รายงานวิจัยฉบับสมบูรณ์

โครงการโคลนและศึกษาการแสดงออกของยีน
ที่เกี่ยวข้องกับการเกิดโรคเปลือกแห้งในต้นยางพารา

Cloning and Expression of Genes Potentially Involved in the Onset of the Tapping Panel Dryness (TPD) in the Rubber Tree (*Hevea brasiliensis*)

โดย

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เสร็จโครงการในเดือนสิงหาคม 2545

สัญญาเลขที่ BGJ/12/2543

รายงานวิจัยฉบับสมบูรณ์

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ผู้วิจัย

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สังกัด

ภาควิชาเทคโนโลยีชีวภาพ

คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

ผู้วิจัย ขอขอบพระคุณ สำนักงานกองทุนสนับสนุนการวิจัย เป็นอย่างสูง สำหรับทุนวิจัยองค์ความรู้ใหม่ในการสร้างนักวิจัยระดับปริญญาเอก สัญญาเลขที่ BGJ/12/2543 โครงการโคลนและการศึกษาการแสดงออกของยีนที่เกี่ยวข้องกับการเกิดโรคเปลือกแห้งในต้นยางพารา เพื่อสนับสนุนเพิ่มเติมในการทำการวิจัยของ นางสาวอัญชีรา สุขมากซึ่งเป็นนักศึกษาระดับปริญญาเอก ในหลักสูตรปรัชญาดุษฎีบัณฑิต สาขาเทคโนโลยีชีวภาพ สังกัด ภาควิชาเทคโนโลยีชีวภาพ คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล ที่ได้รับทุนโครงการปริญญาเอกกาญจนาภิเษก สำนักงานกองทุนสนับสนุนการวิจัย รหัส 2.B.MU/42/F.1 โครงการนี้ดำเนินการเป็นเวลา 2 ปี ระหว่าง 1 กันยายน 2543 – 31 สิงหาคม 2545 ซึ่งในส่วนงานวิจัยในโครงการนี้ ผู้วิจัยขอขอบคุณ Dr. Herve Chrestin ที่ GeneTrop Laboratory, IRD (ex-ORSTOM), Montpellier, France ซึ่งได้กรุณาให้คำปรึกษาและแนะนำแนวทางที่เป็นประโยชน์ต่อโครงการวิจัยมาโดยตลอด ขอขอบคุณ ศูนย์วิจัยยาง ฉะเชิงเทรา และ The SOGB industrial rubber plantation in Ivory Coast, Africa ที่ให้ความช่วยเหลือด้านแปลงปลูกพืชทดลอง และการเก็บตัวอย่างมาศึกษาตลอดโครงการวิจัยนี้

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รหัสโครงการ: BGJ/12/2543

ชื่อโครงการ: การโคลนและการศึกษาการแสดงออกของยีนที่เกี่ยวข้องกับการเกิดโรคเปลือกแห้งในต้นยางพารา

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การปลูกยางพาราซึ่งเป็นพืชเศรษฐกิจที่สำคัญของประเทศไทย มักประสบปัญหาเรื่องโรคของต้นยาง โดยเฉพาะโรคเปลือกแห้ง (Tapping Panel Dryness -TPD) ทำให้ผลผลิตลดลงหรือหยุดให้ผลผลิต สาเหตุของอาการเปลือกแห้งยังไม่ปรากฏหลักฐานแน่ชัด เพียงแต่สรุปว่าเป็นความผิดปกติทางสรีรวิทยาซึ่งเกิดขึ้นเองตามธรรมชาติ ความถี่ในการกรีดเอาน้ำยางและการใช้น้ำยากระตุ้นให้น้ำยางไหลมากเกินไป มีความสัมพันธ์กับการเกิดอาการเปลือกแห้ง หากปล่อยให้ต้นยางเป็นโรคเปลือกแห้งจนกระทั่งเกิดอาการเปลือกแห้งแบบถาวร จะทำให้ไม่สามารถทำการกรีดยางได้อีก นอกจากนั้นเนื้อไม้ก็เสียหายด้วย ดังนั้น ข้อมูลพื้นฐานเกี่ยวกับโปรตีนที่เกี่ยวข้องกับโรคเปลือกแห้งจะทำให้เข้าใจองค์ความรู้เกี่ยวกับโรคนี้มากขึ้น ซึ่งจะนำไปสู่การพัฒนาปรับปรุงพันธุ์ยางพาราและระบบกรีดยางที่มีประสิทธิภาพและหลีกเลี่ยงการเกิดอาการเปลือกแห้งแบบถาวรได้ในอนาคต ในโครงการนี้ผู้วิจัยได้ทำการโคลนและศึกษาลักษณะของ cDNA ของยีนที่เกี่ยวข้องกับการตอบสนองต่อความเครียดของต้นยางพารา คือ Cu/Zn-superoxide dismutase (Cu/Zn-SOD), ascorbate peroxidase (APX), และ glutathione peroxidase (GPX) ของยางพารา พบว่า sequence ของ cDNA ที่แยกได้มีความเหมือนกับที่พบในพืชชนิดอื่นที่เคยมีรายงาน และเนื่องจากไม่พบ signal peptide จึงคาดว่า เมื่อถอดรหัสแล้วโปรตีนจะอยู่ใน cytosolic compartment การแสดงออกของ GPX ในต้นยางที่ให้น้ำยางปริมาณมาก จะสูงกว่าอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับต้นยางที่ให้ผลผลิตปริมาณน้อย การกรีดยางมากขึ้นจะทำให้ GPX มีการแสดงออกน้อยลงแม้ดูภายนอกต้นจะเป็นปกติ ในขณะที่ต้นที่แสดงอาการเปลือกแห้ง (TPD) ก็มีการแสดงออกของ GPX น้อยอย่างชัดเจนชี้ให้เห็นว่า เกิดการ uncompensate ของ oxidative stress ในเนื้อเยื่อที่ผลิตน้ำยาง ที่น่าสนใจคือ ต้นที่แสดงอาการเปลือกแห้งร่วมกับ Trunk Phloem Necrosis (TPN) กลับมีการแสดงออกของ GPX สูงเป็นปกติ ข้อมูลนี้ทำให้ทราบว่า ต้นที่แสดงอาการเปลือกแห้งแบบ TPD และ TDN ที่เห็นความแตกต่างทางสรีระน่าจะมีความแตกต่างในเรื่องสาเหตุการเกิดอาการด้วย จากการศึกษาพบว่า เมื่อทำการกรีดหรือกระตุ้นต้นยางให้ผลิตน้ำยางบ่อยเกินไป การแสดงออกของ GPX และ APX จะมากสอดคล้องกัน ซึ่งอธิบายได้ว่า ในสภาวะเครียดเช่นนี้ เซลล์ต้องการทั้งสองเอนไซม์ซึ่งทำหน้าที่คล้ายกันในการกำจัด H_2O_2 และ reactive oxygen นอกจากนั้นเป็นไปได้ว่า GPX ซึ่งมีแอคติวิตี้ของ phospholip hydroperoxide glutathione peroxidase (PHGPX) จะทำงานกับ hydroperoxides และ large thiol compounds ซึ่งไม่สามารถกำจัดได้ด้วยเอนไซม์ในกลุ่ม APX และ catalase เราทราบว่า phospholipid hydroperoxide เป็น substrate ที่เหมาะสมของ PHGPX ใน *in vitro* จากการศึกษานี้ ทำให้เกิดจุดน่าสนใจใหม่ว่า phospholipid ใน biomembrane จะสามารถเป็น substrate ที่เหมาะสมของ PHGPX ใน *in vivo* หรือไม่ จึงควรมีการศึกษาบทบาทของ PHGPX ที่เกี่ยวข้องกับ การเปลี่ยนแปลงทางสรีระของต้นยางพาราเพื่อตอบสนองต่อความเครียดต่อไป

คำหลัก : ยางพารา, สภาวะเครียด, โรคเปลือกแห้ง, Cu/Zn-superoxide dismutase, ascorbate peroxidase, glutathione peroxidase

ABSTRACT

Project Code: BGJ/12/2543

Project Title: Cloning and Expression of Genes Potentially Involved in the Onset of theTapping Panel Dryness (TPD) in the Rubber Tree (*Hevea brasiliensis*)

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Among many problems related to the development of rubber production, the relative low rubber yield per area is the most serious problem. One of the main limiting of the rubber yield is a physiological disorder called Tapping Panel Dryness (TPD), a common disease characterized by the reduction or total cessation of latex flow. The objectives of this research are find out some biochemical and/or molecular markers related to yield and stress response of rubber tree, as well as to investigate the role of oxidative stress in the onset of TPD of rubber tree. Three full-length cDNAs encoding Cu/Zn-superoxide dismutase (Cu/Zn-SOD), ascorbate peroxidase (APX), and glutathione peroxidase (GPX) have been cloned and characterized. They showed high homology with the same enzymes in the other plant species. Absence of signal peptide indicates that the corresponding proteins are addressed in the cytosolic compartment. Only the GPX activity and GPX gene expression were showed to be significantly higher in the latex from healthy tree from the high yielding clone compared to the low yielding clone. Overtapping treatment induced a decrease in both GPX activity and gene expression even in the still healthy tree. Moreover, compared to healthy tree, the tree exhibiting TPD symptom showed decreased in GPX activity and gene expression. These evidences confirm the occurrence of uncompensated oxidative stress in the latex producing tissues. Contrary, TPD trees exhibiting Trunk Phloem Necrosis (TPN) were characterized by both higher in latex GPX activity and gene expression. This result suggested that the 2 symptoms (TPD and TPN) differ in their physiological effects and probably in their origin. In some cases, such as in overexploitation experiment, the APX gene was expressed in the same way as GPX gene. Since these two enzymes (APX and GPX) can detoxify H_2O_2, they may be much more required in condition that cell contains high concentration of H_2O_2 or other ROS. Alternatively, *Hevea* GPX contained phospholip hydroperoxide glutathione peroxidase (PHGPX) activity, which might be restricted to more complex acceptor or donor substrates such different organic hydroperoxides or large thiol compounds that are not metabolized by other antioxidant enzymes such as APX and catalase. Although the best substrates for PHGPX *in vitro* are phospholipid hydroperoxides, it is not clear whether such phospholipid that comprises biomembranes are the natural substrate of the enzyme. The physiological role of *Hevea* PHGPX is a subject of future study.

Keywords : Rubber tree, Oxidative stress, Tapping Panel Dryness, Cu/Zn-superoxide dismutase, ascorbate peroxidase, glutathione peroxidase

INTRODUCTION

Rubber tree is a major economic crop in Thailand. Moreover, the status of rubber production is predicted to increase, due to a replanting program as well as to the expansion of new planting areas in the Northeast region of Thailand. Thailand increased its output from less than 500,000 tons in 1978 to more than 2 millions tons in 2002 and has ranked as the first natural rubber production in the world since 1991 (Loyen 1998). Among many problems related to the development of rubber production, the relatively low rubber yield per area and increasing labor cost are the most serious problems.

The three main physiological limiting factors of rubber yield are :

- the duration of the latex flow, which is limited by coagulation process,
- the metabolic potential to regenerate the lost latex between two tappings,
- A physiological disorder: the Tapping Panel Dryness (TPD), a common disease characterized by the reduction or total cessation of latex flow.

The widely used stimulation of *Hevea* bark with ethylene generators (Ethephon), can transitorily overcome the first two limiting factors. But over-exploitation, especially overstimulation can induce TPD. TPD was reported for the first time in Brazil in 1887 in *Hevea* in the Amazon forest and at the beginning of the century in plantations in Asia (Rutgers and Dammerman, 1914). The symptom range from partial dryness with no browning of the tapping cut, browning and thickening of the bark and cracking and deformation of the bark in some instances. The syndrome is characterized by the appearance of tylosoid and the coagulation of latex *in situ* (de Faÿ, 1981; de Faÿ and Hebant, 1980; Paranjothy *et al.*, 1976), abnormal behavior of the parenchyma cell adjoining the laticifers and general increase in synthesis of polyphenol (Rand, 1921). A detailed review of the histological, histochemical and cytological study of the disease bark was presented by de Faÿ and Jacob (1989).

TPD usually develops as follow. Its onset is often marked by a phase of excessive late dripping with a simultaneous drop in dry rubber content. After a while, the volume of latex per tapping gradually declines, and this is attributed to a reduction in the colloidal stability of the latex leading to damage of the lutoid particles, flocculation of rubber particles and plugging of the latex vessels. As this process continues, the entire laticiferous system in the vicinity of the tapping cut becomes clogged, and latex flow stops. This phenomenon is

normally localized, but may slowly spread across the whole tapping panel. Subsequently, secondary symptoms such an abnormal bark growth may develop.

A number of studies of biochemical and physiochemical features associated with TPD have been summarized (Sethuraj, 1988). There is a sharp increase in bursting index, the ratio of free acid phosphotase activity to total acid phosphatase activity, which is indicator of intactness of lutoid. Lutoid stability and hence latex stability are affected. Moreover, there are complex enzymatic changes and a reduction in serum, protein and RNA. The changes in levels of certain ions and in mineral ratios are observed. It can be added the indications that TPD is often associated with an abnormal decrease in levels of sucrose reflecting with changes in metabolic activity (Jacob and Prevot, 1989) and copper ions (Yeang, 1989), which are known to help stabilize rubber particle.

It is well known that sensitivity 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 (GT 1, RRIM 600) have less susceptibility, while those with the lowest activity (PR 107, PR 261, AV 2027) are the least susceptible (Commere *et al.*, 1989; Eschbach *et al.*, 1989; Luckman, 1989; Sivakumaran and Haridas, 1989; Somnark *et al.*, 1994; Sukolrod, 1994). Apart from genetic effects, it should also be emphasized that some environmental or cultural factors may effect the TPD syndrome, for example the wind damage, the stock and scission relationship, the way of tapping, the ecoclimatic factor (most probably drought and soil composition) (Commere *et al.*, 1989).

A considerable amount of work for exploring the cause and mechanism has been carried out. (Nandris and Chrestin, 1991; Nandris *et al.*, 1991). The hypothesis of pathogen involvement has not been ruled out but has yet to be proved, though rickettsiae has been found in some very particular cases, in disease tissues. 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.

The two types of TPD have been reviewed by Jacob *et al.* (1994) :

1. The irreversible necrotic symptom or Trunk Phloem Necrosis (TPN)

It appears randomly for unknown reasons in a plantation and then spread, often along the lanes of trees. The bark of affected trees often shows dark necrotic areas. Histochemical and cytological studies have shown complete disorganization of the bark tissues, with formation of tylosoid (invagination of disorganization of parenchyma cells into

the latex cells) and hyperplastic tissues, in situ latex coagulation, lignification of the cell walls and accumulation of tannins. Even though this kind of necrotic TPD seems not to be directly attributable to overexploitation, exploitation of the trees favors its development.

2. The overexploitation-induced TPD (TPD)

It has been said that trees succumb to TPD when the level of exploitation exceeds the physiological capability of the tree to regenerate latex (Sethuraj, 1988). Indeed, the incidence of TPD is increased by a higher tapping frequency (overtapping-induced TPD) and/or by excessive use of ethephon as a stimulant of latex production (overstimulation-induced TPD). This kind of TPD may or may not be accompanied by necrosis of the bark tissues. It may be reversible if the trees are allowed to rest.

Biochemical experiments brought the first indications of the oxidative stress involvement in TPD; in healthy trees stimulated with ethephon, the activity of NAD(P)H oxidase was found to increase but was compensated by an increase in SOD and catalase activities. The pool of reduced thiols decreased (which reflects their consumption in response to oxidative stress) but then returned to its initial level. When trees were submitted to overstimulation (up to 8 treatments per year), some trees developed typical symptoms of bark dryness. In these (partially) dry trees, the activity of the NAD(P)H oxidase was much higher, but the SOD and catalase activities as well as the stock of reduced thiols decreased and never regained their initial levels. It was also observed that the "bursting index" (which reflects the fragility of the lutoid membranes) was considerably higher in TPD trees than in healthy trees. Also it was reported a lipid peroxidation activity in the latex from TPD tress. The hypothesis is that deficiencies in the antioxidant system resulted in excessive degradation of the lutoid membrane and consequently abnormal release of coagulating factors. This cause the rubber particles aggregation and early plugging of the laticifers upon tapping and even *in situ* latex coagulation which may completely obstruct the latex cell net. (Chrestin, 1989).

Northern blot analysis using homologous c-DNA probes indicated that ethylene stimulation could induce over-expression of catalase and MnSOD genes in the latex. In the contrary onset and development of overstimulation-induced TPD was associated with a decrease in both catalase and MnSOD in the latex from the diseased trees (Kongswadworakul *et al.*,1997).

The objectives of this research are find out some biochemical and/or molecular markers related to yield and stress response of rubber tree, as well as to investigate the role of oxidative stress in the onset of TPD of rubber tree. The strategy is, first, to quantify some major enzymatic activities involved in the process of ROS scavenging in the latex from control healthy trees, spontaneous and ethylene-induced TPD trees. The cDNA encoding the enzymes that will have been evidenced to be involved in TPD will be cloned and then used as probe for gene expression analysis according to the physiological status of the laticifers.

These studies may lead to the proposal of new tools (biochemical or molecular markers) for rubber improvement and research programs, as well as for the latex (or bark) diagnosis of the physiological status of the tapping panel in rubber plantation. Furthermore, the knowledge acquired at the physiological and molecular level on rubber yield limiting factors should be of great help to select the key targets for the future genetic modification of the rubber tree.

MATERIALS AND METHODS

Plant material

Hevea brasiliensis : RRIM 600, AVROS 2037, PB 235, PB 260, and PB 314 clones from experimental plots in in RRIT, Center of Chachoengsao, Thailand and in the SOGB industrial rubber plantation in Ivory Coast, Africa, were used in this experiment.

Clonal typology : Three clones known for their different yield and metabolism potential were chosen to study the eventual relationships between yield potential, enzymatic activity and gene expression in the latex cells (18 to 20-year-old trees).

Control (not stimulated) or Ethrel stimulated trees (more than 3 months after the last stimulation), homogenous in girth, were selected from these 3 clones. Their rubber production was checked which allowed to setup an inter- and intra-clonal yield classification. Their health status was verified allowing their classification as healthy or diseased (TPD or TPN) trees.

Incidence of Trunk Phloem Necrosis : Six apparently healthy and 6 TPN trees (12-year-old homogeneous in girth) of the PB 260 clone, which is very sensitive to TPN, were selected.

All trees were tapped every 5 days and had not been stimulated for more than one year. Their latex was collected for biochemical analysis.

Effects of over-exploitation : This experiment was set up in order to try to induce physiological fatigue, able to lead, in the long term, to TPD symptoms. Three batches of 20 homogeneous (girth and yield) apparently healthy (no TPD nor TPN symptom) trees were set and submitted to different treatments: i) control (normal exploitation): tapped every 5 days, with 4 stimulations (5% Ethrel)/year, applied on the tapping cut; ii) over-tapping: tapped every 2 and 3 days, without stimulation; iii) over-stimulation: tapped as the control, but stimulated every 2 weeks with 5% Ethrel on the tapping cut.

Eventual onset of bark disease symptoms was checked every 3 months by visual observation of the tapping cut of each tree: extension of the dry area associated TPD, or TPN with the development of necrotic area close to the cambium. Rubber yield was checked on the tree to tree basis, at the initiation and after 1 year of the experiment, by weighting the cumulated air-dry rubber production of 6 or 8 tappings and was expressed as gram of dry rubber/tree/tapping (g/t/t). Latex was collected individually from 6 trees per treatment (healthy and eventually exhibiting symptoms of bark disease) for RNA extraction and biochemical analysis after 12 months experiment.

Kinetics of "bark opening" and ethylene stimulation effects : This experiment was performed to study the effect of tree "opening" (starting exploitation of trees through the first tapping) and the kinetics of exogenous ethylene effect on some biochemical characteristics and gene expression in the latex cells. As virgin (never tapped) trees, ready to be opened for exploitation were needed, the experiment was set up in the only plot available at this time, planted with 6-year-old trees of the PB 314 clone, which has yield and metabolism potential close to the PB 235. Six batches of 6 homogenous (girth) virgin trees were set up : one as a control (scrapped bark/no stimulation) and the others were treated on a 1 cm large lightly scrapped bark band, just beneath the future tapping cut, with 5% Ethrel at 6, 12, 24, 36, 48 hours, respectively, before the first tapping as described by Pujade-Renaud *et al.*, 1997. All trees were tapped on a half spiral cut and the latex was collected on the same day for RNA extraction and biochemical analysis. Trees were subsequently tapped every two days, and the latex was collected and analyzed on the 1^{st}, 2^{nd}, 3^{rd}, and 5^{th} tapping.

Methods

1. cDNA library construction

Total RNA was extracted from latex. Poly(A^+) RNA was isolated on oligo-dT cellulose column. cDNA was synthesized from 2.5 µg of Poly(A^+) RNA from latex of ethylene-induced TPD trees to increase the chances to isolate cDNA of genes overexpressed by ethylene and TPD symptom. The kit that was used in this experiment is the ZAP Express™ cDNA synthesis kit (Stratagene, USA) because it allows orientated cloning of the synthesized cDNA : ecru adapters were added at the 5'end of the cDNA and XhoI primers at the 3' end, in order to force ligation of the cDNA into the ecru-XhoI compatible ends of the vector in an orientated way.

In order to check the quality of the double-stranded cDNA, a PCR amplification was performed using 2 primers designed to specifically amplify a 0.2 kb fragment from the 5' end of a 1.5 kb clone coding for glutamine synthetase (Pujade-Renaud *et al.*, 1997). A 0.2 kb fragment could indeed be amplified using the double-stranded cDNA as template, which means that the elongation of the 1st strand cDNA was at least 1.5kb. The average size of the mRNA in a plant cell is about 1 kb. Therefore we can expected that the majority of the latex mRNA has been correctly converted into 1st strand cDNA.

