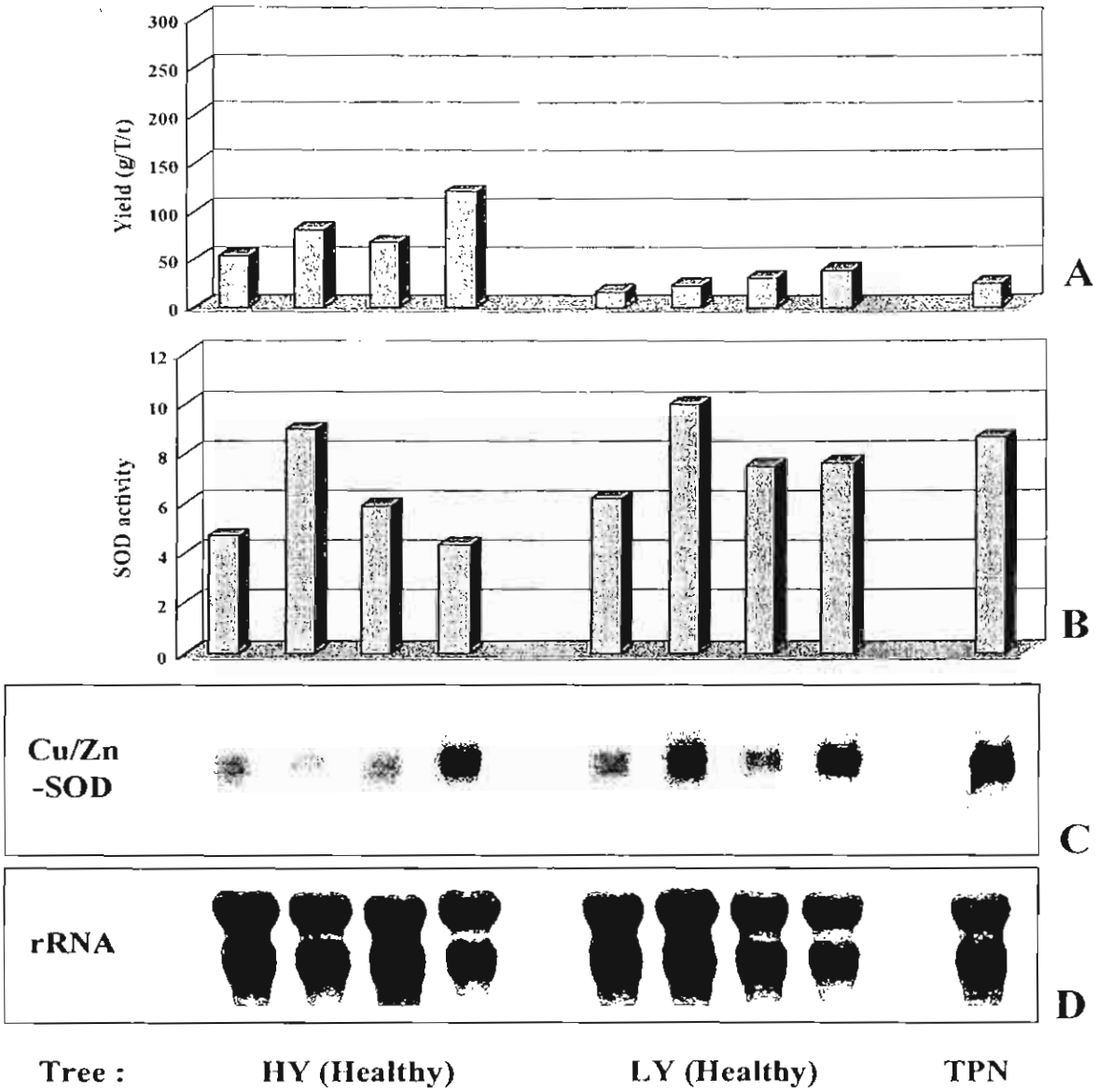

Figure 10. Intra-clone (Avros 2037) relationships between the variation of rubber yield, total SOD activity and Cu/Zn-SOD gene expression in the latex from high yielding and low yielding healthy trees.

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.

