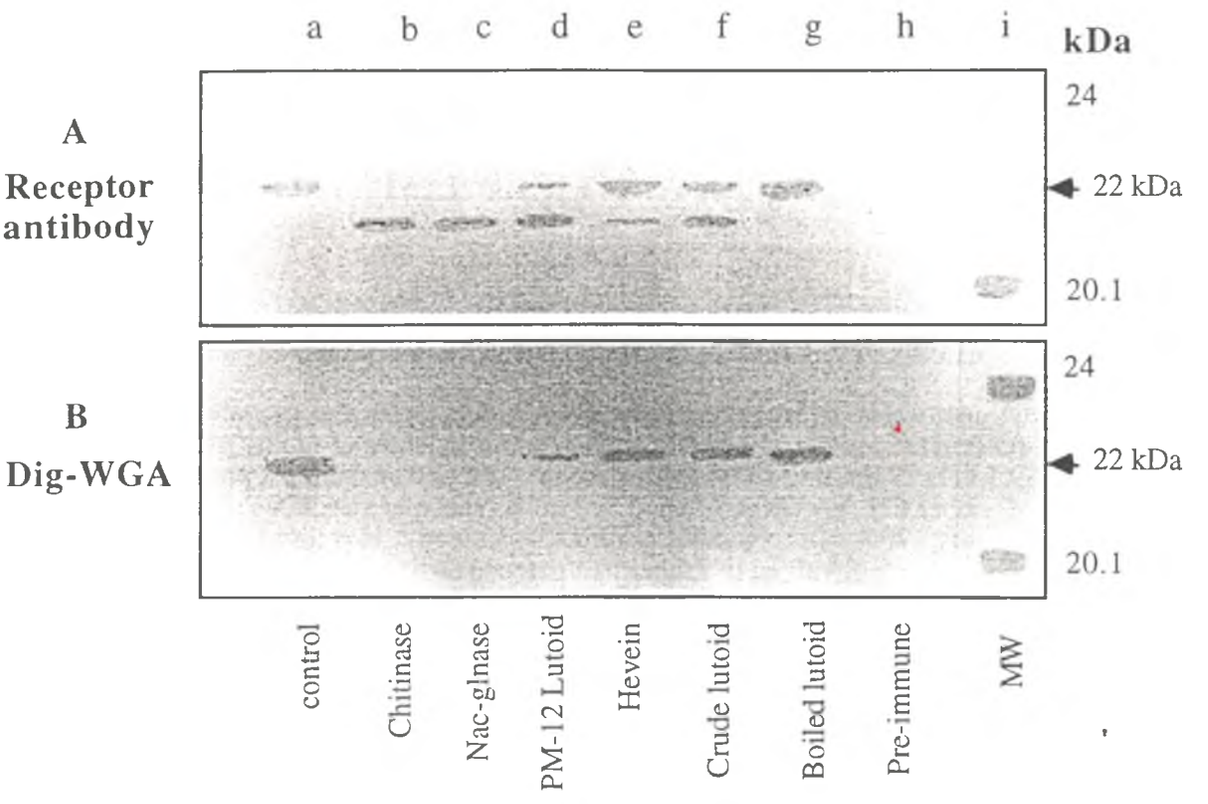


Figure 1 : Effects of various exogenous enzymes and of lutoids extracts on the glycosylated status of the hevein receptor protein from the latex rubber particles (Western blotting):

A - Detection with the antibody raised against the hevein receptor.
B - Detection with the digoxigenin labelled wheat germ agglutinin (WGA)

a : control (crude rubber particles protein extract);
b : treatment 30 min with commercial exochitinase;
c : treatment with commercial N-acetyl glucosaminidase;
d : incubation 30 min with the lutoids sap, ultrafiltrated on PM-12 in order to eliminate hevein (upper part).
e : incubation 30 min with the lutoids sap ultrafliltrate (+ hevein) (passed-through PM 12);
f : incubation with crude lutoids sap;
g : incubated with boiled lutoids sap;
h : detection with preimmune rabbit serum;
i : Molecular weight protein markers (Amidoblack)

Other rounds of screening with a new cDNA library under construction with higher quality RNA and in more secondary structure denaturing conditions (methyl mercuric hydroxide), should allow us to fish out the full length cDNA clone.
Nevertheless our 750 bp long partial cDNA can be used as an homologous probe for studies on the hevein-receptor expression in the latex .

d - Chitinase:

Exochitinases, which are present in large amount in the latex vacuoles, are able (see results: § 2), as well as N-Acetyl-glucosaminidase, to remove the N-Acetyl-glucosamine moyety of the hevein receptor protein, and therefore are supposed to inhibit the interaction between hevein and its receptor, hence to delay or inhibit latex coagulation.
We have cloned (in collaboration wit IMCB-Singapore), through PCR, a fragment of a chitinase cDNA using our cDNA library as template and 2 heterologous 23 mers primers designed from consensus sequences deduced from heterologous plant chitinase genes or cDNA. The PCR parameters were as following: 1 min denaturation at 95°C; 1 min annealing at 52°C; 1 min polymerization at 74°C / 30 cycles). Two DNA bands could be obtained, one very bright ≈ 200bp, and 1 very faint ≈ 550 bp. The bright one (≈ 200 bp) was eluted and ligated in the pMosBlue Ti vector then introduced in *EColi* (DH5α). Its sequence showed up 92 % homology with other plant Chitinase genes. This chitinase insert could be used as an homologous probe for Northern blot analysis.

2) Further studies on the action of Chitinase and N-Acetyl-glucosaminidase:

a - Deglycosylation of the hevein receptor:

We had previously purified the 22 kDa membrane receptor-protein of hevein, and shown that it could be deglycosylated by exogenous chitinase (Gidrol *et al.* 1994). We have made further studies (in collaboration with IMCB-Singapore) to evaluate the efficiency of latex enzymes and stimulation, on the glycosylation of the hevein receptor.

Using the specific antibody we had previouly raised (in IMCB-Singapore) against the hevein receptor as a probe, Western blot analysis of the proteins from the rubber particles membrane clearly showed (Fig.1 A) that commercial exochitinase (b), N-acetylglucosaminidase (c), as well as latex intravacuolar sap (intralutoids serum) which is known to countain both chitinases and N-acetylglucosamini-dase, when ultrafiltrated and washed on PM 12 to eliminate most of the low MW hevein (d), resulted in a decrease of the molecular weight (around - 0.7 to 1 kDa) of the hevein receptor: the untreated receptor (a) exhibit a single band at 22 kDa, while the commercial enzyme-treated receptor reveals a single band ≈ 1 kDa beneath the native receptor (b,c). The hevein receptor treated with a PM 12 ultrafiltrated lutoid extracts (minus hevein) exhibits 2 bands (d): a minor one ≈ 22 kDa and a major one ≈ 21kDa.
When incubated with the PM 12 passed-through fraction (e)(containing Hevein but neither chitinase nor N-acetyl glucosaminidase) or with a 5 min boiled crude lutoid sap extract (g) the receptor exhibited a unic band at 22 kDa.
When incubated with crude lutoids sap (endogenous chitinases + hevein), the hevein receptor exhibits 2 bands of ≈ equal intensity (e), probably because hevein could compete with the endogenous chitinases for fixation on the same sugar moiety of the hevein receptor, leading to its partial "protection".

The use of digoxigenin labbelled wheat germ agglutinin as a probe (fig 1 B), which can detect N-acetylglucosamine only, shows that the glycosylated native receptor is detected as a single intense band (a), and that treatments with exogenous (b,c) or endogenous enzymes (d and f) induce the desappearance or a dramatic decrease in the intensity of the signal, corresponding to a total (b and c) or partial (d and f) deglycosylation of the hevein receptor. The ≈ 21 kDa protein cannot be detected with the WGA probe, confirming that it lost its N-acetyl-

Action of bark stimulation with Ethrel (ethylene generator) on the glycosylated status of the hevein receptor

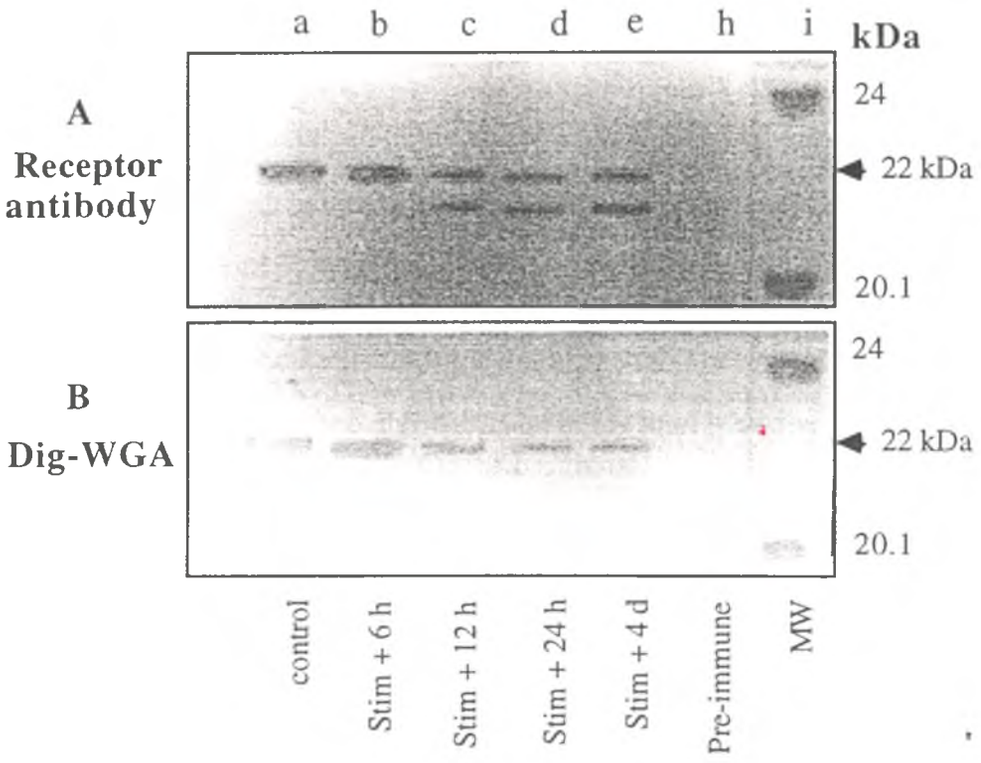


Figure 2 : Effects of bark stimulation with Ethrel (an ethylene generator) on the glycosylation of the hevein-receptor protein from the latex rubber particles (Western blotting):

A - Detection with the antibody raised against the hevein-receptor.
B - Detection with the digoxigenin labelled wheat germ agglutinin (WGA)

a : control: rubber particles proteins : no treatment /1st tapping);
b : 6 hours after bark stimulation with Ethrel (1st tapping)
c : 12 hours after treatment (1st tapping)
d : 24 hours after treatment (1st tapping)
e : 4 days after treatment (3rd tapping)
h : detection with preimmune rabbit serum;
i : Molecular weight protein markers (Amidoblack)

Effects of tapping and of bark stimulation of virgin trees on the expression of the genes involved in the coagulation of latex.

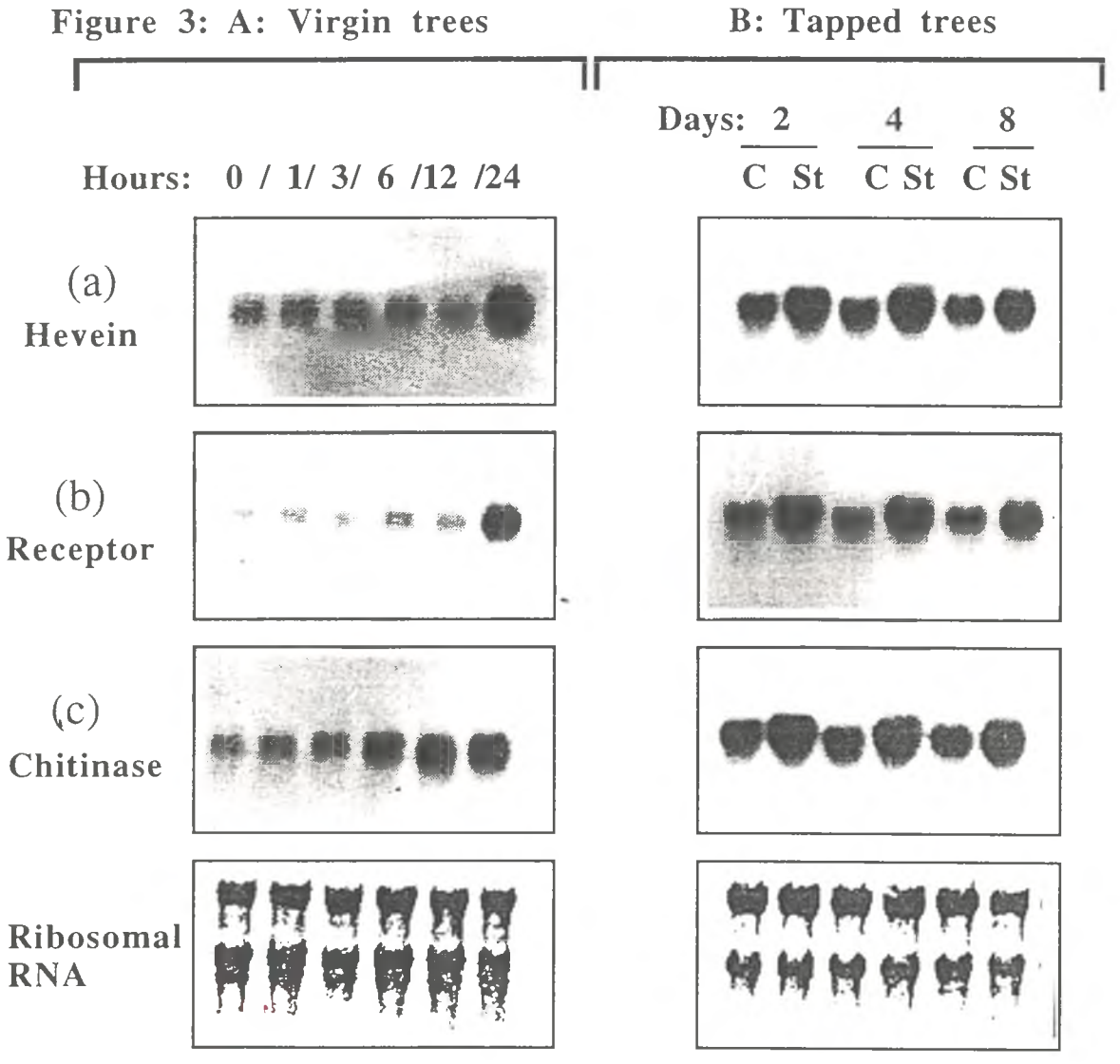


Figure 3: Effects of tapping (wounding/regeneration: B) and kinetic effects of a stimulation (A) with Ethrel, on the expression of hevein (a), hevein receptor (b) and chitinase (c) genes, in the latex cells of virgin trees.

Northern blotting: 10µg total RNA from latex/65°C hybridization and washings (0.5-0.1 x SSC). Ribosomal RNA stained with Methylene blue.
Hours: hrs after stimulation; D: days after stimulation; C: control; St: stimulated trees.The virgin (never tapped) trees were tapped all together for the first time, then after they were all tapped every 2 days.(Exposure: hevein = 6 hrs, receptor and Chitinase = over night)

Expression of the genes involved in latex coagulation in the latex cells of two clones with different characteristics of their latex flow. Effects of Stimulation with Ethrel.

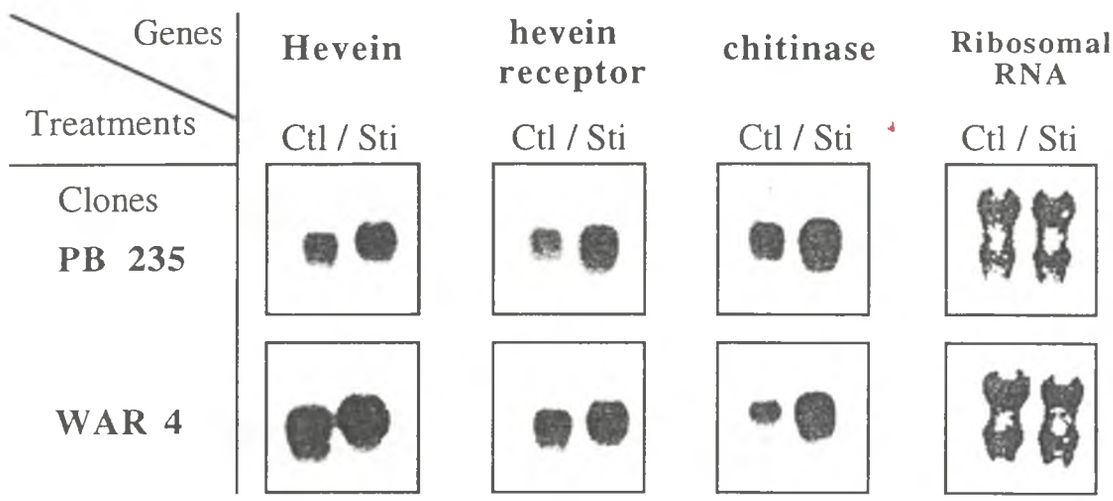


Figure 4: Northen blot analysis of the expression of the hevein, the hevein receptor and chitinase genes in the latex cells of the rubber tree clones: PB235 (high yielding/easy latex flow) and WAR4 (poor yielding/short latex flow)

Ctl: Control not stimulated; Sti: Stimulated 48 hours before tapping. All trees have been regularly tapped for at least 6 years.
(Exposure: hevein 6 hours; others: overnight)

glucosamine moiety. The presence of hevein (e and f) confered a partial protection on the sugar moiety. Hence hevein and Chitinases (or N-acetyl-glucosaminidase), which are located in the same vacuolar compartment, can compete (upon the bursting of the lutoids) for the same site of interaction: the N-acetyl-glucosamine moiety of the hevein receptor. Therefore one can hypothetize that the relative amount and affinity of the chitinases, N-acetylglucosaminidase ("anticoagulating factor) and hevein (aggregation factor) for the glycosylated hevein receptor, when released in the latex cytosol, might at least partly determine the efficiency of rubber coagulation.

b - Ethrel stimulation induces partial deglycosylation of the hevein-receptor:

From Fig 2A, it can be seen that the hevein receptor from the stimulated trees exhibits 2 bands 12 h (c) and 24 h (d) after stimulation, the intensity of the ≈ 21 kDa band increasing with time after stimulation, while the one from control trees (a) and 6 h after stimuation exhibited only one band at 22 kDa, as detected with the receptor antibodies. When using labelled WGA (Fig 2 B), only the 22 kDa band could be detected, with a decreasing intensity 12 and 24 hours after the stimulation treatment. This confirms that stimulation can induce, between 6 and 12 hours after the treatment, an increase in the activity of latex chitinases (and N-acetylglucosaminidase?), resulting in partial deglycosylation of the hevein-receptor, hence delayed coagulation.

3) Tapping and Stimulation can induce the overexpression of the 3 major genes involved in coagulation.

From Northern blot analysis using latex total RNA from virgin trees (Fig 3 A) which had or not been stimulated with Ethrel, it can be seen that the 3 genes: hevein (3A-a), the hevein receptor (3A-b) and chitinase (3A-c) are constitutively expressed in the latex cells before any wounding (tapping) (see time "0").

Stimulation with ethylene induces, in the latex cells, even of untapped trees (no regeneration processes), an over-expression of these 3 genes. But the earlier (between 6 and 12 hours for chitinase compared to 24 hours for hevein and its receptor) and higher over-expression of chitinase can explain the partial deglycosylation of the hevein receptor as mentioned above (Fig 2) and the resulting delay in coagulation.

From Fig 3 B, it can be seen that upon successive tappings, the wounding effects and/or latex regeneration process between 2 tappings, induces *per se* the overexpression of the 3 genes, but the effect is more pronounced for chitinase, which can explain the well known effect of the more and more facilitate latex flow upon the successive first tappings. Successive tappings of stimulated trees exhibit immediate maximum over-expression of the 3 genes, always more marked for the chitinase genes. The over-expression of these 3 genes lasted more than 8 days after a stimulation with Ethrel. Other experiments did show that the effect was still visible after 2 weeks (in general the mean duration of the yield increase after stimulation).

4) Is the expression of the genes involved in coagulation a clonal characteristic?

We checked the expression of the 3 genes involved in the coagulation process as clonal characteristics. For this, we started (in collaboration with IMCB) preliminary experiments checking 2 clones with extreme characteristics as far as their latex flow is concerned (Table 1):

- 12 year-old regularly tapped PB235 trees (RNA previously extracted in IMCB-Singapore from PB235 trees in South Malaisia), a good yielding clone with typically good flow characteristics, with a low plugging index (Table1).
- 13 year-old tapped WAR-4 trees (RNA previously collected in Ivory Coast), a very low yielding clone in Africa with problems of flow, high capability of coagulation and high plugging index (table 1).

Table 1 - Mean Plugging index of PB235 and War-4 clones (a mean of 6 homogeneous trees/clone) before (-) and 48 hours after (+) stimulation treatment. The value are those from the trees, the latex of which had been collected and pooled for RNA extraction and Northern analysis.

Clone	initial flow rate (ml/min)		total latex volume (ml)		Plugging Index	
stimulation	-	+	-	+	-	+
PB235	6.2	*7.7*	147.2	*214.0*	4.2	*3.6*
WAR 4	2.7	*5.5*	21.8	*77.4*	12.4	*7.1*

Northern blot analysis (Fig 4) showed that hevein and the hevein receptor were constitutively higherly expressed, while chitinases are far less expressed, in the latex cells from the WAR-4 trees compared with the PB235 trees (no stimulation).

Stimulation induced a comparatively higher expression of chitinase in the latex of the WAR-4 trees, clone which is known to better respond (increased of yield ≥ 3,5) to ethylene than PB235 (≈ 1.5)(see table 1).

Same experiments will be done within the next few months to verify these clonal relationships between the characteristics of the latex flow, coagulation rate and gene expression in the latex cells, with some well typed clones in Asia (Thailand), in order to state if the expression of these genes can be considered as yield marker for selection purposes.

5) Hevein and the hevein receptor gene expression is laticifer specific.

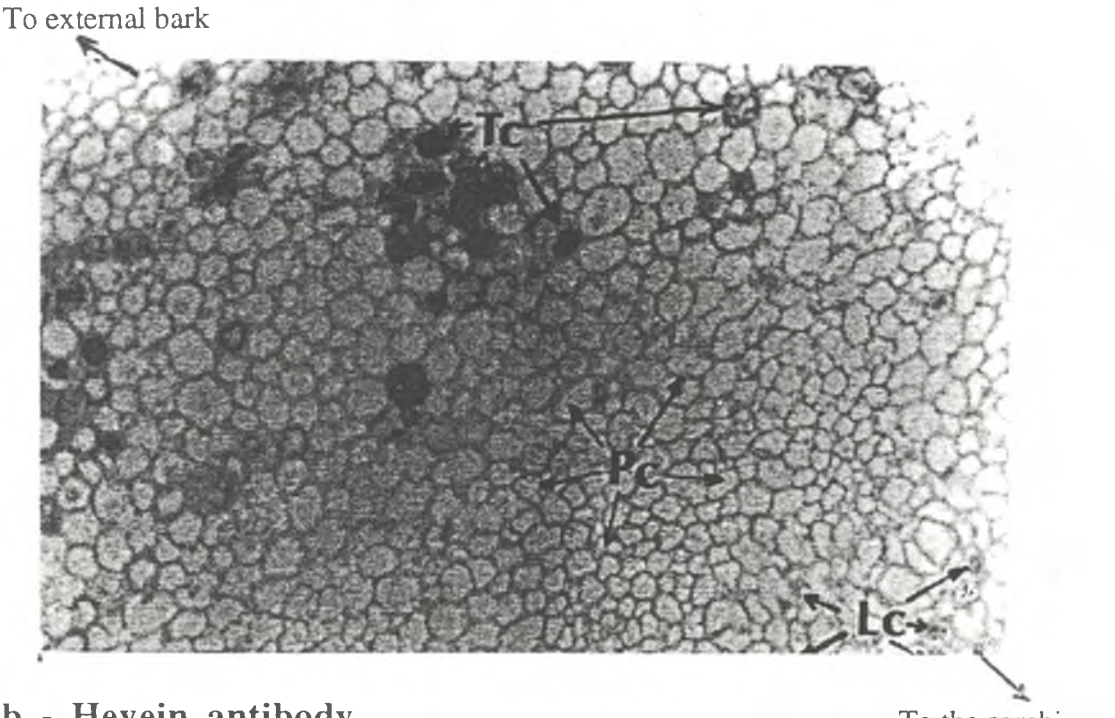
We had already demonstrated (in IMCB) by immunohistocytology using ultra-thin sections of young stems and specific antibodies raised against hevein and its receptor protein, that the expression of these genes (in term of translation product) is laticifer specific (Fig 5). These proteins are present in the latex cells only.

We could confirm that the expression of these 2 genes (at the transcription level) is also latex specific. By Northern blot analysis, using total RNA extracted from the latex and from the leaves (RRIM 600 clone) we could show that the mRNA corresponding to hevein and its receptor protein accumulate in the latex cells only (Fig 6 a and b). The few contaminating traces of both mRNAs in the RNA from leaves probably come from the fact that laticifers differenciate in the secondary tissue (nerves) of the leaves, so there is always some latex contaminations in leaves extracts.