The cDNA was cloned in a Lamda ZAP (pBluscript SK(+/-)) phagmid vector using the ZAP Express™ cDNA Gigapack® II Gold cloning kit (Stratagene). Then, the library in lamda vectors was amplified to make a large, stable quantity of a high titer stock of the library. After the amplification, the titer of this cDNA library was about $8x10^8$ pfu/ml.

2. Cloning of full-length latex cDNAs encoding a cytosolic Cu/Zn superoxide dismutase (Cu/Zn-SOD), ascorbate peroxidase (APX), and glutathione peroxidase (GPX)

2.1 Synthesis of the partial cDNA fragments coding for Cu/Zn SOD, APX, GPX

The degenerated oligonucleotide primers were designed on the basis of well-conserved amino acid sequences of Cu/Zn superoxide dismutase, ascorbate peroxidase and glutathione peroxidase from other plants. PCR was performed using our latex specific cDNA

library from ethephon-stimulated rubber trees or double stranded latex cDNA as template. The purified cDNA fragments generated by PCR were ligated into the pGEM-T Easy vector purchased from Promega (USA). PGEM-T Easy processing a single 3'deoxythyamine (T) overhangs at both ends facilitates the insertion of the PCR products ending with a single 5'deoxyadenine (A). After transformation, the nucleotide sequence of cloned PCR fragments was determined by dideoxynlucleotide chain termination method by using the primer T7 and subsequently compared with the published sequence database, GENBANK by BLAST Search at www.ncbi.nlm.nih.gov/.

2.2 cDNA screening

The library was screened using the PCR-amplified cDNA fragment that had been ^{32}P-labeled by random priming using the Rediprime kit from Amersham. Hybridization were performed overnight at 65^{o}C, in 5X SSC (0.3M NaCl, 30 mM trisodium-citrate, pH 7), 10X Denhardt's reagent (0.2% ficoll, 0.2% PVP, 0.2% BSA), 7% SDS and 20 mM sodium phosphate buffer (pH 7.2). Final washes were carried out at 65^{o}C in 1X SSC and 1% SDS. For the positive clones, the cDNA-containing phagemid (pBK-CMV) was excised using the ExAssist helper phage (Stratagene) and subsequently amplified in XLOLR *E. coli* cells.

On the other hand, the positive clones could be amplified by PCR using T3 and T7 primers. Then, PCR products were cloned into pGEM-T Easy vector and transformed to *E.coli* DH5α competent cell. This transformant could be further characterized.

3. Study of gene expression in *Hevea* latex by northern blot analysis

Samples of 10 µg total RNA were separated in a 1.2% agarose/formaldehyde gel (Sambrook *et al.*, 1989) and transferred onto nylon membranes (Hybond N^{+}, Amersham). Sample loading was checked by staining RNA with methylene blue, after transfer. The membranes were hybridized overnight at 65 ^{o}C with a partial or a full-length cDNA specific probes of gene of interest (Cu/Zn-SOD, APX, GPX) that had been ^{32}P-labeled by random priming using the Rediprime kit from Amersham. Hybridization was performed in 5X SSC (0.3M NaCl, 30 mM trisodium-citrate, pH 7), 10X Denhardt's reagent (0.2% ficoll, 0.2% PVP, 0.2% BSA), 7% SDS and 20 mM sodium phosphate buffer (pH 7.2). The membranes were washed sequentially in the appropriate volume of 3X SSC, 2X SSC, 1X SSC plus 1%SDS at

65°C for 30 min each and 0.5X SSC, 0.1X SSC plus 0.5%SDS at 65°C for 30 min each. The washed membranes were wrapped with plastic wrap and exposed to the x-ray film (Kodak) in a cassette containing an intensifying screen, at -80°C for an appropriate period depending upon the radioactivity of the probe. Hybridization signal was observed by developing the exposed film in the developer and fixer, respectively.

Gene expression in *Hevea* latex was studied in response to ethylene stimulation, tapping intensity, in different case of TPD (spontaneous necrotic TPD, over-exploitation-induced TPD), clones, and yields.

4. Enzyme activity assay

The cytosolic serum (C-serum) was isolated from deep-frozen latex (stored at -80°C) by two times of 60,000g centrifugation for 45 min at 4°C and frozen at -20°C for storage before enzyme analysis.

4.1 Cu/Zn-superoxide dismutase activity assay

SOD activity was determined by quantitative method which is based on the inhibition of nitroblue tetrazolium (NBT) reduction by the superoxide anions generated from the xanthine-xanthine oxidase reaction (McCord and Fridovich, 1977).

The assay mixture consisted of 1.5 ml solution (solution A) containing 200 µl of 0.1 M phosphate buffer (K_2HPO_4, KH_2PO_4) pH 7.8, 1000 µl of 1 mM xanthine, 200 µl of 2 mM NBT, 100 µl xanthine oxidase (300 mU/ml). Prior to addition of the enzyme extract, the absorbance of solution A at 560 nm was followed for 3 min at a 30-second interval to establish a baseline slope. The enzyme extract (10 µl) was then added to solution A and absorbance measurement was continued every 30 second for another 3 min. The result slope from 4 min to 6 min was then compared to the slope of the first 1 min to 3 min to determine if the test solution could reduce the rate of NBT reduction (Absorbance at 560 nm (A_{560} increase). Each sample was assayed at least three times.

To calculate the unit of SOD activity in the assay fraction, the rate of A_{560} increase from 4 to 6 min was subtracted from the initial rate from 1 to 3 min. The resultant was then

multiple by dilution factor of the enzyme extract. These calculated units of SOD activity were then normalized for protein content by dividing by mg protein in the 10 µl sample assayed.

4.2 Ascorbate peroxidase activity assay

Ascorbate peroxidase (APX) activity was determined by method of Nakano and Asada (1981) with some modification. This procedure measures the rate of oxidation of ascorbic acid at 290 nm (ε = 2.8 mM^{-1} cm^{-1}). The typical reaction mixture (1ml final volume) contained 50 mM phosphate buffer (pH 6.25), 0.5mM Ascorbic acid, 0.29 mM H_2O_2 and a rate limiting amount of *Hevea* APX. The nonenzymatic oxidation rate of Ascorbate was determined in the absence of *Hevea* APX and was subtracted from the one determined in the presence of all components. A unit of APX activity is expressed as the amount of enzyme required to oxidize 1 µmol of ascorbic acid/min/ml. Protein concentrations were determined using the dye binding assay of Bradford (1976).

4.3 Gutathione peroxidase activity assay

Glutathione peroxidase activity was determined by the coupled enzyme method of Paglia and Valentine (1967) with some modifications. This procedure measure the rate of oxidation of NADPH at 340 nm in the presence of Glutathione reductase with catalyzes the reduction of GSSG produced by GPX activity. The typical reaction mixture (1 ml final volume) contained 50 mM Phosphate buffer (pH 7.5), 0.16 mM NADPH, 1.0 mM GSH, 1.0 IU Glutathione reductase, 0.15 mM H_2O_2, and a rate limiting amount of *Hevea* GPX. The reaction was initiated by the addition of hydrogenperoxide, and the disappearance of NADPH was followed spectrophotometrically. The nonenzymatic oxidation rate of GSH was determined in the absence of *Hevea* GPX. The NADPH peroxidase was also determined in the absence of Glutathione reductase and GSH. Then the nonezymatic rate and NADPH peroxidase rate was subtracted from the rate determined in the presence of all components. A unit of GPX activity is expressed as the amount of enzyme required to oxidize 1 µmol of NADPH/min/ml under the described assay conditions (Duan *et al.*, 1988). Protein concentrations were determined using the dye binding assay of Bradford (1976).

Result

1. Cloning and characterization of a full-length latex cDNA coding for each Cu/Zn-SOD, APX, and GPX

1.1 Screening of the latex cDNA library

The *Hevea* partial cDNA fragment probes, CZS3, APX400, and GP6 synthesized by PCR amplification were used to screen the latex cDNA library by the same procedure in order to fish out the full-length cDNA coding for Cu/Zn-SOD, APX, and GPX, respectively. About 50,000 recombinant phages were transferred onto nylon membrane. The target cDNA inserted in the ZAP phage vector was detected by hybridization with the appropriate probe labeled with the α^{32}P-dCTP. Labeling and hybridization were performed as described in Materials and Methods.

From the primary screening, 20 positive clones (hybridized by the probe) were isolated and purified. Secondary and tertiary screening were performed in order to isolate only single well isolated positive plaques.

After tertiary screening, twenty positive recombinant clones that gave strong hybridization signals were selected for further characterization by PCR. PCR amplification was performed directly on recombinant phages using the primer T3 and T7 which bind on phagemid sequences farming the insert. Three clones contained insert of the expected size were selected for sequencing to determine whether they were full-length cDNA of each gene by using T3 and T7 as the primer for the cycling sequencing reaction. The full nucleotide sequence was determined by the didoxynuclotide chain termination method. From the sequences obtained with primer T3 and T7 (5' end and 3' end of the insert), new primer were designed, used for further sequencing, and so on, until both strand were completely sequenced.

2. Sequence analysis of the isolated cDNA

2.1 A full-length latex cDNA coding for Cu/Zn-SOD

The longest full-length cDNA clone, named CZS11.1.1, was obtained, and completely sequenced on both strands. The nucleotide and deduced amino acid sequences of CZS11.1.1 are presented in Fig1. The cDNA, CZS11.1.1 was 738 bp in length and contained

an open reading frame of 456 bp-long, followed by 188 bp 3' untranslated sequence terminated by a string of A residues. Putative polyadenylation signal, AAUAA at the position 551 to 554 was found about 180 bp upstream from the 3' end of cDNA. The cDNA encoded for the protein of 152 amino acids in length and have a calculated molecular mass of 15.6 kDa and the theoretical pI of 5.67. This sequence was submitted to Genbank Accession No. AF457209.

From the amino acid sequence comparison, CZS11.1.1 showed a high degree of identity with the cytosolic Cu/Zn superoxide dismutase in various plant species. CZS11.1.1 shares the highest homology at 72% and 71% with Cu/Zn superoxide dismutase from *Populus tremuloides* (AF016893) and *Manihot esculenta* (AF170297), respectively (Table 1). Further analysis of this deduced amino acid sequences found that this protein contain two domains of Cu/Zn-SOD signatures, signature 1: GFHVHTFGDTT (position 43-53) and signature 2: GNAGDRIACGII (position 137-148) which are important for activity of this enzyme (Fig. 1).

Moreover, computer analysis of the predicted protein CZS11.1.1 did not reveal any consensus sequence for secreted protein (i.e. signal peptide), which supports the idea that Cu/Zn-SOD is a cytosolic protein. The CZS11.1.1 protein contained three consensus sequences for a N-myristoylation signal (in position 32-37, 36-41, 55-60). Boutin (1997) suggested that myristoylation is the first step of the mechanism by which a protein associates with membrane.

2.2 A full-length latex cDNA coding for APX

The longest full-length cDNA clone, named APX15.1.1, was obtained, and completely sequenced on both strands. The nucleotide and deduced amino acid sequences of APX15.1.1 are presented in Fig. 2. The sequence analyses reveal that APX15.1.1 contained totally 1058 bp with a 753 bp-long ORF sequence followed by 174-bp 3' untranslated sequence terminated by a string of A residues. Putative polyadenylation signal, AAUAA, at the position 1010 to 1014 is found about 50 bp upstream from the 3'-end of cDNA. The encoded protein would be 250 amino acids in length and have a calculated molecular mass of 27.4 kDa which is in good agreement with the MW of other plant APX described by Kim and Chuang (1998) and a theoretical pI of 5.78. This sequence was submitted to Genbank Accession No. AF457210.

Amino acid sequence comparison indicated that APX15.1.1 showed a high degree of identity with the cytosolic ascorbate peroxidase of various plant species. APX15.1.1 had the highest similarity to *Fragaria x ananassa* APX (79%, AF158652), *Pisum sativum* (77%, X62077), *Vigna unguiculata* (76%, U61379), *Nicotiana tabacum* (76%, U15933), *Glycine max* (75%, AF127804), *Capsicum annuum* (75%, X81376) as shown in Table 2. Therefore, it can be concluded that this APX15.1.1 cloned from rubber tree latex does correspond to a full-length APX cDNA.

Further analyses of this deduced amino acid sequence seemed to predict that the protein was devoid of a signal peptide and contained four putative N- myristoylation sites (in positions 45-50, 54-59,73-78, 197-202). The fact that the cloned cDNA does not have a transit peptide support the idea of the existence of this enzyme in the cytosol.

It was found to have the conserved amino acid of APX, H163, D207, and W179 (Fig. 2, underlined). These amino acids are known to make a part of the active site of APX, which is composed of His-Asp-Try catalytic triad, as determined by the three-dimensional structure (Patterson and Poulos, 1995).

2.3 A full-length latex cDNA coding for GPX

The longest "Full-length" cDNA clone, designed as GPX6.2.1 was obtained, and completely sequenced on both strands. The nucleotide and deduced amino acid sequences of GPX6.2.1 are presented in Fig. 3. The nucleotide-sequence analyses revealed that the complete clone GPX6.2.1 contained 890 bp with a 5' untranslated region of 119 bp, an ORF of 531 bp, and 3′ untranslated region of 240 bp terminated by a string of A residues. Putative polyadenylation signal, AAUAA, at the position 682 to 686 is found about 210 bp upstream from the 3'-end of cDNA. The encoded protein would be 176 amino acids in length and have a calculated molecular mass of 19.3 kDa and a theoretical pI of 5.23, which corresponds to the expected size of other plant GPX so far identified (Lopez *et al*, 1996; Holland *et al*., 1993, Criqui et al., 1992). This sequence was submitted to Genbank Accession No. AF242650.

Amino acid sequence comparison indicated that GPX6.2.1 showed a high degree of identity with glutathione peroxidase of various plant species. GPX6.2.1 shares the highest level (86%) of sequence identity with the phospholipid hydroperoxide glutathione peroxidase-like protein in *Arabidopsis thailiana* (AF030132) and glutathione peroxidase-like protein in *Hordeum vulgare* (AJ238697) (Table 3).