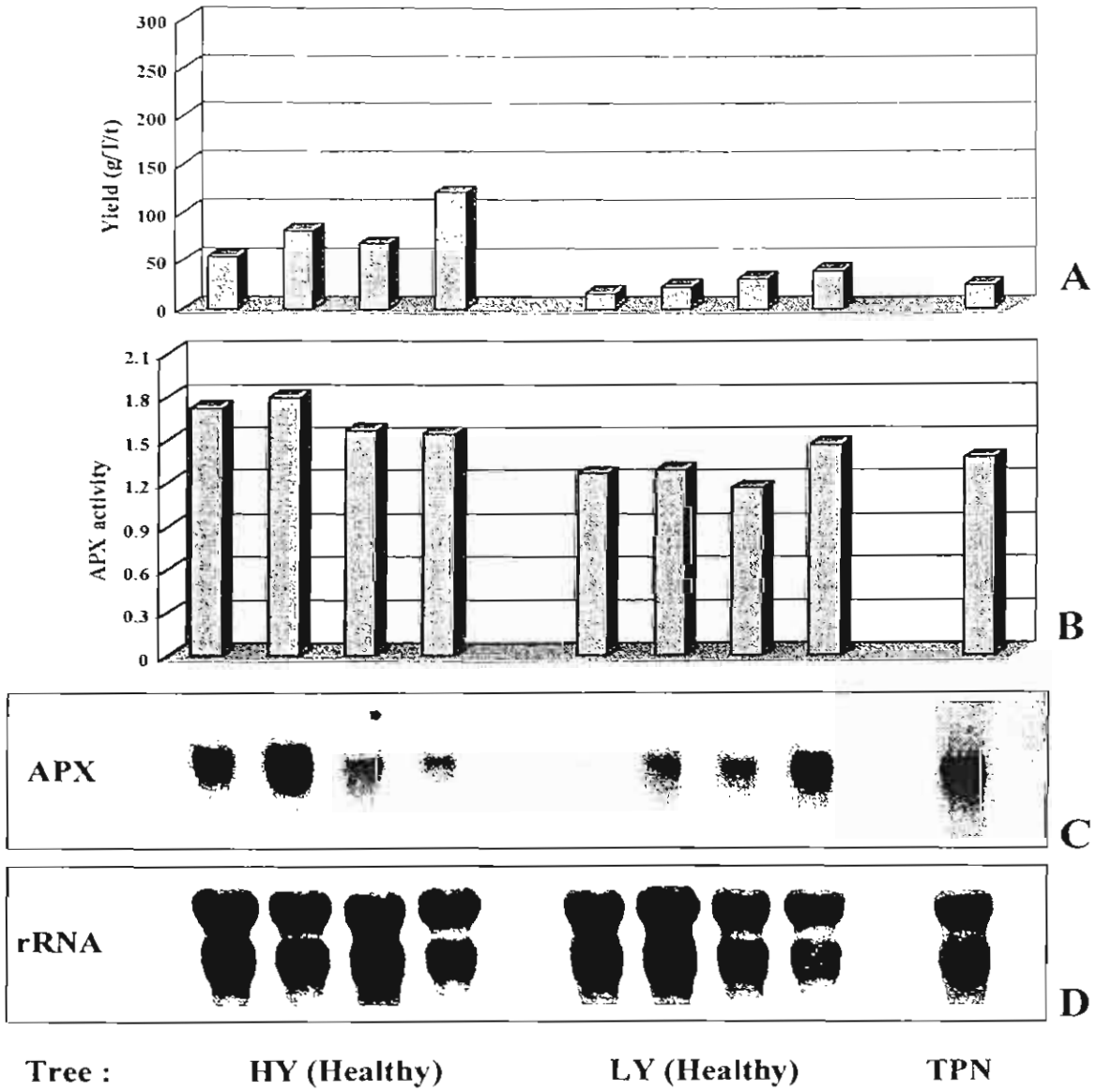

Figure 11. Intra-clone (Avros 2037) relationships between the variation of rubber yield, APX activity and gene expression in the latex from high yielding and low yielding healthy trees.

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.