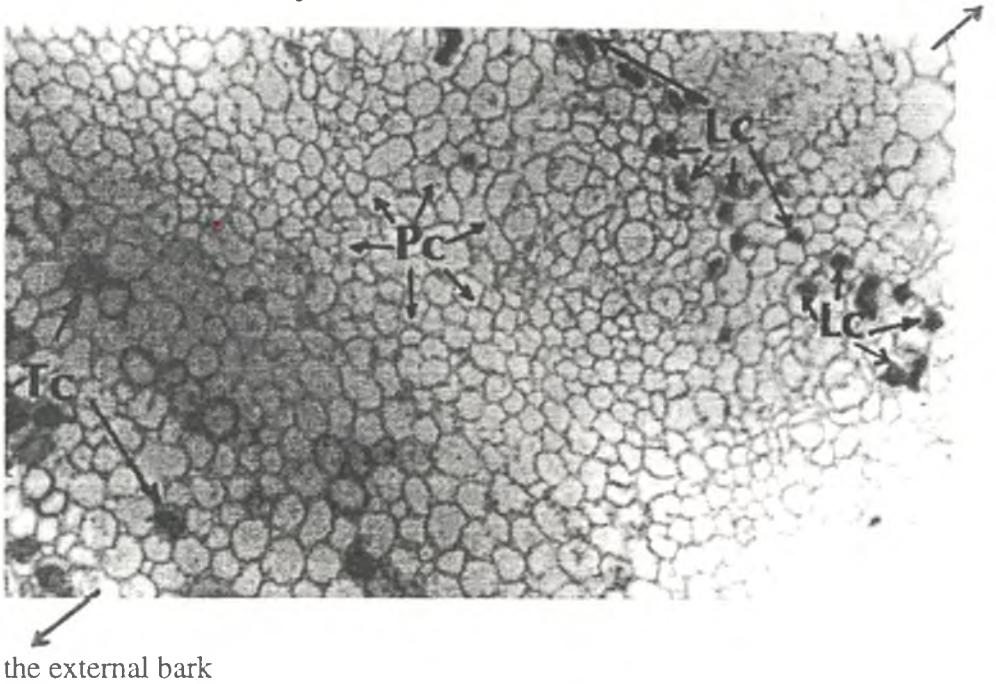
The fact to find out genes which are specifically expressed in an organ or in a specialized tissue (such as the latex cells) is very important for plant genetic engineering in general.
The expression of hevein and the hevein-receptor genes being latex cells specific means that these two genes must be under the control of latex specific targeted promotors, with strong efficiency (these genes are highly expressed in regularly tapped trees).
Cloning and characterizing these genes promotor regions will be of high interest for the futur program towards the genetic engineering of the rubber tree, in order to control the expression of any desired gene in the latex cells only, without perturbing the physiology of other organs or tissues.
We have started the cloning of the promotor region of the hevein gene (Chapter: Results III).

Figure 5 A: Laticifers specific expression of the Hevein gene (cyto-histo-immunolocalization)

a - Preimmune serum



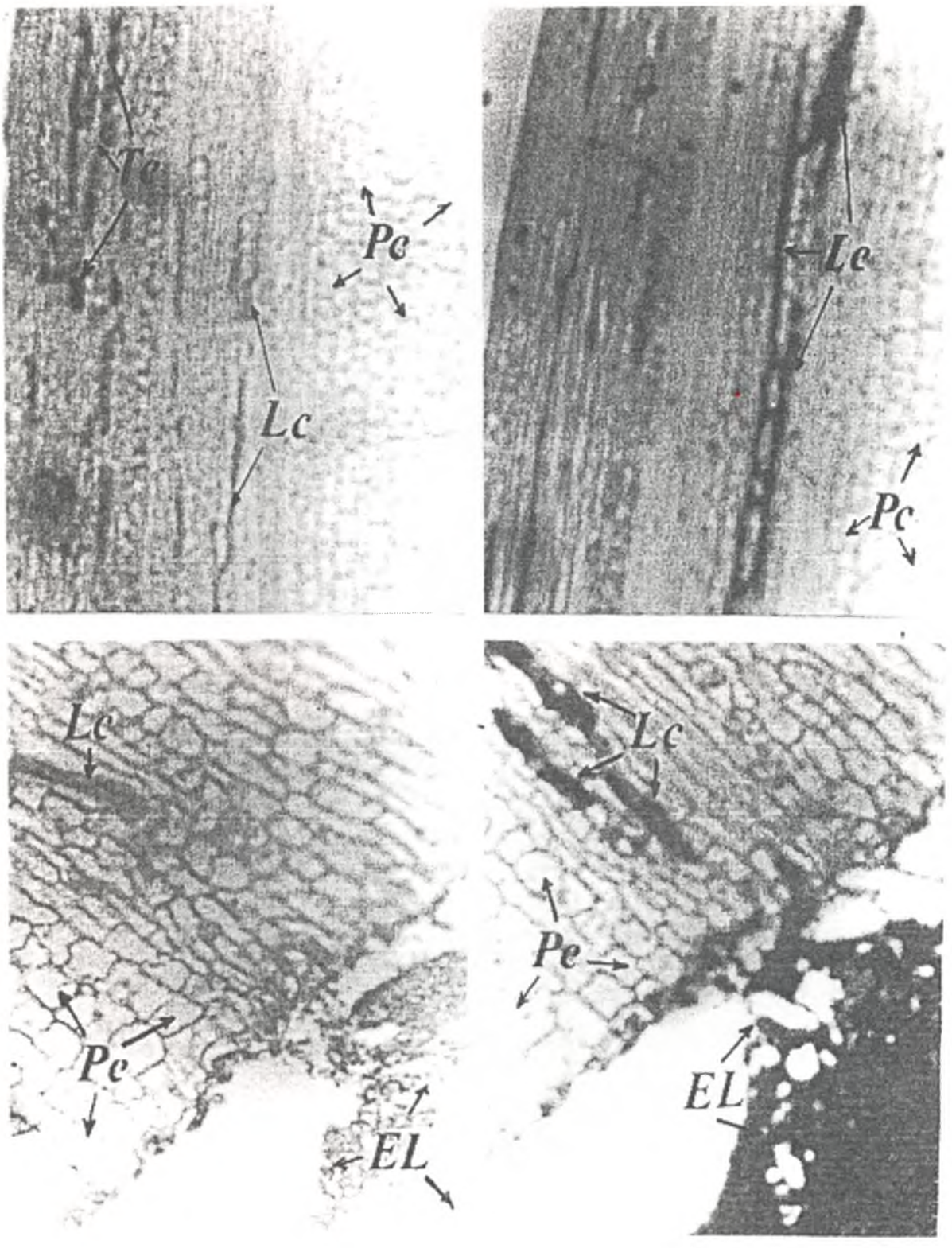
b - Hevein antibody



(Lc: Latex cells with coagulated latex inside; Pc: Parenchyma cells in the inner phloem; Tc: Tannin and stone cells near the epidermis / transverse thin sections of a young stem of rubber tree)

Figure 5 B : Laticifers specific expression of the Hevein-receptor gene (cyto-histo-immunolocalization)

a - Preimmune serum **b - Hevein receptor antibody**



(Lc: Latex cells with coagulated latex inside; Pc: Parenchyma cells (inner phloem); Tc: Tannin cells near the epidermis; EL: Exuded Latex (coagulated) / Longitudinal cut of a young stem)

Laticifers specific expression of the Hevein and of the Hevein receptor genes

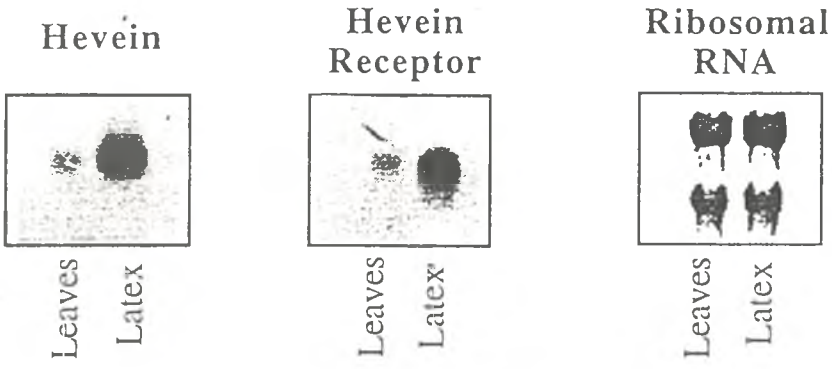


Figure 6 : Northern blot analysis of the tissue specific expression of the Hevein and of the Hevein receptor genes.

(10 µg total RNA from latex or leaves, hybridization and washings: 65°C/0.1 SSC / 0.5% SDS. Exposure = 2 hours). Ribosomal RNAs were stained with Methylene Biue.

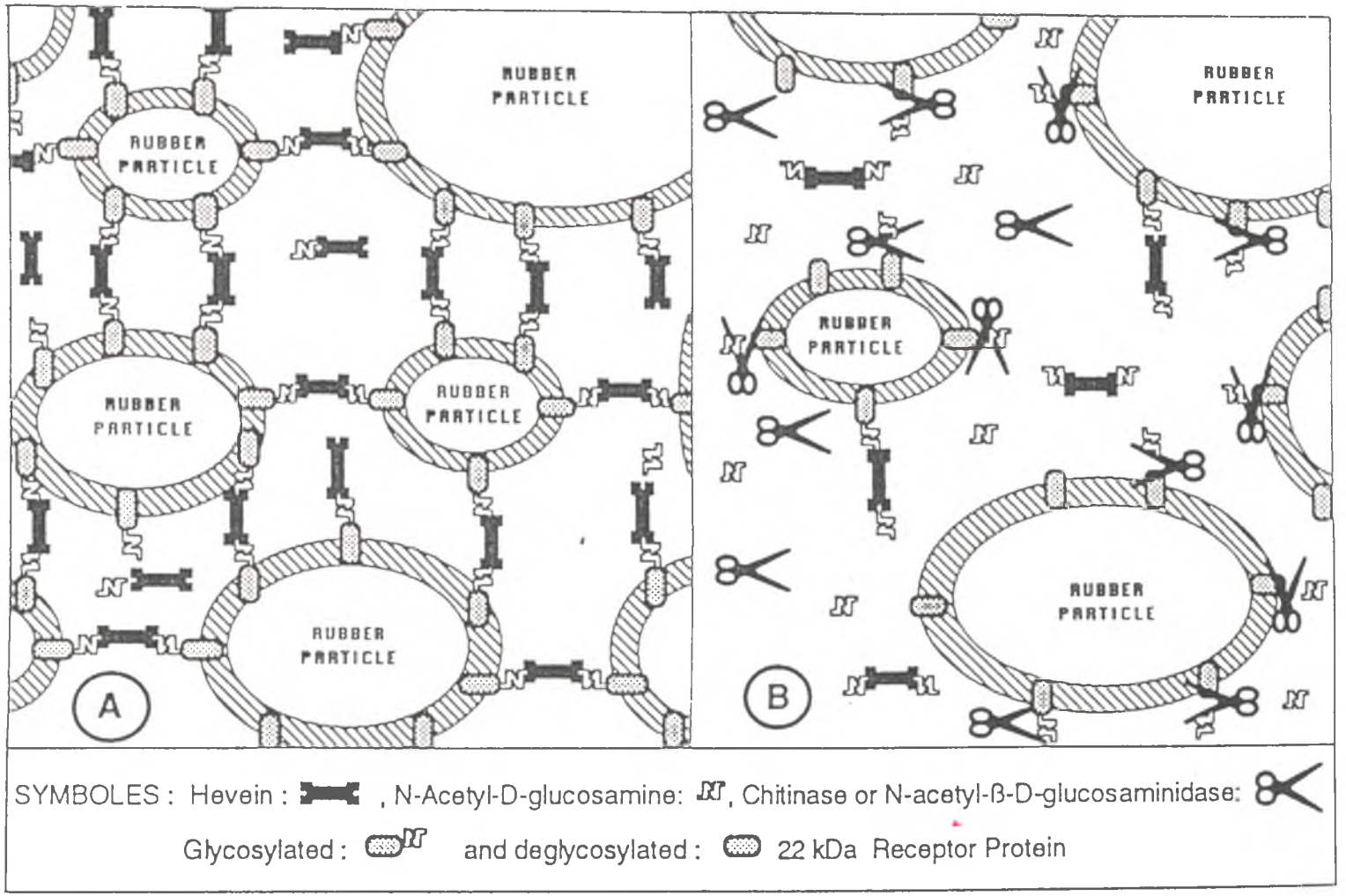


FIG. 7: **Model for coagulation of latex.** *A*, efficient coagulation of latex occurs at the wounded sites of the laticifers after tapping or *in situ* in case of dry bark disease. Hevein, freed by the bursting of the lutoids bodies, is present in high concentration (together with Ca^{2+}) in the latex cells cytosol. The lectin creates multivalent bridges between rubber particles, through its binding to the GlcNAc moiety of the glycosylated 22-kDa receptor, protein located at the surface of the rubber particles. This stage is associated with low chitinase activity and low level of free GlcNAc in the latex cytosol. Rubber particles are agglutinated, and the latex is coagulated. *B*, coagulation is inhibited, in normal condition, inside the latex cells. The hevein, mostly compartmentalized in the stable lutoids, is present in low concentration in the latex cytosol. The high chitinase activity provokes the deglycosylation of the 22-kDa receptor protein, increasing in turn the concentration of free GlcNAc, which can saturate the binding sites of hevein still present in the cytosol. Negative charge-charge repulsion keeps the rubber particles in suspension in the cytosol and allows stability of latex.

6) Conclusions and prospects about latex coagulation

As stated in the introduction part, coagulation process is determined by protein interactions: hevein and its receptor provoque coagulation, chitinases (and N acetyl-glucosaminidase) delay or inhibit coagulation by the removal of the N-acetyl-glucosamine moiety of the hevein receptor.
It could be seen using ethylene stimulation, as a tool for increasing latex flow (hence rubber yield), that ethylene induced an overexpression of these 3 major genes involved in coagulation. But the overexpression of chitinase happened earlier (and with a higher extent) inducing concomitantly (between 6 and 12 hours) at least partial deglycosylation of the hevein receptor, leading to delayed coagulation, hence prolonged latex flow. Figure 7 (a & b) illustrates the molecular basis of latex coagulation and the effects of Ethrel stimulation at its level.

Considering rubber tree clones with extreme characteristics as far as their latex flow and coagulation propensity are concerned, the clones with higher propensity for rapid coagulation exhibit higher expression of hevein and its receptor (pro-coagulants) and, on the contrary, far less basic (constitutive) expression of chitinases (anti-coagulants). The expression of chitinase was far more increased after ethylene stimulation in this clone with high plugging index, concomitantly with a higher increase in yield (in %) as compared with rubber clones with easier latex flow.

Even if it is highly probable, the relationship between expression of the "coagulation genes" and propensity to coagulation has to be verified, through the comparison of several other rubber tree clones with known latex flow characteristics (+ effects of ethylene stimulation), before stating that these 3 genes could serve as usefull markers of high latex yield. Such experiments will be performed starting end of 1995 to 1996 with the opening of a clonal trial in the RRC Chachoengsao and Chantaburi in Thailand.

An other way to verify this hypothesis could be to genetically engineer rubber trees to get some kind of "hemophylic clones" ,through over expression of chitinases or N-acetylglucoasminidase in the cytosol, or/and decreased expression of hevein or of its receptor protein through the antisens technology...

Finally, the hevein and its receptor genes are highly and specifically expressed in the latex cells. The corresponding tissue (laticifers) specific strong promotors should be cloned in the view of future genetic engineering of the rubber tree.

Effects of tapping and of bark stimulation of virgin trees on the expression of some genes involved in the regeneration of latex.

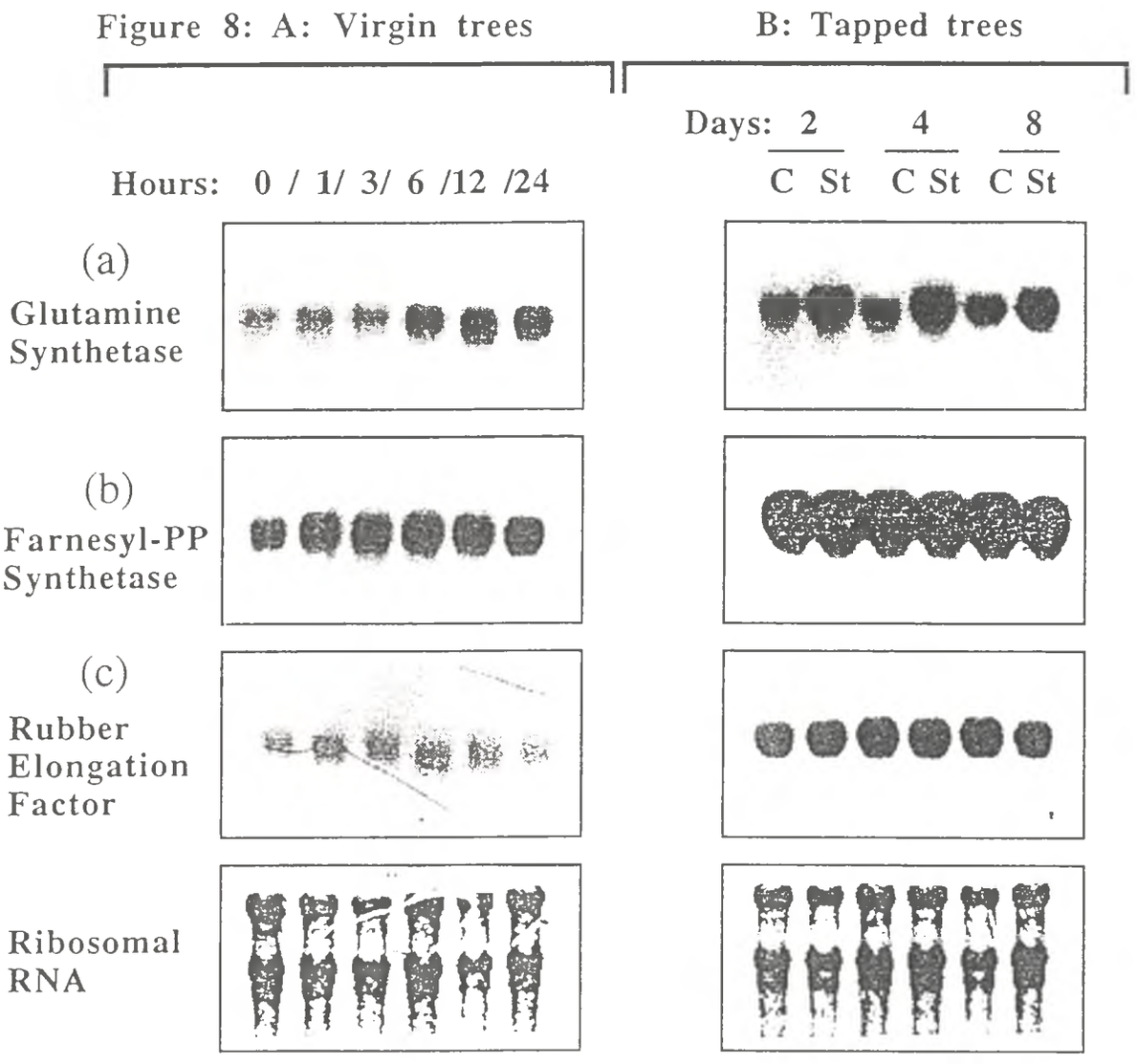


Figure 8: Effects of tapping (wounding/regeneration: B) and kinetic effects of an Ethrel stimulation (A), on the expression of glutamine synthetase (a), Farnesyl-pyrophosphate synthetase (b) and of Rubber elongation factor (c) genes, in the latex cells of virgin trees.

Northern blotting: 10µg total RNA from latex/65°C hybridization and washings (0.5-0.1 x SSC). Ribosomal RNA stained with Methylene blue.
Hours: hrs after stimulation; D: days after stimulation; C: control; St: stimulated trees.The virgin (never tapped) trees were tapped all together for the first time, then after they were all tapped every 2 days.(Exposure: GS = 16 hrs, FPPS and REF = 2 hours)

II - THE REGENERATION OF LATEX

1) Cellular regeneration: nitrogenous compounds (proteins, nucleic acids,...)

a - Interest of Glutamine synthetase

Glutamine synthetase (GS) is a key enzyme for ammonium assimilation in higher plants. It catalyses the incorporation of ammonia into organic molecules which are involved in the synthesis of aminoacids, nucleotides, and consequently in the synthesis of proteins, nucleic acids, and other nitrogenous compounds. Therefore, GS is a key enzyme in the regeneration of most cellular components.

In rubber tree, the latex is expelled during tapping. An efficient metabolic machinary is therefore necessary to ensure complete and quick regeneration of this exported latex cells cytoplasm (latex) between consecutive tappings. In this context, the GS activity is of major importance, for the reconstitution of all nitrogenous compounds.

b - Cloning and characterization of the latex cytosolic GS full-length cDNA

A cDNA of a cytosolic GS (cGS) from the latex has been cloned (V. Pujade Renaud, 1993). Genomic DNA was extracted from rubber tree leaves (genotype RRIM600) and purified by ultracentrifugation in cesium chloride. Samples were digested with EcoR I, Hind III, Sac I, BamH I, and Xba I and the restriction fragments separated on gel by electrophoresis, then transfered onto nylon membranes. Southern blot hybridization with the full length GS cDNA or subfragments (5' and 3' ends as well as internal portion) demonstrated than GS in rubber tree is encoded by a small multigene family, one of them might be laticifer-specific.

c - Tapping and stimulation can induce an early overexpression of GS.

Our previous work, using an heterologous probe, demonstrated that treatment of the trees with the ethylene generator, ethephon, was able to increase, within 48 h after (and during several tappings after) the treatment, both the activity and the mRNA level of glutamine synthetase, concomitantly with an increase in the production of latex (Pujade-Renaud et al, 1994).

From Northern blot analysis using latex total RNA from virgin trees (Fig 8 A-a) which had or not been stimulated with Ethrel, it can be seen that the cGS gene is constitutively expressed in the latex cells before any wounding (1st tapping) (see time "0"), but at a low level.

Stimulation with ethylene induces, in the latex cells, an early over expression the cGS gene. To our knowledge the cGS gene is the earliest (between 3 and 6 hours after the treatment) overexpressed gene, in the latex cells, after an ethylene treatment of the bark surface.
From Fig 8 B-a, it can be seen that upon successive tappings, the wounding effect (eventually through the endogenous synthesis of ethylene) and/or latex regeneration process between 2 tappings, also induces *per se* the overexpression of the cGS gene in the latex cells, and that this positive effect of ethylene can be prolonged over 4 tappings (8 days). Other experiments showed that the duration of the stimulation effects lasted more than 12 days, in parallel to the increase of latex production which can last up to 18 days after ethylene treatment.

As stated above, to our knowledge the latex cGS gene is the earliest overexpressed gene after an ethylene treatment of the bark. The expression of this gene may be directly under the control of ethylene. We have started the cloning of cGS genes in order to isolate and characterize their promotor and more specifically to determine which part of the promotor is responsible for the regulation by ethylene. Such promotor sequences, inserted in a vector for rubber tree transformation, might be of great interest to increase in a controled manner the expression of a transgene, using ethephon (see Chapter III).

2) The regeneration of rubber.

a - Importance of the farnesyl pyrophosphate synthetase and of the Rubber Elongation Factor in The regeneration of Rubber.

The major carbon compound lost in the exuded latex after tapping is rubber (high molecular weight 500-2000 kDa cis-polyisoprene chains), which accounts for 30-40% of the total latex volume, and about 90% of the latex dry matter. Hence rubber is the major compound (in term of quantity) to be regenerate between 2 tappings.

Farnesyl pyrophosphate synthetase (FPPS), also called rubber or prenyl transferase, is a key enzyme in the synthesis - regeneration - of the *cis*-polyisoprene chains (natural rubber). It catalyzes the synthesis of the first cis-poly-isoprene "primer" Farnesy-PP (FPP) from Dimethyl allyl-PP + Isopentenyl-PP (IPP), then with the help of the Rubber Elongation Factor (REF) catalyse the elongation of the cis-polyisoprene chains by adding molecules of IPP (in cis-configuration). Hence, FPPS is a key enzyme in the regeneration of rubber because it both primes and participates to the elongation of the cis-polyisoprene chains.

b - Cloning of the FPPS and REF partial cDNAs.

The cDNA from the latex FPPS has recently been cloned in IMCB/Singapore (K. Adiwilaga & A. Kush, 1995). The REF cDNA had also already been cloned in UK (Attaniaka et al., 1991). We could obtain our own FPPS (about 950 bp corresponding to 90% of the ORF) and REF (a 425 bp fragment corresponding to the ORF) clones through PCR.

c - Tapping, but not ethylenic stimulation, can induce overexpression of FPPS and REF.

Northern analysis (Fig 8 A-b & c) showed that virgin untapped trees already exhibit a very high level of FPPS and REF mRNA expression, but etephon bark treatment did not induce any significant change in the latex FPPS and REF genes expression, even after 24 hours or one week following the treatment (Fig 8 B-b & c).

In contrast successive tappings induced a significative over expression of both genes. The high level of constitutive expression of these 2 genes support the idea that these 2 genes are under the control of very strong promotors, but which are not controled by ethylene. The higher overexpression of these 2 genes after the first and consecutive tappings can be explained by a general induction of genes involved in the activation of the general metabolism required for the regeneration of latex between consecutive tappings.

d - The Expression of REF is most probably laticifers specific.

We could show (Fig 9 a) that the REF mRNA can be found in the latex cells only, that is to say that its expression is laticifer-specific. As mentioned before, the slight traces found in the leaves could be attributed to some obligatory latex contamination in the leaves extracts. As this gene is constituvely highly expressed, and in the latex cells only, it may be also interesting to clone its promotor which may be one of the "strongest" controling gene expression in the latex cells.