The amino acid sequences deduced from this cDNA contain two conserved domains (the glutathione peroxidase signature, PCNQF and WNFSK) which are found in most plant and mammalian GPX (Fig. 3). The two GPX active sites were found at the position 30-45 (GKVLLIVNVASQCGLT) and position 67-74 (LAFPCNQF). The three critical amino acids (cysteine, glutamine and tryptophan) constituting the catalytic core of the GPX enzyme were conserved in latex GPX protein. Further analyses of this deduced amino acid sequence seemed to predict that the protein was devoid of a signal peptide and contained three putative N-myristoylation sites (in positions 43-48, 122-127, 160-165) which support the idea that GPX6.2.1 protein is a cytosolic or membrane bound protein. Moreover, there were 2 sites of ASN-Glycosylation at the position 48-51 (NYTE) and position 132-135 (NFSK).

```
                                                                  ***
  1 - CTCCACCTTATTTGCAGCTTTTTAATTTTCATCTTTAGATCACATAGAACAATGTTGAAG - 60
  1                                                       M  L  K     3
 61 - GCCGTTGCTGTTATTACCAGCAGTGAGGGCATTAGCGGGAAAATCTTCTTCACCCAAGAA - 120
  4   A  V  A  V  I  T  S  S  E  G  I  S  G  K  I  F  F  T  Q  E     23
121 - GGAGATGGTCCAACCACCGTAACTGGAAGTGTTTCTGGCCTTAAGCCGGGGCTTCATGGT - 180
 24   G  D  G  P  T  T  V  T  G  S  V  S  G  L  K  P  G  L  H  G     43
181 - TTCCATGTTCATACCTTTGGAGACACAACAAATGGCTGCTTGTCGACTGGGCTACATTTC - 240
 44   F  H  V  H  T  F  G  D  T  T  N  G  C  L  S  T  G  L  H  F     63
241 - AACCCTGCTAGCAAAGATCACGGTGGCCCTGAGGATGAGAATCGTCATGCTGGTGATCTG - 300
 64   N  P  A  S  K  D  H  G  G  P  E  D  E  N  R  H  A  G  D  L     83
301 - GGAAATGTCAATGTTGGTGATGATGGCACTGCTAATTTCACAATTGTTGACAAGCATATT - 360
 84   G  N  V  N  V  G  D  D  G  T  A  N  F  T  I  V  D  K  H  I    103
361 - CCTCTTTCTGGCCCACATTCCATTGCAGGAAGGTCAGTTGTTTTTCACGAAGGTCGTGAT - 420
104   P  L  S  G  P  H  S  I  A  G  R  S  V  V  F  H  E  G  R  D    123
421 - GATCTTGGCAAGGGGGGACATGAACTCAGCAAAATCACTGGAAATGCTGGTGACAGAATA - 480
124   D  L  G  K  G  G  H  E  L  S  K  I  T  G  N  A  G  D  R  I    143
481 - GCATGTGGTATCATTGGTTTGCAAGAATAGGTTGATTCTCCAGAGAATCATACTAAGAGA - 540
144   A  C  G  I  I  G  L  Q  E  ***                                152
541 - AAGGCAGCTGAATAATTGTGTATTTGCTAGCTGGACAAAATGTTACAAGACCTTCACTCA - 600

601 - TTTTGAATAAAATTAAGAACAAATCGTACTATGCTTTTTGCTGGTTTGATTTTGTGTGAT - 660

661 - GCAAAATTTTGTATGCTTTGTTTGATCTCATAAATTTAAGCTTCCTCTTGGTTTGCTTCA - 720

721 - AAAAAAAAAAAAAAAAA                                            - 738
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Figure 1. The nucleotide sequence of the Cu/Zn superoxide dismutase (Cu/Zn-SOD) cDNA from *Hevea brasiliensis*. The deduced amino acid sequence of Cu/Zn-SOD cDNA is shown below the nucleotide. The codons with asterisks are putative translation start codon (ATG) and stop codon (TAG). The amino acids containing two domains of Cu/Zn-SOD signature at position 43-53 and 137-148 were underlined. The putative polyadenylation signal, AAUAA is framed.

Table 1. Percentage of sequence identity between the *Hevea brasiliensis* Cu/Zn-SOD (AF457209) and other plant Cu/Zn-SOD sequences.

Plant Material	GenBank Accession number	% of identity	
		Nucleotide sequence	Amino acid sequence
Populus tremuloides	AF016893	96	72
Manihot esculenta	AF170297	86	71
Zea mays	X17565	81	71
Paulownia kawakamii	AF037359	80	71
Nicotiana plumbaginifolia	X55974	80	70
Ipomoea batatas	X73139	84	70
Sandersonia aurantiaca	AF479059	nd	70
Ananas comosus	AJ250667	81	70
Glycine max	JW0084	nd	69
Lycopersicon esculentum	X87372	77	69
Mesembryanthemum Crystallinum	U80069	79	68
Oryza sativa	L19434	80	68
Olea europaea	AJ428575	79	68
Capsicum annuum	AF009734	81	68
Arabidopsis thailiana	AY091168	80	68
Zantedeschia aethiopica	AF054150	92	66
Carica papaya	Y13610	81	66

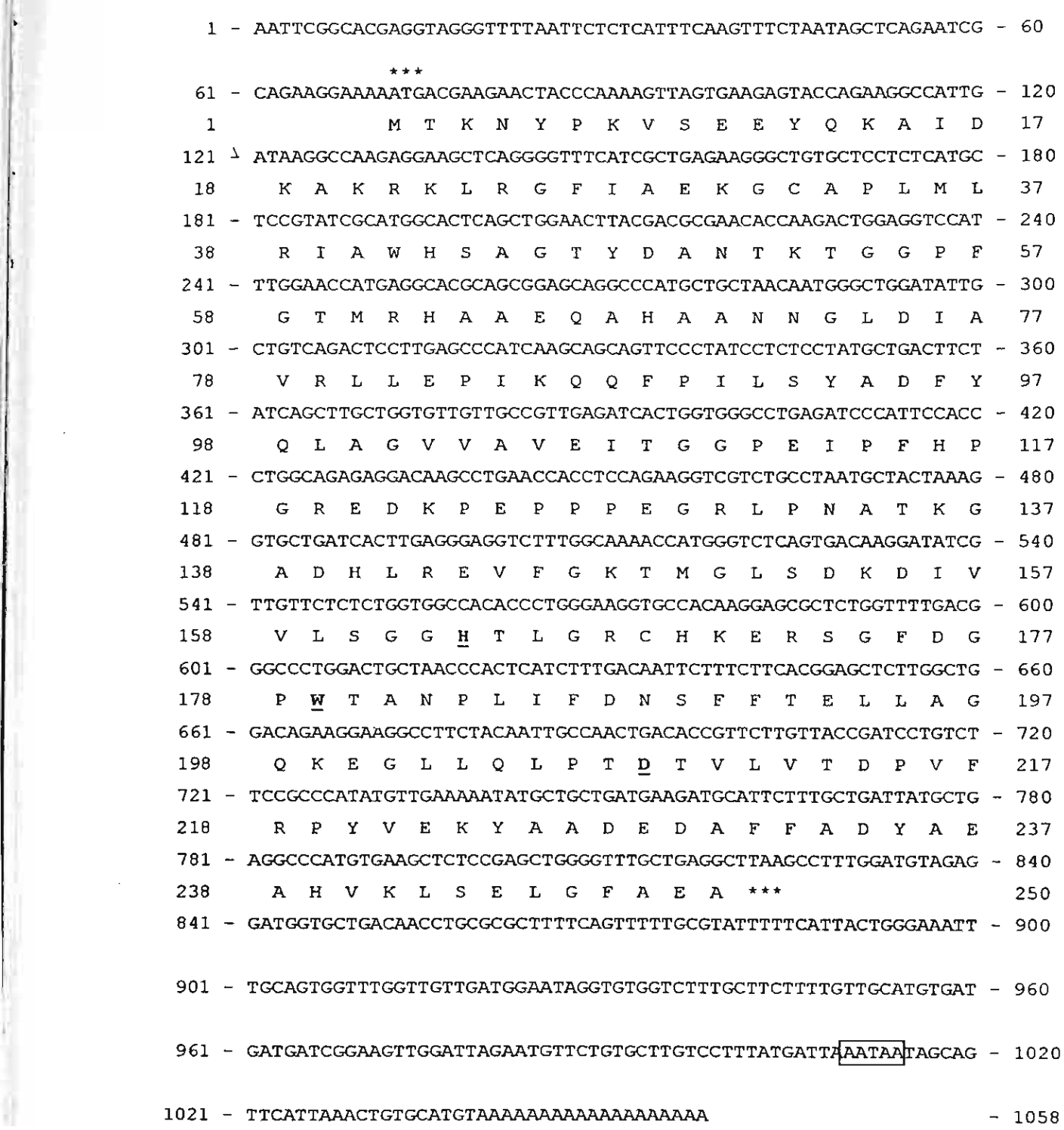

Figure 2. The nucleotide sequence of the ascorbate peroxidase (APX) cDNA from *Hevea brasiliensis*. The deduced amino acid sequence of APX cDNA is shown below the nucleotide. The codons with asterisks are putative translation start codon (ATG) and stop codon (TAA). The three conserved amino acid (H163, D207 and W179)which are known to make a part of active site of APX, composed of His-Asp-Try catalytic triad, as determined by the three-dimensional structure (130), are underlined. The putative polyadenylation signal, AAUAA is framed.

Table 2. Percentage of sequence identity between the *Hevea brasiliensis* APX (AF457210) and other plant APX sequences.

Plant Material	GenBank Accession number	% of identity	
		Nucleotide sequence	Amino acid sequence
Fragaria x ananassa	AF158652	82	79
	AF158653	82	79
Pisum sativum	X62077	82	77
	M93051	82	77
Vigna unguiculata	U61379	81	76
Nicotiana tabacum	U15933	79	76
	D85912	79	76
Cucumis sativus	D88649	79	76
Glycine max	AF127804	84	75
	L10292	84	75
Capsicum annuum	X81376	80	75
Hordeum vulgare	AF411228	79	75
Zantedeschia aethiopica	AF378131	78	75
Pimpinella brachyarpa	AF159380	78	75
Lycopersicon esculentum	Y16773	nd	74
Oryza sativa	AB053297	81	73
Zea mays	Z34934	78	72
Brassia oleracea	AB078599	86	72
Spinacia oleracea	D85864	nd	72

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1   - TCAATTTCGATCAAACCCTTTTGCGTTTTCTTCGCAATTTATCGGTTCCTTTGGTTTCTC - 60
                                                                  *
61  - AGTCCATCACAACAGGAGATTCAAGGCCTGCGTTGTGTTCTTTGAGATTTCATCATACCA - 120
1                                                               M   1
                **
121 - TGGCTAGCCAATCCGAACCTAAATCAGTCTATGACTTCACTGTTAAGGATGCTAGGGGAA   180
2   -   A  S  Q  S  E  F  K  S  V  Y  D  F  T  V  K  D  A  R  G  N   21
181 - ACGATGTTGATCTCAGCACTTACAAGGGGAAGGTTCTCTTGATTGTTAATGTTGCTTCAC   240
22      D  V  D  L  S  T  Y  K  G  K  V  L  L  I  V  N  V  A  S  Q   41
241 - AATGTGGCTTGACAAACTCGAACTACACTGAGCTGACTCAGTTGTACCAGAAATATAAGG - 300
42      C  G  L  T  N  S  N  Y  T  E  L  T  Q  L  Y  Q  K  Y  K  D   61
301 - ACCAAGGCTTGGAGATTCTGGCTTTCCCCTGTAATCAGTTTGGATCTCAGGAGCCAGGTA - 360
62      Q  G  L  E  I  L  A  F  P  C  N  Q  F  G  S  Q  E  P  G  T   81
361 - CCAATGAACAGATTGTGGAGTTTGCTTGTACTCGTTTCAAGGCTGAGTATCCAATATTTG - 420
82      N  E  Q  I  V  E  F  A  C  T  R  F  K  A  E  Y  P  I  F  D  101
421 - ACAAGGTTGATGTGAATGGAAACAATGCTGCTCCTCTTTACAAATTCTTGAAGTCTAGCA - 480
102     K  V  D  V  N  G  N  N  A  A  P  L  Y  K  F  L  K  S  S  K  121
481 - AAGGTGGAATTTTTGGGGACAACATCAAGTGGAATTTTTCCAAGTTTCTGGTTGATAAAG - 540
122     G  G  I  F  G  D  N  I  K  W  N  F  S  K  F  L  V  D  K  D  141
541   ATGGCAATGTTGTTGACCGTTATGCCCCCACTACGGTCCCCTCTCAGCATCGAGAAGGAT   600
142     G  N  V  V  D  R  Y  A  P  T  T  V  P  S  Q  H  R  E  G  C  161
601 - GTGAAGAAACTGCTGGGGATTGCATGAGTGCGCAAGGCAGGCCTAATTAAATTATGGGAG - 660
162     E  E  T  A  G  D  C  M  S  A  Q  G  R  P  N  ***             176
661 - ATAAGTGAGGATATCACTATGAATAAGCACCATGAAGGGATGTACTTTATAGGATCTTTT - 720

721 - TCCCTTCTAAAATCATGTTAGGATGTGCATTGAAGTTAATCCTTTGTACTTTCTGTACTC - 780

781 - TTTCATGATTTCTTTTTCTTTTGGTTCGAATCTGAGTGATTTAATATTTAGACTTTCTAA - 840

841 - GGTATCATGGTATGATTAAAACGTATCTTTTTAAAAAAAAAAAAAAAAAA            - 890
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Figure 3. The nucleotide sequence of the glutathione peroxidase (GPX) cDNA from *Hevea brasiliensis*. The deduced amino acid sequence of GPX cDNA is shown below the nucleotide. The codons with asterisks are putative translation start codon (ATG) and stop codon (TAA). The amino acids containing two conserved domains of the GPX signature (PCNQF and WNSFK) are underlined. The putative polyadenylation signal, AAUAA is framed.

Table 3. Percentage of sequence identity between the *Hevea brasiliensis* GPX (AF242650) and other plant GPX sequences.

Plant Material	GenBank Accession number	% of identity	
		Nucleotide sequence	Amino acid sequence
Arabidopsis thaliana	AF030132	81	86
	AY077655	81	84
Hordeum vulgare	AJ238697	80	86
Citrus sinensis	X66377	83	85
Oryza sativa	AY100689	81	83
Mesembryanthemum Crystallinum	AJ315976	80	78
Nicotiana sytvestris	X60219	83	78
Spinacia oleracea	D63425	79	77
Nicotiana tabacum	AB041518	82	77
Gossupium hirsutum	AF037051	83	77
Lycopersicon charantia	Y14762	82	76
Momordica charabtia	AF346906	79	75
Zantedeschia aethiopica	AF053311	nd	66

3. Enzyme analysis and gene expression analysis in latex

3.1 Kinetic effect of ethylene treatment and bark opening

This experiment was performed to study the effect of tree "opening" (starting exploitation of trees through the first tapping) and the kinetic of exogenous ethylene effect on some biochemical characteristics and gene expression in the latex cells. 6-year-old trees of the PB 314 rubber clone were used in this study. Six batches of 6 homogeneous (girth) virgin trees were set up: one as a control (scrapped bark without stimulation) and the others were treated with 5% Ethrel at 6, 12, 24, 36, 48 hours, respectively, before the first tapping. The latex was collected on the same day on the 1st, 2nd, 3rd, 4th, and 5th tapping .

Effect on rubber yield

Upon tree "opening" (1st tapping), the rubber yield is low for the control trees and the positive effect of ethylene stimulation is hardly discernible in 24 hours after application of Ethrel, then highly significant in 36 and 48 hours after the treatment (Fig. 4-6A). Upon the successive tappings, the yield of the control trees increase progressively from 29 g dry rubber/Tree/tapping at the first tapping to 81 g/T/t at the fifth tapping. The yield difference between control tree and stimulated tree increases on the 2nd tapping, reaches a maximum on the 3rd tapping, and then decrease on the 5th tapping. One can conclude from these results that the stimulation treatment was efficient.

Effect on latex cytosolic total SOD activity and Cu/Zn-SOD gene expression

A single Ethrel stimulation did not have significant effect on latex cytosolic total SOD activity while the successive tappings could slightly induce increase of total SOD activity observed in the 5th tapping. In contrary, it was found that the Cu/Zn-SOD mRNA level was high in the resting cell of virgin tree. Ethylene decreases the accumulation of Cu/Zn-SOD mRNA between 6-12 hours after treatment. The effect of ethylene stimulation still remained to the fifth tapping which control trees showed higher accumulation of Cu/Zn-SOD mRNA than stimulated trees. Moreover, when compared between the 1st and the 5th tapping, the result showed that the number of tappings had slightly effect on *Hevea* Cu/Zn-SOD gene expression. (Fig. 4 C)

Effect on latex cytosolic APX activity and gene expression

Either tree opening and successive tappings, or a single Ethrel stimulation tended to have significantly effect on both latex cytosolic APX activity and APX gene expression (Fig. 5B,C). The differences of the enzyme activity and the gene expression between control and

stimulated trees were observed in the 2nd tapping and the 3rd tapping. It was found that the APX mRNA level was very low in the resting cells of virgin trees. Ethylene slightly induced the accumulation of APX mRNA between 36-48 hours after stimulation. However, the ethylene effect and probably opening effect were obviously observed more in the 2nd tapping than in the 1st tapping, both in case of APX activity and APX gene expression (Fig. 5B,C).

Effect on latex cytosolic GPX activity and gene expression

Even at the first tapping, the unstimulated trees from the high yielding clone (PB 314, Fig. 6B), show high latex cytosol GPX activity, similar to the other high yielding clone (PB 235, Fig. 9B). Successive tappings did not seem to bring about much changes in the latex GPX activity of these control trees, although a slight increase might be noted especially upon the 2nd and 3rd tapping. In contrary, bark opening and successive tappings, induced a noticeable increase in the GPX gene expression in the laticifers. This last effect was significant since, whatever the tapping, RNA loading was rather equal for the corresponding samples (Fig. 6D). The kinetic effects of a single stimulation with Ethrel were not so evident. Upon the first tapping, the trees, which had been stimulated 36 to 48 hours before the first tapping, and also on the second tapping, the ones which had been treated 6 and 12 hours before the opening (as tapped every 2 days, this means 54 and 60 h after the Ethrel treatment), appeared to exhibit both a decrease in their latex GPX activity and in the expression of the GPX gene. If relevant, these results may correspond to late (36-60 h) and transient depressive effect of ethylene on GPX. Afterwards, both GPX activity and gene expression did not look to differ significantly between control and stimulated trees.

