Chapter 7.II

BIOCHEMICAL ASPECTS OF BARK DRYNESS INDUCED BY OVERSTIMULATION OF RUBBER TREES WITH ETHREL®

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I. INTRODUCTION

Overexploitation of *Hevea* (by excessive tapping as well as overstimulation) can lead to a stoppage of flow caused by the physiological disorder known as "bark dryness" or "Brown Bast". Extensive studies of these "pathophysiological syndrome" have been carried out and a great number of hypotheses, such as impairment of phloem transport,[28,57] depletion of latex nutrients,[18,88] adverse water relations,[110,114] and wound reactions[90,95] have been put forward to account for the different physiological or histological phenomena in the phloem, or more specifically in latex vessels.

However, the results obtained on the organic nutrition of laticifers and on deviations from the normal mineral balance in latex in relation to the onset and development of dryness appear to be controversial to varying degrees[7,18,80,81,127] and do not appear to account for the rapid induction of dryness by methods (e.g., sealed punctures[111]) in which there is no excessive drainage of latex and subsequent lack of metabolites at the tapping panel. Furthermore, histological examination has shown that sieve plates appear to be normal,[37,81] and there is no severe depletion of starch reserves in diseased bark,[37] particularly in overstimulated trees, suggesting that nutrient depletion in latex vessels should not be regarded as the single prime cause of the onset of bark dryness.

Histological features such as cross-walls, the invasion of latex by tyloses from neighboring parenchyma cells, flocculation, and partial or complete coagulation of latex within the vessels,[37,80,81] as well as the adherence of rubber particles to more or less damaged lutoids and other membrane structures,[80,81] suggest that disorganization of membrane structures and cell wall, especially inside latex vessels, is associated with the onset and development of dryness. Moreover, ultracentrifugation of latex from partially dry trees did show virtual disappearance of Frey-Wyssling complexes, flocculation, and considerable reduction in bottom fraction (which included lutoids).[14,22,25,81,127] In addition, isopycnic centrifugation of the bottom fraction of latex from diseased trees revealed considerable lightening of residual lutoids.[22,25,36]

Finally, abnormal instability of latex organelles from partially dry bark was clearly revealed by demonstration of a relation between the onset and development of dryness and an abnormally high bursting index (BI) of lutoids (Figure 1A) and abnormal release of *o*-diphenoloxidase activities (normally compartmentalized within the Frey-Wyssling particles)[31] released into the cytosol of latex from diseased trees.

Taken as a whole, these observations strongly suggest that deterioration of the stability of all the membrane structures such as lutoids and Frey-Wyssling particles,[22,33,80,81] which normally compartmentalize nearly all the latex coagulating factors, lead to the destabilization and hence the coagulation of latex *in situ*, which develops and spreads along the latex vessels, might be one of the primary causes of the onset of bark dryness.

Much attention has been paid over the past 6 years to seeking biochemical disorders within latex vessels which are likely to damage membranes leading to subsequent degeneration of latex vessels.

This chapter focuses on the existence of an endogenous NAD(P)H oxidase in lutoids which generates toxic forms of oxygen ($O\dot{}_2^-$, H_2O_2, $OH^{\cdot}$,etc.) responsible for the peroxidative degradation of organelle membranes in the latex from diseased trees (Figure 1B). It is shown that the induction of bark dryness through deliberate overstimulation with Ethrel® results in an imbalance in peroxidative activities; this has harmful effects on membranes and on protective scavenging activities in latex and results in the destabilization of organelles and in lysis.