The latex FPPS c-DNA probe hybridizes with both latex and leaves mRNAs, although FPPS expression is much higher in the latex cells (Fig 9 b). Are there different isoforms (latex and leaves) with sufficient homology to hybridize with the same probe ? Anyway, they hybridize at the same level ≈ 1,3 kb , so it's impossible, using this technique, to certify that FPPS is laticifer specific.

Tissue specific expression of the Farnesyl-PP Synthetase and of the Rubber Elongation Factor genes?

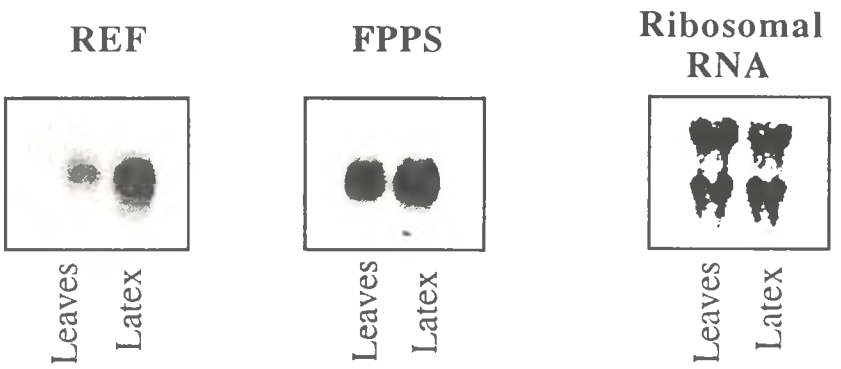


Figure 9 : Northern blot analysis of the tissue specific expression of the Rubber Elongation Factor (REF) and of the Farnesyl-pyrophosphate Synthetase (FPPS) genes.

(10 µg total RNA from latex or leaves, hybridization and washings: 65°C/0.1 SSC / 0.5% SDS. Exposure = 2 hours). Ribosomal RNAs were stained with Methylene Blue.

3) Conclusion and prospects about latex regeneration

We have studied the expression of 3 genes that are involved in latex regeneration : glutamine synthetase involved in ammonium incorporation into nitrogenous compounds and therefore necessary for proteins and nucleic acids regeneration, the Rubber Elongation Factor and the Farnesyl Pyrophosphate Synthetase, both involved in rubber synthesis (regeneration).

Only the cGS gene which is overexpressed after stimulation, concomitantly with the classical increase in latex yield, might be considered as a potential yield-marker, although clonal characteristics linking yield and GS expression remain to be checked.

The REF and FPPS genes are not induced after stimulation with ethylene. It is proposed that the expression of most of the genes involved in the synthesis of rubber, among which REF and FPPS, is not a limiting factor in such specialized tissue constitutively synthetizing 90% of its dry matter as natural rubber.

Nevertheless, complementary studies have to be done, comparing the expression of these genes involved in latex regeneration, with the clonal yield potential and response to stimulation.

The latex cGS gene was the only gene ever seen to be induced so early after a bark ethylene treatment. The promoting region of this gene has to be studied to check if it includes *cis*-acting sequences necessary for the regulation by ethylene and wether trans-acting factors involved in such a regulation can be identified.

III - TAPPING PANEL DRYNESS (TPD)

A - INTRODUCTION

1) Widespread nature and seriousness of the TPD desease:

TPD is ultimately characterized by the definitive cessation of latex flow upon tapping, due to the degeneration of the latex cells in the bark of the rubber tree. It is a complex phenomenon, the cause of remaining still unknown, despite the volume of work undertaken over many years.

It is a very serious matter, since it has been estimated that TPD is annually responsible for the loss of at least 10% of the production (value ≈ US$ 200 million)(1). In some plots, the number of TPD deseased trees can exceed 50%, which is economically very damaging

During the last International Rubber Research Development Board (IRRDB) meetings in Penang (June 1989), Manila (1991), then in Jakarta (1992) and in Cochin (India,1994), it was agreed that "the number 1 priority subject in the IRRDB program is Tapping Panel Dryness, given the widespread nature of this phenomenon and its seriousness" (1).
Since TPD is known to be linked to inadequate tapping system (over exploitation) for the various rubber tree clones, it is felt as a kind of "guilty desease" and, most probably for that, it is extremely difficult to get precise figures about the incidence of TPD in the various rubber producing countries. Often the data cannot be precisely known especially from the small-holders rubber plantations, and even if they are known in the larger scale estates, in most cases they will remain highly confidential.

In Indonesian Lonsum Ind. Co. Ltd commercial plantations (2.572 ha), taking into account all the planted rubber tree clones in all the estates with various ecological conditions together, it was reported in 1989 (2) that a mean of 15 to 23.5 % of the 6-14 year old trees (after 1-9 years exploitation) suffered of total TPD desease. No indication was given about the incidence of partial dryness which is classically the same order of magnitude.

In Malaysian commercial estates, taking into account all the states (with various ecological conditions) together, and the 3 more planted rubber clones: 2 precocious high yielding clones PB 260 and PB 235 (more sensitive to TPD) and 1 medium yielding clone RRIM 600 (less sensitive to TPD), it was reported in 1989 (3) that respectively about 10% and 8.5 % of the PB clones, and 4.5% of the RRIM 600 ten-twelve year old trees (after 5-7 years exploitation) suffered of total TPD Desease. No indication was given about the incidence of partial dryness.

In Thailand, very few global data are available, probably because most of the rubber plantations (≈92%) are smallholdings. The only recent data available from a study on 16 smallholdings (6200 trees 11-14 year-old) in the South area indicates that an average of about 11% (ranging from 6.2 to 19%) of the planted trees were stopped because of problems at the tapping cut and tapping panel (mostly TPD)(4). More over, in small plots checking in smallholdings, 17 to 70% of the tapped trees showed some symptoms of partial TPD (4). Some preliminary results (5) from 3 RRIT experimental stations (7320 trees 12-14 year-old) show that only 3% of the trees were affected by TPD. The clones RRIM 600 and GT 1 are the less suceptible (1-2.5%) while the clones KRS138 and the newly recommended PB 260 are more affected (respectively 10 and 8%). From small plots experimental trials in the RRIT experimental stations in the East and North-East (6a), after 7 years of tapping in the Rayong area, less than 3% of the RRIM 600 trees and 14.7 % of the PB5/63 trees were stopped because of total TPD. In Nong Kai area, after 9 years of tapping, 18.7% of the RRIM 600 trees, 28.8% of the GT1 trees and 32% of the Tjr1 trees were stopped because of total TPD.

In Côte d'Ivoire (Africa), the incidence of TPD has been recorded almost since the start of planting in the entire country. Nowadays around 50.000 ha have been planted exclusively in areas with adequate ecological conditions (rain > 1,500 mm/year and no more than 2-3 month dry season < 50 mm/month). The number of trees presently tapped is about 16 millions, of which 160,000 (1%) new trees turn affected by TPD (completely improductive) each year, and about the same amont display symptomes of partial TPD. From the beginning of rubber exploitation in Cote d'Ivoire (about 25 years), more than 2 million of trees had to be stopped because of TPD desease (7).

2) Nature of TPD

TPD has been described as "an abnormal physiological phenomenon induced by tapping... When the level of exploitation of the tree exceeds the physiological capability of the tree to regenerate latex, the tree succumbs to TPD" (1). Indeed the incidence of TPD is normally increased by a higher tapping frequency and/or excessive yield stimulation with ethylene releasers (Ethrel) (6b, 7, 8, 9, 10).

It is well known that sensivity to TPD is a genetic (clonal) characteristic. The rubber clones can be classified in 3 groups: those in the most susceptible class (PB 235, PB 260) are precocious high yielding clones with high metabolic activity of their latex cells; those with intermediate activity (GT1, RRIM600) have less susceptibility, while those with the lowest activity (PR107, PR261, AV2027) are the least susceptible (2, 3, 5, 6a, 7, 8).

Apart from clonal (genetic) effects, there are other factors which influence TPD such as ecoclimatic conditions (most probably drought and soil composition)(2, 3, 5, 6a, 7, 8). Until now, all the numerous attempts to identify a pathogene that might be involved in TPD have failed (11, 12)

It is most probable that TPD is a universal phenomenon, the incidence of which is governed by some complex interactions between the clonal sensitivity, exploitation intensity, and environmental factors.
Despite the considerable volume of work, little information has emerged which is of practical value for the prevention and/or management of TPD. The main outpout from this part of our program should be to develop diagnostic tests to forecast the impending incidence of TPD.

Two methodological approaches were developped by our team in an attempt to find out TPD molecular markers and understand the origin of the TPD deseases:

- The "blind" approach, which aims to find out protein markers of TPD, through comparative studies by 1 or 2D-electophoresis of the latex proteins from healthy and TPD trees.
- The "specific" approach by Northern blot analysis (specific genes expression), with particular regards to oxidative stress, which had previously been biochemically characterized as far as overstimulation-induced TPD is concerned.

B - THE "BLIND" APPROACH / PROTEIN MARKERS OF TPD

One can distinguish at least 2 possible classes of TPD: the spontaneous TPD occuring abruptly, without any explanation, and the overexploitation (stress)-induced TPD (overtapping, overstimulation, or the combination of both of them).

The characterization of proteins which may be induced or repressed before or during the onset of TPD may lead to the setting up of diagnostic tools (immuno-diagnostic kits) and, through the cloning of the corresponding c-DNAs, to get back to the genes in order to understand the mechanism of the onset of TPD.

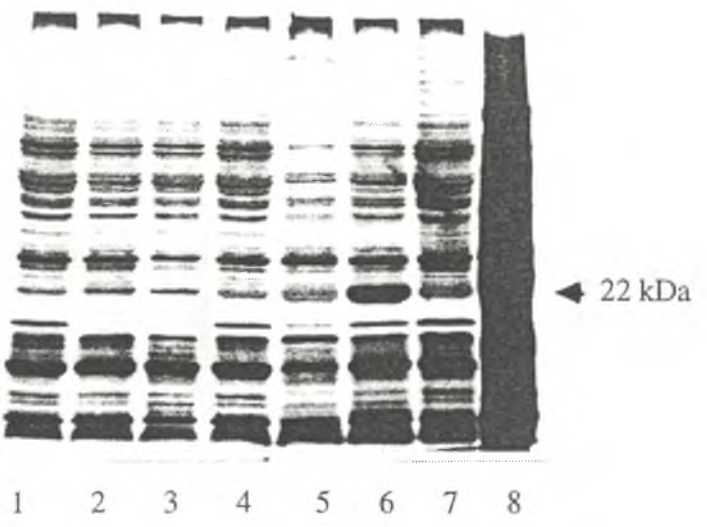


Figure 10: One-Dimension SDS-PAG Electrophoresis of latex cytosolic proteins of healthy trees and spontaneous TPD trees

Healthy trees (lane 1) TPD trees class1 (lane 2 and3) class 3 (lane 4 and 5) and class 5 (lane 6 and 7). MW lane 8. (Silver staining)

Fig. 11-a

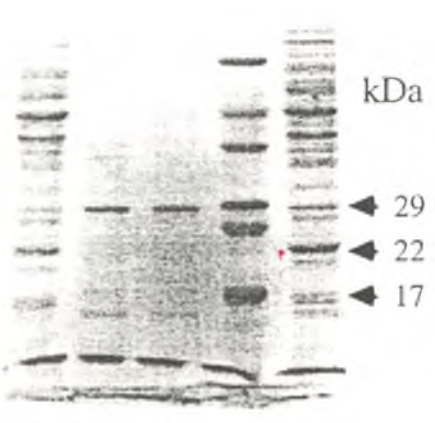


Fig. 11- b

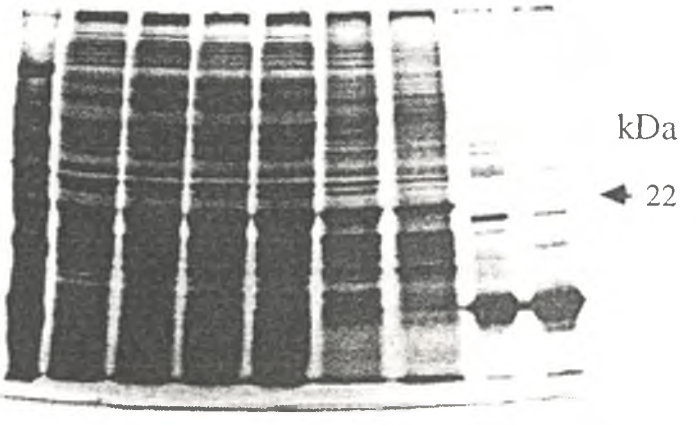


Figure 11: Electrophoresis of the proteins from the latex cytosol, from the lutoids and from the rubber particles membrane:

(Fig 11-a) One-dimension-SDS-PAG Electrophoresis of proteins from the latex cytosolic (lane 1 and 5) and lutoidic (lane 3 and 5) compartments from control (lane 1 and 2 and spontaneous TPD (lane 3 and 5) trees (PB 5/51)

(Fig 11-b) : SDS-PAGE of proteins from the latex cytosol (lane 2 to 7) and the rubber particles membrane (lane 8-9). Control (lane 7 and 8), TPD (lane 2 to 6 and 9)(All silver staining)

Fig. 12 a

Fig. 12 b

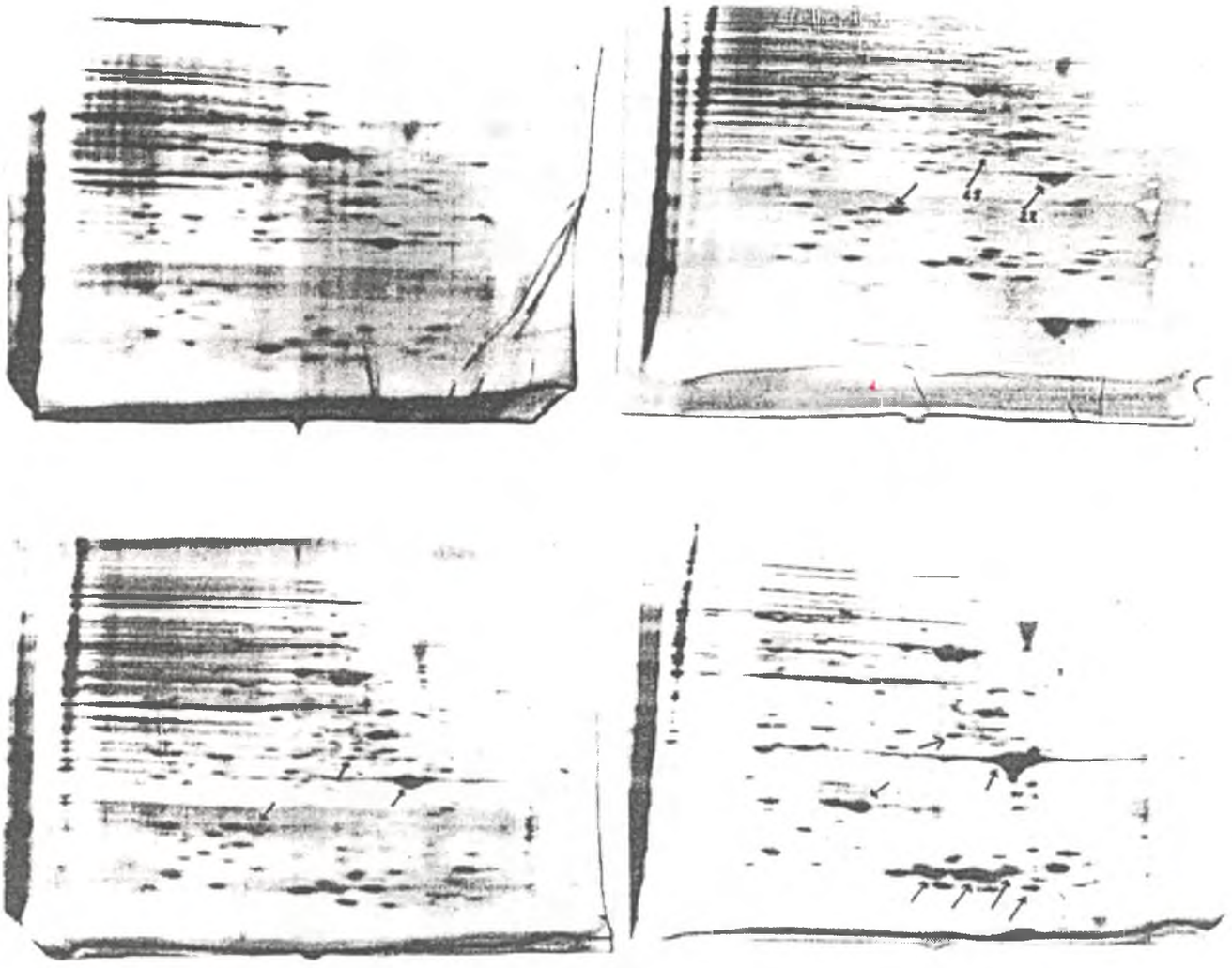


Fig.12 c

Fig. 12 d

Figure 12 : Two-D-SDS-PAG Electrophoresis of the cytosolic proteins from the latex of control and Spontaneous TPD trees.

Control (fig. a) and TPD class 1 (b), class 3 (c) class 5 (d) RRIM 600 trees.(IEF: pH 5-7 / Acrylamide 12.5%)(Silver staning).

1) Spontaneous TPD

a - RRIM 600 Clone / Low tapping pannel:

Among the 530 RRIM 600 trees checked (see table 2 below) 16.5% of these 12 year old trees had preliminary or declared irreversible symptoms of TPD after 4 years tapping (every 2 days/no stimulation/stop of tapping during from February to April each year): 10% with typical TPD symptoms (dry spots or zones on the tapping cut) and 4,5% having more than 50% up to 100% of their tapping cut dryed.

Class	% dry-cut length	symptomes	Nb trees	%/total
0	0	Healthy trees / good latex flow	443	83.5
1	< 20 %	Difficulties of flow/to 20% scattered dry spots	56	10.5
2	20≤%<40	Completely dry zones on the tapping cut.	7	1.3
3	40≤%<60	" "	6	1.1
4	60≤%<80	" "	7	1.4
5	80≤% ≤100	" / to completely dry cut	11	2.1

Table 2: Repartition of the RRIM 600 trees among the TPD classes (1994/lower tapping pannel)

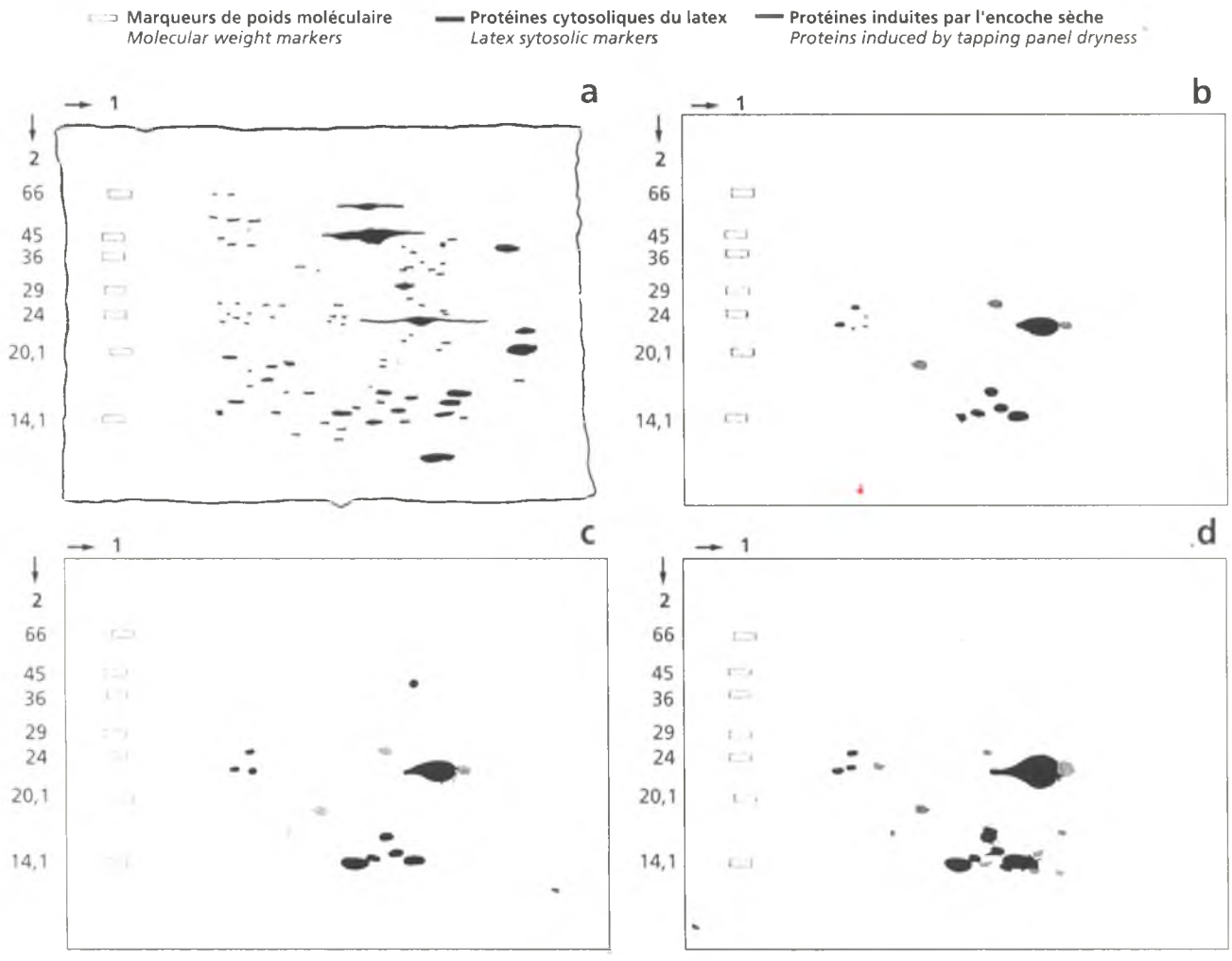
Comparison of the 1-D-SDS-PAGE (fig 10) exhibited an over expression of some cytosolic proteins in the MW range between 14 to 30 kDa in the latex from TPD trees. Especially, a 22 kDa protein was overexpressed in the TPD trees compaired with the control, in correlation with the seriousness of the TPD desease. There is also some increase in the expression of a ≈29 kDa protein, but it is not clear wether these proteins are from cytosolic origin, or could originate from the organels fraction (lutoids bursting?)(fig 11).
There is no clear difference between the pattern of proteins from the organels and the rubber particles membranes of healthy and spontaneous TPD trees (fig 11).

2-D-SDS-PAGE electrophoresis (IEF pH 5-7) showed differences in the pattern of the RRIM 600 latex cytosolic proteins (fig 12 a - e) with decreased amount for some of them (especially an acidic ≈19 kDa protein), and increased amount (22 kDa), and at last 4 proteins in the 14-19 kDa MW range) or *de novo* (?) synthesis (at least a 29 kDa) of some proteins. The increase in the expession of the 22 kDa protein is clearly linked to the severity of the desease.

Scheme 1 (a-d) is a comprehensive picture, representative (190 gels) of the general systematic changes (overexpresssion only) scored in the pattern of the latex cytosolic proteins from healthy and spontaneous or induced TPD trees (RRIM 600),with various severity of TPD symptoms.

Compared to the pattern Sch. 1-a (control: completely healthy trees), pattern Sch. 1-b (class 1 with roughly ≤ 10% dry cut length) shows that at this early stage some proteins are altready overexpressed (arrow : o): 4 in the 14-20 MW and 5-6 PHi ranges. One is a major protein of 22 kDa MW with pHi around 5.5 (called PDF 22a), and 2 others are minor proteins with a molecular weight between 24 and 29 kDa, pHi ≈ 7. Three new proteins (arrows: n) can be detected: one with a MW ≈ 19 kDa and pHi ≈ 6.5; a second with a MW ≈ 27-29 kDa and pHi ≈ 6 (PDF 29); and a third a protein with a MW ≈ 22 kDa (PDF 22b) and a pHi ≈ 5.5, close to the big PDF 22 spot but a little bit more acidic .

Pattern Sch. 1-c exhibits the class 3 TPD (≈ 50% dry cut length) characteristics, with a general increased amount of most of the already overexpressed proteins shown in class 1. A new 18 kDa protein, pHi ≈ 6.5 can be detected (low expression).
The pattern Sch. 1-d is characteristic of severe TPD desease (class 5 with ≥ 80% dry cut length) and differs from the previous ones. Six new proteins were detected in the range of 14-20 kDa



Scheme 1: **Two-dimensional chromatography of latex cytosolic proteins from a healthy tree (a) and from trees with increasing severity of the tapping panel dryness disease (length dry cut b: 10%, c: 50%, d: 90%).**

Diagram (a) shows all the stained cytosolic proteins from the latex of healthy trees. In the other diagrams (b, c, d) only those proteins increasing in amount, in line with disease severity are shown in black, and those *de novo* induced only in the event of tapping panel dryness are shown in grey. Protein patterns obtained with 40µg of proteins loaded on a pH 5-7 gel for the first dimension; migration time 3½ hours at 750 Volts. The second dimension was run on 12.5% polyacrylamide gel, with a 15 mA current per gel. The molecular weight markers are on the left-hand side.