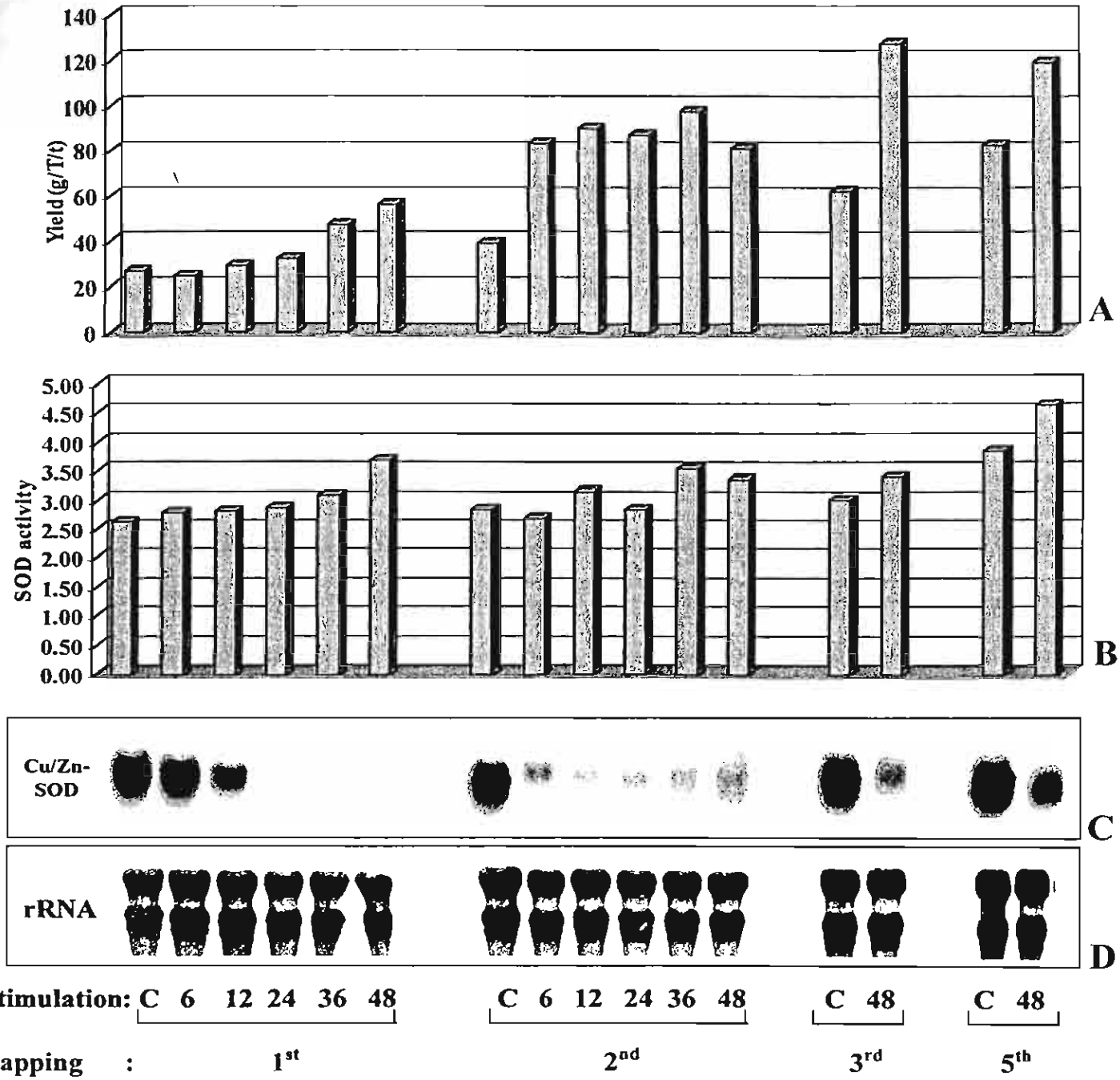

Figure 4. Kinetic effect of yield stimulation with Ethrel and successive tappings on rubber yield, cytosolic SOD activity and Cu/Zn-SOD gene expression in the latex of virgin trees (clone PB 314).

Six batches of 6 homogeneous virgin trees were selected. One batch was not stimulated as a control (C), the 5 other batches were stimulated 6,12, 24, 36, 48 hrs, respectively before the first tapping. The trees were all tapped on the same day, and the latex was collected upon the 1st, 2nd, 3rd, and 5th tapping for anyalysis of the enzyme activity and gene expression. A: yield (g dry rubber/Tapping/tree); B: latex cytosolic total SOD activity; C: Cu/Zn-SOD gene expression in latex by Northern blot analysis; D: methylene blue staining of rRNA as a control of loading homogeneity.

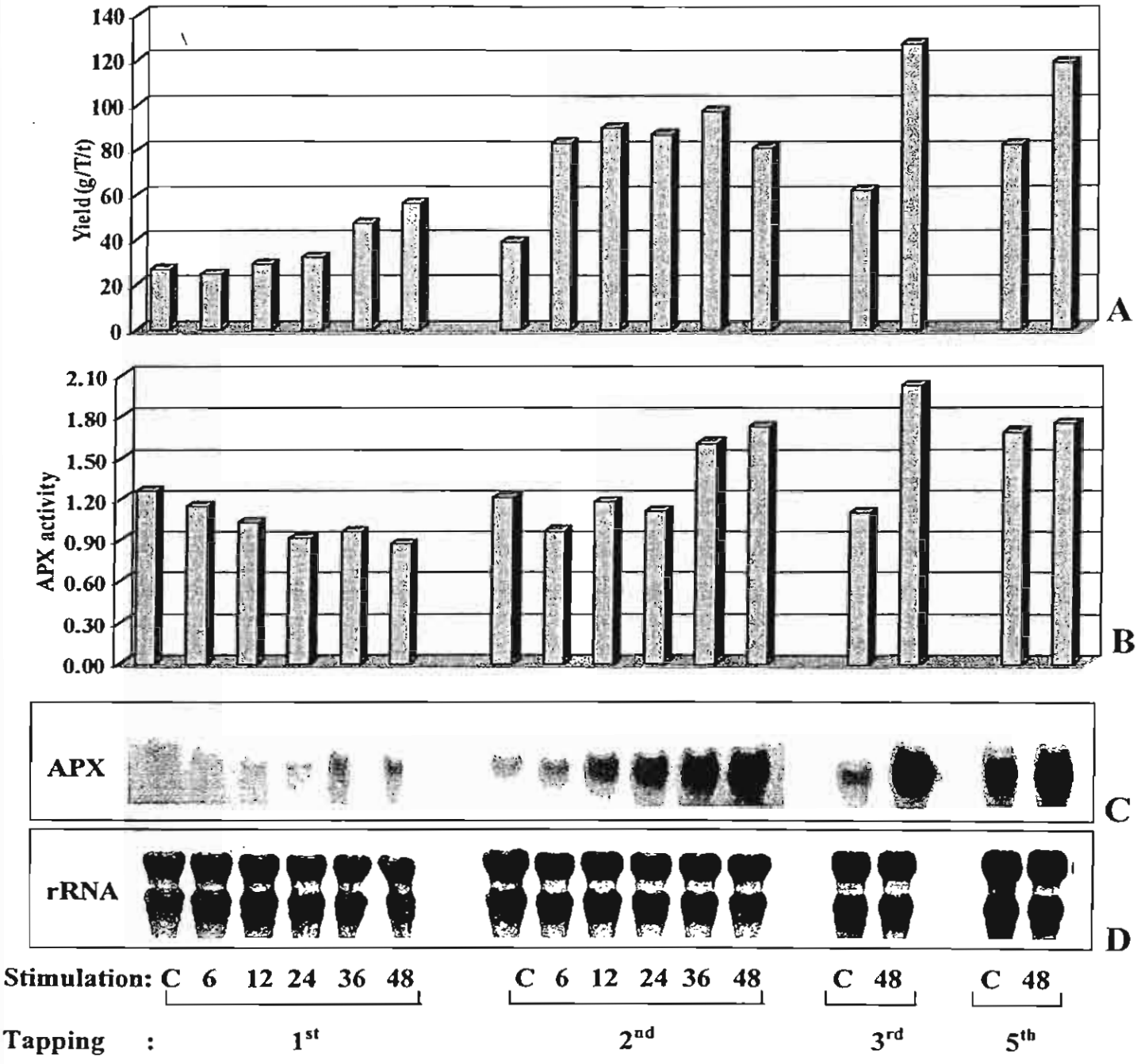

Figure 5. Kinetic effect of yield stimulation with Ethrel and successive tappings on rubber yield, cytosolic APX activity and gene expression in the latex of virgin trees (clone PB 314).

The samples were the same as in Fig. 4. A: yield (g dry rubber/Tapping/tree); B: latex cytosolic APX activity; C: APX gene expression in latex by Northern blot analysis; D: methylene blue staining of rRNA as a control of loading homogeneity.

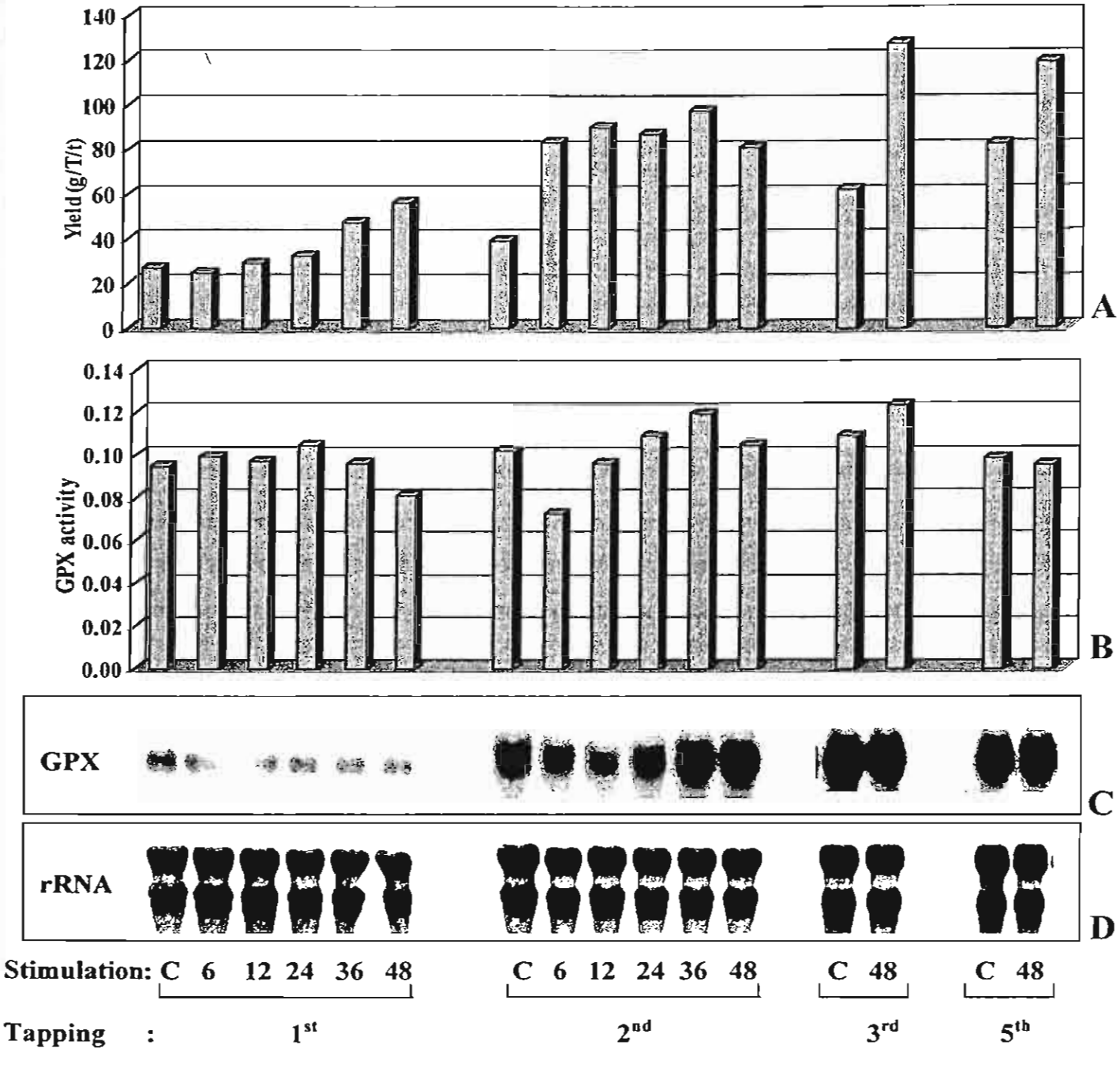

Figure 6. Kinetic effect of yield stimulation with Ethrel and successive tappings on rubber yield, cytosolic GPX activity and gene expression in the latex of virgin trees (clone PB 314).

The samples were the same as in Fig. 4. A: yield (g dry rubber/Tapping/tree); B: latex cytosolic GPX activity; C: GPX gene expression in latex by Northern blot analysis; D: methylene blue staining of rRNA as a control of loading homogeneity.

3.2 Inter-clone relationships between yield, latex cytosolic enzyme activity and gene expression

Considering the homogeneous (girth) healthy non-stimulated high yielding trees selected from the PB 235, PB 260 and Avros 2037 clones, we could verify (Table 4 and Fig.7-9A) that PB 235 is far the highest yielding clone, which very highly significantly differed from the other 2 lower yielding clones.

In parallel, cytosolic GPX activity of PB 235 clone was shown to be highest and significantly differed from PB 260 and Avros 2037 clones (Fig. 9B). PB 260 and Avros 2037 showed the similar level of GPX activity. PB 260 contained the lowest activity of total SOD, compared to the other two clones (Fig. 7B). Among these three clones, there were not significant differences of APX activity (Fig 8B).

Northern blot analysis showed that the expression of APX and GPX depends on clones of rubber tree. The result indicated that in most case, GPX gene expression appeared to be highest in the latex of healthy non-stimulated (high yielding) trees from PB 235 clone, compared to PB 260 and Avros 2037 (Fig. 9C). Meanwhile, APX gene expression was highest in PB 260 but lowest in PB 235, although the APX activity was not significantly different in these three rubber clones (Fig. 8C).

Table 10. Statistical analysis of the inter-clone variation for yield and latex enzyme activity

A total of 30 regularly tapped (Control) healthy trees, belonging to the highest producing class for each clone, were selected, and known for their different yield and metabolism potential. Their rubber yield (g/T/t) was checked on tree to tree basis over 4 tappings. This table reports the recorded mean value (± confident interval at 5%). PB 235 is very highly significantly different (***) from the other 2 clones (PB 260, Avros 2037) in mean yield and GPX activity.

Clone	Mean Yield (g/T/t)	Mean activity		
		SOD	APX	GPX
PB 260	84.1 ± 11.7	3.02 ± 0.44	1.60 ± 0.16	0.045 ± 0.008
PB 235	201.6 ± 45.6***	5.96 ± 0.16	1.61 ± 0.15	0.109 ± 0.010***
Avros 2037	63.4 ± 26.4	5.99 ± 2.11	1.66 ± 0.12	0.065 ± 0.020

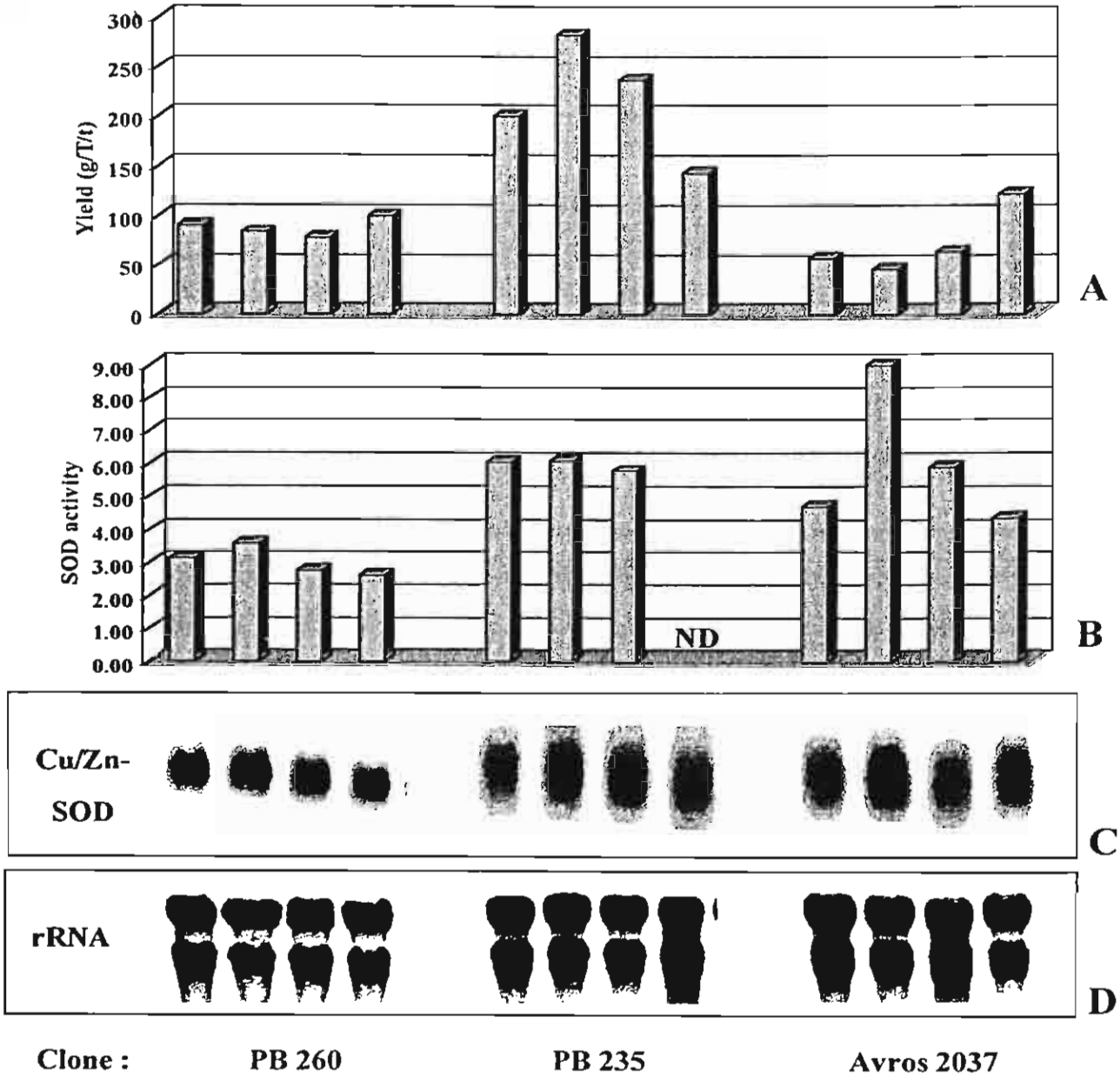

Figure 7. Inter-clone variation of rubber yield, SOD activity and Cu/Zn-SOD gene expression in the latex.

The latex was collected from the 4 highest yielding unstimulated trees of 3 rubber clones that exhibit different yield potential: PB 235 (high yielding clone), PB 260 and Avros 2037 (low yielding clone). A: yield (g dry rubber/Tapping/tree); B: latex cytosolic SOD activity; C: Cu/Zn-SOD gene expression in latex by Northern blot analysis; D: methylene blue staining of rRNA as a control of loading homogeneity.

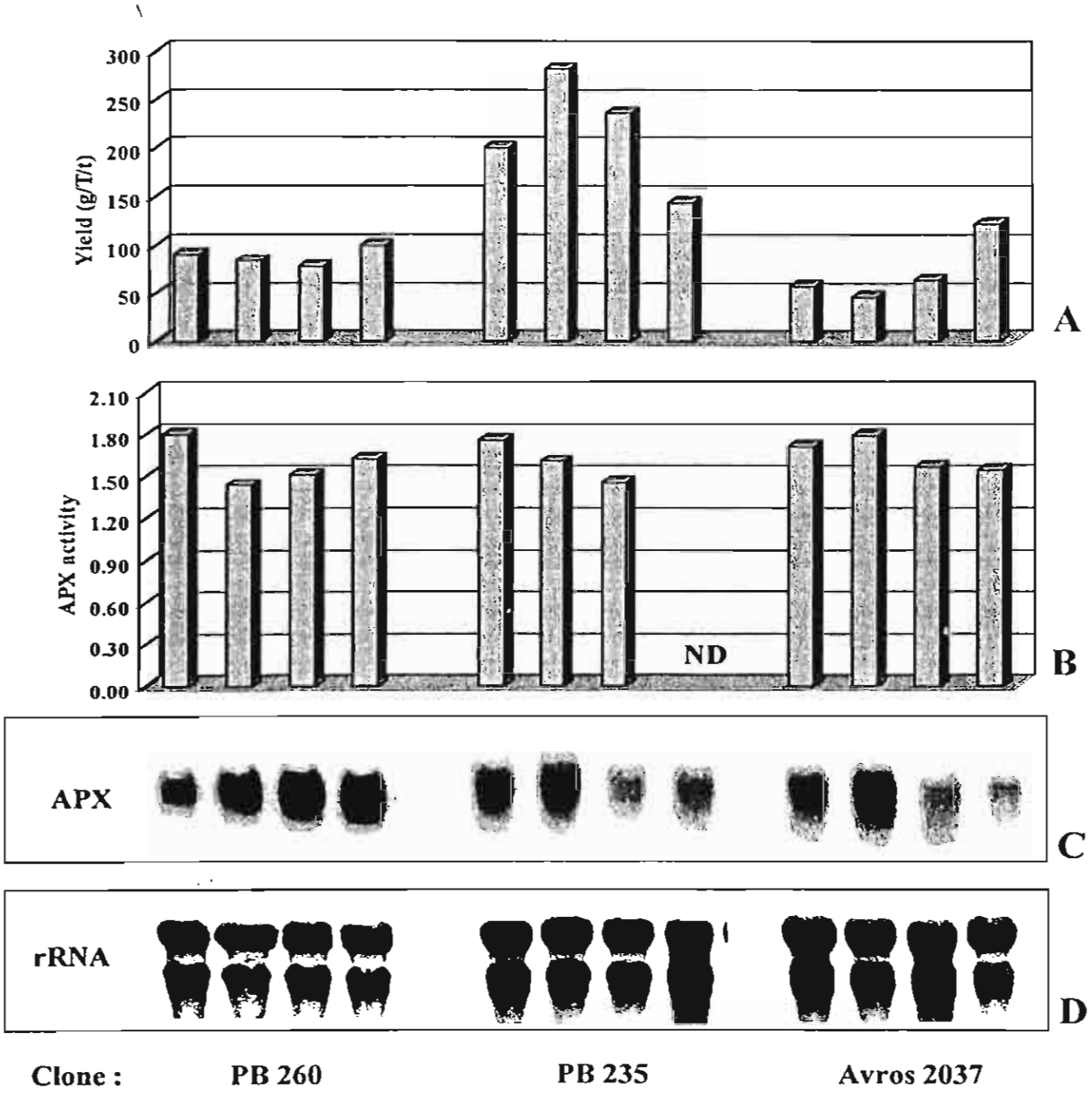

Figure 8. Inter-clone variation of rubber yield, APX activity and gene expression in the latex.

The latex was collected from the 4 highest yielding unstimulated trees of 3 rubber clones that exhibit different yield potential: PB 235 (high yielding clone), PB 260 and Avros 2037 (low yielding clone). A: yield (g dry rubber/Tapping/tree); B: latex cytosolic APX activity; C: APX gene expression in latex by Northern blot analysis; D: methylene blue staining of rRNA as a control of loading homogeneity.

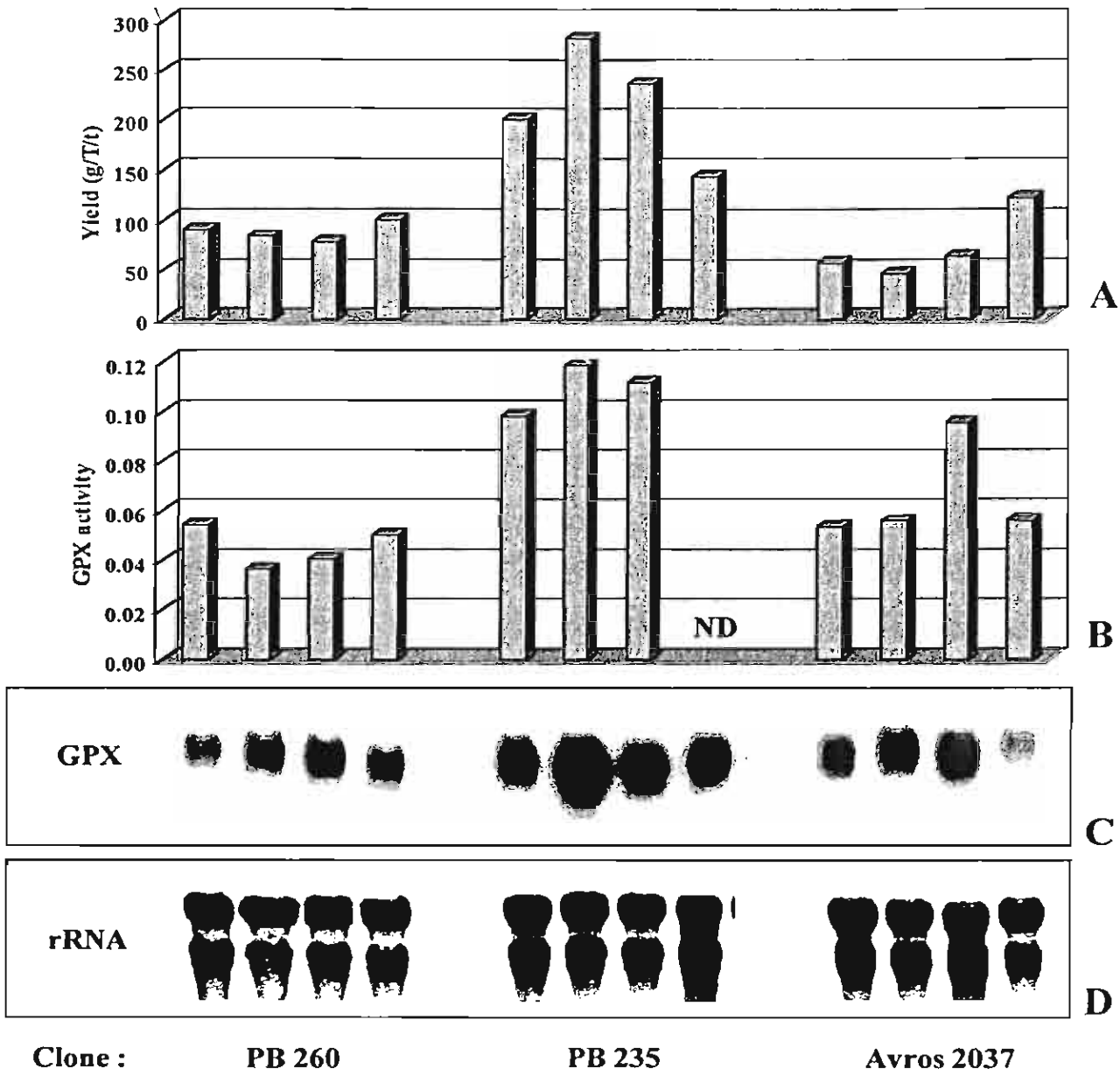

Figure 9 Inter-clone variation of rubber yield, GPX activity and gene expression in the latex.

The latex was collected from the 4 highest yielding unstimulated trees of 3 rubber clones that exhibit different yield potential: PB 235 (high yielding clone), PB 260 and Avros 2037 (low yielding clone). A: yield (g dry rubber/Tapping/tree); B: latex cytosolic GPX activity; C: GPX gene expression in latex by Northern blot analysis; D: methylene blue staining of rRNA as a control of loading homogeneity.

3.3 Intra-clone relationships between yield, health status, latex cytosolic enzyme activity and gene expression

3.3.1 Avros 2037

From the selected trees of the Avros 2037 rubber clone, all trees were healthy except only one tree showed low yielding with Trunk Phloem Necrosis (TPN) symptoms. This diseased tree turned to be TPN just only a few weeks after latex collection for analysis. The healthy trees were separated into two distinct highly significantly different classes of low yielding and high yielding trees (Table 5, Fig. 10-12 A).

From the enzyme analysis, the result indicated that the group of high yielding trees showed significantly higher latex cytosolic APX and GPX activity, compared to the low yielding trees. Only the tree which later exhibit early symptom of Trunk Phloem Necrosis (TPN) showed abnormally higher latex GPX activity. There were high variations of total SOD activity between trees, however it looked like that total SOD activity was a little bit higher in low yielding trees, compared to high yielding trees. (Table 5, Fig. 10-12 B).

The results of northern blot analysis of APX and GPX gene expression were correlated to the results of APX and GPX enzyme activity, respectively. Fig. 11-12 C indicated that most of the high yielding trees tended to exhibit higher expression of the APX and GPX gene, compared to low yielding tree. Only the diseased tree, contained TPN symptom and low yield, exhibited high latex cytosolic GPX activity and the highest GPX gene expression. The expression of Cu/Zn-SOD gene was not significantly different between high and low yielding trees (Fig. 10 C).

Table 5. Statistical analysis of the intra-clone (Avros 2037) variations for yield and latex enzyme activity.

Among a total of 24 rubber trees (clone Avros 2037), which were regular tapped and contained homogeneous girth, the 5 highest and 5 lowest yielding trees were selected and separated into 2 yield classes (HY, LY). One of the low yielding trees showed TPN symptoms. The high yielding trees show significantly (*) higher APX and GPX activity, compared to the low yielding ones.

Yield Class	Number of trees	Mean Yield (g/T/t)	SOD Activity	APX Activity	GPX Activity
High yield (HY)-Healthy	5	96.1 ± 31.3	5.99 ± 2.11	1.66 ± 0.12	0.065 ± 0.020
Low yield (LY)-Healthy	4	30.7 ± 10.2*	7.85 ± 1.58	1.30 ± 0.12*	0.042 ± 0.008*
Low yield (LY)-TPN	1	21.1	8.67	1.38	0.1348

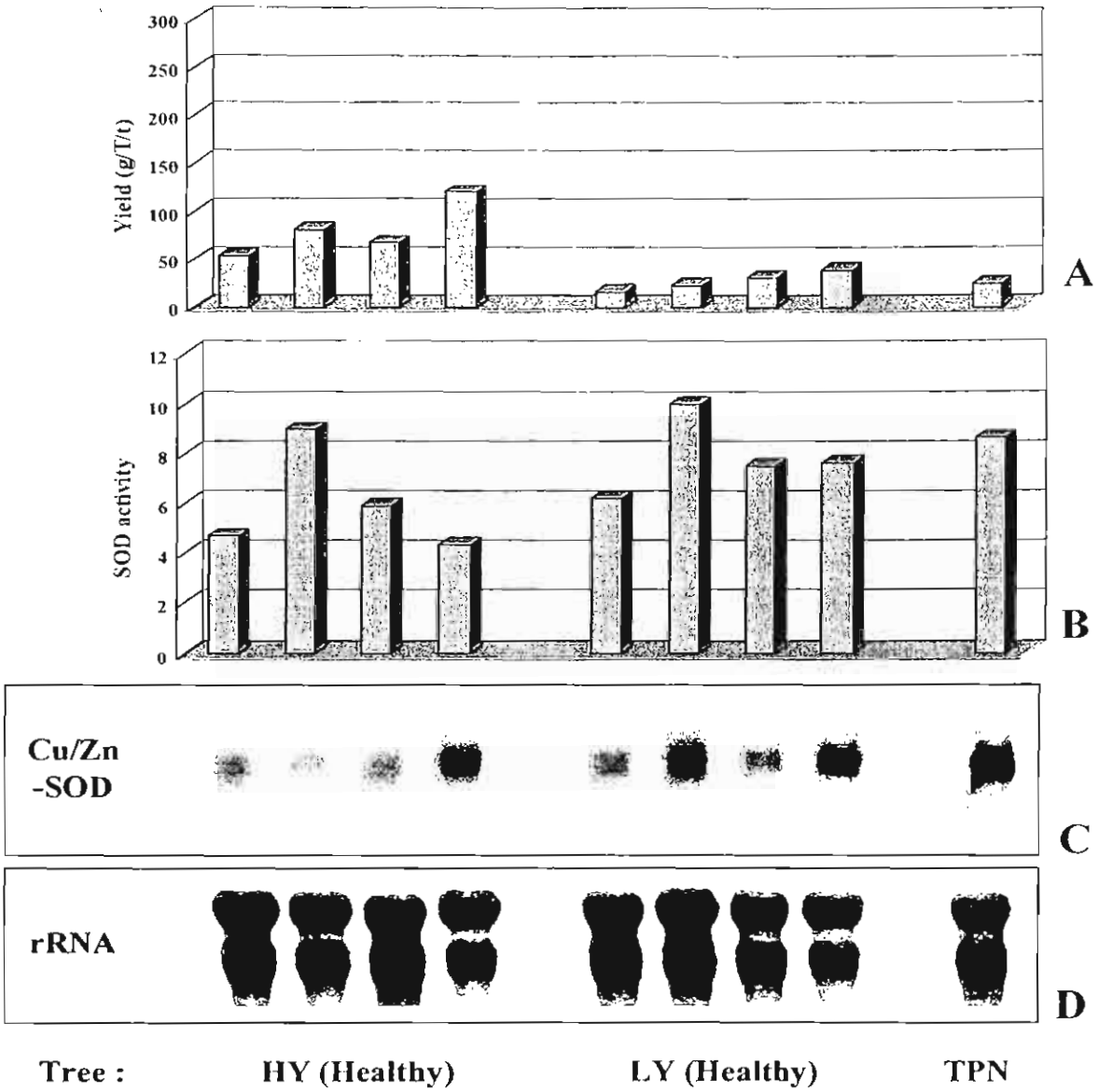

Figure 10. Intra-clone (Avros 2037) relationships between the variation of rubber yield, total SOD activity and Cu/Zn-SOD gene expression in the latex from high yielding and low yielding healthy trees.

The latex was collected from 4 highest yielding (HY) healthy trees and the 5 lowest yielding (LY) trees, of which 4 healthy trees and 1 tree exhibited Trunk Phloem Necrosis (TPN) symptom. A: yield (g dry rubber/Tapping/tree); B: latex cytosolic SOD activity; C: Cu/Zn-SOD gene expression in latex by Northern blot analysis; D: methylene blue staining of rRNA as a control of loading homogeneity.

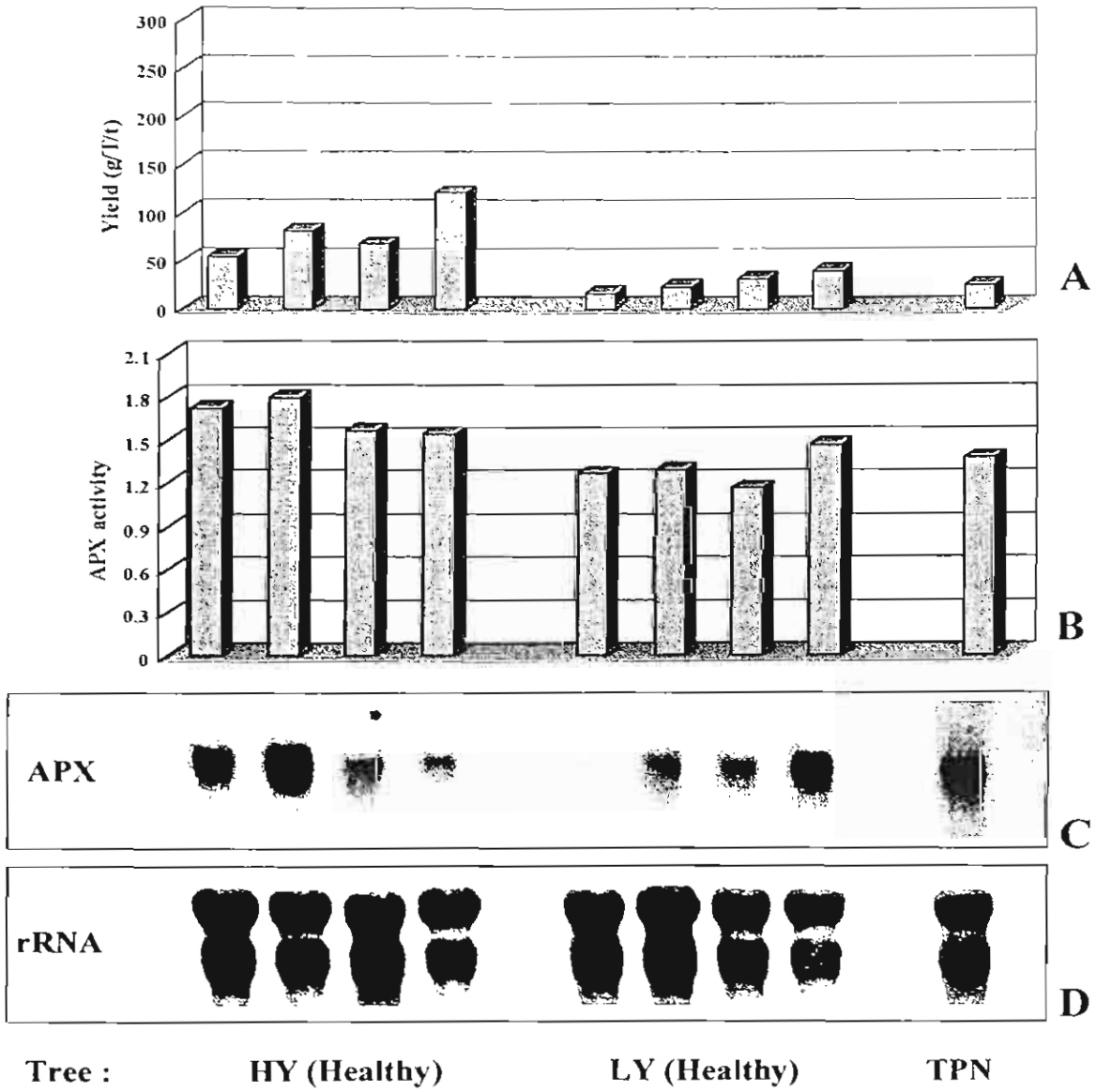

Figure 11. Intra-clone (Avros 2037) relationships between the variation of rubber yield, APX activity and gene expression in the latex from high yielding and low yielding healthy trees.

The latex was collected from 4 highest yielding (HY) healthy trees and the 5 lowest yielding (LY) trees, of which 4 healthy trees and 1 tree exhibited Trunk Phloem Necrosis (TPN) symptom. A: yield (g dry rubber/Tapping/tree); B: latex cytosolic APX activity; C: APX gene expression in latex by Northern blot analysis; D: methylene blue staining of rRNA as a control of loading homogeneity.

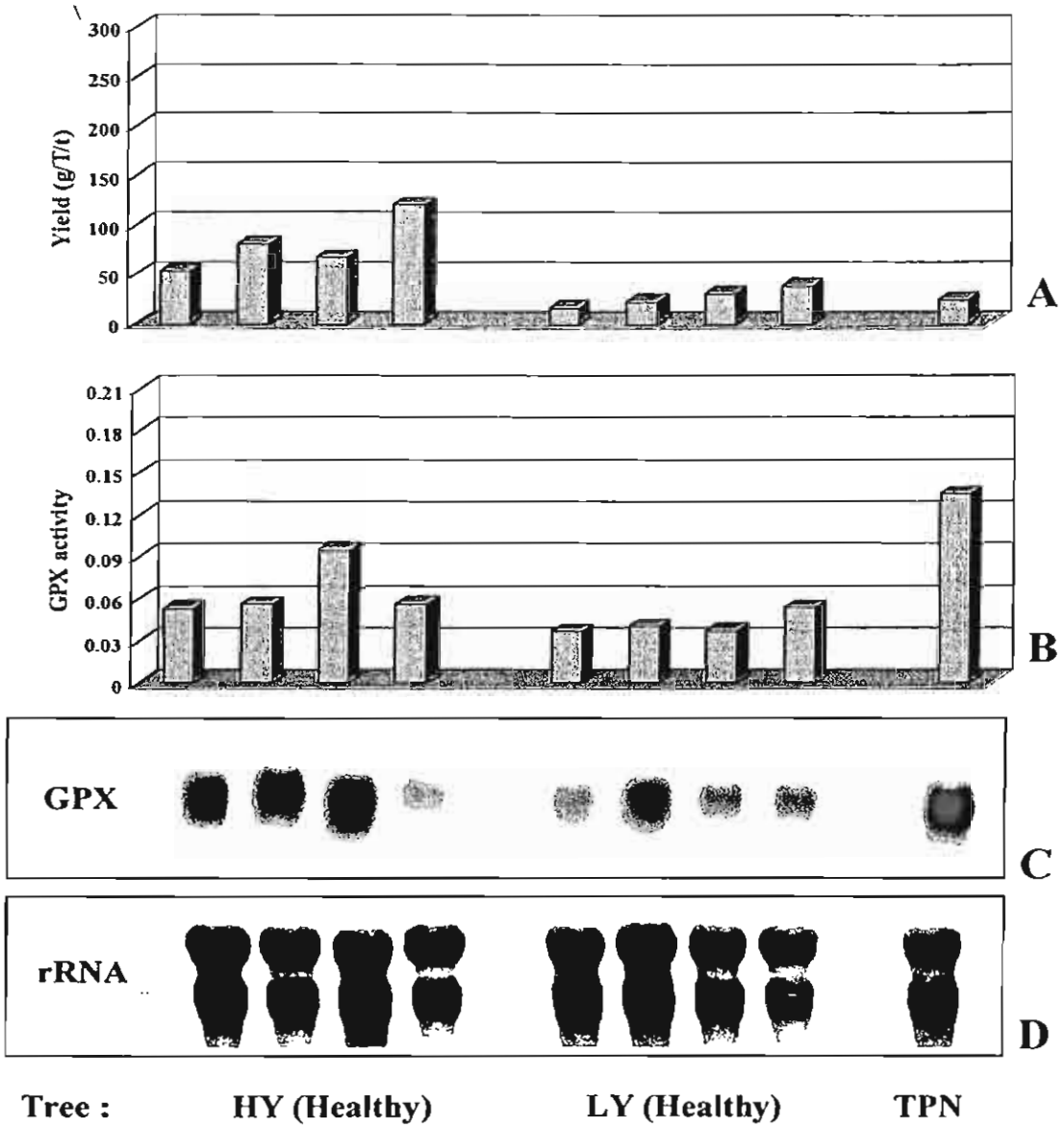

Figure 12. Intra-clone (Avros 2037) relationships between the variation of rubber yield, GPX activity and gene expression in the latex from high yielding and low yielding healthy trees.

The latex was collected from 4 highest yielding (HY) healthy trees and the 5 lowest yielding (LY) trees, of which 4 healthy trees and 1 tree exhibited Trunk Phloem Necrosis (TPN). symptom. A: yield (g dry rubber/Tapping/tree); B: latex cytosolic GPX activity; C: GPX gene expression in latex by Northern blot analysis; D: methylene blue staining of rRNA as a control of loading homogeneity.

3.3.2 PB 235

Unlike Avros 2037, from the selected tree of PB 235, all high yielding trees were shown to be healthy but all the low yielding trees were diseased. Most of the diseased trees exhibited only typical symptoms of bark dryness (TPD) and one of them showed Trunk Phloem Necrotic (TPN) symptoms. The healthy and diseased trees were classified in highly significantly different groups of high and low yielding trees (Table 6, Fig. 13-15 A). The yield classes were fit with the health status of the trees.

Table 5 and Fig. 15 B showed that high yielding healthy trees from PB 235 clone exhibited very significantly higher GPX activity than low yielding TPD trees. Northern blot analysis (Fig. 15 C) indicated that GPX gene expression tended to be slightly higher in the latex from the high yielding healthy trees, compared to the low yielding-TPD trees. Similar to Avros 2037, the only tree exhibiting typical TPN symptom showed the highest GPX activity and relatively high GPX gene expression.

For the latex cytosolic SOD and APX activity, No significant differences could be observed between high and low yielding trees of PB 235. However, APX gene expression tended to be higher in high yielding healthy trees but lower in low yielding TPD trees. (Fig. 13-14)

Table 6. Statistical analysis of the intra-clone (PB 235) variations for yield, health status and latex enzyme activity.

Among a total of 24 rubber trees (clone PB 235), which were regular tapped and contained homogeneous girth, the 5 highest and 5 lowest yielding trees were selected and separated into 2 yield classes (HY, LY). All the low yielding trees showed symptoms of bark disease (4 TPD and 1 TPN). The high yielding healthy trees show significantly (**) higher GPX activity than the low yielding ones with TPD symptoms. Only one tree with TPN symptoms showed abnormally higher latex GPX activity.

Yield Class	Number of trees	Mean Yield (g/T/t)	SOD Activity	APX Activity	GPX Activity
High yield (HY)-Healthy	5	201.6 ± 45.6	5.96 ± 0.16	1.61 ± 0.15	0.109 ± 0.010
Low yield (LY)-TPD	4	20.3 ± 11.8**	5.31 ± 1.25	1.53 ± 0.18	0.049 ± 0.007**
Low yield (LY)-TPN	1	21.3	5.97	1.8	0.185

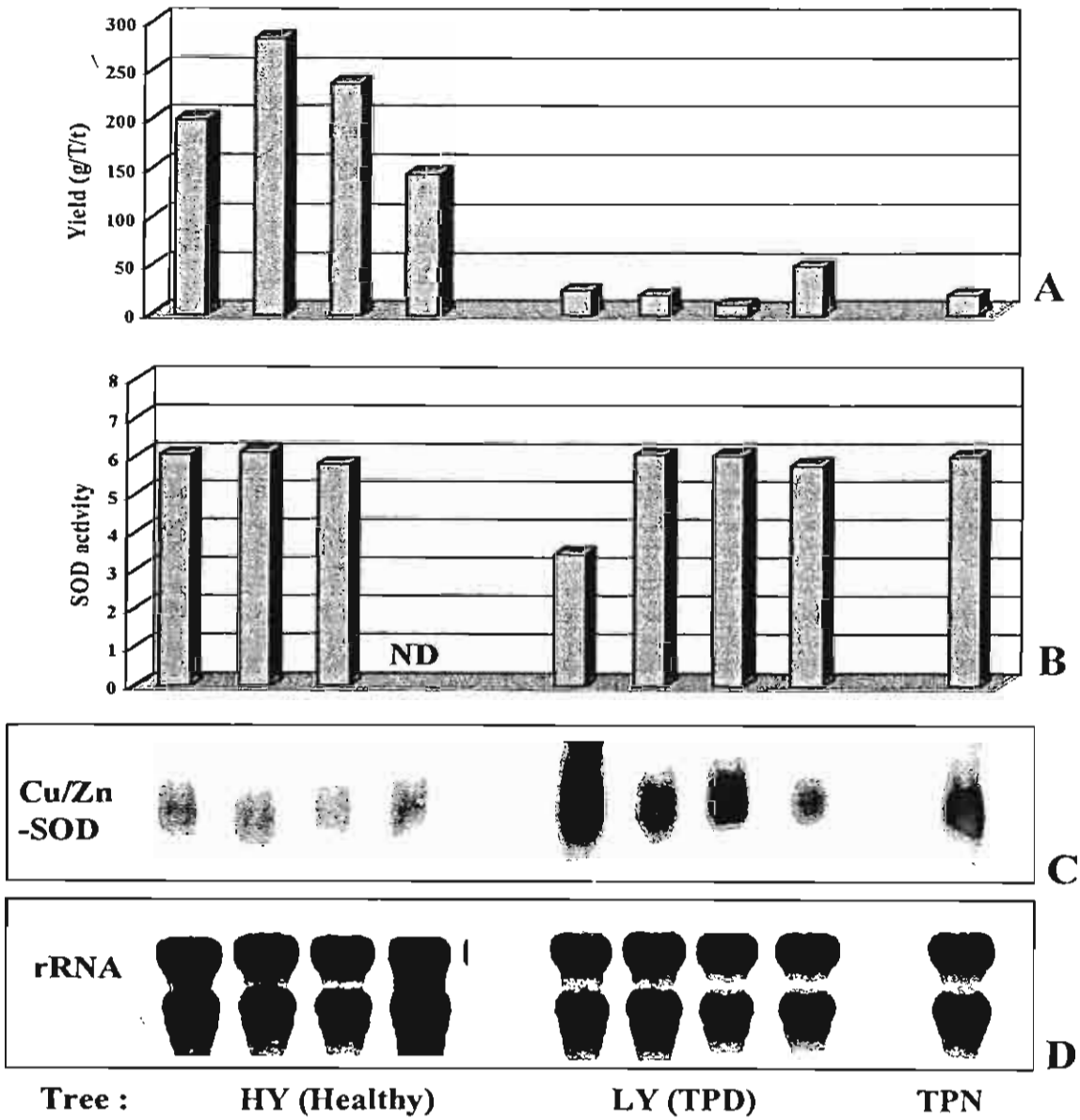

Figure 13. Intra-clone (PB 235) relationships between the variation of rubber yield, total SOD activity and Cu/Zn-SOD gene expression in the latex from high yielding healthy trees and low yielding diseased trees.

The latex was collected from 4 highest yielding (HY) healthy trees and the 5 lowest yielding (LY) trees, of which 4 trees exhibited TPD symptom and 1 tree exhibited Trunk Phloem Necrosis (TPN) symptom. A: yield (g dry rubber/Tapping/tree); B: latex cytosolic SOD activity; C: Cu/Zn-SOD gene expression in latex by Northern blot analysis; D: methylene blue staining of rRNA as a control of loading homogeneity.