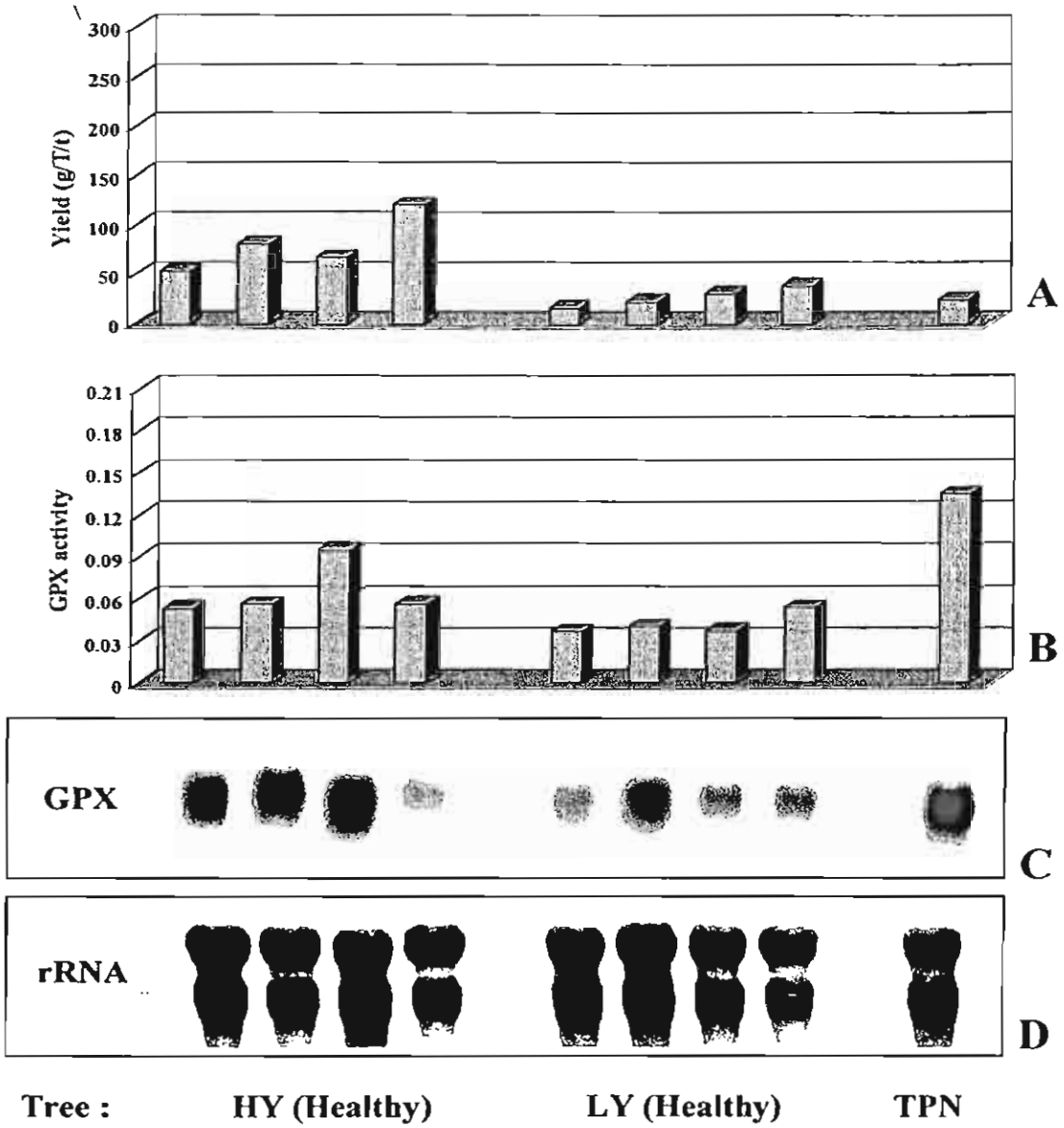

Figure 12. Intra-clone (Avros 2037) relationships between the variation of rubber yield, GPX activity and gene expression in the latex from high yielding and low yielding healthy trees.