Fig. 13 a Fig. 13 b

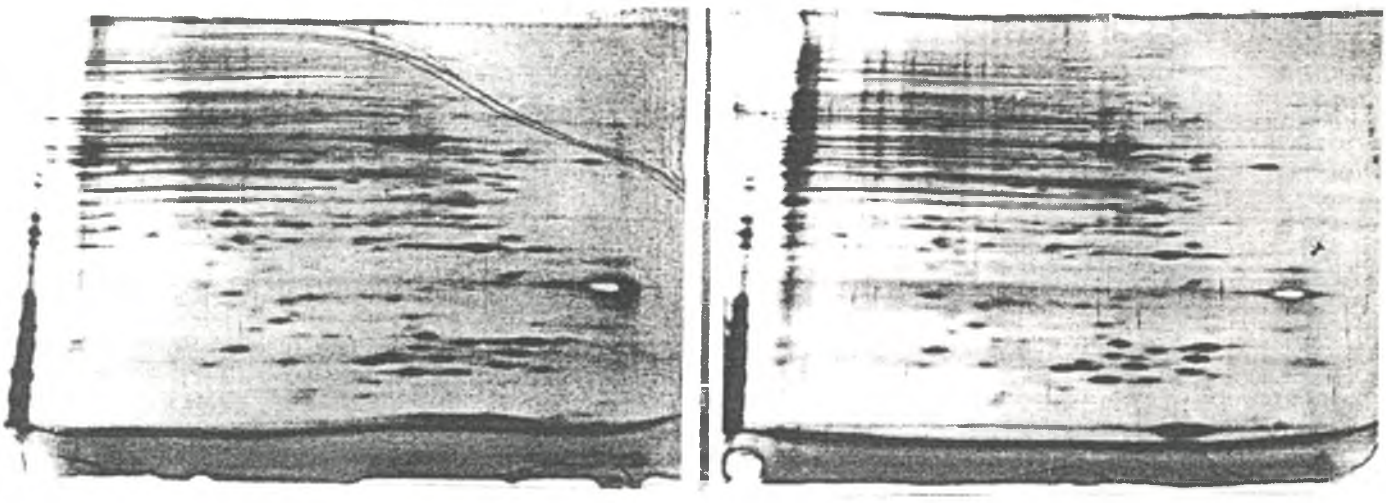


Figure 13: Accumulation of low molecular weight is not due to the action of endogenous proteases during latex collection or storing.
2-D-SDS PAG Electrophoresis of the latex cytosolic proteins from RRIM 600 trees after 90 min preservation of the latex at 4°C (Fig. a) or 27°C (Fig b). (Silver staining).

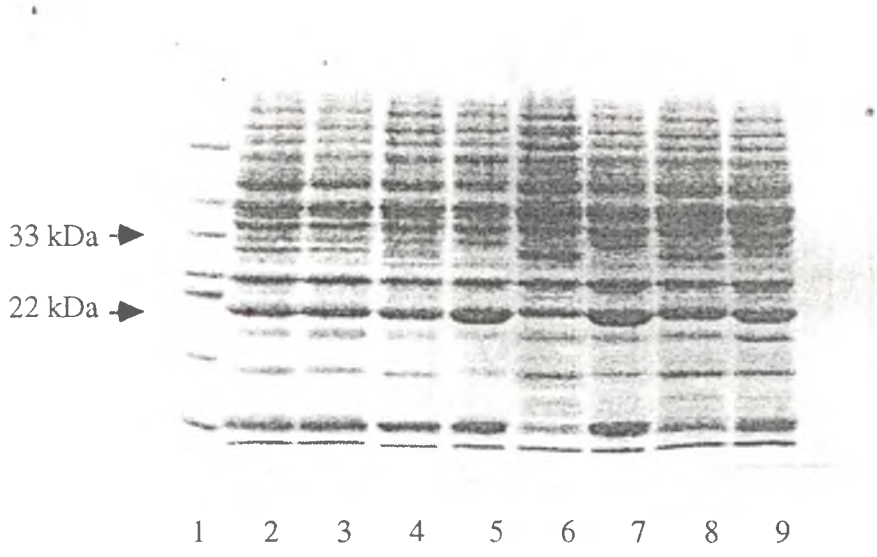


Figure 14: One-D-SDS-PAG Electrophoresis of the latex cytosolic proteins of 4 trees before and after getting TPD symptoma.
The latex was collected from 4 RRIM 600 trees before (latex collection on 02 June 1995 : lane 2, 4, 6 and 8) and after they got spontaneous TPD class ≥ 3 symptoma (lane: 3, 5, 7 and 9 /latex collection on 05 October 1995) (Coomasssie-blue staining).

Fig. 15 a

Fig. 15 b

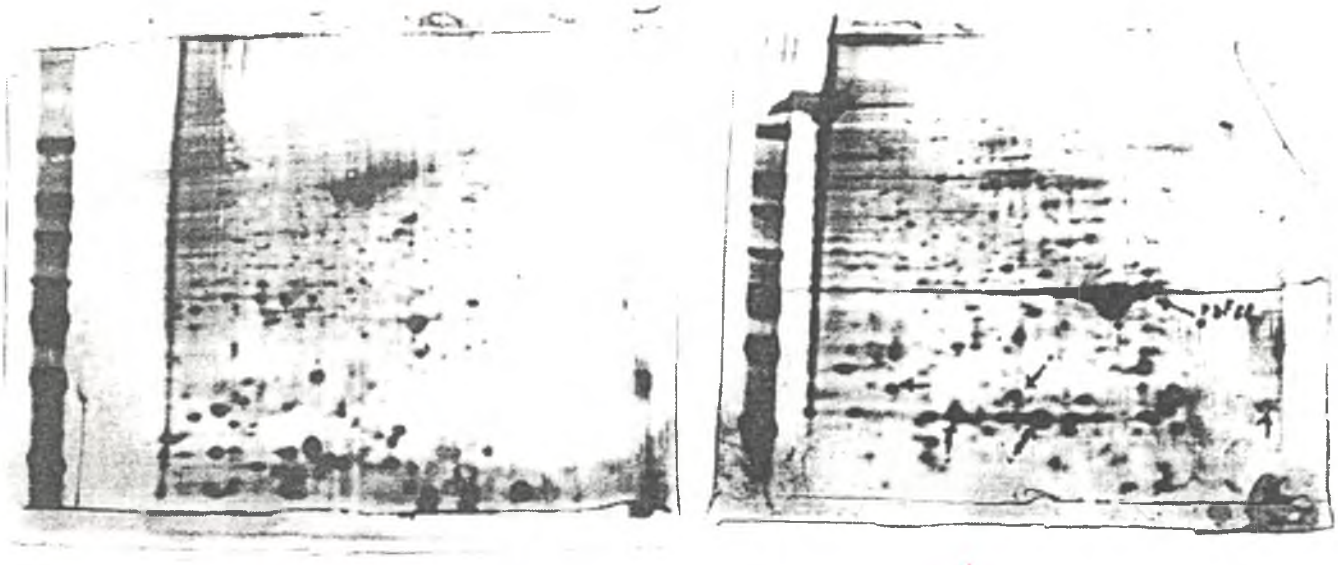


Figure 15: Two-D-SDS PAG Electrophoresis of the cytosolic proteins of latex from PB 5/51 control and spontaneous TPD trees.

control (Fig. a) and TPD class ≥ 3 trees (Fig. b).(IEF = 3-10 /Acrylamide 12.5% Silver staining)

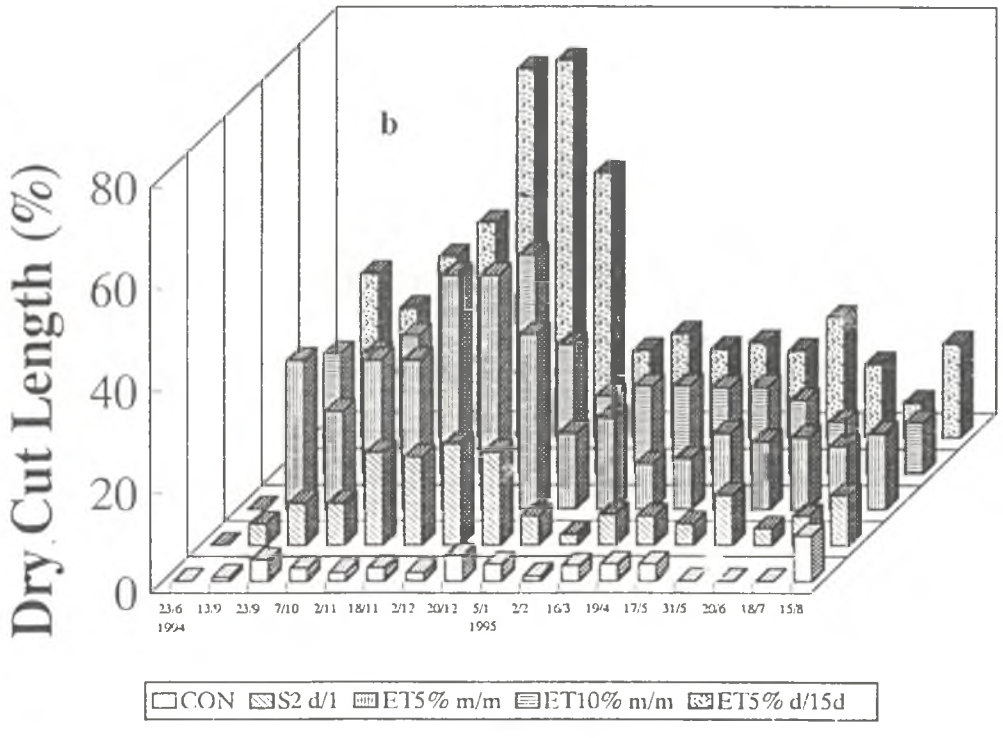


Figure 16: Kinetic evolution of the TPD symptoma of RRIM 600 trees during 15 months experiment:
Dry cut length is expressed in % of the tapping cut length (cm): control (S2 d/2), over-tapped (S2 d/1) and overstimulated (ET 5% every month, ET10% every month, ET5% every 15 days)

with pHi ranging from 5.5-6.5; four of them being grouped in the 14-17 kDa MW range. An other protein can be detected with a MW ≈ 22 kDa but neutral (6.5<pHi ≤7)

To check if some of the low molecular weight proteins (< 30 kDa), encountered in the latex cytosol of TPD trees, might either originate from high bursting of the organels or from some endogenous protease activity (cleaving higher MW protein), the whole latex from healthy and TPD trees was either preserved on ice for 90 min, or incubated at room temperature for the same time, before centrifubation at 4°C and elecrophoresis. Figure 13 (a - b) shows that there is no obvious difference between the pattern of the cytosolic proteins from latex incubated either at 4°C or 27 °C.

b - RRIM-600 / high tapping pannel.

As the tapping cut was quite low after 4 years tapping, the tapping pannel was changed to the opposite higher pannel (1rst opening). Checking the TPD symptoms after the 10 first tappings exhibited very few deseased trees (see table 3), and the latex was collected (03 June 95) from control trees (never got any visible TPD symptoms) and the trees which had previously exhibited TPD symptoms. After 4 months tapping (S/2 d2, no stimulation) some trees (table 3), most of them having previously got TPD on the lower pannel, exhibited again TPD symptoms on the higher new tapping cut. Their latex was collected (05 October 95) again and checked by 1 and 2-D protein pattern analysis.

The 1-D protein patter of individual latex analysis showed (fig. 14) that the trees that had previously developped TPD symptoms at the lower pannel, and which looked healthy at the opening of the higher pannel, then got again TPD symptoms 4 month later, clearly lost some time 1 or more often 2 protein bands around 33-34 kDa, and systematically accumulate the 22 kDa protein.

Class	% dry-cut length	June 1995		October 1995	
		Nb trees	%/total	Nb trees	%/total
0	0	377	77	251	52
1	< 20 %	98	20	167	35
2	20≤%<40	10	2.4	31	6.4
3	40≤%<60	2	0.4	12	2.3
4	60≤%<80	0	0	16	3.3
5	80≤% ≤100	1	0.2	5	1

Table 4 : Repartition of the RRIM 600 trees among the TPD classes (higher tapping Pannel) after change of the tapping pannel (03 June and 05 October 1995).

c - PB 5/51 Clone / Low tapping pannel

PB 5/51 as most of the PB origin clones are very sensitive to TPD and stresses in general. 2-D-SDS-PAGE electrophoresis (IEF pH 3-10) of the cytosolic proteins from the PB 5/51 latex (Fig 15 a,b), always exhibited a wide increase in the expression of the 22 kDa protein in TPD trees, which seemed to split into 2 different pHi in seriously deseased trees (classes 3 to 5), most probably because of phosphorylation or glycosylation or other post-translational processing.
The 29 kDa protein is also always present in the cytosol of TPD trees, while absent or undetectable in the latex cytosol from healthy trees.
There are many changes in the pattern of the 14-19 kDa MW range proteins, but comparison of several PB 5/51 gels could not lead to clear generalization as far as these very low-molecular weight proteins are concerned. More work has to be done to get valuable statistical generalization on these very low molecular weight protein markers.

Fig. 17 a

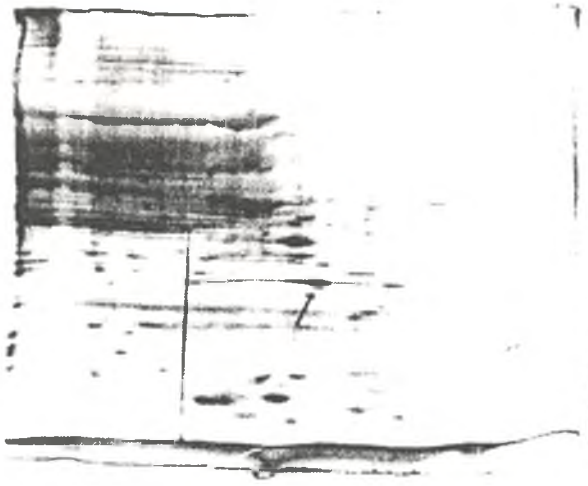


Fig. 17 b

Fig. 17 c

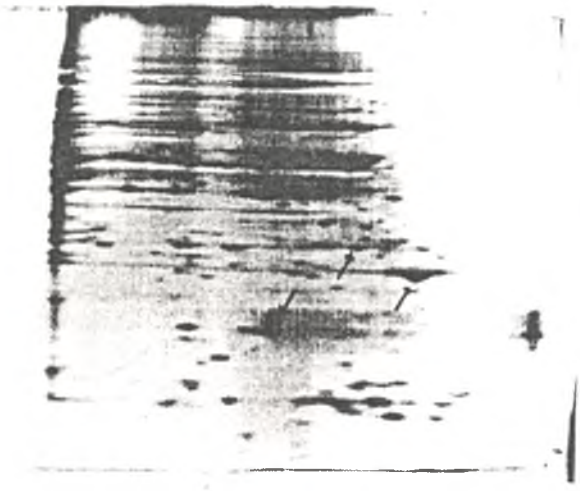


Fig. 17 d

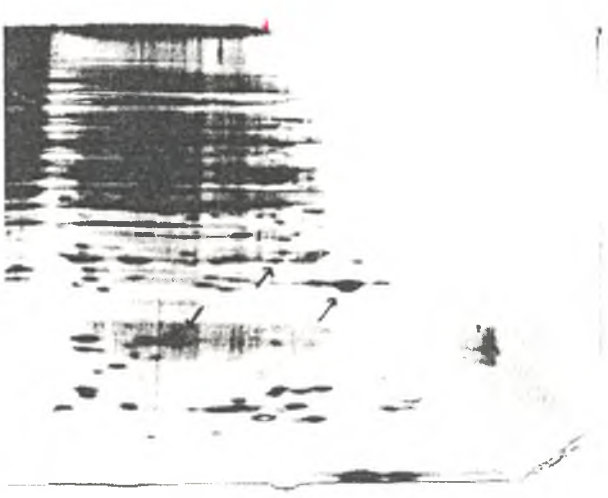


Fig. 17 e

Figure 17: Two-D-SDS PAG Electrophoresis of the cytosolic proteins of the latex from RRIM 600 control and TPD trees.
control (Fig. a: S/2 d/2) and TPD class ≥ 3 trees : fig. b: ET 5% m/m; fig. c: ET 5% d/15d; fig. d: ET 10% m/m; Fig. e: S/2 d/1.(IEF = 5-7 / Silver staining).

2) Induced TPD (14 month experiment)

From Fig. 16, it can be seen that overstimulation, after 3 months experiment, could induce transient reversible TPD (September-December). Only 3 trees reached and remained class 5 (almost completely dry). After the transient desease, for most of the trees, the TPD symptoms decreased but remained always higher than the control tree. This transient induction of TPD with over exploitation is well-known while not yet well understood. It is supposed to originate from the superimposition of several stresses such as overexploitation + prolonged rainy season (roots flooding and/or insufficient light/and or relatively low temperature).
After 14 month daily tapping (overtapping), only few trees got TPD symptoms (one class 4), as compared with overstimulated trees.

Comparison of 2D-SDS-PAGE latex cytosolic pattern of control and induced TPD trees (Fig 17 a, b) showed that, at the same date (season), PDF 22a was always more expressed in the partial TPD trees, whatever the severity of the desease.
Some other protein markers were also common to spontaneous and induced transient TPD such as : PDF 29, PDF 19 and *de novo* expressed PDF 22b. The same pattern could be observed with the latex of induced TPD, whatever the treatment: overstimulation (D: fig 17b, E: fig 17c , F: fig 17d) and overtapping (few trees but exhibiting the markers fig 17 e).

3) TPD/stress protein markers

a) Statistics and general observations:

Some of the protein markers are common to spontaneous and induced TPD, even if the latter is reversible TPD. They generally appear at the early stage of the TPD desease (class 1):
The examination of more than two hundred sixty 2D-SDS-PAGE of latex from healthy and spontaneous or induced TPD trees (roughly 120 trees, RRIM 600 and PB5/51 clones, tapped in various seasons) could allow to score the % of trees exhibiting the marker and the relative apparent intensity of the various protein markers (table 5) :

Protein markers (kDa)	22		29		15-19 (4 proteins)	
%	*trees*	intensity	*trees*	intensity	*trees*	intensity
TPD Class:						
0	*88*	≤ 20	*96*	0≤ i <5	*94*	0≤ i <20
1	*92*	≥ 50	*88*	≥ 50	*81*	≥ 40
3	*96*	≥ 80	*100*	≥ 80	*100*	≥ 60
5	*100*	80 ≤ i ≤100	*100*	80 ≤ i ≤100	*100*	80 ≤ i ≤100

Table 5: Relashionships between the seriousness of TPD and the relative apparent "intensity" (amount: range 0 for absence to 100 for maximum intensity) of the various TPD marker proteins in the latex cytosol / % of trees of each class getting these characteristics.

It can be seen that, starting in class one, 80-92 of the trees exhibit a higher amount of all the 6 considered protein markers. Therefore it can be concluded that at least this set of 6 cytosolic proteins can be considered as serious candidates as early markers of TPD and overexploitation-induced stress. They have to be purified in order to raise antibodies, as tools to set up a molecular diagnostic of overexploitation and TPD risks.

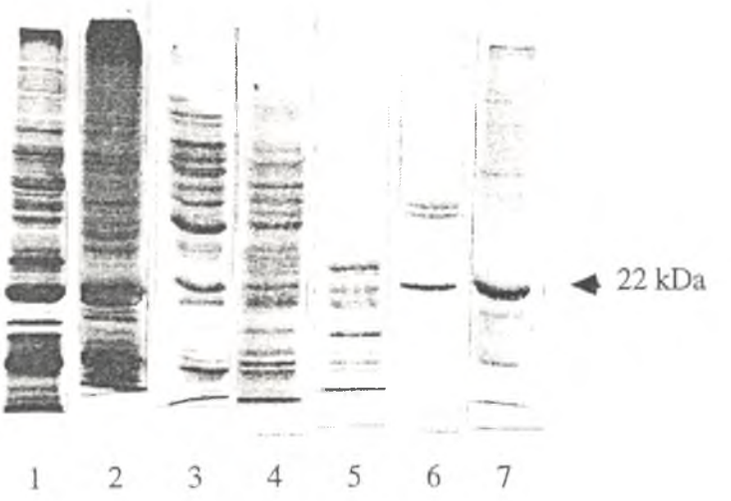


Figure 18-A: Checking the purification of the latex cytosolic 22 kDa protein by One-Dimension SDS-PAG Electrophoresis.
Crude cytosol (lane 1), 40% (NH4)2SO4 precipitate in water (2), pass-through HIC column (3), column wash with 1.7 M (NH4)2SO4 (4), wash with Na2HPO4 pH 7.0 at 4°C (5), wash with 1% Triton X-100 (6), elute with 3% Triton X-100 (concentrated)(Silver staining).

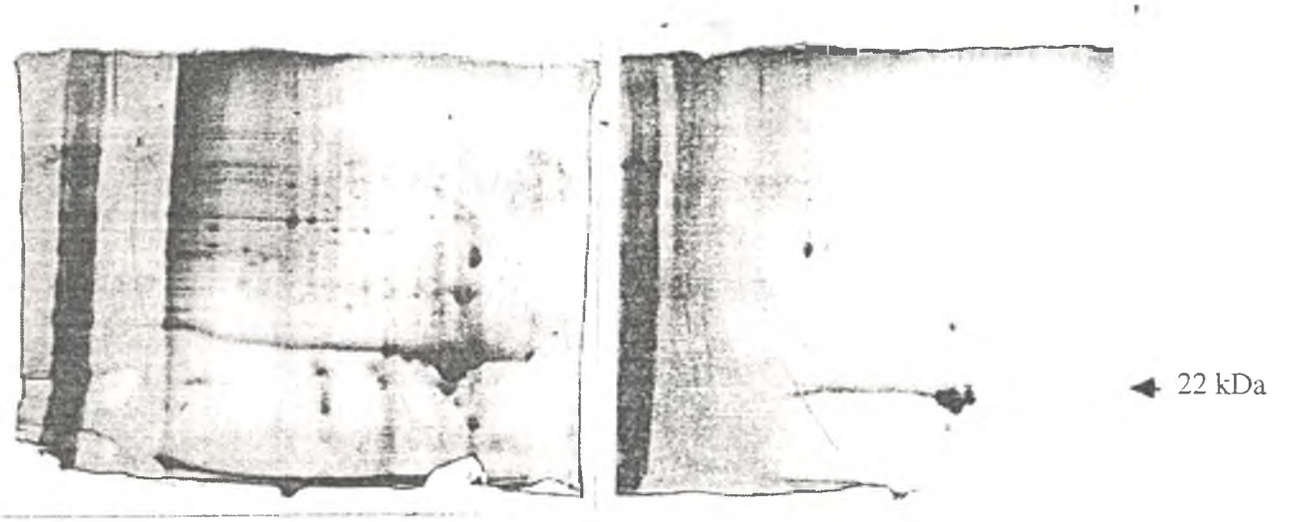


Fig. 18 a

Fig. 18 b

Figure 118-B:Checking the purification of the 22 kDa latex cytosolic protein from TPD trees by 2-D--SDS-PAGE.

Fig. a: Ammonium sulfate (40%) precipitate dissolved in mQ water.
Fig. b: elution from HIC column with 3% Triton X-100.

b) Towards the purification of the PDF 22 protein TPD marker:

We have started the purification of the probably easiest protein marker to be purified (≈22 kDa), because of its aboundance in the latex of TPD trees. It is a highly time consuming process because, as we don't know the nature and function of these potential protein markers, we cannot monitor the purification through the simple measurement of any enzyme activity, hence we have to verify the presence of the studied protein through 1 and 2 D-SDS PAGE, at each purification step.
At this time, the following steps have been set up:

- latex centrifugation 10.000 x g, 1 hour, 4°C;
- rubber particles and organels discarded;
- ultracentrifugation of the cytosol 50.000 x g / 1 hour / 4°C
- precipitation of cytosolic proteins with 40% ammonium sulfate (shake 3 h / room temperature)
- centrifugation 7000 x g / 30 min./ 4°C;
- resuspend the pellet in mQ water;
- pass through a phenyl-agarose (HIC) column, equilibrated in 1.7 M (NH4)2 SO4, 50 mM NaH2PO4 , pH 7.0 at 4°C;
- rinse HIC-agarose with 50 mM NaH2PO4 , pH 7 at 4°C;
- elute protein with 50 mM NaH2PO4 , pH 7 + 3 % Triton X 100 at 4°C;
- remove Triton X-100 by passing through an activated charcoal column at 4°C
- overnight dialysis against 50 mM Tris HCl, pH 8 at 4°C.

These preliminary steps lead to an about 80% purification of the protein, still with contaminents which remain to be separated (fig 18 A & B). Only a highly purified protein has to be be used for microsequencing and raising specific polyclonal antibodies.

Parallely we started the purification of this protein by simply punctionning the stained protein spot (with Methylene blue or amidoblack) on 2-D-SDS PAGE large gels. These cumulated extracts (around 50 spots) where injected to a rabbit, and served also as booster 3 weeks and 1 month later. The preliminary assay using these antisera will started within few weeks. Some of these spots have recently been sent to a specialized laboratory for peptides microsequencing.

The use of the 19kDa markers seems to be more promising, as it is *de novo* early expressed upon the onset of TPD, but it will most probably be far more difficult to purify as it is present in far smaller quantity. We will adopt the same strategy (classical purification of the native protein and parallely purification of the danatured protein by 2D-SDS-PAGE/punctionning off the stained spot).

C- THE "SPECIFIC" APPROACH: EXPRESSION OF SPECIFIC GENES.

The specific approach supposes the existence of some hypothesis based on previously determined biochemical and/or histological evidences of phenomenon linked to the onset and development of TPD.