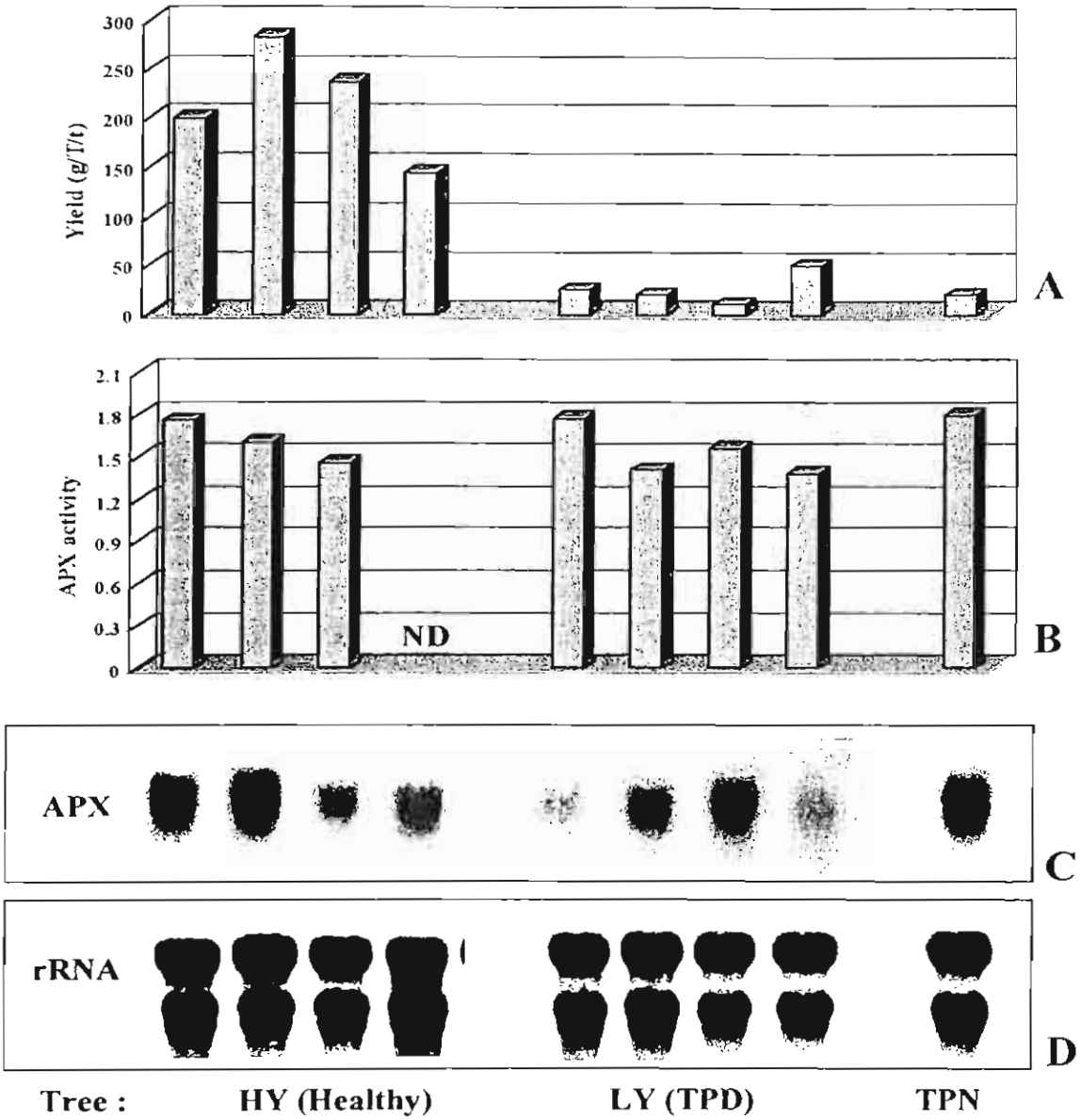

Figure 14. Intra-clone (PB 235) relationships between the variation of rubber yield, total APX activity and gene expression in the latex from high yielding healthy trees and low yielding diseased trees.

The latex was collected from 4 highest yielding (HY) healthy trees and the 5 lowest yielding (LY) trees, of which 4 trees exhibited TPD symptom and 1 tree exhibited Trunk Phloem Necrosis (TPN) symptom. A: yield (g dry rubber/Tapping/tree); B: latex cytosolic APX activity; C: APX gene expression in latex by Northern blot analysis; D: methylene blue staining of rRNA as a control of loading homogeneity.

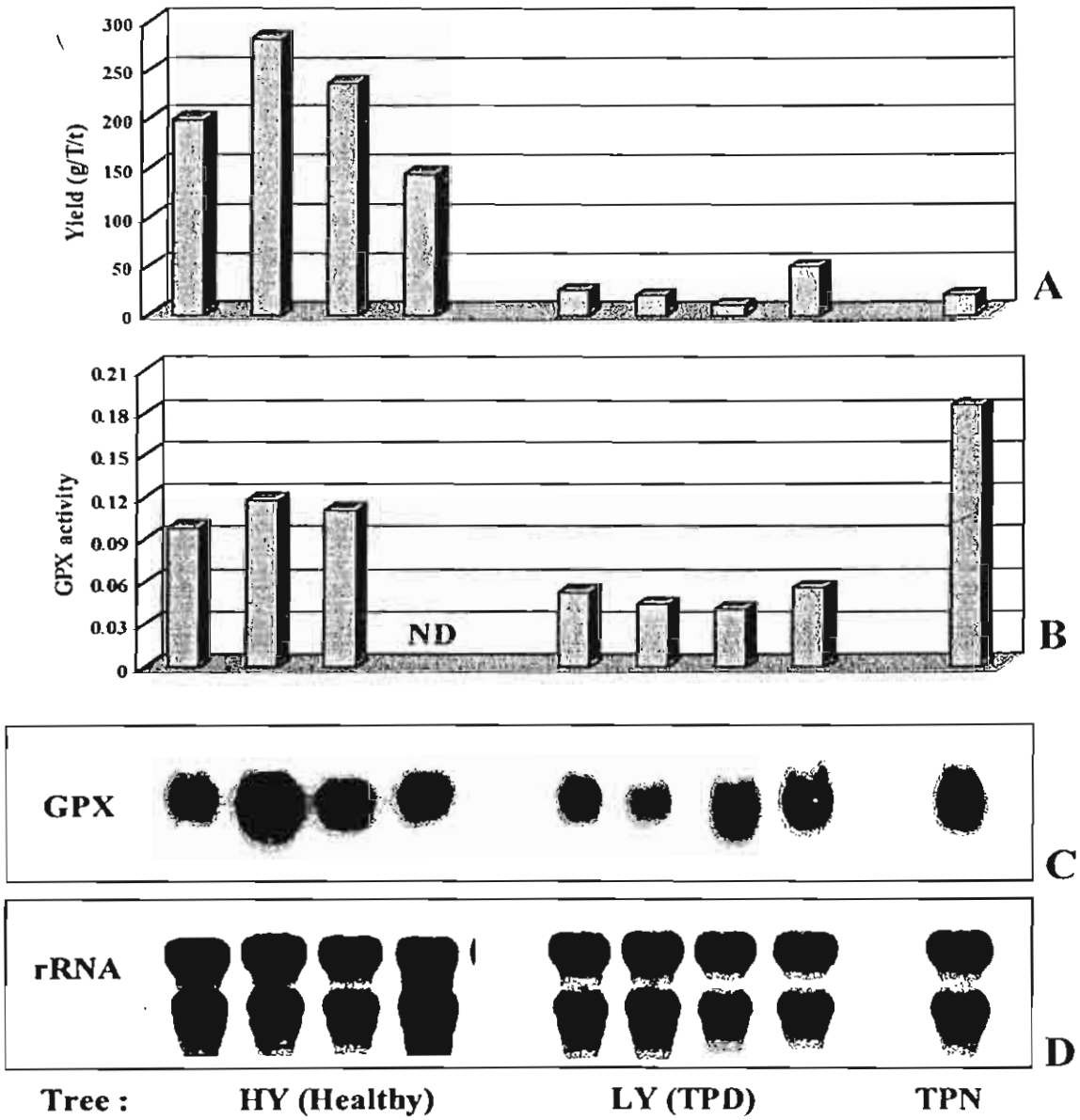

Figure 15. Intra-clone (PB 235) relationships between the variation of rubber yield, total GPX activity and gene expression in the latex from high yielding healthy trees and low yielding diseased trees.

The latex was collected from 4 highest yielding (HY) healthy trees and the 5 lowest yielding (LY) trees, of which 4 trees exhibited TPD symptom and 1 tree exhibited Trunk Phloem Necrosis (TPN) symptom. A: yield (g dry rubber/Tapping/tree); B: latex cytosolic GPX activity; C: GPX gene expression in latex by Northern blot analysis; D: methylene blue staining of rRNA as a control of loading homogeneity.

3.4 Incidence of Trunk Phloem Necrosis (TPN) on latex GPX activity

This experiment settled with the highly TPN sensitive PB 260 clone, shows the incidence of TPN on rubber yield for the still latex producing diseased trees. These trees exhibiting typical late phase of TPN symptoms (extended necrotic area from nearby the cambium to the external bark) showed statistically significant higher latex cytosolic GPX in their latex than the healthy trees (Table 7 and Fig. 16). Unfortunately latex RNA from this experiment could not be extracted because of latex coagulation in the sampling tubes upon storing.

Table 7. Statistical analysis of the intra-clone variations for yield, health status and latex GPX activity for the rubber clone PB 260.

Among a total of 24 regularly tapped homogeneous (girth) trees of the rubber clone PB 260, the 5 highest yielding healthy trees and 6 low yielding ones exhibiting typical symptoms of Trunk Phloem Necrosis (TPN) were selected. Rubber yield and latex GPX activity were analyzed. The low yielding trees with TPN symptoms showed significantly (*) higher latex cytosolic GPX activity than the high yielding healthy ones.

Yield Class	Number	Mean Yield (g/T/t)	Mean latex GPX activity
High (HY) healthy	5	82.2 ± 15.1	0.048 ± 0.006
Low (LY) TPN	6	19.8 ± 6.2***	0.065 ± 0.009*

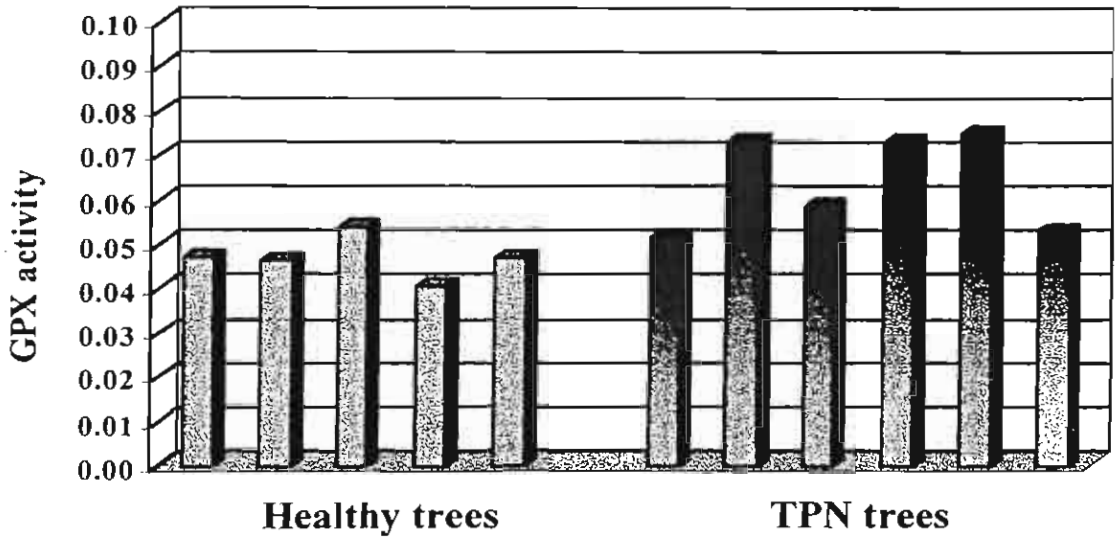

Figure 16. Cytosolic GPX specific activity in the latex from healthy and diseased (TPN) trees of the rubber clone PB 260.

The latex was collected from healthy trees and diseased trees exhibiting typical symptoms of Trunk Phloem Necrosis (TPN). Their latex cytosolic GPX specific activities were determined as described in Materials and Methods.

3.5 Effect of over-exploitation (over-tapping and over-stimulation)

Effect on yield and onset of bark diseases

At the beginning of the experiment, all trees (clone PB 260) were selected for their apparent good health (neither TPD nor TPN apparent symptom) and their relatively homogeneous girth. As under normal exploitation for 3 years, their yield was within a mean of 94 ± 14 g dry rubber/Tree/tapping. After one-year experiment, the yield/Tree/tapping and the number of diseased trees per treatment were reported in Table 8-A . Considering only the still healthy trees for each treatment, it can be seen that, after one year, control trees exhibited the same level of rubber yield/tapping as at the beginning of the experiment. The over-stimulated trees did not respond any more to Ethrel stimulation and even tended to give lower yield/tapping (not significant in statistics). The over-tapped trees exhibited significantly lower yield/tapping compared to control and over stimulated trees (Table 8-B). The healthy trees, which had been selected for the biochemical and molecular analysis of their latex, did not significantly differ in their yield characteristics from the total batch of trees per treatment (Table 8-A). From these results it could be seen that over-tapping showed the highest negative incidence on rubber yield for the PB 260 clone.

Over one year experiment, in whatever treatment, only 1-2 trees per treatment exhibited some early stage of TPD symptoms, showing seriously impaired latex flow however the dryness had never exceeded 20% of the total tapping cut length. Two trees per treatment exhibited typical inner phloem necrotic (TPN) symptoms. Two trees giving no more latex (100% dry tapping cut) were found in the control group and another one was found in the over-exploited groups. Taking into account, only the trees which still produced latex (Table 8-C), the mean yield of the diseased trees from whatever treatment, was 43.2 ± 19.9 g/tree/tapping, being significantly lower from the still healthy trees. Anyway, it can be deduced that, in the PB 260 clone, one-year over-exploitation treatments was not enough to induce significant difference between the incidence of laticifers disease (TPD) and the inner phloem disease (TPN).

Effects of treatment on latex enzyme activity and gene expression in relation with yield and health status

From Table 8-B and Fig. 19 A and B, it is shown that the over-tapping treatment could induce a global highly significant decrease in the latex GPX activity of whatever health status of the trees, correlated to the decrease in yield. Comparing with the still healthy tree and TPN trees in whatever treatment, the trees exhibiting TPD symptom were characterized by a

significant lower cytosolic GPX activity in their latex (table 8-C, Fig 19 B). Once again, compared to healthy trees, the trees showing early stage of TPN symptoms, but still producing some latex (in the over-exploitation group), tended to contain higher GPX activity. In this experiment, Northern blot analysis indicated that the latex cytosolic activity was rather well correlated to the expression of the GPX gene in the laticifers (Fig. 19C).

Meanwhile, APX activity tended to slightly decrease with the overexploitation treatment (over-stimulation and over-tapping) (Table 8-B, Fig. 18B). APX gene expression was lowest in the overtapped-tree compared to control and overstimulated tree. But there were not significantly differences between healthy and diseased tree.

In contrary, total SOD activity tended to slightly increase with the overexploitation (Table 14-B, Fig. 17B), but the Cu/Zn-SOD gene expression tended to decrease especially in the over-tapped tree, compared to normal exploited trees (control trees) (Fig. 17C).

Table 8. Statistical analysis of rubber yield, health status and latex cytosolic enzyme activity of trees submitted to various treatments after one year experiment.

Rubber trees (clone PB 260) were either submitted to normal exploitation (control) or to over-exploitation through over-stimulation or over-tapping

8-A: Rubber yield (g/Tree/tapping) and the health status of all trees (20 trees/treatment) and of the selected trees (6 trees/treatment) were recorded. The table reports the yield (± confidence interval at 5%) of still apparently healthy trees and the number of diseased trees per treatment after one-year experiment: TPN: among 2 diseased trees, either 2 [(a)] trees or 1 [(b)] tree were completely dry. Statistics : (**): highly significant at 5%.

8-B: Effects of the treatments on rubber yield and latex cytosolic GPX activity considering all latex yielding trees in whatever health status or considering only healthy trees. Overtapped trees differed highly significantly (**) from the control and over-stimulated ones for yield and latex GPX activity.

8-C: Effects of the health status on rubber yield and latex cytosolic GPX activity considering all latex yielding trees inwhatever treatment: trees exhibiting symptoms of Tapping Panel Dryness (TPD) differ significantly (*) from the healthy or TPN trees for yield and latex GPX activity. Here again, latex from TPN trees tended to exhibit GPX activity higher than high yielding healthy trees (even not significant) and than TPD trees (*).

Table 14-A	All healthy trees	Selected healthy trees	Diseased trees	
Table 8-A	Mean Yield/Tap	Mean Yield/Tap	TPN	TPD
Control	98.5 ± 8.3	97.7 ± 18.2	2[a]	1
Over-stimulated	90.2 ± 14.3	91.8 ± 14.5	2[b]	1
Over-tapped	66.9 ± 7.4 **	53.4 ± 4.0**	2[b]	2

Table 8-B	All trees in whatever health status			
Treatment	Mean yield/Tap	Mean SOD	Mean APX	Mean GPX
Control	90.3 ± 20.9	3.27 ± 0.69	1.51 ± 0.19	0.042 ± 0.011
Over-stimulated	85.1 ± 12.7	3.81 ± 1.01	1.36 ± 0.39	0.070 ± 0.013
Over-tapped	49.6 ± 17.5*	5.12 ± 1.92	1.24 ± 0.22	0.026 ± 0.011**
	Only healthy trees			
Treatment	Mean yield/Tap	Mean SOD	Mean APX	Mean GPX
Control	97.7 ± 18.2	3.02 ± 0.38	1.54 ± 0.19	0.045 ± 0.007
Over-stimulated	91.8 ± 14.5	3.99 ± 1.25	1.47 ± 0.28	0.053 ± 0.012
Over-tapped	53.4 ± 4.0**	4.56 ± 0.34	1.25 ± 0.12	0.025 ± 0.007**

Table 8-C	Latex giving trees in whatever treatment			
Health status	Mean yield/Tap	Mean SOD	Mean APX	Mean GPX
Healthy	84.7 ± 13.6	3.73 ± 0.97	1.44 ± 0.23	0.043 ± 0.014
Necrosis (TPN)	80.5 ± 7.9	3.72 ± 0.09	1.21 ± 0.49	0.058 ± 0.018
Dryness (TPD)	43.2 ± 19.9*	5.26 ± 2.52	1.22 ± 0.35	0.024 ± 0.012*

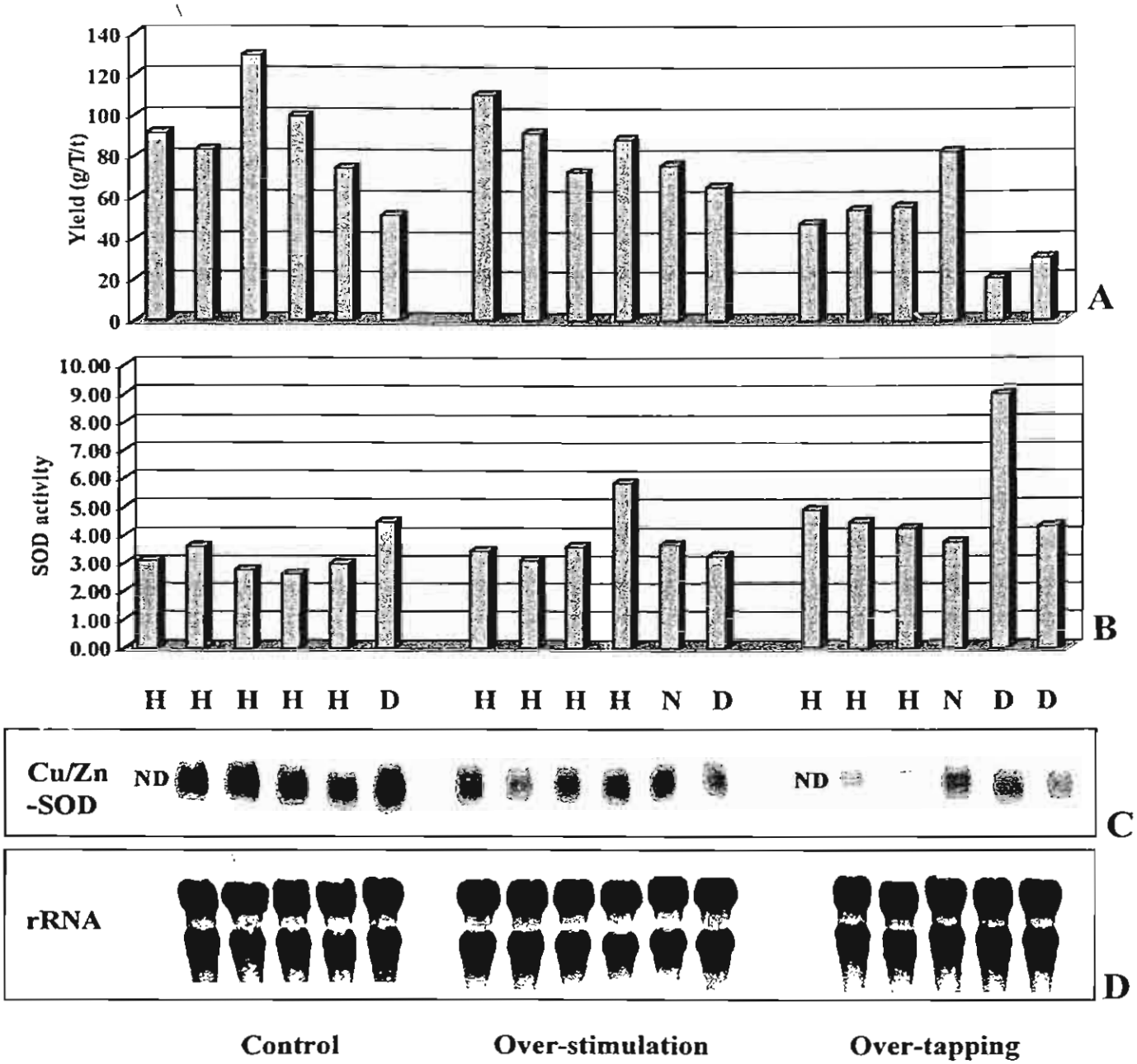

Figure 17. Effect of over-exploitation on rubber yield, SOD activity and Cu/Zn-SOD gene expression in the latex of healthy and diseased tree from PB 260 clone.

A: yield (g dry rubber/Tapping/tree); B: latex cytosolic SOD activity; C: Cu/Zn-SOD gene expression in latex by Northern blot analysis; D: methylene blue staining of rRNA as a control of loading homogeneity. H: healthy tree; N: tree with typical symptom of Trunk Phloem Necrosis (early stage of TPN), D: tree with TPD symptom.

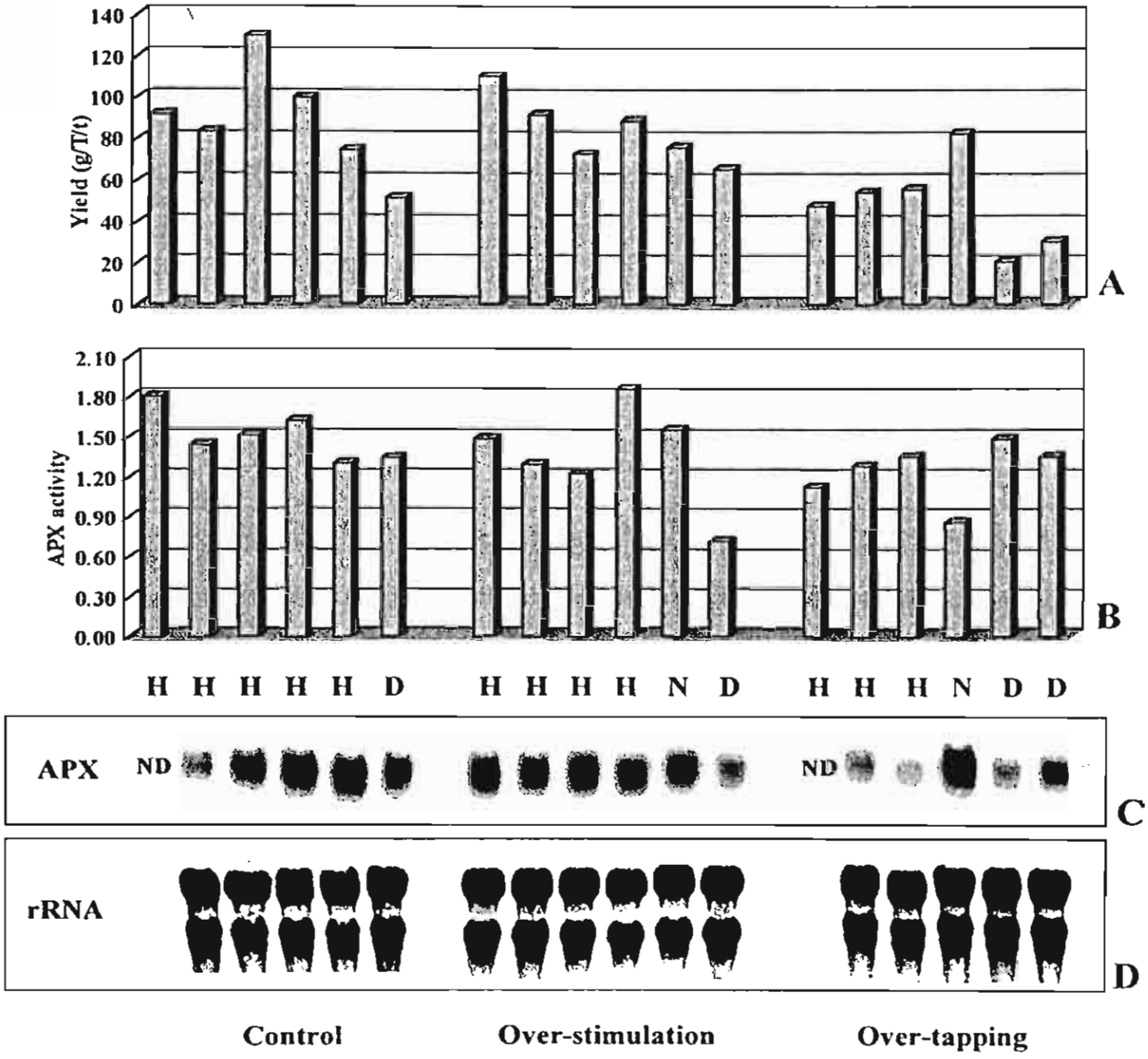

Figure 18. Effect of over-exploitation on rubber yield, APX activity and gene expression in the latex of healthy and diseased tree from PB 260 clone.

A: yield (g dry rubber/Tapping/tree); B: latex cytosolic APX activity; C: APX gene expression in latex by Northern blot analysis; D: methylene blue staining of rRNA as a control of loading homogeneity. H: healthy tree; N: tree with typical symptom of Trunk Phloem Necrosis (early stage of TPN), D: tree with TPD symptom.

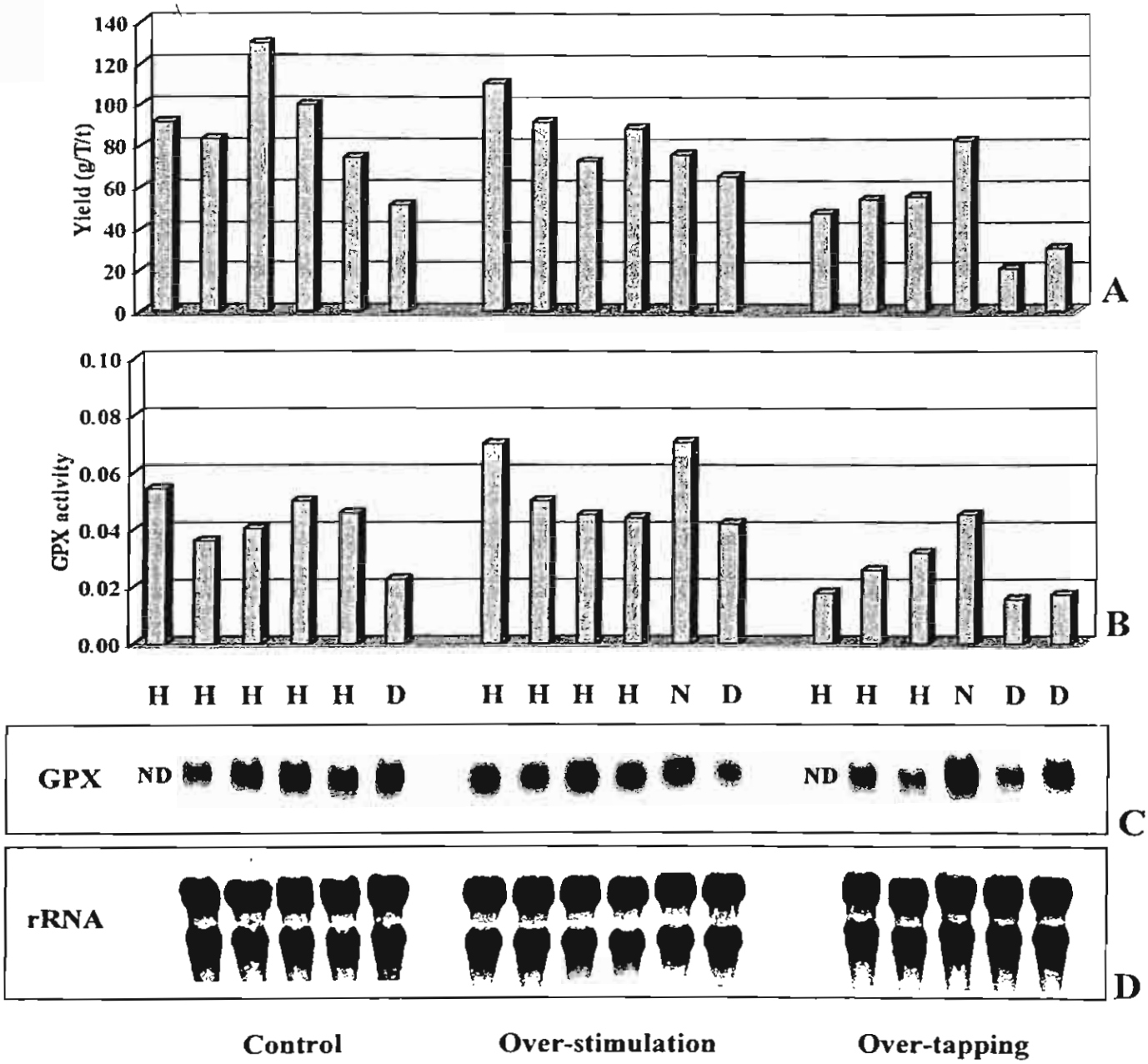

Figure 19. Effect of over-exploitation on rubber yield, GPX activity and gene expression in the latex of healthy and diseased tree from PB 260 clone.

A: yield (g dry rubber/Tapping/tree); B: latex cytosolic GPX activity; C: GPX gene expression in latex by Northern blot analysis; D: methylene blue staining of rRNA as a control of loading homogeneity. H: healthy tree; N: tree with typical symptom of Trunk Phloem Necrosis (early stage of TPN), D: tree with TPD symptom.

3.6 Latex GPX has behavior as PHGPX

As sequencing of the *Hevea* GPX cDNA clone indicated it may correspond to a PHGPX, we tested if a latex cytosolic GPX could use an organic hydroperoxide as substrate. Comparison of both GPX and PHGPX activities using H_2O_2 and phosphatidylcholine hydroperoxide (PH) as electron donors, respectively (Table 15) shows that the activity of latex cytosolic samples (PB 235 and Avros 2037 clones) was about 30% higher in the presence of the phospholipid hydroperoxide. High yielding trees from the clone PB 235 exhibited 2 times more maximum activity for both GPX and PHGPX compared to AVROS 2037, indicating that both activities might result from the action of the same enzyme. The maximum PHGPX rate was already reached at a PH concentration of 20µM, while, in the same conditions, 150 µM H_2O_2 were necessary for maximum GPX activity. However, probably due to non reproducible preparation and non optimal storing conditions of the PH substrate, the PHGPX measurements were poorly reproducible from one day to another, and standard deviation was always 2 to 3 times higher when measuring PHGPX compared to GPX activities (Table 9).

Table 15. Glutathione peroxidase (GPX) and phospholipid hydroperoxide glutathione peroxidase (PHGPX) activities in the latex cytosol.