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

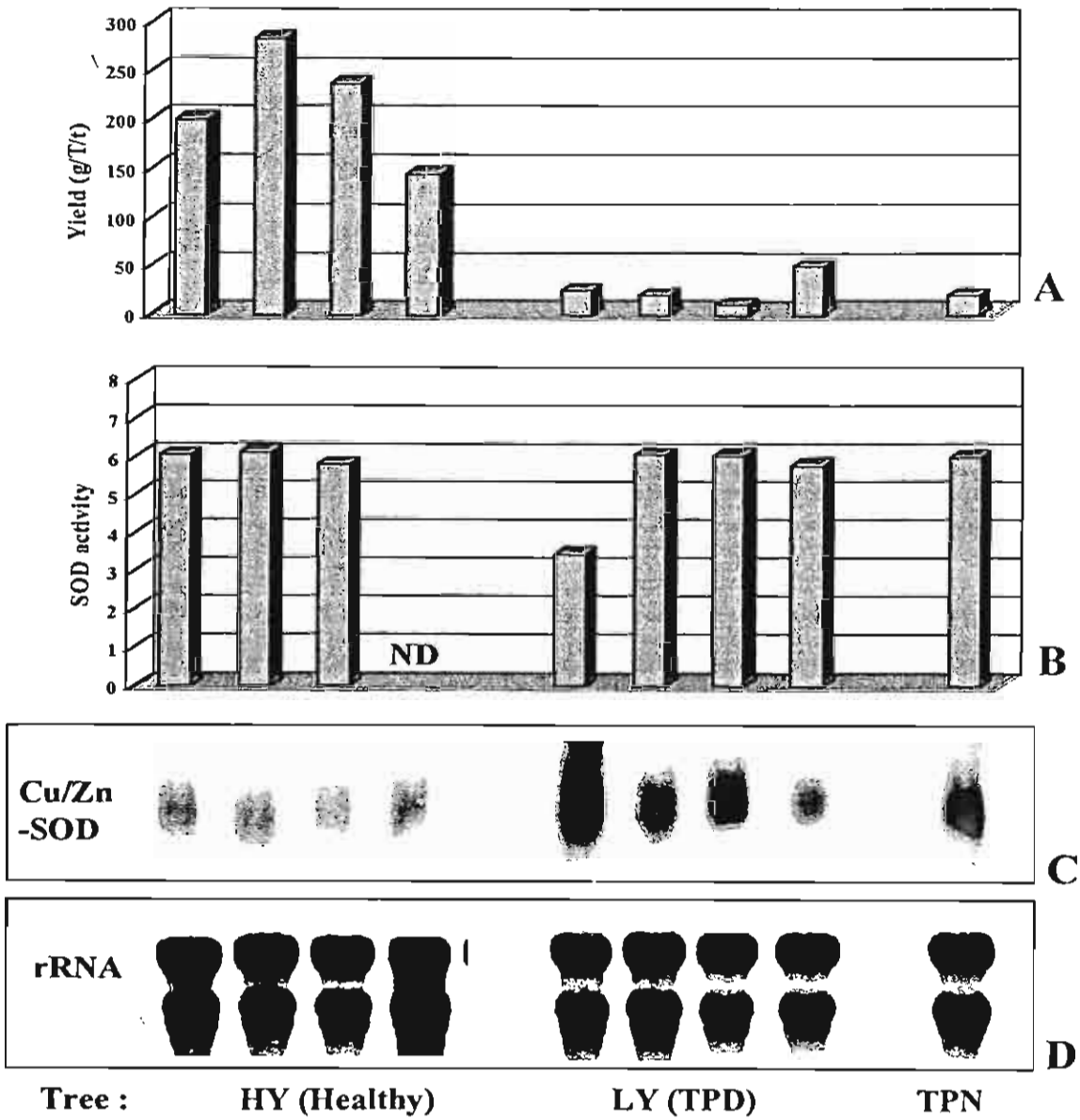

Figure 13. Intra-clone (PB 235) relationships between the variation of rubber yield, total SOD activity and Cu/Zn-SOD gene expression in the latex from high yielding healthy trees and low yielding diseased trees.

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.