It is well known that the ultimate phase of TPD is characterized by the coagulation of latex within the latex cells, and this fact had already been verified by electron-microscopy. So we had first to check if the latex from TPD trees might have got some pecularities as far as their propensity to early (up to in-situ) coagulation is concerned

Further, we had previously demonstrated through latex biochemical and physiological analysis, that at least overstimulation-induced TPD was linked to the occurence of an "oxidative stress" inside the latex cells. This oxidative stress was shown to result in lipid peroxidation hence in membrane damages, leading to the bursting of the lutoids (vacuoles) and to the consecutive release of the coagulating factors (hevein) they normally compartimentalize in the latex cells.

Gene Expression in the latex cells of healthy and TPD trees (PB 5/51 clone)

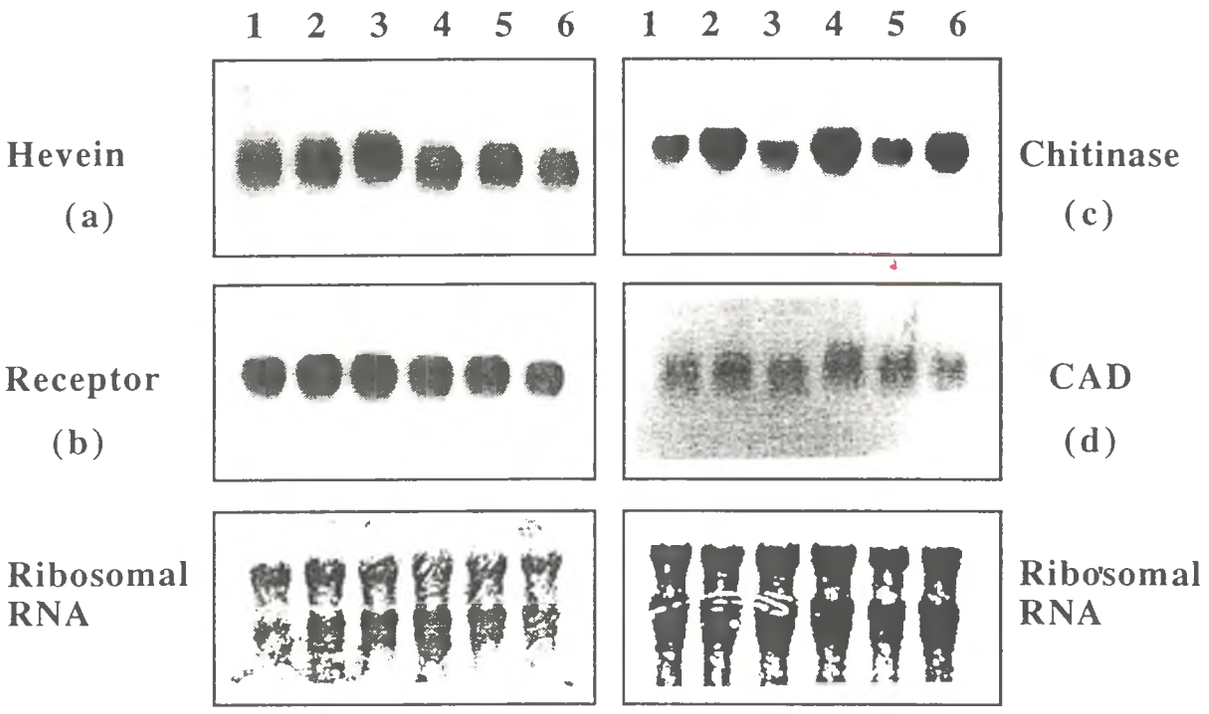


Figure 19 : Expression of the genes involved in coagulation, in the latex cells of control and spontaneous TPD trees.
Control (lanes 1, 3, 5) and spontaneous TPD (2, 4, 6) trees. A tree per tree experiment with TPD class ≥ 2 (clone PB 5/51)(10 µg total RNA loaded). Washing at 65°C with 0.1 x SSC for hevein and its receptor; 60°C with 1 x SSC for chitinase and Cynamyl Alcohol Dehydrogenase (CAD).
Exposure:hevein = 2 hours; chitinase and hevein recepetor = over-night; CAD = 2 days

Genes Expression in the latex cells of healthy and spontaneous or stimulation-induced-TPD trees (RRIM 600 clone)

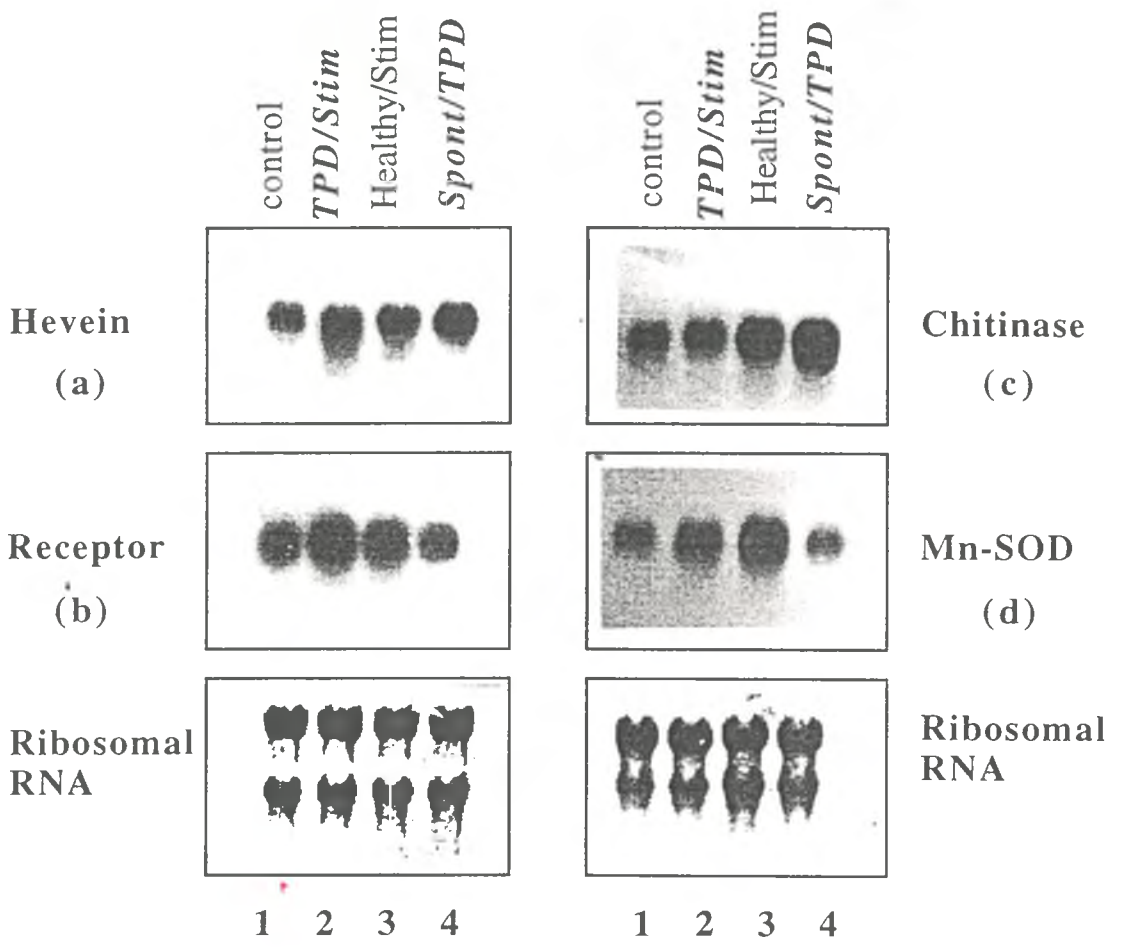


Figure 20: Expression of the genes involved in coagulation and oxidative stress, in the latex cells of control, spontaneous TPD, stimulated healthy and stimulation-induced-TPD trees.

Control (lane 1), spontaneous TPD (lane 4) stimulated healthy (lane 3) and stimulation-induced-TPD trees (lane 2).
A pooled latex experiment with (class ≥ 2) TPD (clone: RRIM 600)(10 µg total RNA loaded). Washing at 65°C with 0.1 x SSC for hevein, its receptor and Mn-Superoxide Dismutase (Mn-SOD)/ 60°C with 1 x SSC for chitinase. (Exposure: hevein = 2 hours; chitinase, receptor and Mn-SOD = over night)

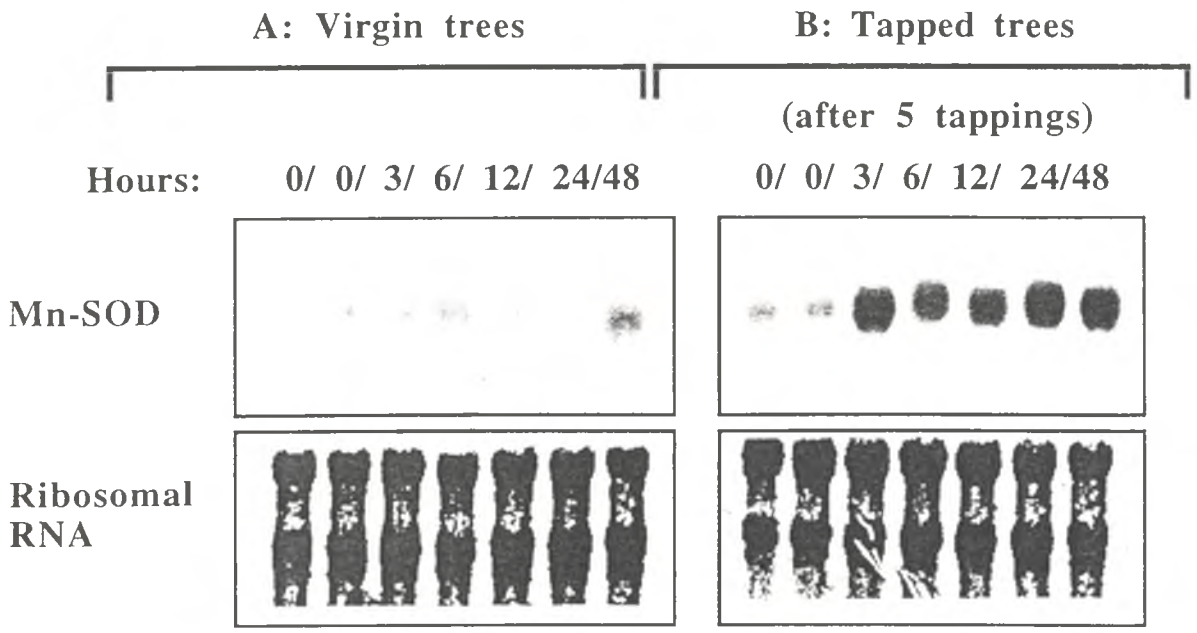


Figure 21: Effects of tapping (wounding/regeneration) and kinetic effects of Ethrel stimulation, on the expression of Mn-Superoxide Dismutase gene in the latex cells of virgin trees.
Northern blotting : 10µg total RNA from latex / hybridization and washings at 65°C (0.1 x SSC). Ribosomal RNA stained with Methylene blue.
Hours: hours after stimulation; 0: controls; 3 to 48 hrs after the stimulation treatment (Exposure: Mn-SOD = 1 day).

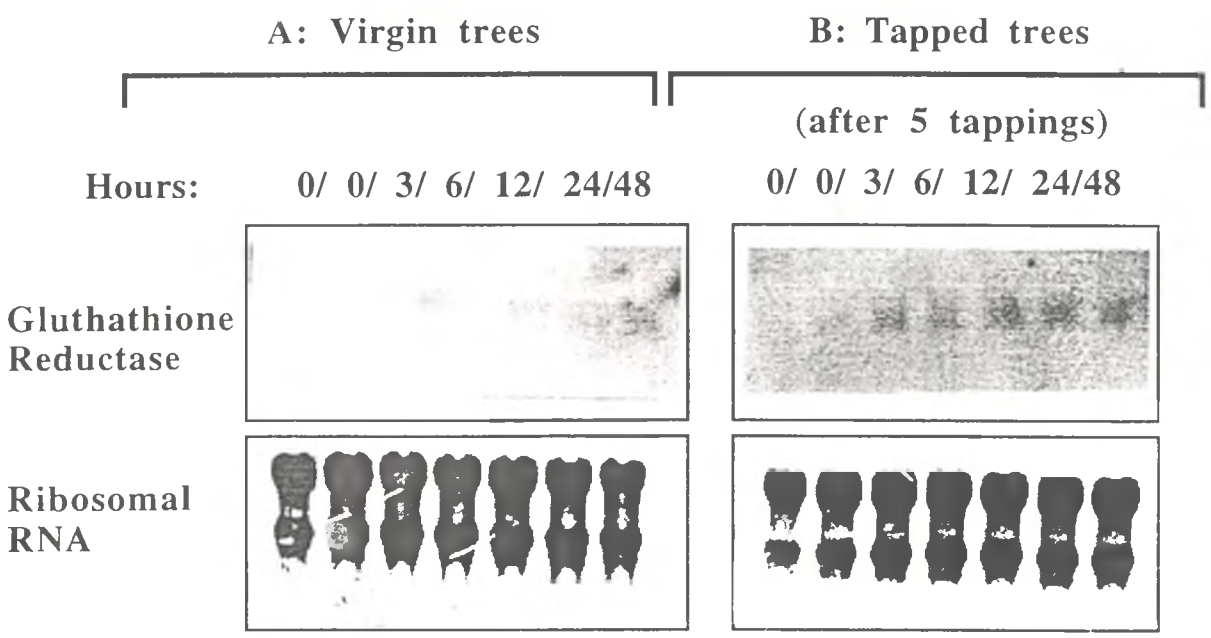


Figure 22: Effect of tapping (B:wounding/regeneration) and kinetic effects of Ethrel stimulation (A), on the expression of Gluthathione Reductase gene in the latex cells of virgin trees.
Northern blotting : 10µg total RNA from latex / hybridization and washings at 65°C (0.1 x SSC). Ribosomal RNA stained with Methylene blue.
Hours. hours after stimulation; 0: controls; 3 to 48 hrs after the stimulation treatment (Exposure: GR = 4 days).

1) Spontaneous TPD :

We could show by Northern blotting (tree per tree) that, compared with control trees of PB 5/51 clones, the latex from trees with TPD symptoms did not exhibit clear differences as far as the expression of the hevein, and its receptor is concerned(fig. 19 a,b), whereas the expression of chitinase gene was increased in the latex of trees with spontaneous TPD (Fig. 19 c and 20 c).

On the contrary, Northern blotting of RNA from pooled latex (of 6 trees/TPD class 0 and ≥3) of RRIM 600 clone show that hevein is slightly overexpressed in the latex of the spontaneous TPD trees while the expression of the hevein receptor remains unchanged when comparing latex from healthy and spontaneous TPD trees (fig 20 a & b).

As far as the internal bark from TPD trees tends ultimately to turn brownish, we tested heterologous 2 c-DNA probes of enzymes involved in the phenylpropanoid pathway : Cinamyl alcohol dehydrogenase (CAD) and phenylalanine ammonia lyase (PAL). We could show that there was no or very slight difference between the latex of control and TPD (slight increase?) trees (PB 5/51 and RRIM-600), as far as the expression of the PAL (not shown) and the CAD genes (fig 19 d) are concerned.

Finally, fig. 20 (d) shows that spontaneous TPD exhibit some slight decrease in the expression of the scavenging enzyme of the Mn-SOD (mitochondrial SOD) gene.

2) Overstimulation-induced TPD:

a - Enzymes involved in oxidative stress.

As stated above we had previoulsly shown that overstimulation-induced TPD was linked to the onset of an oxidative stress within the latex cells. This oxidative stress has been characterized as an over generation of toxic reactive oxygen species (ROS: superoxide anions and derivatives), with a concommitant abnormal decrease in the scavenging enzymes activity such as superoxide ismutase and catalase, and a decrease in reduced thiol content). But it was impossible until now to stipulate wether this decrease in these scavenging enzymes activities originated from impaired transcription/translation or from postranslationnal misregulation.

i) *Superoxide dismutases (SOD):*

Superoxide Dismutases are ubiquitous enzymes involved in the scavenging of ROS through the dismutation of superoxide anions (SA) in perhydrol which will be destroyed by catalases.

We have cloned through PCR (see M. Sc. results) a Mn-SOD (mitochondrial) gene fragment from the latex, which could be used as a homologous probe for Northern blotting. Stimulation with ethylene induced an overexpression of the Mn-SOD gene in untapped latex cells 24-48 hours after the treatment (fig 21 a). Successive tappings of stimulated trees induced an even more marked overexpression of the Mn-SOD gene in the latex cells (Fig. 21 b).

The latex from overstimulation-induced TPD trees (Fig 20 d) is characterized by a marked decrease in the Mn-SOD expression, while the still healthy overstimulated trees (internal control) exhibit, even 3 weeks after the last stimulation, a marked overexpression of the Mn-SOD gene.

Using heterologous probes of Cu-Zn-SOD did not give any interpretable result. We are now attempting to clone (through PCR) c-DNA or gene fragments coding for Cu-Zn SOD to get homologous probes to be used in Northern blotting analysis.

ii) *catalase:*

As for Cu-Zn SOD, using heterologous catalase probes did not give any interpretable results. We are now attempting to clone (through PCR) c-DNA or gene fragments, coding for catalases, to get homologous probes to be used in Northern blotting analysis.

iii) *Glutathione reductase (GR):*

Glutathione (GSH), a thiol tripeptide, is an ubiquitous chemical scavenger of ROS, hence is oxidized in GSSG by most of the ROS. GSSG can be reduced again into GSH through the action of GR in the presence of NADPH.

Using oligonucleotides designed from the analysis of consensus sequences (given by Dr Skorn's group) we could clone a 400 bp gene fragment presenting good sequence homology with other published GR sequences (see M. Sc. results). The use of this probe could show that, as usually in most plant tissues, this gene is very poorly expressed or have a very low expression turnover in the latex cells (a systematical one week film exposure at least is necessary to discern faint hybridization patterns).
Using this probe, It could be seen that ethylene stimulation-induced, in untapped latex cells (virgin trees) a slight overexpression of the GR gene after 24 hours (Fig 22 a) and a marked overexpression upon successive tappings of the stimulated trees (Fig. 22 b).

For the moment we could not get reproducible and well characterized results comparing GR expression in the latex of control and spontaneous or stimulation-induced TPD (not shown).

b - Genes involved in coagulation:

Northern blot analysis of RNA (RRIM 600) shows that, as for spontaneous TPD, expression of the hevein gene is slightly increased in the latex from stimulation-induced TPD (compared to stimulated still healthy trees (Fig. 20 a). Contrarily to spontaneous TPD, expression of the hevein receptor is also increased in the latex of stimulation-induced TPD (Fig. 20 b).

Also contrarily to the latex cells of spontaneous TPD (never stimulated) which exhibited increased expression of chitinase gene, the latex from stimulation-induced TPD exhibited the same level of chitinase expression as the control non-stimulated trees, and lower expression compared to the internal control still healthy stimulated trees (Fig. 20 c).

c - Oxidative stress and coagulation :

Although all these results have to be confirmed at a wider scale, as far as stimulation-induced TPD is concerned, the increase in the expression of the hevein and of the hevein receptor genes (pro-coagulants), together with a relative decrease in the expression of the chitinase gene (anti-coagulant) should result in a higher coagulability of the latex.

Further, the decrease in Mn-SOD gene expression (fig. 20 d), in latex cells with high metabolic activity due to stimulation, should result in some ROS accumulation and consecutive membrane (lipids) damages as earlier demonstrated, leading to fragilization of the lutoids which compartmentalize the coagulation factors. All these phenomenons together should result in latex flow difficulties (which have been for long well documented as a characteristic upon the onset of TPD) and even to *in situ* coagulation of latex, with definitive cessation of flow.

D - CONCLUSIONS AND PROSPECTS ABOUT TPD MARKERS

1) Protein markers:

We have shown that there are differences in the protein pattern of latex from spontaneous as well as induced TPD. Most of these differences are common to both types of TPD and it may be difficult, using these protein markers, to distinguish between the different types of TPD and therefore difficult to state the TPD origin. Whatever, some of these proteins have to be regarded as putative early markers of stress leading to TPD.

We will go on purifying some of the proteins that are overexpressed (22 kDa and ≈17 kDa) or which desappear (≈ 33-35 kDa) in the latex during the onset of natural or induced TPD, and try to do so for the *de novo* expressed 29 KDa protein (very low amount). These purified proteins will be used to raise specific antibodies and for peptide microsequencing.
The antibodies will be tested for immunological early detection of TPD and for attempting to clone the corresponding c-DNA through a latex (TPD) expression c-DNA library, and get back to the genes.
The peptide microsequences will be used to design specific oligonucleotides in order to clone the corresponding c-DNA either through PCR or through the screening of our cDNA libraries. The c-DNA clones obtained will be tested for their ability to be used as specific markers of overexploitation and induction of TPD.
The same probes will be tested as putative markers of sensitivity to TPD in the rubber wood gardens and gerplasm.

2) Genes expression:

Interestingly, spontaneous TPD seemed to exhibit different expression of some genes compared to overstimulation-induced TPD. This was the case for the hevein receptor gene which was overexpressed in the latex of stimulation-induced TPD compared to the still healthy stimulated trees, while there was less difference between control and spontaneous TPD. This was also the case of the chitinase gene which was underexpressed in the latex cells of overstimulation-induced TPD, as compared with the stimulated still healthy and control non-stimulated trees, while markedly over expressed in the latex of spontenous TPD. Endly Mn-SOD gene was far more markedly underexpessed in the latex of stimulation-induced TPD compared to still healthy stimulated trees, than in the latex of spontaneous TPD compared to control non stimulated trees. All these characteristics concerning gene expression in stimulation-induced TPD let think that the latex producing tissues of theses trees do not respond any more to ethylene stimulation.

Therefore, contrarily to proteins markers which could be used in a diagnostic approach as "generic" markers of stress leading to TPD (forcasting TPD risks), Northern (Dot) blots diagnostic using the hevein receptor, the chitinase and the Mn-SOD probes could give informations about the type of TPD (spontaneous or induced by overexploitation), therefore allow giving specific recommandations as far as the tapping systems are concerned.

We will continue this specific approach, and attempt to validate these (and some other such as Cu-Zn SOD, GR and catalase) putative stress/TPD markers, through series of tree per tree as well as pooled samples and clonal analysis by latex RNA Northern blotting and dot blotting.

RESULTS : 2nd PART

THE MAIN RESULTS OBTAINED BY STUDENTS

ON THE MOLECULAR BIOLOGY OF RUBBER

I - STUDIES ON DIFFERENTIAL EXPRESSION OF GENES POTENTIALLY INVOLVED IN THE ONSET OF THE TAPPING PANEL DRYNESS (TPD) IN *HEVEA BRASILIENSIS.*

Student name : **Miss Panida Kongsawadworakul (M. Sc. Student)**

Advisers : Prof. Dr Jarunya Narangajavana
Co-advisers: Drs Hervé Chrestin and Valérie Pujade-Renaud

Duration: Lab work: May 1995 - September 1997

The aim of this program is to find out molecular markers for early detection of TPD risks and for understanding of the origins of TPD.

The strategy consists mainly in using the specific approach, essentially according to the hypothesis of the occurence of an oxidative stress within the latex cells upon overexploitation of rubber leading to TPD. We had previously biochemically characterized such a situation. The main question to answer is: "can the decrease of the main ROS scavenging enzymes activity be attributed to deregulation at the transcription/translation level (gene expression) or to post translationnal regulation". The c-DNA or gene fragments corresponding to these enzymes will be cloned to serve as probes in the studies of regulation of their gene expression.

The blind approach will also be used, especially for the cloning of c-DNA corresponding to overexpressed proteins, through the use of oligonucleotides which will be designed from the petptides microsequencing of TPD protein matrkers.

1) Mn-Superoxide Dismutase (Mn-SOD)

As stated before, Superoxide Dismutases are ubiquitous enzymes involved in the scavenging of ROS through the dismutation of superoxide anions (SA) in perhydrol which will be destroyed by catalases.
There are classically 3 sorts of SOD in plants:

- Cu-Zn SOD, in the cytosol, where SA can be generated from numerous redox activities.
- Fe-SOD, which is compartmentalized within the chloroplasts where SA can be generated from diversion of the normal electron flow along the photosystems I and II.
- Mn-SOD which is compartimentalized within mitochondria, where SA can be generated from the diversion of part of the normal electron flow from the respiratory chain .

Using heterologous Cu-Zn SOD probes (tobacco or Arabidopsis) did not give any intrepretable results because of highly nonspecific binding at the ribosomal RNA or no binding at all, depending on the stringency of washings (not shown). We are now attempting to clone an homologous c-DNA or genomic probe through PCR. We recently obtained a ≈ 500 bp fragment which will be sequenced to acertain that it is from the desired gene.

For the moment we did not check for Fe-SOD (chloroplastic) expression in the latex cells which are not photosynthetic tissues.

a - Cloning of a Mn-SOD gene fragment from rubber

Two oligonucleotides were designed according to the sequence of a genomic rubber tree clone coding for Mn SOD. They were both internal to Exon I and separated by 294 bp.
Genomic DNA from rubber tree leaves (genotype RRIM600) was used as template. The PCR parameters were as follow:

- Denaturation: 94°C 1.0 min
- Annealing: 60°C 1.0 min
- Elongation: 72°C 1.0 min
- Number of cycles: 30

Analysis of the PCR products on gel showed a very clear unic band of the expected size. It was eluted from the gel, ligated in the pMosblue T vector (Amersham) and introduced into *EColi* (DH5α). All clones recovered carried an insert of the expected size (≈ 250 bp). Preliminary sequencing confirmed that it was indeed part of the Mn-SOD gene.

b - Expression of Mn-SOD in the latex cells :

This Mn-SOD gene fragment could be used as a homologous probe for Northern blotting with latex total RNA.

i) *Effects of tapping and stimulation on latex cells Mn-SOD expression*

Fig 21 (a) shows that Mn-SOD is very poorly expressed in the resting latex cells of virgin trees. This can be understood since these resting untapped latex cells are characterized by a very low metabolic activity (no cytoplasm regeneration). Latex regeneration between tappings (Fig 21 b) induced an overexpression of the Mn-SOD.