Latex cytosolic samples from the clones PB 235 and AVROS 2037 were tested for their GPX and PHGPX activities, using H_2O_2 and phosphatidylcholine hydroperoxide (PH), respectively as substrate. The assays were performed at 2 concentrations of the electron donors, in the presence of GSH, NADPH and glutathione reductase, as described in Materials and Methods. The activities were followed by monitoring the NADPH oxidation at OD_{340nm}. One unit activity was expressed as the amount of protein required to oxidize 1 µmole of $NADPH.min^{-1}.ml^{-1}$ (a mean of 3 samples from the highest yielding trees/clone).

Clone activity	Substrate concentration (µM)			
	H_2O_2		Phosphatidylcholine hydroperoxide	
	20	150	20	40
PB 235 (units)	0.082 ± 0.010	0.112 ± 0.012	0.153 ± 0.025	0.148 ± 0.024
AVROS 2037 (units)	0.043 ± 0.012	0.056 ± 0.014	0.075 ± 0.027	0.073 ± 0.026

DISCUSSION

It is generally admitted that at least 2 kinds of TPD have to be distinguish: the irreversible Tapping Panel Necrosis (Trunk Phloem Necrosis or TPN), and the reversible overexploitation-induced TPD. In the later case, membrane destabilization leading to bursting of the lutoid and consecutive *in situ* latex coagulation, has been proposed to be associated with the occurrence of an uncompensated oxidative stress within the latex cells (Chestin, 1989). In Chrestin's experiments, trees submitted to overstimulation fell into 2 categories: those which remained healthy all along the experiment and those which developed the bark dryness syndromes. In case of still healthy-overstimulated tress, the increased production of oxyradical species was compensated by a transient increase both in activity of protective enzymes SOD and catalase, and in the content of the scavenging reduced thiols. But in case of overstimulated trees with TPD symptoms, the protective elements (enzymatic and nonenzymatic) were no longer increased in response to stimulation. On the contrary, their level even dropped below the initial level, giving way to peroxidative lipid degradation.

The objective of the present work was to investigate whether modifications in the expression of some genes involved in the protection against oxidative stress could account for the unbalancing of the protective metabolism towards deleterious effects in different cases of TPD and whether these genes are related to yield and stress response of rubber tree.

To test this hypothesis, three full-length cDNA clones of Cu/Zn-SOD, APX, and GPX were first obtained and used as the specific probes to study their gene expression.

Cu/Zn superoxide dismutase (Cu/Zn-SOD)

There are classically 3 sorts of SOD in plants; Mn-SOD, Cu/Zn-SOD, and Fe-SOD. Mn-SOD is mostly compartmentalized in mitochondria whereas the Cu/Zn-SOD and Fe-SOD are cytosolic or chloroplastic. Study of all these three genes would be necessary to get precise information on SOD gene expression in rubber tree latex. In this work, we report the cytosolic Cu/Zn-SOD cDNA sequence from rubber tree. Sequence analysis (Fig. 1) showed that it consisted of 738 nucleotides, potentially encoding a 152 amino acid protein with a calculated molecular weight of 15.6 kDa and pI 5.67. No signal peptide was found in the *Hevea* Cu/Zn-SOD sequence, suggesting that it encode a cytosolic Cu/Zn-SOD. Further sequence analysis showed that its deduced amino acid sequences contain two domains of Cu/Zn-SOD signatures, GFHVHTFGDTT (position 43-53) and GNAGDRIACGII (position 137-148). Comparison of the nucleotide and deduced amino acid sequence of *Hevea* Cu/Zn-

SOD cDNA with other plants Cu/Zn-SOD sequence recorded in several databases showed that *Hevea* Cu/Zn-SOD had highest score of homology to the *Populus tremuloides* Cu/Zn-SOD cDNA, with 96% and 72% homology in nucleotides and amino acids, respectively. Southern analysis of genomic DNA from leaf indicated that there is more than one copy gene of Cu/Zn-SOD in *Hevea*. For *Hevea* Mn-SOD cDNAs, they were isolated by Miao and Gaynor (1993). For the moment, we did not check for Fe-SOD (chloroplastic) expression in the latex cells, which are not photosynthetic tissue.

Ascorbate peroxidase (APX)

It has been reported that APX exist as several plastidal or cytosolic isozymes. We isolated and sequenced a full-length APX cDNA, encoding a cytosolic isoform in *Hevea* latex. This cDNA (Fig.2) consisted of 1058 nucleotides, contained an ORF of 753 bp. The predicted amino acid length and protein size is 250 residues and 27.4 kDa, respectively. The deduced amino acid sequences share nearly 80% similarity with those of other plant cytosolic APX reported to date. Furthermore, the isolated *Hevea* APX cDNA has the conserved sequences or regions, such as catalytic triad, that are important for the maintenance of structure and function in all APX. Meanwhile, it does not have any sequence similar to transit peptide of plastidal isoforms.

Glutathione peroxidase (GPX)

Although glutathione peroxidase is considered as one of the key enzymes involved in scavenging oxygen radicals in animals, this enzyme was poorly known in plants until recently. We report in this work on the isolation and characterization of the GPX-like encoding cDNA named GPX6.2.1 from *Hevea* latex (Fig. 3). Amino acid sequences deduced from the cloned cDNA showed the two conserved domains PCNQF (glutathione peroxidase signature) and WNFSK, containing Q and W residues, two of the three residues (W, Q and selenocysteine) known to be critical for GPX catalytic activity. Concerning the selenocysteine residue, the *Hevea* GPX sequence shows a UGU codon, in place of the UGA codon, which characterizes the selenocysteine-containing GPX in mammal (Chu *et al.*, 1993). This suggesting that *Hevea* GPX protein is not a selenium-dependent protein, as well as the other plant GPX so far identified (Holland *et al.*, 1993; Criqui *et al.*, 1992). Moreover, the presence of selenocysteine in any plant proteins has not been identified by either protein or DNA sequence analysis (Holland *et al.*, 1993; Criqui *et al.*, 1992). In animals, the replacement of the catalytic selenocysteine by cysteine is known to result in a drastic decrease of the

enzyme activity (Eshdat *et al.*, 1997, Maiorino *et al.*, 1995). The substitution of sulfur by selenium in selenocysteine confers on this amino acid a greater nucleophilic power and a low pK than that of cystein. Thus, selenium-dependent GPX acquires a more powerful redox potential towards its substrate (Eshdat *et al.*, 1997). Therefore, being selenium-independent enzyme, *Hevea* GPX and other plant GPX are expected to have much lower activity than animal GPX.

The amino acid sequences deduced from the ORF review that *Hevea* GPX encoded a protein of 176 amino acid with a calculated molecular weight of 19.3 kDa and a theoretical pI of 5.23. The protein product shared similarity with other plant GPX-protein and especially phospholipid hydroperoxide glutathione peroxidase (PHGPX). PHGPX is a monomeric enzyme (molecular mass of around 20 kDa) that reduces hydroperoxide derivatives of lipids (phospholipid hydroperoxides and cholesterol hydroperoxides) inserted membranes. Further analyses of *Hevea* latex GPX revealed that it contains three putative N-myristoylation sites (in position 43-48, 122-127, 160-165) and one of them (in position 122-127) was found to be in the same position as in the mammalian PHGPX. Myristoylation is the first step of the mechanism by which a protein associates with a membrane (Boutin, 1997). These results suggest that *Hevea* GPX is more closely related to PHGPX than to other types of GPXs. In addition computer analyses of *Hevea* GPX seemed to predict that the protein is devoid of a signal peptide or transmembrane regions, and is also unlikely to be a chloroplast protein. Thus *Hevea* GPX is most likely a cytosolic protein or may be able to bind to membrane structures facing the cytosolic compartment.

In the GPX activity analysis, we could verify that latex cytosolic samples could use both H_2O_2 and phosphatidyl-choline hydroperoxide as electron donors. The fact that the PHGPX/GPX activity ratio (~ 1.35) of PB 235 and AVROS 2037 were identical, indicated that the same enzyme may be able to catalyze both reactions. However, in our conditions, even the PHGPX activity was shown to be slightly higher (+ 35%) than GPX activity, it was rather low even compared to other plant PHGPX (Holland *et al.*, 1993; Criqui *et al.*, 1992). Purification of the latex cytosolic PHGPX need to be undertaken for further biochemical characterization, in particular to verify its affinity for both H_2O_2 and organic hydroperoxides, as well as its ability to bind to latex organelles membranes in one or another condition.

These three full-length cDNA clones were used as specific probes in Northern blot analysis.

Enzyme activity and gene expression analysis in latex

Relationships between yield, health status, enzyme activity, gene expression were studied from various clones of rubber tree such as PB 235, PB 260, Avros 2037. Different cases of TPD were investigated including spontaneous necrotic TPD or trunk phloem necrosis (TPN), and overexploitation-induced TPD (both overtapping and overstimulation-induced TPD). In overexploitation experiment, it appeared that it was not so easy to induce the disease after 1-2 years of overexploitation, only a few trees became real sick after treatment. It seems that the genotype studied in this experiment (PB260) was rather resistant to TPD in the condition (soil, climate, etc) encountered at the SOGB industrial rubber plantation in Ivory Coast, Africa.

Kinetic effect of ethylene treatment and bark opening

Ethylene is known to have a general stimulatory effect on the metabolism of the latex cell (Coupe and Chrestin, 1989) and to trigger the overexpression of some specific genes in the latex (Goyvaert *et al.*, 1991; Kush *et al.*, 1990; Miao and Gaynor, 1993; Pujade-Ranaud *et al.*, 1997; Suberto *et al.*, 1996). Thus, before investigating the effect of overstimulation, it was interesting to study the effect of a normal single ethylene treatment on the expression of our genes of interest. This experiment was performed on virgin trees in order to avoid any side effect due to tapping.

The level of APX mRNA was very low in the resting latex cells of virgin tree (untapped and unstimulated tree) (Fig. 5). This maybe described since the resting untapped latex cells of virgin tree are characterized by a very low metabolic activity (as there is no need for cytoplasm regeneration).

In virgin trees, stimulation with ethylene slightly induced an accumulation of APX mRNA (Fig. 5) in the latex between 36-48 hrs after the treatment. However, the ethylene effect and probably opening effect were obviously observed in the 2nd tapping. The result showed that there is probably a high metabolic activation by ethylene and additional tapping. Successive tappings of stimulated trees induced an even more marked over-expression of the APX gene in the latex cell. It is not known whether this is a direct response to wounding (generation to endogenous ethylene) or an indirect effect associated with the metabolic activation required for latex regeneration upon successive tappings. In many plant systems, wounding is known to trigger the synthesis of endogenous ethylene, which in turn is involved in the regulation of gene expression. Thus, endogenous ethylene potentially produced in response to the tapping wound may act as an intermediary messenger for the stimulation of

APX gene expression. Because APX reduces H_2O_2 into water, this may indicate that a high level of APX is required for removing increased level of H_2O_2 in the stimulated trees owning to higher metabolic activity (hence high oxidation processes) in the latex cells. This northern blot experiment, performed on virgin trees, demonstrates that ethylene induced APX transcript accumulation in rubber tree latex independently of tapping-induced stimulation that may occur in regular exploitation conditions.

Contrary to some other genes (Pujade-Ranaud *et al.*, 1997), single stimulation had little or no effect on latex GPX activity and GPX gene expression in the resting latex cells of virgin trees (Fig. 6). It has to be mentioned that the PB 314 clone, used in this experiment, is a high yielding one as PB 235, which contained high constitutive GPX activity and gene expression. It may be conclude that GPX activity is already at its maximum rate and cannot exceed this constitutive level. Nevertheless this short-term kinetics experiment could show that, as the small rubber particle protein (SRPP) gene, the GPX1 gene expression was not directly induced by ethylene treatment (Oh et al., 1999) but induced by tappings. Its expression was very low in the resting latex cells of virgin tree but increased after the trees had been tapped, without significant change in GPX activity. It may be concluded that increase in the tapping-induced-laticifers regenerative metabolism (Coupe and Chrestin, 1989) can trigger GPX gene expression and enzyme turnover. It looked like that the associated GPX activity remained mainly under the control of post-transcriptional regulation.