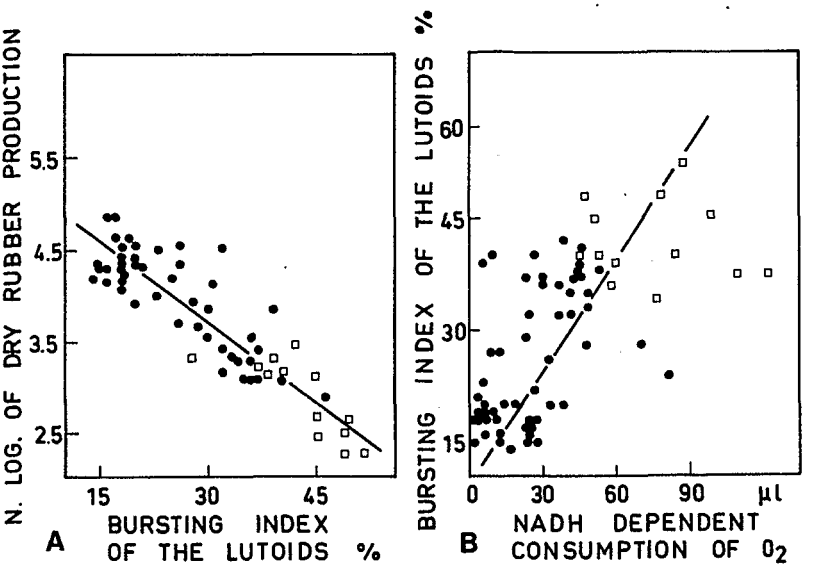


FIGURE 1. Biochemical symptom of bark dryness. (A) Relationship between the BI of the lutoids (%) and the latex yield of apparently healthy or partially dry-bark trees. Latex yield was expressed as the Naperian logarithm grams dry rubber/tree/tapping; (□) "dry cut syndrome", partially dry trees; (●) "healthy" high and low-yielding trees. (B) Correlation between the BI of the lutoids and their NAD(P)H-dependent O_2 consumption, as measured polarographically. Symbols as in A.

II. EVIDENCE FOR A LUTOIDIC NAD(P)H OXIDASE THAT GENERATES SUPEROXIDE ANIONS

A. Partial Characterization of the Enzyme

An enzymatic NAD(P)H oxidase which generates toxic forms of oxygen and which is thus likely to account for membrane instability was revealed in lutoid tonoplast from partially dry trees (Section 2).[22,23,25,33,36] This activity was measured either polarographically in function of oxygen consumption dependent on reduced pyridine nucleotides (Figure 2) or spectrophotometrically by recording the oxidation of NAD(P)H.

The partial characterization of this lutoid enzyme showed that it was not sensitive to the classic inhibitors of bacterial or mitochondrial respiratory chains, but was unexpectedly markedly activated by hydroxamic acids, the well-known inhibitors of the alternate mitochondrial pathway (Table 1).[104]

It was shown that this redox system could accept electrons from either NADH or NADPH apparently with similar efficiency.[22] The oxidase was strongly affected by physiological concentrations of Fe^{3+} (25 µ*M*) or Cu^{2+} (100 µ*M*), especially in the presence of chelating agents[22,23] such as ADP or EDTA, which are assumed to facilitate electron transfer from the reduced enzyme to molecular oxygen via metal cations.[77]

The NAD(P)H oxidase was also shown to be greatly activated by exogenous phenolic, quinonic, or semiquinonic compounds which, as well as Cu^{2+} or Fe^{3+}/ADP, were proposed to act as physiological activators or electron carriers to molecular oxygen *in situ*.[23]

The NAD(P)H-dependent consumption of oxygen was significantly inhibited by the addition of superoxide dismutase (SOD) (Figure 2), indicating that the consumption of molecular oxygen led to linear formation of superoxide radicals ($O_2^{\cdot-}$) (undetectable by polarography but detectable by SOD-inhibitable appearance of formazan in the presence of nitroblue-tetrazolium salts).[8] As the addition of exogenous catalase resulted in a significant release of molecular oxygen into the medium, it was concluded that some H_2O_2 had accu-

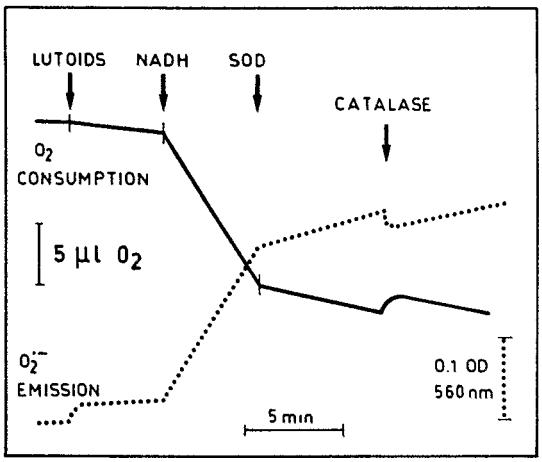


FIGURE 2. Characterization of a NADH dependent O_2 consumption. Parallel polarographic determination of the NADH-dependent O_2 consumption, and evidence of SOD inhibitable emission of $O_2^{\cdot-}$ as followed by the direct reduction of nitro-blue tetrazolium in formazan. (Adapted from Chrestin, H., *Caoutch. Plast.*, 647/648, 75, 1985.)