Stimulation with Ethylene induced an over expression of the Mn-SOD gene 24-48 hours after the treatment (fig 21 a). The superimposition of the 2 stresses (exogenous ethylene and tappings/latex regeneration) induced an very high overexpression of the Mn-SOD gene (Fig 21-b), showing that there is a very high metabolic/energy demand for the regeneration of the latex cells cytoplasm, especially under ethylene yield stimulation.

ii) *Expression of Mn-SOD in TPD trees latex cells.*

Fig. 20 (d) shows that spontaneous TPD exhibits some slight decrease in the expression the gene coding for the scavenging enzyme Mn-SOD.

The latex from overstimulation-induced TPD trees (Fig 20 d) is characterized by a marked decrease in the Mn-SOD expression, while the still healthy overstimulated trees (internal control) exhibit, even 3 weeks after the last stimulation, a marked overexpression of the Mn-SOD gene. It appears that in trees developping TPD symptomes due to overstimulation, Mn-SOD gene cannot respond any-more to further stimulalion.

2) Cu-Zn Superoxide Dismutase (Cu-Zn SOD)

a - Cloning of Cu-Zn SOD gene fragments from rubber

Two oligonucleotides were designed according to two consensus sequences of 6 plant Cu-Zn-SOD.

Genomic DNA from rubber tree leaves (genotype RRIM600) was used as template. The PCR parameters were as follow:

- Denaturation: 94°C 1.0 min
- Annealing: 47°C 1.0 min
- Elongation: 72°C 1.30 min
- Number of cycles: 30

Analysis of the PCR products by gel electrophoresis showed 2 bands, one of the expected size (≈ 220 bp) and a longer one (≈ 500 bp). Both were eluted from the gel, ligated in the pMosblue Ti vector (Amersham) and introduced into *EColi* (DH5α). They actually are under cloning and will be sequenced before eventually being used as homologous probes.

3) Glutathione Reductase (GR)

As stated above, glutathione (GSH), a thiol tripeptide, is an ubiquitous chemical scavenger of ROS; hence it is oxidized in GSSG by most of the ROS. GSSG can be reduced again into GSH through the action of GR in the presence of NADPH. As it had previously be seen, the latex from TPD trees are characterized by abnormally low concentration of GSH, which could originate from decreased expression of GR.

a - Cloning of a gene fragment of rubber gluthathion reductase (GR):

This work has been done in collaboration with the laboratory of Dr. Skorn, at the Chulabhorn Research Institut. In this laboratory, degenerated oligonucleotides corresponding to conserved sequences of glutathion reductase genes (*gor* genes) have been designed. PCR on DNA from different bacterial strains using the two oligonucleotides as primers allowed the amplification of a 400 bp fragment in every case.

To get the equivalent from rubber tree, genomic DNA was extracted from leaves of rubber trees and used as a template for PCR amplification using the two oligonucleotides as primers.

The PCR parameters were as follow:

- Denaturation: 94°C 1.0 min
- Annealing: 53°C 1.0 min
- Elongation: 72°C 1.5 min
- Number of cycles: 35

A gel control of the PCR products revealed a smear, with one clear band around 400 bp, which was consequently eluted from the gel, purified with phenol-chloroform, and cloned in the pMosBlue T vector (Amersham). Three kinds of clones were obtained carrying inserts of 400 bp, 470 bp and about 1 kb respectively.

One clone with a 400 bp insert was partially sequenced. By comparing the deduced amino acid sequence with published GR sequences, it appeared that this clone presentedè significant homology with GR sequences from other organisms:

- 33.6% identity, in a 125 amino acid overlap, with a pea GR enzyme.
- 35.9% identity, in a 131 amino acids overlap, with a GR enzyme from E. Coli .
- 36.1% identity, in a 133 amino acid overlap, with a human GR enzyme.

This brings good evidence that the sequenced clone is indeed glutathion reductase. It would be interesting to check as well the other clones by sequencing, as they might correspond to isoforms or to other proteins sharing homology with glutathion reductase.

b - Expression of GR in the latex cells :

The cloned GR gene fragment was used as a probe for northern blot experiment on total RNA extracted from latex

i) *Effects of tapping and stimulation on latex cells GR expression*

It could be shown (fig 22 a & b) that, as usually in most plant tissues, the GR gene is very poorly expressed or have a very low expression turnover in the latex cells (a systematical at least one week film exposure is necessary to discern faint hybridization patterns even with high labelling and specific activity of the probe).

It could be seen that ethylene stimulation induced, in untapped latex cells (virgin trees) a slight overexpression of this GR gene after 24 hours (Fig 22 a).
Tappings alone (latex regeneration) induced a slight increase in GR expression. Successive tappings (4 tappings) of stimulated trees induced a marked GR overexpression (Fig 22 b). This could indicate that a high level of GR is required for the recycling of GSSG to GSH in the stimulated tapped trees, owing to the high metabolic activity (hence high oxidation processes) demanded for the regeneration of the exported latex.

ii) *Expression of GR in TPD trees latex cells.*

For the moment, probably because of the very low quantities of GR messengers in the latex, we could not get reproducible and well characterized results comparing GR expression in the latex from control and spontaneous or stimulation-induced TPD (not shown).

4) Conclusions and prospects.

Interesting results have already been obtained within these first 6 month of lab and field work. They confirm that TPD trees, especially those induced by overstimulation, are characterized by some oxidative stresses in the latex producing tissues.

After verification of the sequence of the "Cu-Zn-SOD" inserts, they will be used as probes for checking expression of this gene during the onset of TPD.

Same will be done with catalase genes, the produce of which is of considerable importance in scavenging ROS and protection against oxidative stresses.

Further, the 22 kDa protein which has been shown to be highly overexpressed in TPD latex cells have been purified through 2D-SDS-PAGE and recently sent for microsequencing. These sequences will serve to design oligonucleotides which will be used for cloning the corresponding cDNA, either by PCR or by screening a TPD specific cDNA library that we will prepare for this purpose, and get back to the gene (function and regulation).

II - SMALL SCALE PRAPARATION AND ANALYSIS OF DNA AND RNA FROM RUBBER TREE.

Student name : **Miss Pritsana Chomchai (B. Sc. Student)**

Advisers : Prof. Dr Jarunya Narangajavana
Co-adviser: Dr Hervé Chrestin

Duration: Lab work: 2 months fragmented between june 1994 and March 1995

1) Aims

The actual studies in the molecular biology of rubber are made using "large" amounts of plant material, that make complicated procedure and waste money and time: extraction of RNA from at least 6 ml of latex or RNA and DNA from at least 5 gr of leaves. For futur use of markers probes for early selection, or TPD diagnostic, or even DNA methylation, and for routine "Latex Molecular Diagnostic" we will need to use small samples.
This project consisted in an attempt to develop a method for mini-preparations of DNA and RNA from various tissue of the rubber tree (latex, leaves, callus and seed integuments).

2) Results and discussion

a - Extraction of DNA:

Genomic DNA was extracted from young leaves, inner and outer integuments of young seeds, GT 1 cultivar, by modified published techniques for the extraction of DNA from plants which produce latex and/or phenolic compounds. Starting from 1 to 1,5 gr of leaves, the best selected method could yield after two precipitations genomic DNA more than 350 µg/g leaf, with a puification ratio OD_{260} / $OD_{280} \approx 2$.
The same selected method was used to purify genomic DNA from embryogenic callus and inner or outer-integuments of young seeds (only the inner integument can be used for embryogenic callus induction) from GT 1 and RRIM 600 cultivars. It could be shown that the method gave also satisfactory DNA yield with integuments (210 to 320 µg/g) with satisfactory degree of purification (OD_{260} / $OD_{280} \approx$ 1,6 to 2), whereas this method was not suitable to extract DNA from callus.
Verification by 0.7% agarose gel electrophoresis could show that the DNA extracts were of good quality (no smear = no large degradation).
Analysis of Southern hybridization showed that the rubber tree DNA extracted from leaves and seeds integuments can hybridize fairly well with the homologous hevein probe.

b - Extraction of RNA

RNA extracted from rubber tree leaf tissues gave rather good yield (≈ 190 µg/g) with a ratio OD_{260} / $OD_{280} \approx$ 1,8.

RNA small scale extraction from 0,75 ml latex could yield up to ≈ 12 µg RNA (≈ 16 µg/ml latex), which is little but enough to perform at least one or two dot or Northern blot analysis.

Verification by 0.7% agarose gel electrophoresis showed that the RNA from latex was especielly of very good quality (ribosomal-RNA bands without smear). But northern blot analysis was not successfull using the nonradioactive labelled hevein probe.

Further development for small scale RNA preparation are needed. Northern hybridization should be done with a radioactive labelled probe (it is well documented now that the available nonradioactive DNA labelling systems are not efficient for Northern hybridization).

3) Conclusions and prospects

The methods for DNA extraction from callus have to be improved for studies on the effect of DNA methylation on embryogenesis induction.
The small scale latex RNA extraction technique can be used for latex molecular diagnostic. It gives a RNA extract of very good quality, but may be probably further improved to get better yield (starting from 6 ml latex yields from 25 to 30 µg/ml latex).
Radioactive labelled probes will probably have to be used for Northern or RNA Dot blot analysis.

III - DIRECT TRANSFERT OF FOREIGN DNA INTO HEVEA PROTOPLASTS

Student name : **Mr Supat Dara (B. Sc. Student)**

Adviser : Prof. Dr Jarunya Narangajavana
Co-adviser: Dr Valerie Pujade-Renaud

Duration: Lab work: 2 months fragmented between june 1994 and March 1995

1) Aims

The future genetic engineering of the rubber tree will first need studies on transient expression to characterize specific promotors that will be included upstream reporter genes such as GS or LUC in the desired vectors.

Electroporation is one of the direct gene transfer techniques in plant protoplasts, allowing rapid transient expression studies. These first attempts in the genetic engineering of Hevea protoplasts will also give preleminary informations for further studies (kanamycin resistance, etc...).

2) Results and discussion.

Using the technique set up by CIRAD, we could induce embryogenic callus from the inner integument of young seeds, with the "induction medium". These callus could devide and further develop to embryo by using the "Expression medium". The callus was found to resist and grow in liquid and solid medium in the presence of up to 50 µg/ml kanamycin. Thus this concentration (50 µg/ml) or more of Kanamycin can be used for selection of transformants which contain a kanamycin resitance gene as selectable marker.

Parameters for protoplast preparation and electroporation have been optimized using GUS (ß glucuronidase) as reporter gene. Plasmid pBI 121 was used as a model for transformation experiments. PBI 121 was composed of the 35S promotor upstream the GUS gene, and the kanamycin resistance gene. The vector was transfered into Hevea leaf or cell suspension protoplasts.

Hevea cell suspensions could be obtained from embryogenic callus and maintained by sub-culturing every 5 days. Protoplasts were prepared from these cell suspensions by an optimized technique derived from the one set up by E. Cazaux *et al.* (University of Montpellier II/France) through digestion with an enzyme solution composed of cellulase 1.2 % and macerozyme 0.4% for 4hr and were futher heat shocked for 5 min before electroporation. Optimun condition for electroporation was two times low voltage impulse.

After incubation at 25°C in dark for 24 hours with from 0 to 150 µg/ml kanamycin, these electroporated protoplasts were subsequently asssayed for GUS activity by fluorometric and/or checked for resistance to kanamycin as results for transient expression tests.

In these conditions control non transformed (electroporated but without Plasmid pBI 121) protoplasts did not exhibit any GUS activity while up to 33 pmol/min/mg protein GUS specific activity could be measured in protoplasts transformed by Plasmid pBI 121. Futher, the transformed protoplasts could survive in the presence of up to 100 µg/ml kanamycin while control protoplasts could not survive 50 µg/ml.

Attempts failed to regenerate cells or microcallus from protoplast through their embedment into agarose gel and nurse culture technique in the presence of 50 µg kanamycin.

Same experiments where performed with Hevea leaf protoplasts with longer incubation time (4 to 24 hours) for cell wall digestion. Yield and transformation of leaf protoplast were shown to be less efficient than with cell suspension derived protoplasts.

3) Conclusion and prospects

The technique set up for cell suspension derived Hevea protoplasts will be used for studies of "ethylene sensitive" gene promoting regions (Glutamine synthetase, etc...).

For that purpose, transient expression is enough and therefore, regeneration of whole plants from transformed protoplast is not absolutely necessary. However, it would be interesting to further investigate the suitable media and conditions for eventual protoplast regeneration in the future.

RESULTS : 3rd PART

CLONING OF GENE PROMOTORS FROM RUBBER

I - THE HEVEIN PROMOTOR

1) Introduction:

Hevein, a lectin-like protein, is one of the most aboundant proteins in the latex of rubber tree. It is compartimentalized in vacuolar structures called lutoids, where it represents 70% of the soluble proteins. When released in the cytoplasm, upon bursting of the lutoids, this protein is able to bind a 24 kD receptor protein localized at the surface of the rubber particles, leading to the agglutination of these particles (Gidrol *et al.*, 1994).

Such a phenomenon is a major mechanism of latex coagulation, one of the main factor limiting rubber production.

A cDNA encoding hevein has been isolated and completely sequenced (Broekaert *et al.*, 1990). Interestingly, the deduced amino acid sequence of this clone is composed of two regions: the amino-terminal region is identical to hevein (43 amino acids) whereas the carboxyl-terminal portion (144 amino acids) shares homologies with wound-inducible proteins.

Northern blotting using this cDNA as a probe reveals that hevein is highly expressed in latex but at a very low level in leaves or stems (Broekaert *et al.*, 1990). Moreover, immunolocalization techniques was used to confirm that the hevein protein is exclusively present in the laticifers. Stimulation of the trees with ethephon (an ethylene generator used to stimulate latex production) leads to a 2- to 5-fold increase of the amount of mRNA.

Our objective is to clone and characterize the promotor(s) of the hevein gene(s), in order to design useful vectors for future genetic engineering of rubber tree.

Native promotors are thought to allow more efficient expression than foreign promotors such as the Cauliflower Mosaic Virus (CaMV)-35S promotor, a strong constitutive promotor frequently used for transformation. Moreover, constitutive expression can lead to perturbations of the whole plant physiology. A promotor which can target the expression of a foreign gene of interest to a specific tissue (the latex cells in our case), without modifying the expression in the other tissues, is far less stressfull for the whole plant.

Therefore, because it directs very strong expression in a laticifer-specific way, the hevein promotor is a very interesting candidate for the designing of vectors for the genetic transformation of rubber tree.

2) Cloning of hevein promotors:

a - Cloning:

Our first attempt was to clone the hevein promotor by the technique of Reverse PCR. Two oligonucleotides were designed from the sequence of the cDNA, one on the non coding strand, (nucleotides 60 to 85), and the other one on the coding strand (nucleotides 381 to 404).

Figure 23-A/a

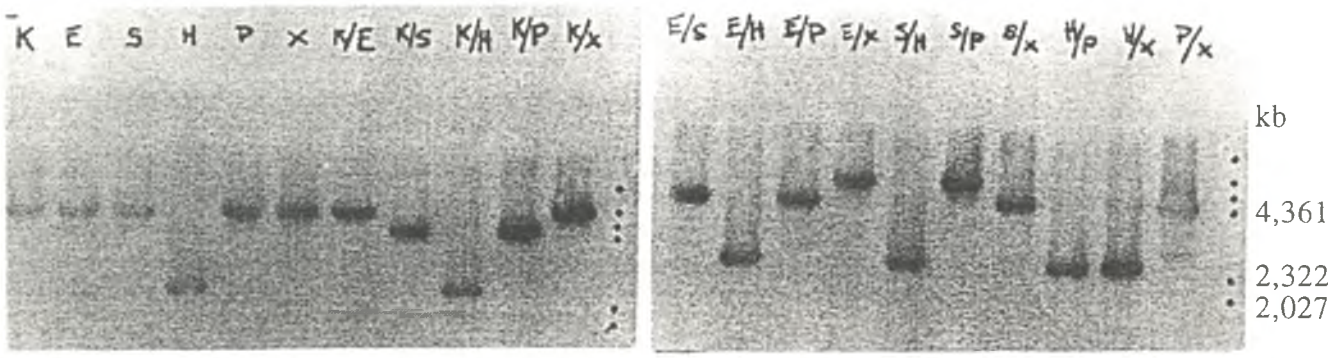


Fig. 23-A/b

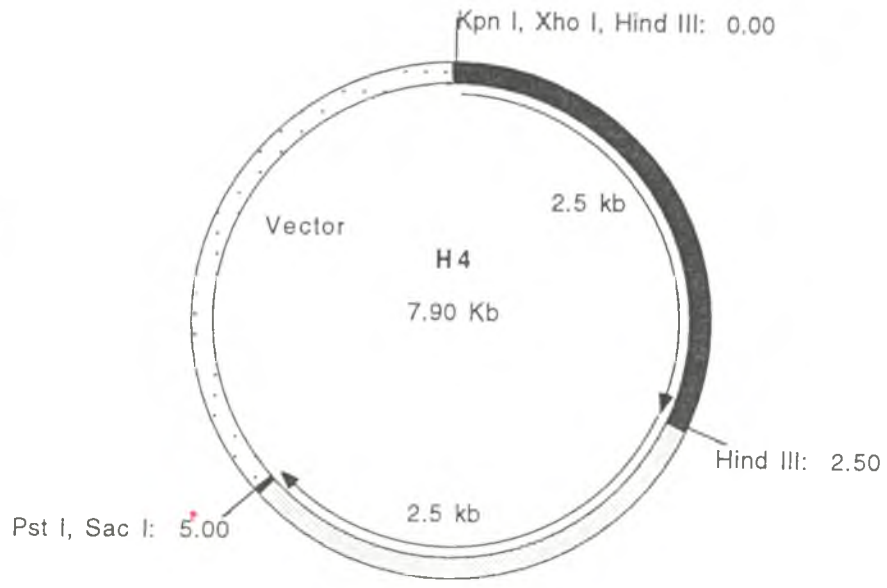


Figure 23-A: Mapping of the hevein clone 9.4.1.1 (H4):
Fig. a : Southern blot: After digestion with the enzymes Sac I (S), Pst I (P), Hind III (H), EcoR I (E), Xho I (X) and Kpn I (K), alone or in pairs, the plasmid DNA was transfered to a nylon membrane and hybridized with probes corresponding to different parts of the hevein cDNA. On this figure, the probe used was a 90 bp coresponding to the 5' end of the cDNA.
Fig. b : Restriction map.
In black, the fragment that hybridizes with a 90 bp probe corresponding to the 5' end of the cDNA. In grey, the fragment that hybridizes with a 300 bp probe corresponding to the 3' end of the cDNA.

Genomic DNA was extracted from rubber tree leaves of genotype RRIM600, restricted with enzymes (EcoRV or HindIII, which generate fragments of about 2 kb) and ligated to circularize the fragments on themselves. PCR amplification was performed on this circularized DNA using the two foregoing oligonucleotides. The PCR products were then cloned in the pMosBlue vector. However, very few clones were obtained, of very short size (less than 200 bp of promotor) and the one we sequenced showed very little homology with the hevein cDNA (only at the sites corresponding to the primers).

Because of the poor results obtained by Reverse PCR, a more classical approach: the screening of a genomic library, was undergone.

A genomic library of DNA extracted from rubber tree leaves (genotype RRIM600) and cloned in the Lambda-Zap vector, was screened with the full length hevein cDNA. After three rounds of plating, 18 plaques emerged with a significant hybridization signal. For each of these clones, the insert-containing phagemid was excised and multiplied in bacteria.

b - Mapping:

Clones were digested with a set of restriction enzymes (EcoRI, XhoI, HindIII, KpnI, Sac I) alone or in combination. The fragments obtained were separated by electrophoresis and transfered onto nylon membranes for analysis by Southern blotting. The membranes were hybridized with different portions of the hevein cDNA clone, either a 90 bp fragment corresponding to the 5' end, or a 300 bp fragment corresponding to the 3' end.

This brought several informations:

- The size of the inserts ranges between 3 and 5 kb (approximation).
- The pattern of restriction fragments is different for every clone except 2 which are identical..
- Out of the 18 clones:
    - 7 hybridize both with the 5' and the 3' fragments of the hevein cDNA clone
    - 10 hybridize only with the 5' probe
    - 1 hybridizes only with the 3' probe.

The 7 clones hybridizing with both probes are full length clones encoding hevein, whereas the others are partial. However, all 7 clones have different restriction patterns. This diversity in patterns could be accounted for the diversity in the length of the inserts (as the genomic library was constructed from partially digested genomic DNA), but it is most probable that we encounter here a multigene family.

Fig. 23-A shows an example of mapping for one of the clones we obtained.

c - Sequencing:

To verify wether or not the 7 full length clones carry the hevein coding sequence with its promotor, we started sequencing them, using an internal oligonucleotide primer chosen at the 5' end of the coding sequence (Figure).

So far, 6 clones out of 7 were partially sequenced, on one strand, and 350 bp to 400 bp could be determined.

All clones have very strong identity with the hevein cDNA (upstream the primer). However, two classes of genes can clearly be distinghished from their sequence upstream the cDNA.

- The first group is composed of clone 10.5.1.1 (called H1), clone 8.5.1.1 (H2), and clone 8.5.2.1 (H3) , with a few differences in 10.5.1.1 that need to be confirmed by further sequencing on both strands.
- The second group is composed of clone 7.6.4.1 (H4), clone 9.4.1.1 (H5) and clone 9.6.1.1 (H6).
- The clone 11.2.2.1 (H7) still has to be sequenced

This interesting result indicates that their are at least two genes coding for hevein in rubber tree, and that their expression is directed by two different promotors (probably differentially regulated).

d- Subcloning:

For each clone H1 to H6, digestion with Hind III was performed. The fragment recognized by the 90 bp probe corresponding to the 5' end of the cDNA was isolated on gel, purified, and sucloned in a pBlueScript vector (in the Hind III site). The size of these fragments are:

H1: 1.3 kb
H2: 1.1 kb
H3: 1.1 kb
H4: 2.5 kb
H5: 1.8 kb
H6: 1.8 kb

The clone H7 will be digested with Xho I. The 6.8 kb fragment containing both the vector and a 4 kb portion of the gene homologous to the 90 bp 5' probe will be purified and self-ligated.

This subcloning will facilitate further manipulation of the clones in order to:
- fully sequence them
- further characterize the efficiency of the promotor

3) Conclusion and prospects:

Further characterization of the cloned promoters is necessary to answer the following questions:
- are the promotors able to allow the expression of foreign genes in the latex cells?
- is their expression tissue specific?
- which portion of the promotors is necessary and enough to control the tissue specificity? The sensitivity to ethylene?

Our strategy will be to make constructs with deleted portions of the promotors cloned in front of a reporter gene, to introduce them *in planta* using particle gun or *Agrobacterium* transformation, and to measure transient expression of the reporter gene.
To make deletions of the promoters, either Exonuclease III digestion or a PCR technique will be used.

The reporter gene will be *gus* (β-glucuronidase), or eventually *luc* (luciferase).
The choice of the plant material is critical, as we need tissues bearing laticifers as well as non laticifer tissues, in order to study the tissue specificity. *In vitro* plantlets (microcuting) might be convenient for studies of transient expression using the particle gun. Moreover, we verified that laticifers can be visualised by hysto-chemical techniques at very early stage of development (as early as the embyo stage). Therefore, the use of maintained lines of embryogenic callus could be also very convenient, knowing that the technique of somatic embryogenis developped by the CIRAD team at Montpellier allows the production of a large number of embryos from such callus lines.

So far, we performed very preliminary bombardment experiments on pieces of stem and embryogenic calli, using a vector carrying the *gus* gene behind the CaMV-35S promotor; but technical parameters of the bombardment need to be adjusted more precisely before we can conclude about the feasibility of the technique.

II - GLUTAMINE SYNTHETASE (GS)

1) Introduction:

Glutamine synthetase is a key enzyme for ammonium assimilation in higher plants. It catalyses the incorporation of ammonia into organic molecules which are involved in the synthesis of aminoacids, nucleotidse, and consequently in the synthesis of proteins, nucleic acids, etc...
In rubber tree, the latex is expelled during tapping. An efficient metabolic machinary is therefore necessary to ensure complete and quick regeneration of this exported latex between consecutive tappings. In this context, the GS activity is of major importance, for the reconstitution of all nitrogenous compounds.

Our work demonstrated that treatment of the trees with an ethylene generator, ethephon, was able to increase both the activity and the mRNA level of glutamine synthetase, concomitantly with an increase of the production (Pujade-Renaud *et al*, 1994 and see chapter 1in this report). A cDNA of a cytosolic GS from latex has been cloned (Pujade-Renaud, Thesis) and further characterized by Northen and Southern blotting. Among the genes regulated by ethylene in latex that we could identify so far, GS is the one that shows the **earliest response to ethylene** (as soon as 6h after treatment, with a 10 times stimulation factor after 48 h).

We are now undergoing the **cloning of GS genes** in order to **isolate and characterize their promotor** and more specifically to determine **which part of the promotor is responsible for the regulation by ethylene.** Such promotor sequences, inserted in a vector for rubber tree ransformation, might be of great interest to increase in a controled manner the expression of a transgene, using ethephon.