Interestingly, the expression of Cu/Zn-SOD was rather high in virgin tree and found to decrease after Ethrel stimulation. In opposite to *Hevea* Mn-SOD, of which expression was very low in virgin tree and found to increase after stimulation with Ethrel (Miao and Gaynor, 1993 and Kongsawadworakul *et al.*, 1997). This result suggested that these two genes maybe differently regulated during stress. In addition, some literatures have revealed that the SOD genes are differently regulated during development (Kurepa *et al.*, 1997). The discrepancy between ethylene-induced decrease or increase of gene transcript levels and the constant (not significantly different) activity of the corresponding enzyme suggested that SOD activity is regulated post-transcriptionally, for example, by regulating efficiency of translation and / or protein stability.

Higher metabolic activity means higher production of toxic oxygen species. It is therefore important for the cell to have corresponding higher protective systems. Moderate ethylene treatment stimulates catalase, Mn-SOD, GR (Kongsawadworakul et al., 1997), and APX (in this study) between 24 and 48 hrs after treatment. It should be underlined that a much quicker ethylene-induced gene overexpression (between 6 and 12 hrs) was observed

in the latex of virgin tree in the case of glutamine synthetase (Pujade-Ranaud *et al.*, 1997). This would indicate that overexpression observed for catalase, Mn-SOD, GR and APX may be an indirect consequence of the general metabolic activation induced by ethylene, as more efficient protective system are required in that case to balance an increased production of toxic oxygen species. Overexpression of these genes may be directly activated by increased release of $O_2^{\cdot-}$ and H_2O_2, due to the action of mitochondrial activity, as it is the case for catalase and Mn-SOD induction in the case of oxidative stresses in bacteria and plant (Bowler *et al.*, 1991).

Inter-clonal and intra-clonal variation of rubber tree

Inter-clonal experiment could show that the healthy unstimulated trees from PB235 – the tested highest yielding clone – exhibited significantly higher cytosolic GPX activity, correlated with a higher GPX gene expression in the latex, compared with the two lower yielding clones (PB 260 and Avros 2037) as shown in Fig. 9. Likewise, intra-clonal experiments could show that, compared to healthy low-yielding trees, the healthy high-yielding ones exhibited higher GPX activity, together with a tendency of the GPX gene to be higher expressed in the latex (Fig. 12,15)

Depending on the treatment, overexploitation showed different effects on GPX activity and gene expression of the still healthy trees (Fig. 19). Although not statistically significant, over-stimulation tended to induce higher GPX activity together with a higher GPX gene expression in the latex, and at the meantime, the trees did not respond anymore to stimulation as far as their latex yield was concerned. Contrary, after one-year experiment, overtapping induced a significant decrease in yield, parallel with a highly significant decrease in GPX activity and gene expression in the latex. From all these results dealing with healthy trees, it may be concluded that there is a positive relationship between rubber yield and latex cytosolic GPX activity, and to some extend with GPX gene expression.

Through inter-clonal and intra-clonal studies, rubber yield has been reported to be positively correlated to the metabolism potential and activity in the latex producing tissues (d'Auzac and Jacob 1989; Vichitcholchai *et al.*, 1997). It has been well documented that high metabolism, especially due to high mitochondrial oxidative activity, can induce a release of ROS (Moller, 2001) which are able to induce numerous cell damages, up to cell death, if they are not detoxified (Arora *et al.*, 2002). For rubber tree, direct relationships have been found between inter-clonal as well as intra-clonal variation of yield, of the reduced thiols content and of the organelles membrane stability in the latex (Chrestin, 1989; d'Auzac 1989).

Further, in the latex, a strictly NADPH-dependent glutathione reductase has been characterized. Its specific activity did not look to be limited *per se* for the reduction of GSSG (Prevot *et al.*, 1984). On the other hand, the very low NADPH concentration in the latex cytosol was supposed to be the limiting factor for GSH recycling and rubber biosynthesis (Prevot *et al.*, 1984; Arreguin et al., 2000). Considering all these data together, we propose that the high yielding rubber clone - as well as the higher yielding healthy trees within a given clone - characterized by a relatively high metabolism activity may generate higher ROS in the latex cells. In these high yielding trees, the corresponding potential oxidative stress is supposed to be fully compensated by higher activities and gene expression of ROS scavenging enzymes such as GPX and/or PHGPX, as well as chemicals such as GSH. This should be particularly true since high metabolism in the latex should generate more NADPH for the reduction of GSSG generated by the GPX/PHGPX activities, through the non-limiting activity of the latex cytosolic NADPH-dependent glutathione reductase.

Compared to normal exploitation, in which the tree giving approximate similar rubber yield, the over-stimulated still healthy trees tended to exhibit slightly higher GPX activity and GPX gene expression (Fig. 19). This may indicate that, in this case, the over-stimulation-induced metabolic activation might probably lead to higher release of ROS (Chrestin, 1989) which was still fully compensated by higher scavenging enzyme activities, especially GPX/PHGPX in the latex cells.

In contrary, compared to the normal exploitation, overtapping induced a decrease of rubber yield associated with a significant decrease in GPX activity and GPX gene expression in the latex, even in the still healthy trees. In this case, since the metabolism was not stimulated by Ethrel treatments as for overstimulation (Coupe and Chrestin, 1989), it may be hypothesized that the metabolic effort required to regenerate the excessive loss of latex due to overtapping, more rapidly overstepped the metabolic potential of the trees. Most probably, the latex producing tissues, nearing exhaustion, turned to inefficient regenerative metabolism which impaired gene expression and consecutive lower GPX activity. In the overtapped tree, the expression of Cu/Zn-SOD and APX were also decreased even through their activities were not significantly reduced, compared to normal exploited trees.

Whatever the treatment (normal or overexploitation) and the clone, trees exhibiting bark disease displayed opposite GPX activity and gene expression, depending on the nature of the disease. Trees with typical TPD symptoms (only dryness) were characterized by lower GPX activity and lower GPX expression in the latex. In contrary, compared to control healthy

trees, the ones displaying TPN (necrosis) symptoms exhibited normal or even often higher GPX activity and gene expression.

Latex from overexploitation-induced TPD trees have previously been reported to exhibit higher release of ROS (Chrestin, 1984), associated with lower SOD and catalase activities together with a net decrease in the reduced thiols content (Kongsawadworakul et al., 1997, Chrestin, 1989, Chrestin, 1984). Such an uncompensated oxidative stress was shown to result in enhanced lipid peroxidation and damage to organelles – especially lutoids – membrane within thee latex cells (Chrestin, 1986). The data reported in the present work showed a decrease in the GPX activity and GPX gene expression in the latex from all TPD trees. These are further evidences of an uncompensated oxidative stress occurring at the level of the latex producing tissues in the rubber trees exhibiting symptoms of TPD. As the GPX gene corresponds to an enzyme with PHGPX activity, which contributed in the recycling of membrane lipid-hydroperoxides, thus the decreased expression of GPX gene in the latex from TPD trees undergoing oxidative stress may explain the reported destabilization of the latex organelles.

Contrary to TPD trees, we could show an increase in GPX activity and GPX gene expression in the latex from trees exhibiting typical symptoms of Trunk Phloem Necrosis (TPN). These results, even though obtained from few trees (8 TPD and 10 TPN), give further indications that these two bark diseases (TPD and TPN) are of different origin. In various plant species, PHGPX and GPX-like genes have been reported to be inducible by various biotic or abiotic stresses (Agrawal et al., 2002, Wagner et al., 2002). In the case of TPN, some still unidentified stress is supposed to lead to tissue necrosis. One can hypothesize that the still healthy tissues (still producing some latex), neighboring the areas undergoing necrosis, may be submitted to some inflammatory process and over express ROS scavenging genes including GPX in order to limit the extension of the necrotic areas.

In case of APX and Cu/Zn-SOD, even though they were often not significantly different in their activity and gene expression, compared between rubber clone and yield or healthy and diseased trees, but they still remained high activity and gene expression in the latex cell of normal exploited tree. This evidence suggested that these two enzymes still play important role in protection of latex cell from oxidative stress.

In some cases, such as in high yielding tree and in overexploitation experiment, the APX gene was expressed in the same way as GPX gene. Since these two enzymes (APX and GPX) can detoxify H_2O_2, they may be much more required in condition that cell contains high concentration of H_2O_2 or other ROS. Alternatively, *Hevea* GPX contained PHGPX

activity, which might be restricted to more complex acceptor or donor substrate like different organic hydroperoxides or large thiol compounds that are not metabolized by other antioxidant enzymes such as APX and catalase. Although the best substrates for PHGPX *in vitro* are phospholipid hydroperoxides, it is not clear whether such phospholipid that comprises biomembranes are the natural substrate of the enzyme. The physiological role of *Hevea* PHGPX is a subject of future study.

REFERNCES

Agrawal GK, Rakwal R, Jwa NS, Agrawal VP. Effects of signaling molecules, protein phosphatase inhibitors and blast pathogen (*Magnaporthe grisea*) on the mRNA level of a rice (*Oryza sativa* L.) phospholipid hydroperoxide glutathione peroxidase (OsPHGPX) gene in seedling leaves. Gene. 2002; 283:(1-2) : 227-236.

Arora A, Sairam RK, Srivastava GC. Oxidative stress and antioxidative system in plants. Curent Science. 2002;82:10: 1227-38.

Arreguin B, Arreguin-Espinosa R. Oxidative reactions of the pentose pathway and the origin of reducing equivalents for the biosynthesis of rubber in *Hevea brasiliensis* latex. J. of Chemical Technology and Biotechnology. 2000;75:4:294-298.

Beauchamp C, Fridovich I. Superoxide dismutase: improved assay and an assay applicable to acylamide gels. Anal Biochem 1971;44:276-87.

Boutin JA. Myristoylation. Cell Signaling. 1997;9:15-35.

Bowler C, Slooten L, Vandenbranden S, Rycke RD, Botterman J, Sybesma C, Montagu M, Inze D. Manganese superoxide dismutase can reduce cellular damage mediated by oxygen radicals in transgenic plant. EMBO J 1991;10:1723-32.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;12:248-54.

Chrestin H, Jacob JL, d' Auzac J. Biochemical basis for cessation latex flow and occurrence of physiological bark dryness. Proc Int Rubb Conf; 1985 Oct; Kuala Lumpur. Kuala Lumpur:Commun Int Rubb Conf, 1985:20-42.

Chrestin H, Jacob JL, d' Auzac J. Biochemical basis of cessation of latex flow and occurrence of physiological bark dryness. In : Rajaro JC and Amin L, editors. IRC:1985; RRIM , Kuala Lumpur 1986;3:88-101.

Chrestin H. Biochemical aspects of bark dryness induced by overstimulation of rubber trees with Ethrel. In: d' Auzac J, Jacob JL, Chrestin H, editors. Physiology of rubber tree latex. Boca Raton (FL): CRC Press, 1989:431-41.