Table 1
EFFECTS OF THE CLASSICAL INHIBITORS OF THE RESPIRATORY CHAINS ON THE NADH DEPENDENT CONSUMPTION OF OXYGEN BY LUTOIDS FROM DISEASED TREES[22,25]

	O_2 consumption (µℓ O_2/min/mℓ lutoids)				
Addition:	**None**	**Antimycin A (25 µ*M*)**	**Rotenon (0.1 µ*M*)**	**KCN (1m*M*)**	**Hydroxamic ac. (2.5 m*M*)**
	6.6	6.4	5.2	4.3	11.6

mulated during the action of NADH oxidase (Figure 2). Furthermore, as it was shown that the addition of SOD and then catalase did not result in such significant inhibition in the presence of iron chelates,[22] it was then proposed that under these conditions, the functioning of NADH oxidase led to major accumulation in the medium of forms of oxygen different to superoxides and perhydrol, probably hydroxyl free radicals ($OH^{\cdot}$), owing to some occurrence of Haber-Weiss-like reactions[51] in the presence of metal cations.[8,96]

B. Relations Between NADH-Oxidase Activity and the Yield or Physiological State of the Trees

It was shown that there is a strong correlation between activity of the lutoid NADH-oxidase and the BI of lutoids (Figure 1B); it has previously been shown (Figure 1A) that there was a strong correlation between the latter and the production of rubber and the "physiological state" of the trees.

Lutoids from healthy, high- and medium-yielding trees exhibited only traces, when detectable, of NAD(P)H-dependent consumption of oxygen. Lutoids from very low-yielding trees with no obvious symptoms of typical dryness showed low but detectable NAD(P)H-dependent consumption of oxygen. Only trees with obvious symptoms of dryness exhibited fully effective lutoid NAD(P)H oxidase activity. Hence only the latex from very low-yielding or partially dry trees could be used for studies of the enzyme.

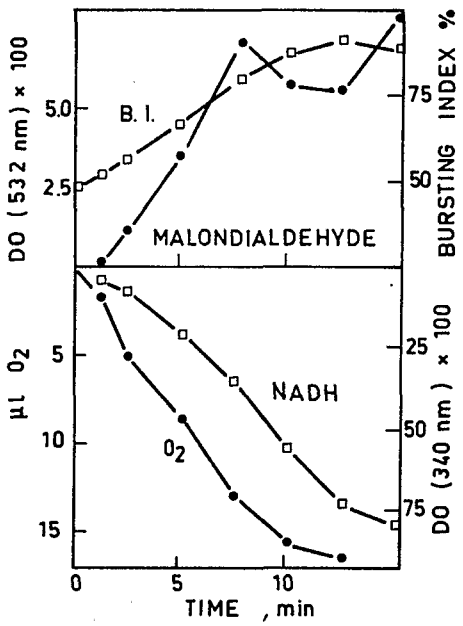


FIGURE 3. Evidence for the degradation of the lutoidic membrane during the functioning of the lutoidic NAD(P)H oxidase in vitro. Lipids peroxidation was monitored following the appearance of malondialdehyde at 532 nm. The BI of the lutoids was measured as described elsewhere (Chapter 4.III). (Adapted from Chrestin, H. and Bangratz, J., *C.R. Acad. Sci. Paris Ser. III*, 296, 101, 1983.)

III. EVIDENCE FOR PEROXIDATION OF LIPIDS AND SUBSEQUENT LYSIS OF ORGANELLES DURING FUNCTIONING OF LUTOID NAD(P)H OXIDASE IN VITRO

The various forms of "toxic oxygen" released ($O_2^{\cdot-}$, H_2O_2, $OH^{\cdot}$) during NAD(P)H-dependent consumption of oxygen, especially in the presence of traces of iron chelates (Figure 3) or Cu^{2+} were shown to lead to rapid peroxidative degradation of the lutoid membrane as well as exogenous unsaturated lipids, as shown by the appearance of thiobarbituric-acid-reactive malondialdehyde (a byproduct of the peroxidation of polyunsaturated lipids).[113]

This peroxidative degradation of unsaturated lipids in latex organelles resulting from the functioning of lutoid NAD(P)H oxidase clearly led to the destabilization of the lutoids themselves, as shown by their increased BI in vitro (Figure 3).

It was then shown (Table 2) that although addition of exogenous SOD alone was effective in scavenging $O_2^{\cdot-}$, it displayed poor protective effects against NAD(P)H-oxidase-dependent peroxidation of lipids and the resulting bursting of lutoids. Catalase which eliminates H_2O_2 is ineffective in scavenging superoxide anions but was shown to be fairly effective in protecting the unsaturated lipids from peroxidative degradation and maintaining lutoid membranes intact.