2) Cloning of GS promotors:

a - Cloning and mapping:

The genomic library previously described for the cloning of the hevein promotors was screened with the full length GS cDNA. After three rounds of platting, 7 clones were recovered.

Preliminary attempts to map the clones was performed, using the enzymes EcoR I, BamH I, Xho I. The restriction fragments were separated by electrophoresis and transfered onto nylon membranes for analysis by Southern blotting. The membranes were hybridized with different portions of the GS cDNA clone, either a 350 bp fragment corresponding to the 5' end or a 370 bp fragment corresponding to the 3' end.
Among the 7 clones, only 4 hybridized with both the 5' and 3' probes. These 4 clones have inserts range between 1.7 and 17 kb and show different restriction maps.
Figure 23-B shows an example of mapping for one of the clones we obtained

b- Subcloning:

- The clone 4.3.2.1 (GS1) is the shortest (1.7 kb insert) and will not be subcloned
- The clone 4.4.2.1 (GS2) has been digested with BamH I and a 4.6 kb fragment containing the vector but also able to hybridize with the 5' probe was purified on gel and then self-ligated.
- The clone 5.2.1.1 (GS3) has been digested with Hind III and a 8.2 kb fragment containing the vector but also able to hybridize with the 5' probe was purified on gel and self-ligated.
- The clone 5.4.4.6 (GS4) has been digested with Xho I and a 11 kb fragment containing the vector but also able to hybridize with the 5' probe was purified on gel and self-ligated.

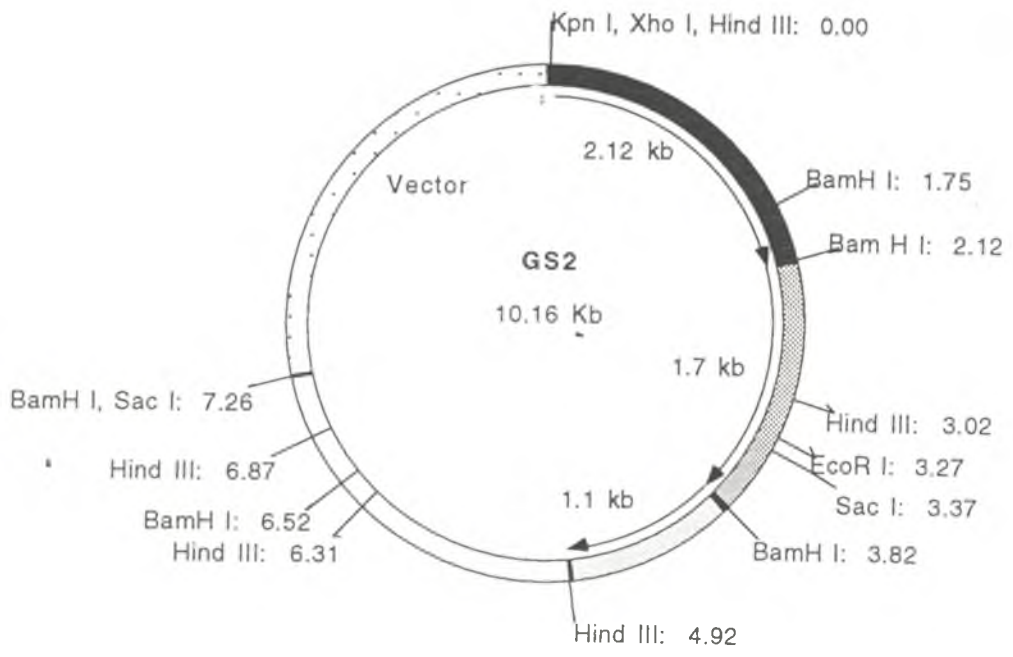


Figure 23-B : Mapping of the GS clone 4.4.2.1 (GS2):
Restriction map: In black, the fragment hybridizing with the 350 bp probe corresponding to the 5' end of the cDNA (should contain the promoting sequence). In dark grey, the fragment hybridizing with the 800 bp probe internal to the open reading frame. In light grey the fragment hybridizing with a 370 bp probe corresponding to the 3' end of the cDNA.

Further subsequencing will be necessary, either by digestion with other enzymes, or by exonuclease III deletion, or by PCR subcloning techniques.

c - Sequencing :

An oligonucleotide has been designed which corresponds to the 5' end of the coding sequence (nucleotides 118 to 141, non coding strand). It will be used as a primer for sequencing, to verify that the 4 clones correspond to GS genes and if they are different or not.

3) Conclusion and prospects:

Deleted portions of the promotor region will be coupled to a reporter gene and introduced in rubber tree cells, to determine which sequences are responsible for the regulation by ethylene. On the contrary to the hevein gene, this analysis can first be performed in protoplasts, as laticifer cells are not necessary. The technique to get protoplasts from rubber tree has been described previously (Eveline Cazaud, PhD, CIRAD). Mr Supat Dara, B. Sc. student under the supervision of Dr. Jarunya Narangajavana, improved the technique at Mahidol university and assessed the transformation of the obtained protoplasts by electroporation (i.e. Report, 2nd Part, § III)). This will be a very usefull tool to carry on the characterization of our promotors.

RESULTS : 4th PART

LATEX DIAGNOSTIC (BIOCHEMICAL)

1) Introduction:

Two groups of production limiting factors can be identified as a function of the knowledge available on the way they affect the productivity.
Knowledge of the first group derives from general observations which mainly involve measurement of production and the rubber content of latex. These production limiting factors, about which knowledge has been acquired globally without full initial understanding of their mechanism, include ecoclimatic factors, clonal characteristics and factors connected with exploitation systems of adult *Hevea*.
In contrast, the discovery of the second category of factors stems from the examination of physical and biochemical analysis of latex; this has led to relatively detailed understanding of the mechanism involved in the physiology of production. The utilization of the basic knowledge leads to the drawing up of a physiological diagnosis, the Latex Diagnostic, of the health of the tapping panel in *Hevea*, definition of typology of the physiological functioning of the clones, and to the utilization of the parameters in the early selection of new *Hevea* clones.

The choice of the parameters to be analyzed depends on the degree of correlation wich can be established between these parameters and production under certain conditions.It has thus been possible to correlate a number of biochemical and physiological parameters in latex with the production and particularly with the two components flow and regeneration (Annexe).

2) Experimentations in the field

We set up two types of trials on clone RRIM 600 starting with virgin (opening) 8 year old trees to induce TPD through overstimulation (1/2S d/2 stim ET 5% m/m, ET 10% m/m and ET 5% d/15d) and overtapping (1/2S d/1), control trees were tapped 1/2S d/2 without stimulation (see Material and Methods). The field experiments (overexploitation) started in July 1994.
The tapping panel of the 1/2S d/1 treatment (overtapping) was changed on 16/06/95 because of the nearly entire consumption of the bark on the first panel after 2 years.

The latex was collected as described in Material and Methods roughly once every month, on the last tapping before the stimulation treatment.

The recorded physiological parameters were: Dry Cut Length (DCL%), Yield (g/tree/tapping) and the parameters of the Latex Diagnostic (LD): ie: dry rubber content (DRC), sucrose content (Suc), thiols content (R-SH) and inorganic phosphorus (Pi). The LD was performed by the team of the Chachoengsao RRC, in close relationship with our french team in Mahidol University, especially with Dr. R. Lacrotte (CIRAD-CP).

3) Results

Considering these treatments, some overexploitation systems, especially overstimulation, could efficiently induce transient TPD and some irreversible TPD.

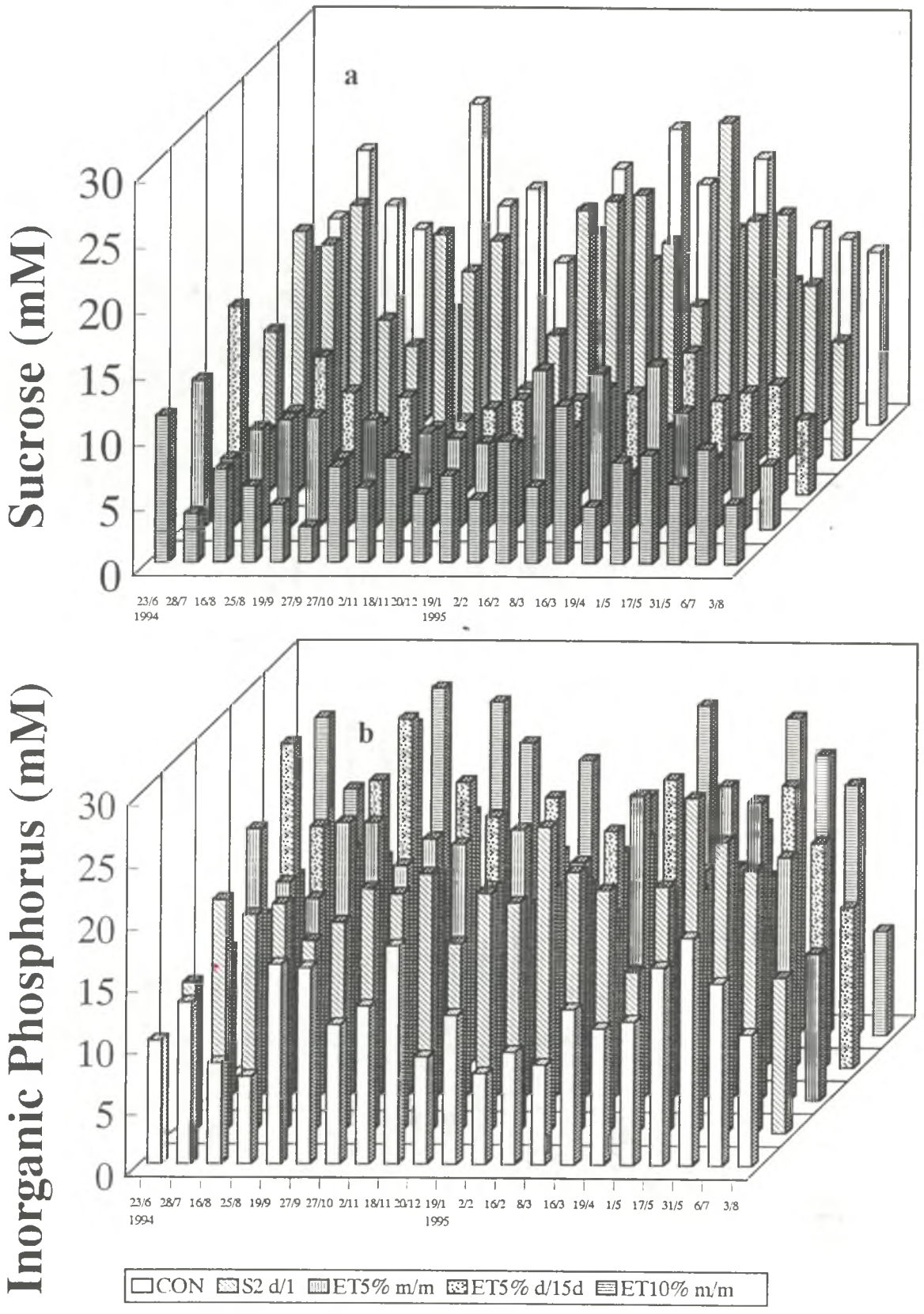


Figure 24: Sucrose (a) and Phosphorus (b) contents in latex according to exploitation.

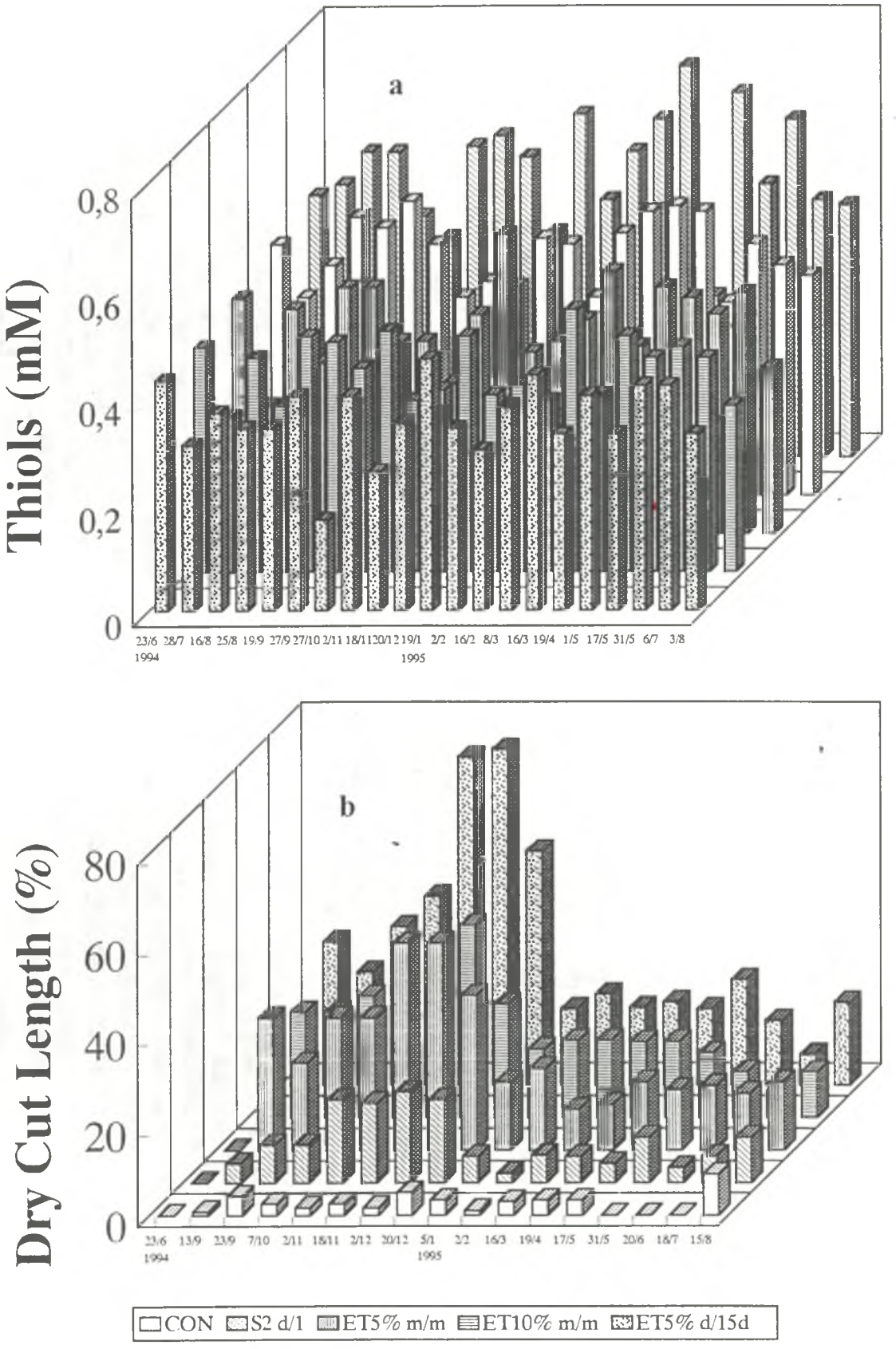


Fig 25: Thiols content (a) and Dry Cut Length (b) according to exploitation.

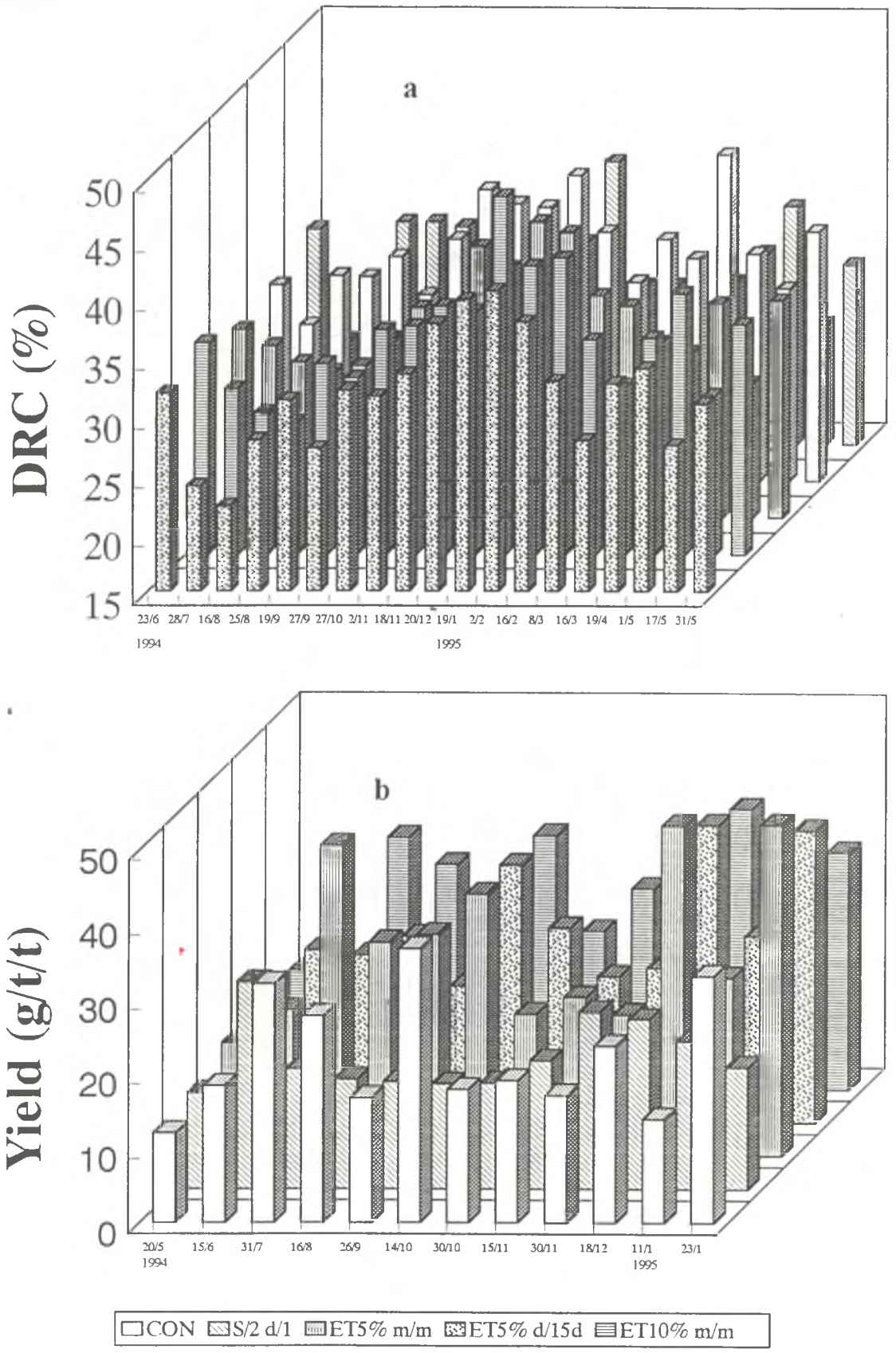


Fig 26: DRC (a) and g/t/t (b) according to exploitation.

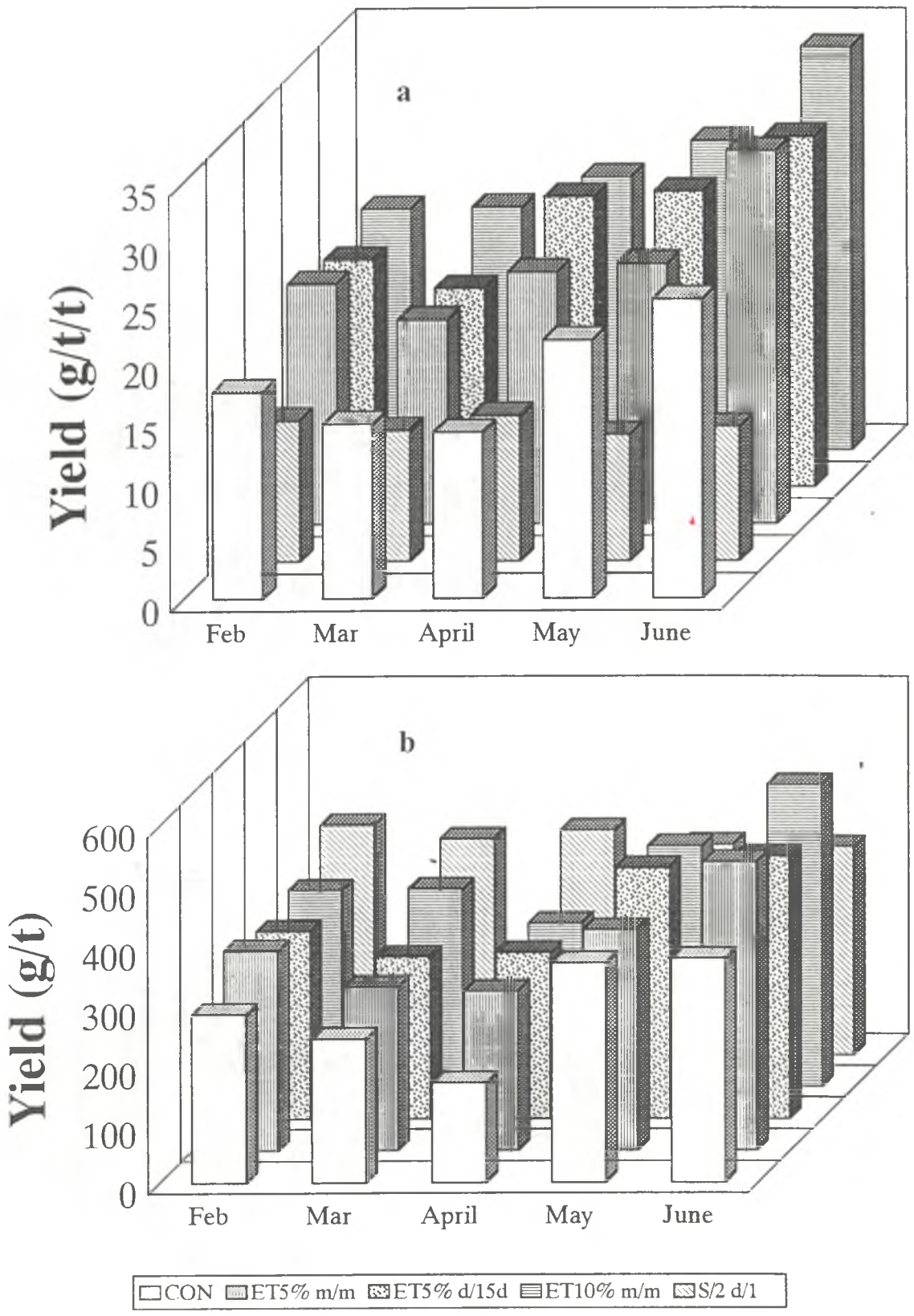


Fig 27: Yield; g/t/t (a) and g/t (b) according to exploitation in 1995.

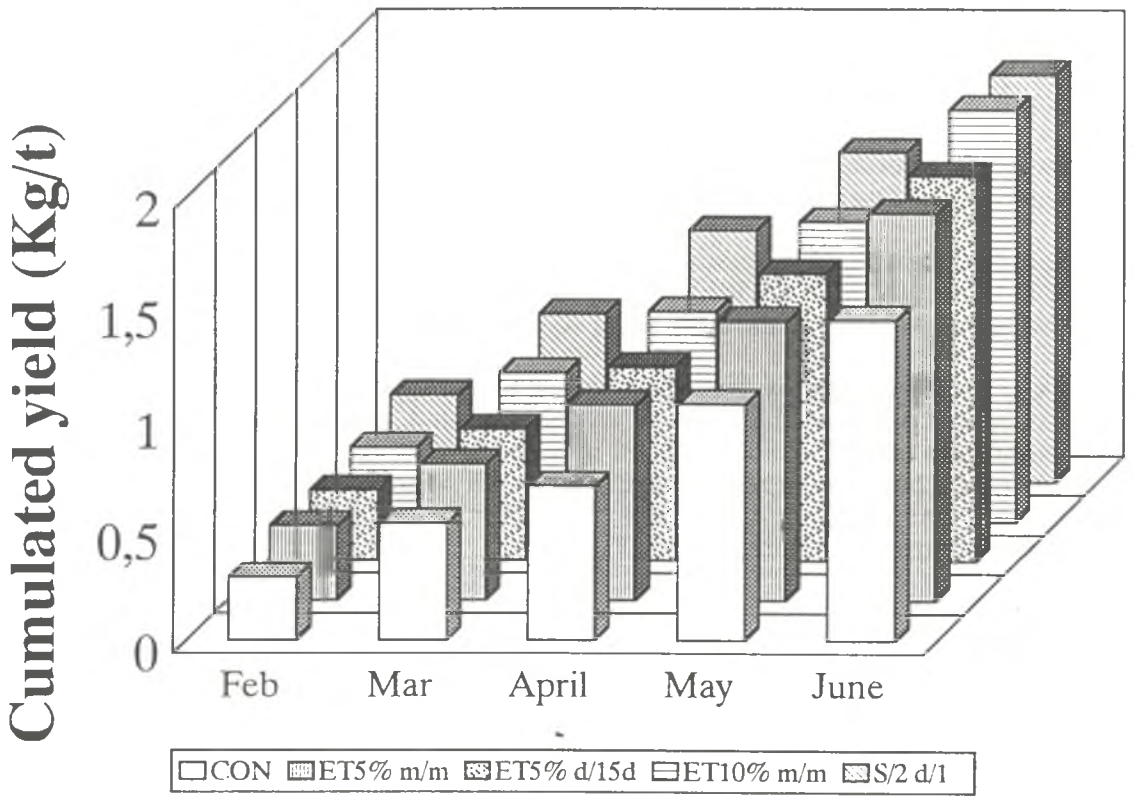


Fig 28: Cumulated yield according to exploitationin 1995.

a - Physiological/biochemical parameters:

- Sucrose content was significantly lower in the latex of stimulated trees. The lowest values were found for the treatments with the highest intensity (ET 10% m/m) and frequency (ET 5% d/15d) of stimulation (Fig. 24 a).
  Daily tapping (d/1) did not lead to decreased sucrose content.