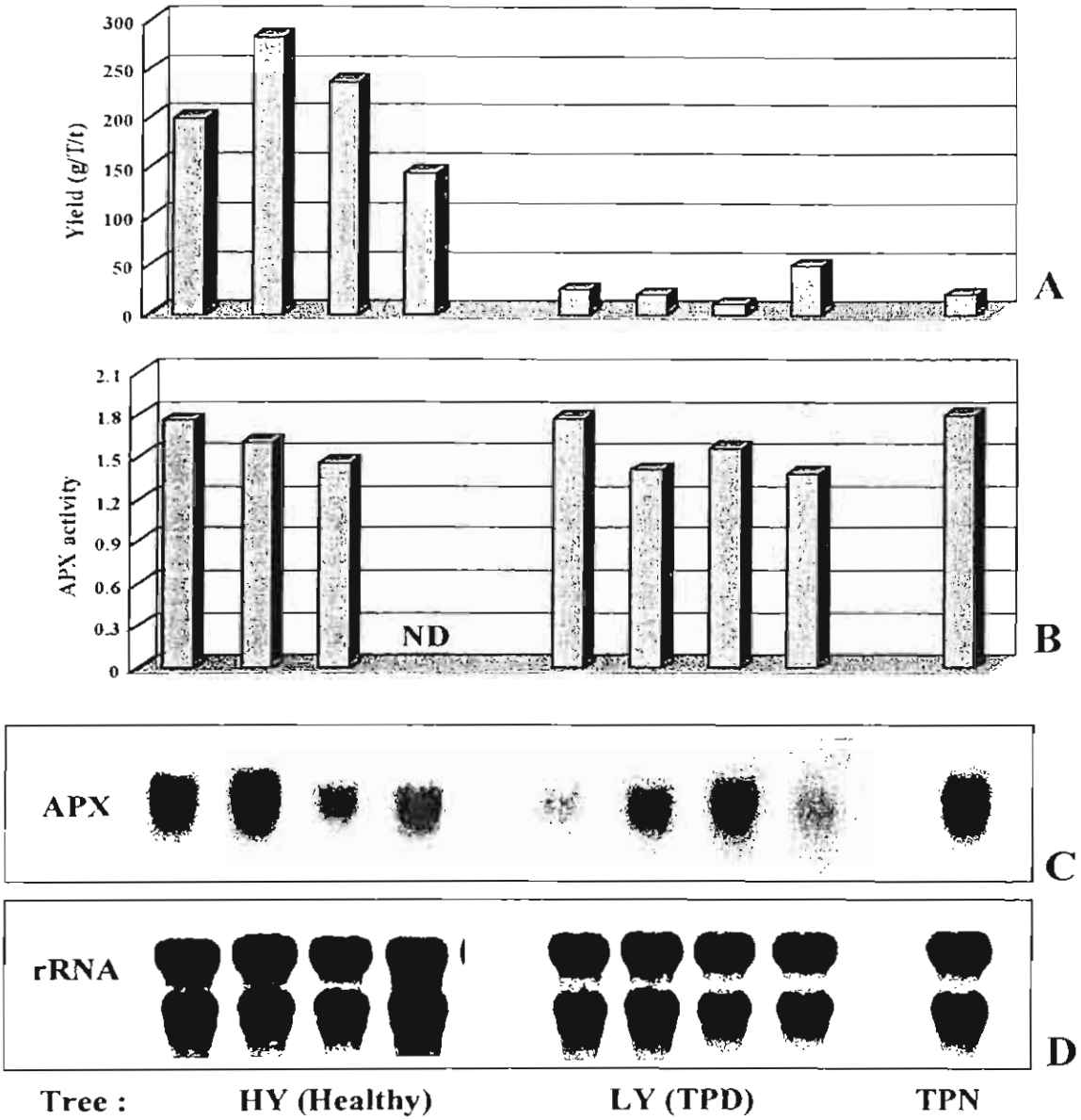

Figure 14. Intra-clone (PB 235) relationships between the variation of rubber yield, total APX activity and gene expression in the latex from high yielding healthy trees and low yielding diseased trees.

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.

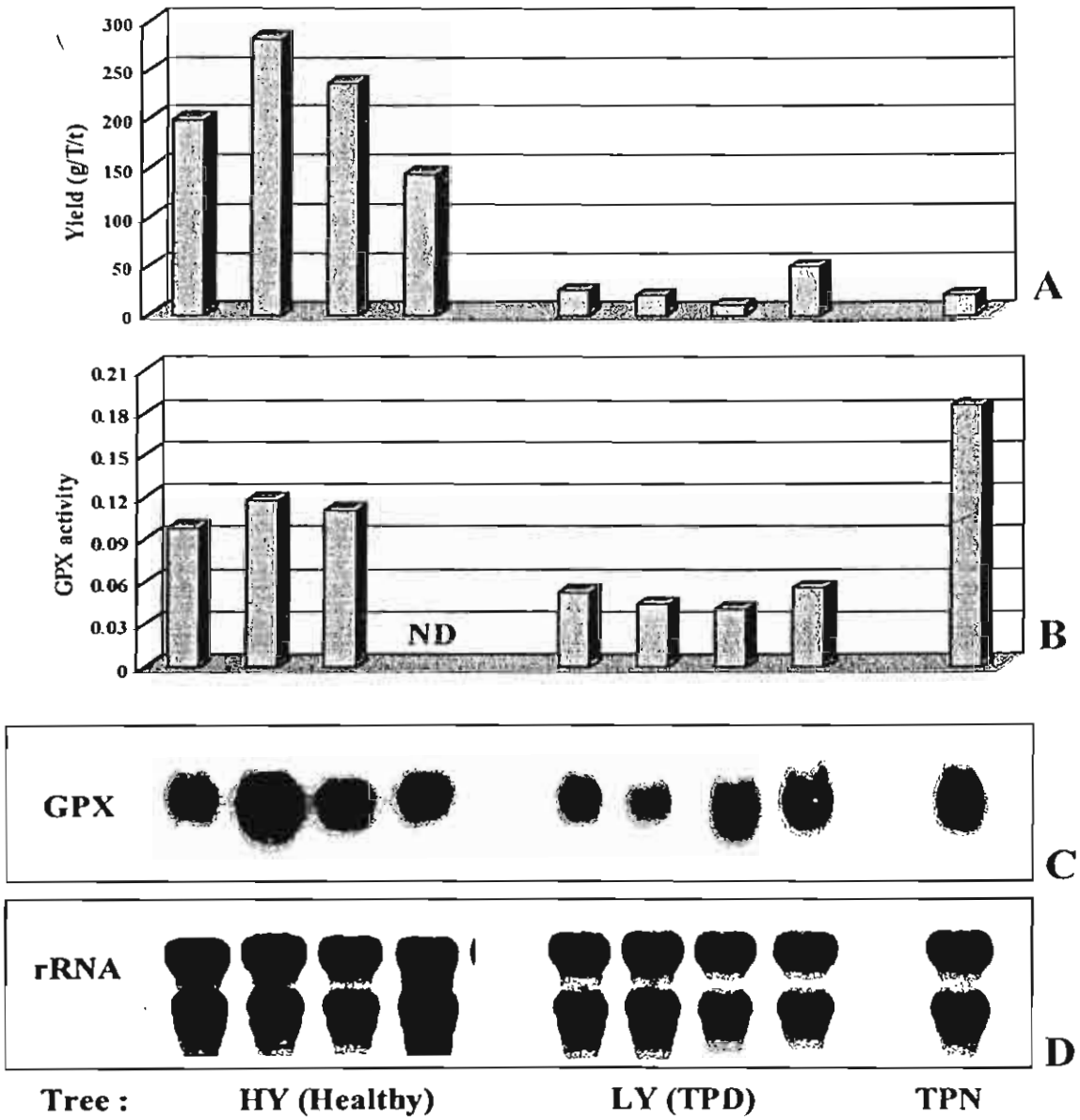

Figure 15. Intra-clone (PB 235) relationships between the variation of rubber yield, total GPX activity and gene expression in the latex from high yielding healthy trees and low yielding diseased trees.