Chrestin H, Pujade-Renaud V, Montoro P, Naranggajavana J, Vichitcholchai N, Teerawatanasuk K, Lacrotte R. Expression of genes invoved in coagulation and regeneration of latex . Clonal variation and effects of yield stimulation with ethrel. proceeding of the seminar on the biochemical and molecular tools for exploitation diagnostic and rubber tree improvement; 1997 October 20-22; Faculty of science,Mahidol university, Bangkok :1997.

Chu FF, Doroshow JH, Esworthy RS. Expression, characterization and tissue-distribution of a new cellular selenium-dependent glutathione peroxidase, GSHPx-GI. J Biol. Chem. 1993;268:2571-2576.

Commere J, Eschbach JM, Serres E. Tapping panel dryness in Cote d' Ivoire. Proceeding of the IRRDB workshop on rubber tree dryness; 1989 June 26-27;Penang: 1989.

Coupé M, Chrestin H. Physico-chemical and biochemical mechanisms of hormonal (ethylene) stimulation. In: d'Auzac J, Jacob JL, Chrestin H, editors. Physiology of the rubber tree latex. Boca Raton (FL): CRC Press, 1989:295-319.

Criqui MC, Jomet E, Parmentier Y, Marbach J, Durr A and Fleck J. Isolation and characterization of a plant cDNA showing similarity to animal glutathione peroxidase. Plant Mol Biol 1992;18:623-27.

d'Auzac J and Jacob JL. Regulation of glycolysis in the latex of *Hevea brasiliensis* . J Rubber Res Inst Malaya 1969 ;21 :417.

d'Auzac J, Jacob JL. The composition of latex from *Hevea brasiliensis* as a laticiferous cytoplasm. In: d'Auzac J, Jacob JL, Chrestin H, editors. Physiology of the rubber tree latex. Boca Raton (FL): CRC Press, 1989:59-96.

d' Auzac J, Ribaillier D. L' ethylene, novel agent stimulant de la production de latex chez l' *Hevea brasilienisi* latex. Physiol Veg 1969; 46 : 857.

de Faÿ E. Histophysiology Comparée des Ecorces Saines et Pathologiques (Maladie des Encoches Seches) de l' *Hevea brasiliensis*, These Doc. third cycle. Universite' Monpellier II (USTL), Montpellier, France:1981.

de Faÿ E, Hebant C. Etude histologique des écorces d'*Hevea brasiliensis* atteintes de la maladies des encoches seches. C R Acad Sci Paris Ser D 1980;291:867.

de Faÿ E, Jacob JL. Anatomical organization of the laticiferuos system in the bark of *Hevea brasiliensis*. In: d'Auzac J, Jacob JL, Chrestin H, editors. Physiology of the rubber tree latex. Boca Raton (FL): CRC Press, 1989:3-14.

de Faÿ E, Jacob JL. The bark dryness disease (Brown-Bast) of *Hevea*, Symtomatology, histological and cytological aspects. In: d' Auzac J, Jacob JL, Chrestin H, editors. Physiology of the rubber tree latex. Boca Raton (FL): CRC Press, 1989:407-30.

Depage N, Drevet J and Boyer N. Molecular cloning and characterization of tomato cDNAs encoding glutathione peroxidase-like proteins. Eur J Biochem 1998;253:445-51.

Duan YJ, Komura S, Szafarz BF, Szafarz D, Yagi K. Purification and Characterization of a novel monomeric glutathione peroxidase from rat liver. J Biol Chem 1988;263:19003-8.

Eschbach JM, Lacrotte R, Serres E. Conditions which favor the onset of Brown Bast (TPD). In: d' Auzac J, Jacob JL, Chrestin H, editors. Physiology of the rubber tree latex. Boca Raton (FL): CRC Press, 1989:431-58.

Eshdat Y, Holland D, Faltin Z, Ben-Hayyim G. Plant glutathione peroxidases. Physiol Plant 1997;100:234-40.

Fridovich I. Measuring the activity of superoxide dismutase: an embarrassment of riches. In : LW Oberly, editor. Superoxide dismutase, Vol 1 CRC Press, Boca Raton, FL 1982 pp 69-77.

Goyvaerts E, Dennis M, Light D, and Chua NH. Cloning and sequencing of cDNA encoding the rubber elongation factor of *Hevea brasiliensis*. Plant physiol 1991;97:317-21.

Holland D, Ben-Hayyim G, Faltin Z, Comoin L, Strosberg AD and Eshdat Y. Molecular characterization of salt-stress-associated protein in citrus : protein and cDNA sequence similarity to mammalian gluthathione peroxidase. Plant Mol Biol 1993; 21:923-27.

Jacob JL Prevot JC. IRRDB Symposia. Penang:1989.

Jacob JL, Prevot JC, Lacrotte R. Tapping Panel Dryness in *Hevea brasiliensis*. Plantations, researche, developpement 1994;1:15-24.

Kongswadworakul P, Pujade-Renaud V, Chrestin H, Montoro P, Lacrotte R, Narangajavana J. Cloning and expression of genes involved in oxidative stress in latex from TPD trees. In : The Biochemical and molecular tools for exploitation diagnostic and rubber tree improvement seminar, Faculty of science, Mahidol university, Bangkok, October 20-22, 1997.

Kurepa J, Herouart D, Van Montagu MV, Inze D. Differential expression of Cu/Zn and Fe-superoxide dismutase genes of tobacco during development, oxidative stress and hormonal treatments. Plant Mol Biol 1997;38:463-70.

Kush A, Goyaverts E, Chye ML, Chua NH. Laticifer-specific gene expression in *Hevea brasiliensis* (rubber tree). Proc Natl Acad Sci USA 1990;87:1787-90.

Lopez F, Vansuyt G, Case-Delbart F, Fourcroy P. Ascorbate peroxidase activity, not the mRNA level, is enhanced in salt-stressed *Raphanus sativa* plants. Physiol Plant 1996;97:13-20.

Loyen G. The future of natural rubber and INRO. Plantations, research, development 1998;5: 261-68.

Luckman M. Tapping panel dryness in Indonesia. Proceeding of the IRRDB workshop on rubber tree dryness; 1989 June 26-27; Penang:1989.

Maiorino M, Aumann KD, Brigélius-Flohé R, Doria D, Van den Heuvel J, MacCarthy J, Roveri A, Ursini F, Flohé L. Probing the presumed catalytic traid of selenium containing peroxidases by mutational analysis of phospholipid hydroperoxide glutathione peroxidase (PHGPX). Biol Chem Hoppe-Seyler 1995;376:651-660.

McCord JM, and Fridovich I. Superoxide dismutase and peroxidase: a positive activity stain applicable to polyacylamide gel electrophoregram. Arch Biochem Biophys 1977;183:511-15.

Moller IM . Plant mitochondria and oxidative stress: Electron transport, NADPH turnover, and metabolism of reactive oxygen species. Annual Review of Plant Physiology and Plant Molecular Biology 2001;52:561-91.

Nakano Y, and Asada K. Hydrogenperoxide is scavenged by ascorbate-specific peroxydase in Spinach chloroplast. Plant cell Physiol 1981;22:867-80.

Nandris D, Thouvenel JC, Nicole M, Chrestin H. The phloem necrosis of *Hevea* trunk in Ivory Coast : 2) Ethiology of the disease. Eur J For Path 1991;21:340-53.

Nandris D. Chrestin H. The phloem necrosis of *Hevea* trunk in Ivory Coast : 1) symtomatology and biochemical characteristics. Eur J For Path 1991;21:325-39.

Oh SK, Kang H, Shin DH, Yang J, Chow KS, Yeang HY, Wagners B, Breiteneder H and Han KH. Isolation, characterization and functional analysis of a novel cDNA clone encoding a small rubber particle protein from *Hevea brasiliensis*. J. Biol. Chem. 1999;274:4:17132-38.

Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 1967;70:158-69.

Paranjothy K, Gomez JB, Yeang H. Physiological aspects of brown bast development. In: International Rubber Conference. Kuala Lumpur, Malaysia 1976;2:181-202.

Patterson WR, and Poulos TL. Crystal structure of recombination pea cytosolic ascorbate peroxidase. Biochemistry 1995;34:4331-41.

Prevot JC, Chrestin H. and Jacob JC. Evidence for a glutathione reductase in the cytosol from the latex of *Hevea brasiliensis*. Mise en evidence d'une glutathion reductase dans le serum cytoplasmique du latex d'Hevea brasiliensis. Comptes-Rendus-des-Sciences-de-l'Academie-des-Sciences,-III-Sciences-de-la-Vie. 1984; 298:2:35-38.

Pujade-Renaud V, Perrot-Rechenmann C, Chrestin H, Lacrotte R, Guern J. Characterization of a full-length cDNA clone encoding glutamine synthase from rubber tree latex. Plant Physio 1994;105:127-32.

Pujade-Renaud V, Perrot-Rechenmann C, Chrestin H, Lacrotte R, guern J. Characterization of a full-length cDNA clone encoding glutamine synthetase from rubber tree latex. Plant Physiol Biochem 1997;35(2):85-93.

Rand RD. Brown Bast disease of plantation rubber; its cause and prevention. Arch Rubber Cult 1921;5:223.

Rutgers AAI, and Dammerman. Disease of *Hevea brasiliensis*. Java Med V H Lab V Plant 1914;10.

Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: A laboratory manual. 2nd ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press, 1989.

Sethuraj MR. IRRDB Symposia. Paris: 1988.

Sivakumaran S, Haridas G. Incidence of tree dryness in precocious high yielding clones in Malaysia. Proceeding of the IRRDB workshop on rubber tree dryness; 1989 June 26-27; Penang: 1989.

Sivasubamanium S, Vanniasingham VM, Tan CT, and Chua NH. Characterization of HEVER, a novel stress-induced gene from *Hevea brasiliensis*. Plant Mol Biol 1995;29:173-8.

Somnark S, Vichitcholchai N, Kosaisewee J, Srisa-arn P, Sornsuk S, Anusiri T, and Maumnual B. Survey on the cause of brown bast disease in non-traditional area (east, northeast, Thailand). 1994. Chachoengsao RRC Research Activity Report no. 33.

Subroto T, Vam Koningsveld GA, Schreuder HA, Soedjanaatmdja UMS, Beintema JJ. Chitinase and β-1,3-glucanase in the lutoid-body fraction of Hevea latex. Phytochemistry 1996;43(1):29-37.

Sukulrad W. Studies on TPD (south, Thailand). 1994. RRIT Research Activity Reports. 321-22.

Taysum DH. Effect of ethylene oxide on the tapping of *Hevea brasiliensis*. Nature 1961;191:1319.

Vichitcholchai N, Kosaiasawe J, Lacrotte R. Latex diagnostic: warning of over-exploitation of *Hevea brasiliensis* in Thailand's non-traditional area. Symposium on natural rubber (Hevea brasiliensis): International Rubber Research and Development Board (IRRDB); Brickendonbury; UK. Vol. II - physiology & exploitation and crop protection & planting methods sessions, Ho Chi Minh City, Vietnam,14-15 October 1997. 1998, 61-65.

Wagner-U; Edwards-R; Dixon-DP; Mauch-F. Probing the diversity of the arabidopsis glutathione S-transferase gene family. Plant Mol. Biol. 2002;49:5: 515-532.

Yeang HY. IRRDB Symposia. Penang:1989.

OUTPUT from this research project

Publications

1. **Sookmark U.**, Pujade-Renaud V., Chrestin H., Lacote R., Naiyanetr J., Sequin M., Romreunsukharom P., and **Narangajavana J.** Characterization of polypeptides accumulated in the latex cytosol of rubber trees affected by the Tapping Panel Dryness syndrome. (Submitted to Plant and Cell Physiology : in the process of minor revision).
2. **Sookmark U.**, Chrestin H., **Narangajavana J.** Cloning and characterization of a glutathione peroxidase cDNA in the latex cells of rubber tree (*Hevea brasiliensis*). Expression related to rubber yield and response to stressing treatment. (In preparation).

Registration of DNA sequences to NCBI database

1. A full-length latex cDNA coding for Cu/Zn superoxide dismutase (Cu/Zn-SOD)
   Genbank Accession No. AF457209

2. A full-length latex cDNA coding for ascorbate peroxidase (APX)
   Genbank Accession No. AF457210

3. A full-length latex cDNA coding for Glutathione peroxidase (GPX)
   Genbank Accession No. AF242650

Presentations

1. **Sookmark U.**, Chrestin H., Srisarn P., **Narangajavana J.** Molecular cloning and characterization of a cDNA encoding glutathione peroxidase from latex of rubber tree (*Hevea brasiliencis*) : Expression in response to ethylene stimulation and TPD disease. In: Proceedings of the 12th Annual Meeting of the Thai Society for Biotechnology, Kanchanaburi, Thailand (2000).
2. Pujade-Renaud V., Lacotte R., **Sookmark U.**, Romruensukharom P., Naiyanetr C., **Narangajavana J.**, and Chrestin H. Accumulation of a polypeptide identified as the latex allergen Hev b3 in the latex cytosol of rubber trees displaying the Tapping

Panel Dryness syndrome. In: Proceedings of the 6th International Congress of Plant Molecular Biology, Que'bec, Canada (2000).

3. Pujade-Renaud V., Montoro P., Kongsawadworakul P., Romruensukharom P., **Narangajavana J.**, and Chrestin H. Cloning of potentially ethylene-inducible and/or laticifer-specific promotors from *Hevea brasiliensis*. In: Proceedings of the 6th International Congress of Plant Molecular Biology, Que'bec, Canada (2000).
4. **Narangajavana J**. Looking for biochemical and molecular markers of yields and tapping dryness disease (TPD) in rubber tree (*Hevea brasiliensis*) . In : Proceeding of IRD's Activities and Perspectives of Collaboration in Thailand and in Asia. Bangkok, Thailand (2001).
5. **Sookmark U.,** Chrestin H., **Narangajavana J.** Biochemical and Molecular Marker related to yield and stress response of rubber tree (*Hevea brasiliensis*). In : proceeding of RGJ-Ph.D. Congress III, Thailand (2002).
6. **Sookmark U.,** Chrestin H., **Narangajavana J.** Biochemical and Molecular Marker related to yield and stress response of rubber tree (*Hevea brasiliensis*). In : proceeding of Agricultural Biotechnology International Conference (ABIC), Saskatoon, Canada 2002

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