- Inorganic Phosphorus was markedly increased in the latex of both stimulated and daily tapped trees. Overstimulation gave the highest Pi values (Fig 24 b).

- Stimulation induced a significant decrease of R-SH, especially the highest frequency of stimulation (ET 5% d/15d). As previously observed in other countries, daily tapping induced an increase of the latex thiols content, when compared to the control (Fig. 25 a)

- DCL increased readily from September (3 months after starting the experiment) to December 1994 in the case of overstimulation treatments, the overfrequency of stimulation being the most efficient in inducing transient TPD. After that, the symptoms decreased but always remained higher in all overexploited trees compared with the control. Daily tapping was poorly efficient in inducing TPD, although DCL remained higher compared with the control (Fig. 25 b)

- DRC of the latex from stimulated trees was lower compared with the control from July to November 1994. After that, DRC increased concomitantly with slower latex flow upon tapping during the winter defoliation period. Then the DRC decreased again with the first rainfalls (Fig 26 a)

b - Yield:

- The monthly yield was first extrapolated from yield checked on one tapping only, every 2 weeks or monthly (Fig 26 b). This can explain the erratic results obtained for yield during this period, even for the control treatment. At the end of January 1995 it was decided that latex production would be checked montly but on all cumulated tapping (all the latex coagulum were monthly grouped/tree and globally weighted tree/tree). Since this time it could be possible to calculate the g/tree/month (g/t) or per tapping (g/t/t) as well as the cumulated real rubber production (kg/t).

- As usual, the yield expressed in g/t/t was higher for the stimulated trees compared to control (Fig. 27 a) but the onset of latex flow problems and of TPD symptomes had a high impact in reducing response to stimulation, leading to small rubber yield differences (February to June 95) in term of g/t/t between control and overstimulated trees. The lowest yield values in term of g/t/t were recorded for the daily tapped trees. It can be supposed that latex regeneration cannot be completed due to the very short harvest time.

- In term of g/t (per month)(Fig. 27 b) it can be seen that yield of contol trees decreased all along the dry hot season corresponding to the defoliation-refoliation period (February to April). The beginning of the rain falls (end of April to june) led to the increase in the yield of control trees. But at this time the response to stimulation is very low due to real problems of latex flow and occurence of TPD symptoms after 6 to 10 months overstimulation. On the contrary the yield (g/t) of the daily tapped trees is quite regular and and often better than the yield of the stimulated trees.

- In term of cumulated yield (Fig. 28/ cumulated from February to June 1995), after one year experiment, the dayly-tapped trees exhibited the best score (+ 27%) together with the the Ethrel 10% monthly stimulated trees (+ 25%), then the either monthly or every 2 weeks Ethrel 5% stimulated trees scored only 20% more yield than the control.

4) Comments:

After 14 months experiment, it was too early to define a seasonnal variation of the parameters recorded, which could allow by comparison to detect the early signs of the induction of TPD. Rainfall was very low between November 1994 and March 1995, and at this time DRC increased for all treatments but could not be paralleled with the other LD parameters with seasonnal variations.
One question is remaining opened: how the decrease of the dry cut length between December 1994 and January 1995 could be explained after the wide increase in DCL measured from September to beginning of December 1994 on all over exploited trees (especially overstimulated trees)? This is a typical case of induction of transitory TPD, and we did not record, at the time we are writing this report (November 1995), such an increase in DCL as seen in 1994.
Whatever, it could be seen that ethylene treatments induced a higher consumption of sugar for latex regeneration purposes and a higher level of oxidation of thiol compounds in the latex corresponding to their ROS scavenging activity as more ROS might derive from metabolism activation by ethylene. As usual, stimulation induced also an increase in the inorganic phosphate concentration in the latex, as a sign of an increase in the energy consumption and availabilty.
This global activation of the metabolism in the latex cells led to a higher production of latex, but not as high as it could be expected. This poor effect of stimulation on yield during these last four months treatments, whereas the latex diagnostic of their latex exhibited the typical effects of stimulation, does show that after one year treatment these overexploited trees exhibit some true physiological fatigue, leading to TPD.
Nevertheless, the Latex Diagnostic parameters abled the warning of overexploitation, especially through overstimulation, leading quickly to the onset of transient TPD and progressively in the longer term to the installation of TPD. Early (3 months/3 to 6 stimulations) after the starting of the experiments the concentrations of sucrose and reduced thiols in the latex from overstimulated trees were too low, as compared with the control or daily tapped trees, to insure normal homeostasis in the latex cells. The high concentration of Pi in the latex of stimulation-induced TPD reveals a very high metabolic activity, consuming too much energy with low extra-gain in production.
In the case of daily tapping during15 month, there were no warning of true overexploitation: the concentrations of sucrose and reduced thiols in the latex remaining the same order of range compared to those from control trees, while Pi concentration remained significantly higher revealing high metabolic activity, because of the high latex regeneration rate needed for such short harvesting time.

5) Conclusion

The Latex (biochemical) Diagnostic (LD) can already be usefull to predict overexploitation through over stimulation and to give recommandations to rubber planters. Nevertheless, within the 15 first months time of this experience "physiological fatigue" did not lead to much definitive TPD, while transient TPD remained difficult to explain.
The Latex Molecular Diagnostic will be a usefull complementary tool for the prediction of TPD induction and differenciation between the different types (spontaneous/induced/transient) of TPD.

CHAPTER III - GENERAL CONCLUSION AND PROSPECTS

I - GENERAL CONCLUSION

As stated in the introduction, there are **2 major intrinsic yield limiting factors** at the level of the latex producing tissues:

- the latex flow limitation by **coagulation** processes at the tapping cut,
- the capacity of **regeneration** of the latex between tappings,

and a major problem impairing rubber yield,with high economical impact:

- the sensitivity towards the **Tapping Panel Dryness** (TPD) desease caused by over exploitation and most probably also by other stresses.

Moreover, **"Stimulation"** with Ethrel (ethylene releaser) is a convenient mean to study gene expression in the latex cells, in relation with increased latex production, and is of great help to find out molecular markers of high latex yield potential.

1) Coagulation:

In this work we could confirm that coagulation of latex is under the control of at least 3 proteins, and therefore under the control of the expression of their corresponding genes. These 3 types of proteins are hevein, a small 2-binding-sites lectin (phytoagglutinin), a ≈ 22kDa glycosilated hevein-receptor-protein bound to the rubber particles membrane, and chitinases. Hevein can bind to the N-acetyl-glucosamine moiety of its receptor protein and therefore bring together the rubber particles leading to the building of a multidimension rubber particle cohesive network known as latex coagulation. This process can be partially - or eventually completely - inhibited by the action of endogenous chitinases or N-acetyl-glucosaminidase, which can cut the glycosylated moiety of the hevein receptor becoming therefore ineffective in binding hevein.

We got c-DNA or genomic clones of these 3 genes. Using these probes, we could show that the 3 genes are constitutively expressed in the non-tapped (virgin) trees, hevein being more expressed that the 2 other genes. Furthermore, tapping alone was shown to induce an over expression of all these 3 genes in the latex cells, more marked for hevein and chitinase.

We could demonstrate that ethylene stimulation - which delay coagulation and therefore increases the duration of the latex flow, hence rubber yield - paradoxically induced an overexpression of these 3 genes involved in coagulation. But the much earlier and higher overexpression (induction) of a chitinase gene in the latex cells after stimulation, which led to effective partial deglycosylation of the hevein receptor (even in virgin trees), could explain the action of ethylene in delaying coagulation processes and increasing the duration of latex flow, and consequently higher production.

Moreover, comparing 2 rubber clones with different latex flow characteristics, such as the high yielding PB-235 clone (low plugging index/prolonged latex flow) and the War-4 low yielding clone (high plugging index/short latex flow), we could demonstrate that, compared with PB-235, the latex of the War-4 clone was characterized by a significantly higher expression of hevein and hevein receptor (pro-coagulants) and above all by a very low constitutive expression of chitinase (anti-coagulant). Ethrel stimulation, which comparatively induced in War-4 clone a much higher decrease in plugging index and increase in the latex yield, induced a much larger increase in chitinase expression in the latex cells of War-4 clone.

Although these results have to be confirmed through the comparison of more clones with various latex flow characteristics, these 3 genes, and particularly hevein and chitinases genes, the products of which compete for the same site (the N-acetyl-glucosamine moiety) of the hevein receptor to induce or inhibit the process of coagulation, are excellent candidates as yield potential molecular markers. If confirmed (2nd period of the project), such markers will be of great help in the rubber breeding programs for early selection of high yielding and/or stimulation responsive rubber clones (parents and progeny).

2) Regeneration of latex:

Regeneration of latex (cell cytoplasm + rubber) depends on many complex biochemical and physiological processes, hence on the expression of numerous genes.

During this first period of the project we focussed on 2 types of genes, the glutamine synthetase (GS) gene, the product of which controls the incorporation of nitrogen in most of nitrogenous organic compounds (proteins, nucleic acids, etc...), and 2 genes involved in rubber synthesis: the farnesyl-pyrophophate synthase (FPPS) which initiates the rubber chains, and the rubber elongation factor (REF) which contributes to the elongation of the cis-polyisoprene chains (rubber).

We got c-DNA clones of these 3 genes. Using these probes we could show that the 3 genes are constitutively expressed in the resting latex cells of non-tapped (virgin) trees. FPPS being higherly expressed than REF, and GS being the least expressed of these 3 genes. Tapping alone also induced a marked over expression of the 3 genes in response to the regeneration demand.

We could show that stimulation with Ethrel, which induced a transient (5 -8 tappings) increase in latex production hence a higher demand for latex regeneration, induced an early (between 3 and 6 hours) overexpression of the GS gene, the duration of which paralleled the duration of overproduction. Interestingly, ethylene did not induce any futher overexpression of FPPS and REF, neither within 24 hours, nor upon the successive tappings. This differential effect of ethylene at the level of genes expression may be explained by the fact that GS is a limiting factor for regeneration purpose, while FPPS and REF, which are already constitutively highly expressed in the latex cells of tapped non-stimulated trees, are not limiting in such a specialized tissue constitutively synthetizing 90% of its dry matter as natural rubber.

The fact that GS only is induced by ethylene stimulation indicates that it could be considered as a potential yield marker. Clonal studies remain to be done in the second period of the project to acertain if these genes can be considered as true markers of yield and/or rubber quality potential.

3) Tapping Panel Dryness and latex physiological diagnostic (Latex Diagnostic)

The Tapping Panel Dryness (TPD) desease results in a transient or definitive cessation of latex production. The symptoms appear abruptly and the desease cannot be easily predicted in the field. It is therefore necessary to find out markers allowing prediction of the desease so that it could be possible to modify the tapping system to prevent the onset of the desease.

We considered two types of Tapping Pannel Dryness (TPD): the spontaneous TPD which can occur randomly in a plantation and then can spread (often along the lanes of trees), and the overexploitation-induced TPD.

a - Induction of TPD through overexploitation:

Fourteen months overexploitation either through overtapping (S/2 d/1) or through overstimulation (ET 5 or 10% evry month, or 5% every 2 weeks), could induce TPD symptoms.

The Latex Diagnostic (LD: measurement of the latex inorganic phosphate, reduced thiol, sucrose and dry rubber content) could allow the warning of overexploitation, especially through overstimulation, leading quickly to the onset of transient TPD and progressively in the longer term to the installation of definitive TPD. After 3 months experiment, the concentrations of sucrose and reduced thiols in the latex from overstimulated trees decreased very low, while high concentration of Pi in the latex of overstimulated trees revealed a very high metabolic activity, consuming too much energy with low (20 - 25%) extra-gain in production.

In the case of daily tapping (overtapping/14 months) there were no warning of true overexploitation: the concentrations of sucrose and reduced thiols in the latex remained the same range as those from control trees, while Pi concentration remained significantly higher than control revealing high metabolic activity, because of the high latex regeneration rate needed for such short harvesting time. Interestingly, overtapping during 14 months did not induce definitive TPD symptoms, showing that trees (RRIM 600) could afford daily tapping at least for some months, but at the expense of a double bark-consumption with only a 27% extra-gain in cumulative latex production.

b - Spontaneous TPD:

Systematic recordings of TPD symptoms on 12 year old RRIM 600 trees revealed that 10 to 12 % of them exhibited TPD symptoms class ≥ 2 and 16 % exhibited preliminary symptoms of TPD (latex flow problems) on the lower panel. Only six month after opening the higher panel 50% of the trees which previously exhibited TPD class ≥ 3 symptoms and 90% of the trees previously exhibiting class 5 symptoms at the lower panel, exhibited even worse TPD symptoms at the newly opened higher tapping panel. This clearly indicates that individual trees can be more sensitive to TPD, and/or that, when installed, the desease will occur again when tapping any part of the bark.

c - Molecular markers of TPD

i - Blind approach : protein pattern analysis

Through a non-specific approach, using 1- and 2-D-SDS-PAG Electrophoresis of the latex proteins, we could show that , compared with control trees, the latex from TPD trees exhibited systematically different patterns of cytosolic proteins: overexpression of a 22 kDa acidic protein and other low molecular weight (14-17 kDa) acidic proteins, a *de novo* expression of a 29 kDa protein, and a decreased expression of a 19 kDa acidic protein as well as two $\approx$ 33 kDa proteins.

It was found that both types of (spontaneous and induced) TPD generally exhibited the same differences. Therefore, one can suppose that at least the overexpressed or *de novo* expressed cytosolic proteins may be stress proteins, warning of some stress leading to TPD. Such markers will be very usefull to predict risks of any kind of TPD, but will not allow to differenciate between spontaneous and induced TPD, if really different.

Because of its large amount in the latex of any TPD trees, the 22 kDa protein is under purification, and a purified fraction has just been injected to rabbits in order to raise specific antibodies to be checked as (efficient) probes for detection of TPD. Same work has to be done with the other potential TPD protein markers in the second period of the project.

ii - Specific approach (gene expression)

Through the specific approach based on two hypothesis able to explain physiological TPD and which previously got some biochemical supports:

- direct *in situ* coagulation of latex due to abnormal expression of genes involved in coagulation ?

- occurence of an oxidative stress due to overexploitation and leading to the decompartmentation of Hevein, hence to the *in situ* coagulation of latex ?

We could show latex from spontaneous TPD trees (PB 5/51 and RRIM 600 clones) exhibited an increased expression of chitinase, while stimulation-induced TPD (RRIM 600) on the contrary exhibited decreased expression of chitinase.

While the latex from PB5/51 spontaneous TPD trees did not show any difference as far as hevein expression is concerned, Hevein was shown to be over expressed in both spontaneous and stimulation-induced TPD.

Furthermore, while the expression of the hevein receptor was not different in the latex of PB 5/51 and RRIM 600 control and spontaneous TPD trees, it was significantly increased in the latex of stimulation-induced TPD trees.

Finally expression of Mn-SOD - a reactive oxigen species (ROS) scavenging enzyme - was shown to decrease in the latex cells of both spontaneous and stimulation-induced TPD trees, while we could show that expression of Mn-SOD is normally highly increased after stimulation. This indicates that the Mn-SOD gene does not respond normally to ethylene stimulation in the latex cells of stimulation-induced TPD.

Based on these results which remain to be statistically verified, it is proposed that the use of chitinase and hevein receptor c-DNA probes to check differential expression of the corresponding genes in the latex of TPD trees, could help in differentiating spontaneous and stimulation-induced TPD, allowing to give recommandations for more adapted (optimum) rubber exploitation systems avoiding TPD risks.

Moreover, based on the results essentially obtained from RRIM-600 clone, both spontaneous and stimulation-induced TPD exhibit a decreased expression of the Mn-SOD gene in their latex, revealing the probable occurence of an uncompensated oxidative-stress in their latex cells. Because of the high metabolism activation induced by ethylene (higher production of ROS due to higher oxidative metabolism) this oxidative stress is very likely to occur at least in the laticifers of stimulation-induced TPD trees.

Finally, latex from spontaneous TPD trees exhibited an increase in the expression of both the hevein and (more marked for) the chitinase gene, while the expression of the hevein receptor remained unchanged. These differential gene expression, and more evidently the marked over expression of the chitinase gene in the latex of spontaneous TPD does not fit with the hypothesis of higher coagulability of latex leading to *in situ* coagulation.

On the contrary, increased expression of both "pro-coagulants": hevein and its receptor, together with decreased expression of the chitinase ("anti-coagulant") genes in the latex cells of stimulation-induced TPD are in favour of a higher "coagulabilty" of the latex of stimulation-induced TPD, leadind to difficulties of latex flow and even *in situ* coagulation in case of decompartimentalization of hevein due to oxidative stress.

4) Promotors

We could previously show by cyto-histo-immunolocalization and now confirm by Northern blotting (tissue specificity of mRNA expression) that hevein and the hevein receptor are specifically expressed in the latex cells only. By Northenr blotting we could show that REF is also specifically expressed in the latex cells (while FPPS probably not). This means that these 3 genes, which are all highly expressed in the laticifers of tapped trees are under the control of laticifers specific strong promotors.

Moreover, we have shown that glutamine synthetase (GS) is the earliest (3-6 hours) gene overexpressed in the latex cells after an ethylene stimulation of rubber yield. This probably means that expression of this genes can be directly regulated by ethylene, and so should include a *cis*-acting sequence upstream its promotor, able to be activated in response to ethylene .

In the view of futur genetic engineering of the rubber tree, we started to clone genomic fragments upstream the 5' end of the hevein and GS genes, in order to clone and characterize a latex specific strong promotor (hevein) and an ethylene regulatory cis-acting sequence (GS). These promotors or *cis*-acting ethylene responsive sequences may be used in the future, to design special gene vectors to be introduced in the rubber tree to get expression of foreign genes of agronomical interest specifically in the latex cells and/or under the control of external stimuli such as ethylene treatments.

II - RECOMMANDATIONS AND PROSPECTS

1) Preliminary recommandations

As reminded in the introduction, all rubber producing countries have more and more to face a common problem: an increasing lack of a highly specialized manpower: the tappers. This comes from the fact that tapper-employees are generally underpayed to maintain higher exploitation profitability, and that, with the actual commonly used tapping systems in Asia (S/2 D/2) each rubber-planted rai consumes too much manpower.

Furthermore, tapping every 2 days, and often every day when the rubber selling rate is high, consumes a lot of bark (the rubber small holder "patrimony") with a small extra-gain in latex production, due to short harvest time. Further more, in the long term overtapping can lead to Tappping Panel Dryness. Our preliminary exprimentations did confirm that high frequency or overdose stimulation treatments, associated with S/2 D/2 tapping systems, lead to marked physiological fatigue and induction of TPD.

All this (lack of manpower and induction of TPD) could be overcome, and the manpower profitability as well as economic span life of rubber trees could be increased at least 2 times, through the use of different tapping systems, to be adapted to each clone and environmental conditions, using decreased tapping frequency (2 tappings/week or even every 4 days) compensated by stimulation of the latex yield by adapted (dose and frequency) ethylene treatment.

2) Prospects

As mentionned in the first DTEC request, this first period ending end of December 1995 allowed us to already find out potential molecular (DNA or protein) markers of high rubber yield and of TPD, as well as starting some studies on hevea gene promotors.
This project has to be extended for at least 2 years (January 1996-December 1997), during which we will go on theses studies in the lab (Mahidol University and CRI), and in the field (in the Rubber Research Centers of The RRIT).

a - Field Experiments: tapping systems, TPD and Ecophysiology:

During the first period, we could initiate small scale experiments, essentially with trees from the most commonly planted RRIM-600 clone, only in the Rubber Research Center (RRC) of Chachoengsao, close to Bangkok.

We could check the kinetics of ethylene stimulation effects, used as a tool for increasing yield, on gene expression in the latex cells. The effects of increasing intensities of tapping systems (tapping frequency versus hormonal stimulation intensity) were checked on yield, Latex Diagnostic, induction of TPD and differential gene expression in the latex cells. Some experiments were also performed in order to compare genes expression and latex protein pattern in the latex from spontaneous TPD on RRIM-600 and PB5/51 trees. Some other experiments were initiated to compare some gene expression between clones with extreme characteristics of yield and latex flow (PB 235 and War-4)

During this second period the already initiated experiments (induction of TPD by overexploitation on the RRIM-600 clone) will be continued.
We will particularly perform clonal studies that could not be done during the first period because of the lack of mature trees (too young) in the Chachoengsao RRC: the effects of various intensity of tapping systems (tapping/stimulation) will be compared between various clones, on production, Latex Diagnostic, latex protein pattern and onset of TPD, expression of genes involved in coagulation, regeneration of latex, onset of TPD and if possible in rubber quality (a clonal trial will be opened end of 1995 in the RRC of Chachoengsao)

b - In the lab (at Mahidol University and Chulaborn Research Institute):

i - *identification and validation of molecular markers to be used as probes in the Latex Molecular Diagnostic (LMD).*

- The actual best candidates as putative yield and stress tolerance c-DNA probe markers (of latex flow: hevein, hevein receptor, chitinase; of latex regeneration: Glutamine synthetase; of oxidative stress: MnSOD, Glutathione reductase) will be used in clonal trials (with/without stimulation) to verify their ability to be used as true "latex yield probes". After we will verify these best candidate markers can be used at an early stage of selection (6 - 24 month young trees from various characteristic clones).

- Other putative yield markers will be tested with heterologous probes. The homologous cDNA clones of the eventual best new putative markers will be cloned from our latex c-DNA libraries, and used in clonal trial tests.

- The homologous cDNA probes of the Rubber Elongation factor (REF) and the Farnesyl Pyrophosphate Synthetase (FPPS) (which determine *cis*-polyisoprene chains initiation and elongation) that we have just cloned through PCR, will be checked for their ability to serve as rubber quality markers (the rubber chains length homogeneity partialy determine rubber quality) as far as the rubber quality (P_0, PRI,...) from the latex of the clones under trial will be characterized by the specialists.

- The best probes will be used for the screening of rubber trees from small holdings in various environment and physiological conditions.

ii - *Tapping Pannel Dryness:*

- The ability of our already cloned oxidative stress homologous cDNA probes (Mn-SOD and GR) to be used as early markers of overexploitation (overtapping and over-stimulation) will be verified.

- We will try to get homologous Cu-Zn SOD and catalase cDNA or gene fragments clones and verify as well their abilty to be used as early markers of overexploitation.

- We will go on purifying some of the proteins that are overexpressed (22 kDa and ≈17 kDa) or which desappear (≈ 33-35 kDa) in the latex during the onset of natural or induced TPD, and try to do so for the *de novo* expressed 29 KDa protein (very low amount). These purified proteins will be used to raise specific antibodies and for peptide microsequencing.
  The obtained antibodies will be tested for immunological early detection of TPD and for attempting to clone the corresponding c-DNA through a latex (TPD) expression c-DNA library and get back to the genes (determine th function of the coresponding proteins).
  The peptide microsequences will be used to design specific oligonucleotides in order to clone the corresponding c-DNA either through PCR or through the screening of our cDNA libraries. The obtained c-DNA clones will be tested for their ability to be used as specific markers of overexploitation and induction of TPD.
  The same probes will be tested as putative markers of sensitivity to TPD in the rubber wood gardens and gerplasm.

iii - Cloning and characterization of putative usefull Hevea gene promotors:

- We have already cloned gene fragments upstream the 5' end of the hevein (0.7 to 1.8 kb) and glutamine synthetase (1.5 to 4 kb) cDNA from our genomic library. Theses clones will be used to characterize the hevein (highly and specifically expressed in the latex cells) and GS (sensitive to ethylene) promotors which may be usefull in the future for the eventual genetic engineering of the rubber tree.

- We will try to clone, in the same way, other gene promotors such as the one corresponding to the hevein receptor gene that is highly and very specifically expressed in the latex cells.

The characterization of laticifer specific gene promotors will need studies through transient gene expression. To do so, specific vectors including the putative hevea gene promotor fragments upstream a gene reporter such as GUS or Luciferase will be designed and transfered by microparticles gun bombardment in pieces of stems and/or somatic embryo (maintained *in vitro*) which already differenciate latex cells. Transient expression will be checked using both the cyto-histoenzymatic and fluorospectrophoto-metric detection.

The characterization of ethylene inducible promotors will be performed through transient expression studies in Hevea leaves or callus protoplasts, after transformation by electroporation according to the methods already set up in our lab.

III - FUTURE OUTPUTS

The obtained molecular probes will be used as "Latex Molecular Diagnostic" tools, through the analysis of small samples of latex (few drops to few mililiters), for :

- the estimation of the physiological status of the latex producing tissues of rubber trees under exploitation, allowing to give recommendations to rubber planters, in order to avoid TPD risks, to get higher yield, better manpower profitability and extended economic life span of rubber trees

- the early estimation (which could be achieved on 10-15 months, instead of 5-6 years old, young trees) of the yielding and rubber quality potentials, as well as the tolerance

to various stresses, of parents and progeny in the rubber breeding programs, will significantly reduce the duration, therefore the operating cost (labor and experimental field area), for the breeding and selection of high yielding, and more adapted clones of rubber.

The planting of such new clones, and later of clones emerging from genetic engineering, associated with the use of suitable tapping systems, should allow to at least double the mean yield/ha of the new areas to be planted or replanted, within 10-20 years.