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*

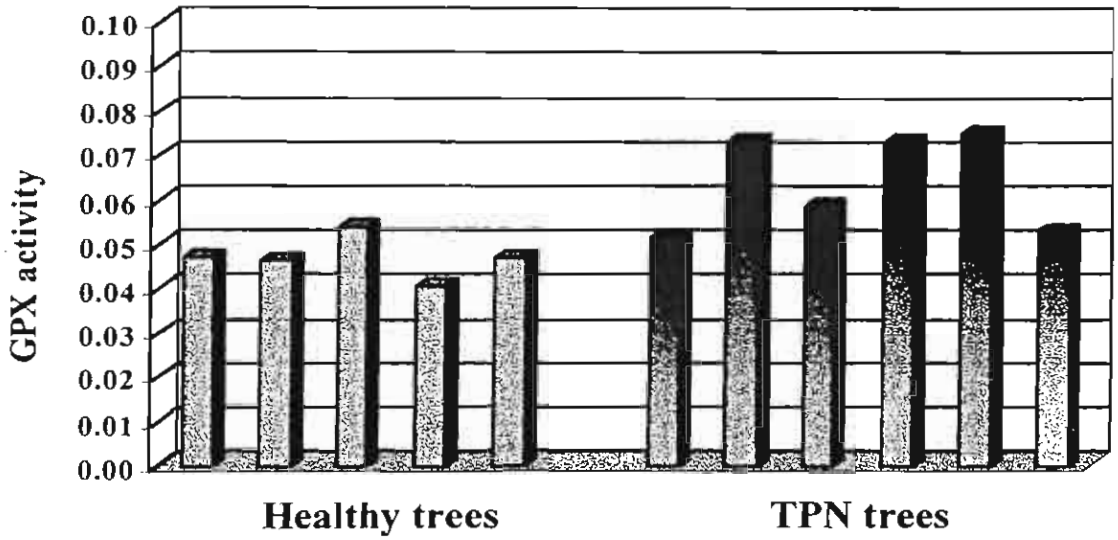

Figure 16. Cytosolic GPX specific activity in the latex from healthy and diseased (TPN) trees of the rubber clone PB 260.

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*

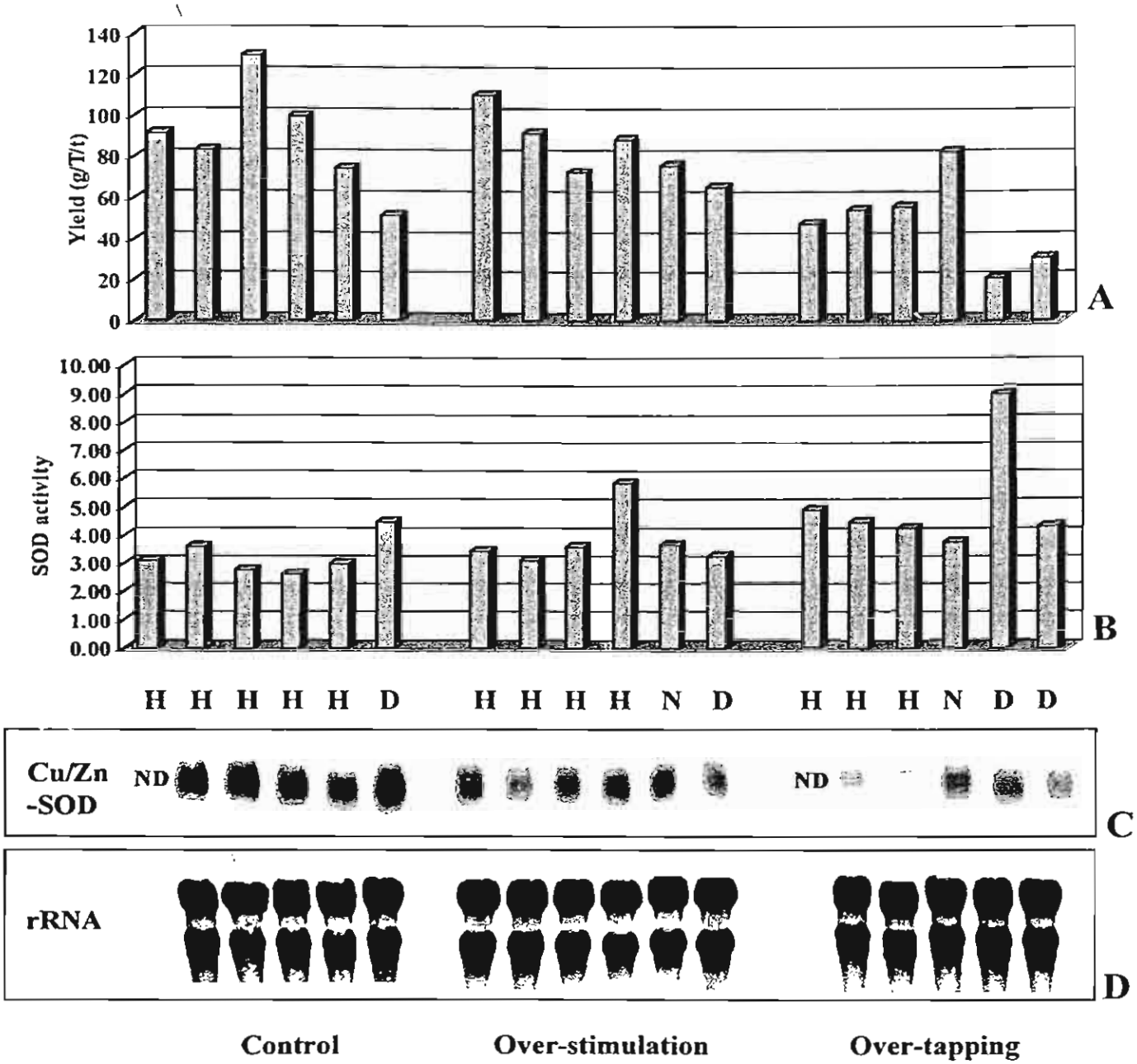

Figure 17. Effect of over-exploitation on rubber yield, SOD activity and Cu/Zn-SOD gene expression in the latex of healthy and diseased tree from PB 260 clone.

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.

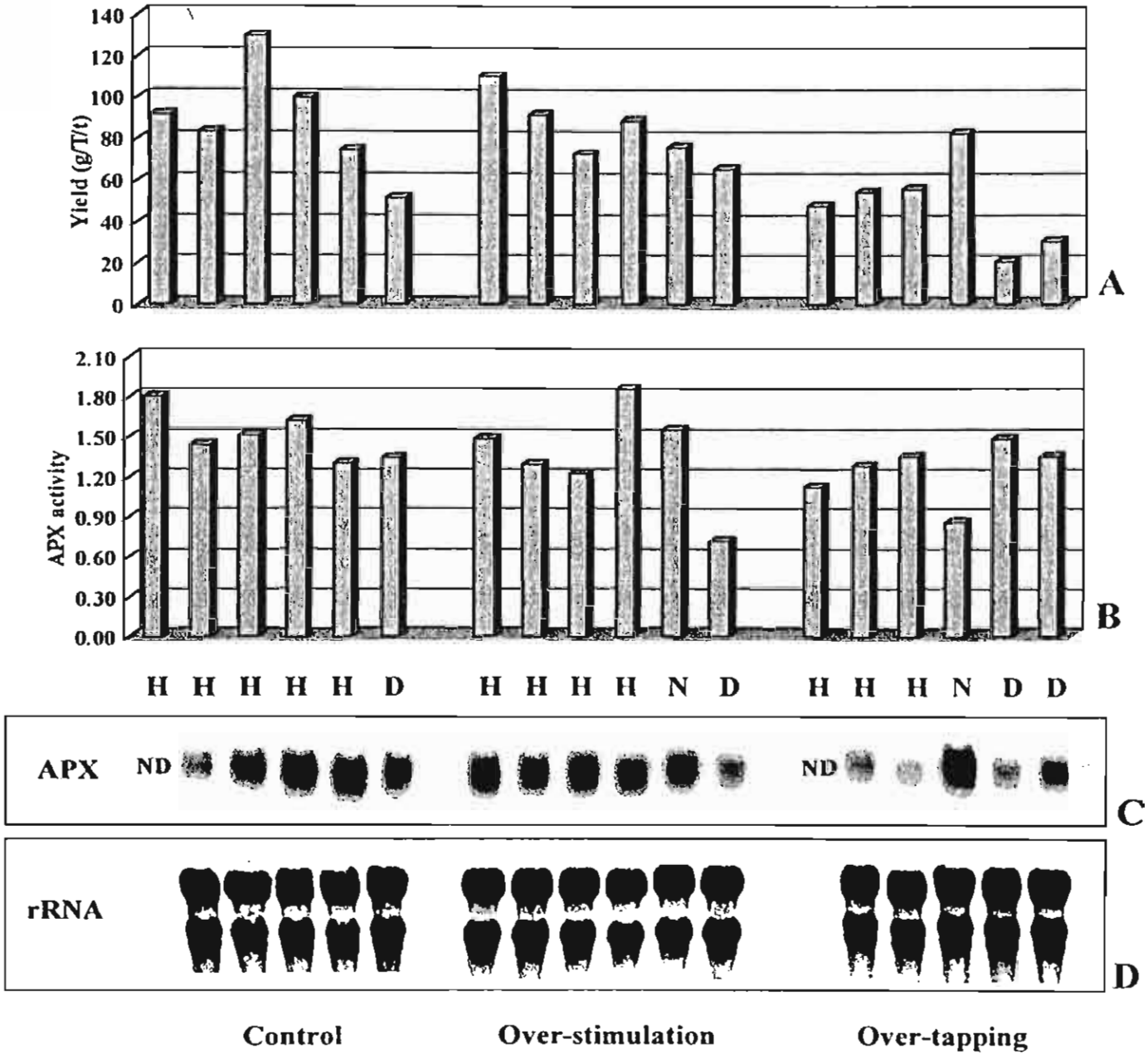

Figure 18. Effect of over-exploitation on rubber yield, APX activity and gene expression in the latex of healthy and diseased tree from PB 260 clone.

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.

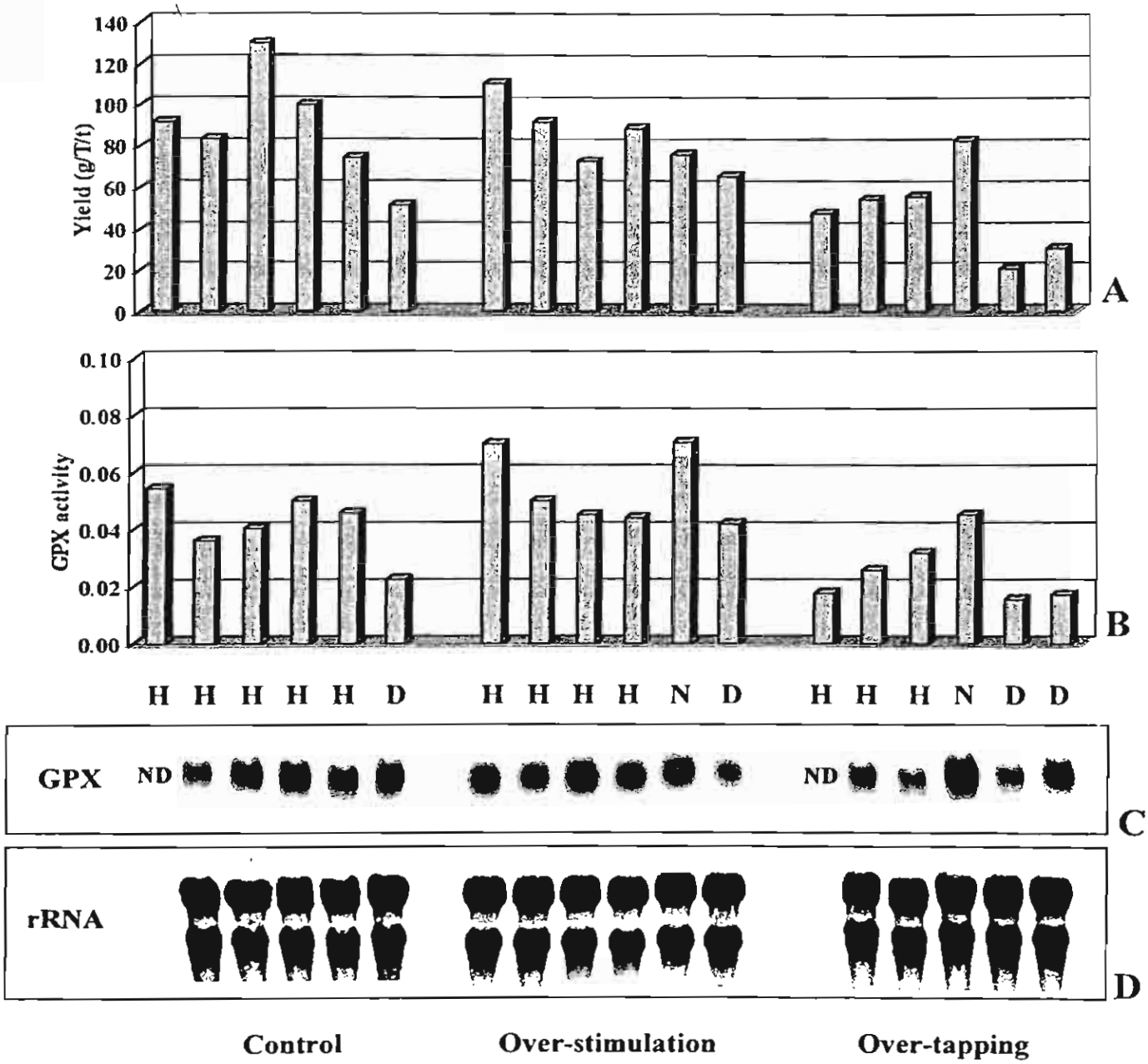

Figure 19. Effect of over-exploitation on rubber yield, GPX activity and gene expression in the latex of healthy and diseased tree from PB 260 clone.

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.

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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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