Mannitol, one of the most efficient scavengers of the highly toxic $OH^{\cdot}$ radicals,[47] also acted as the most effective scavengers of the forms of toxic oxygen released during operation of lutoid NAD(P)H-oxidase (Table 2), as shown by its significant protective effects against lipid peroxidation and also against membrane degradation. This led to suggesting that hydroxyls, released as free radicals by the interaction of $O_2^{\cdot-}$ (produced directly by NAD(P)H oxidase activity), with H_2O_2 (the product of self- or SOD-catalyzed dismutation of superoxides), and H^+, especially in the presence of traces of metal cations,[8,96] were likely to be

Table 2
EFFECTS OF EXOGENOUS SCAVENGERS ON THE NADH-DEPENDENT OXYGEN CONSUMPTION, THE EMISSION OF SUPEROXIDES, THE PEROXYDATION OF UNSATURATED LIPIDS, AND THE BI OF LUTOIDS FROM DISEASED TREES[22,25]

	None	SOD	Catalase	Mannitol	All
O_2 consumption ($\mu\ell$)	14	7.6	7.5	13.5	3.2
Emission $O_2^{\cdot}$ (OD_{560})nm	0.38	0.12	0.31	0.32	0.10
Malondialdehyde (OD_{532})nm	61	58	23	24	13
BI (%)	68	58	43	40	36

the main types of toxic oxygen released by lutoid NAD(P)H oxidase, with harmful effects on unsaturated lipids in membranes.

It should be pointed out that very similar pathways have been described for the NAD(P)H-dependent release of $O_2^{\cdot}$, H_2O_2, and $OH^{\cdot}$, with subsequent membrane damage in mammalian microsomes[20,47] and granulocytes.[5] It was therefore proposed that such degradation and lysis of lutoid vacuo-lysosomal membrane occurs in vivo and might bring about rapid coagulation of latex *in situ* and subsequent degeneration of latex vessels.

IV. IMBALANCE BETWEEN TOXIC PEROXIDATIVE ACTIVITIES AND THE PROTECTIVE SCAVENGING ACTIVITIES: FACTORS IN INSTABILITY OF LUTOIDS IN LATEX FROM PARTIALLY DRY TREES

It was thus demonstrated that lutoids from very low-yielding or partially dry trees exhibit abnormally high lutoid NAD(P)H oxidase activities which lead to abnormal release of toxic forms of oxygen. However, this would not have been thought to result in deleterious effects on membrane structure if the scavenging chemicals and enzymes activities had been perfectly efficient in these latex.

A number of biochemical parameters thought to be involved in membrane damage or protection were therefore analyzed in latex from high-, medium-, and low-yielding trees (with no apparent symptoms of dryness) as well as from partially dry trees.

The factors likely to be involved in membrane degradation taken into account consisted essential of lutoid NAD(P)H oxidase activity (generating toxic forms of oxygen) and peroxidase activities which can generate quinonic free radicals and their products of condensation with high clustering capacity.

The factors likely to protect membranes from the harmful effects of toxic oxygen are provided essentially by both scavenging enzymes such as SOD and a catalase which have been shown to be present in latex,[22,31] and by scavenging chemicals such as thiol compounds and ascorbic acid ("anti-oxygen").

All these parameters were measured independently using latex from 77 rubber trees and processed using different methods of multivariate analysis. The results obtained by Principal Component Analysis (PCA), in which only the biochemical parameters were taken into consideration as active variables (excluding yield data), showed that the first factor (Component or Axis 1) combined high peroxidative activities (NAD(P)H oxidase and peroxidase activities) with low concentrations of reducing scavenging substances in the cytosol (reduced thiols + ascorbate). These parameters were also accompanied by a high lutoid BI.[21,22] These authors then concluded that the first factor (Axis 1) accounted essentially for the toxic oxidative nature (or, on the contrary, the reducing nature) of the latex, and could therefore be interpreted as and named "the Axis of the toxic peroxidative activities".

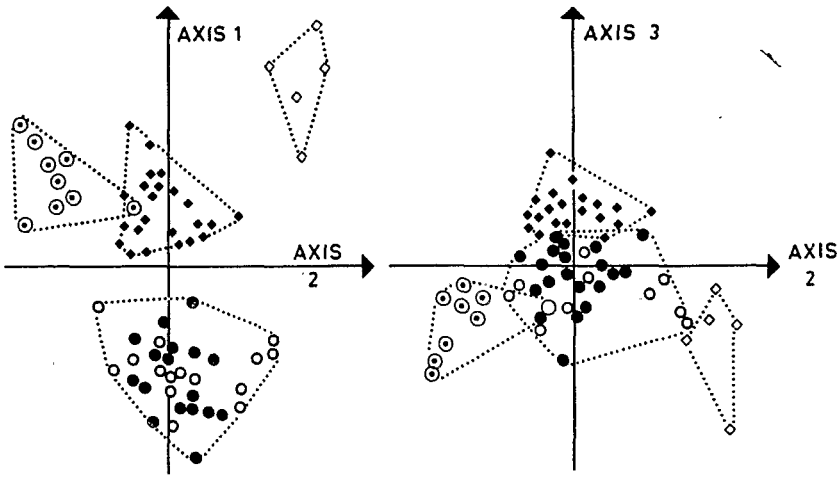


FIGURE 4. Diagrams resulting from PCA of latex from rubber trees with very different productivities. Diagrams representing the projection of each individual *Hevea* in the plans defined by the Axes 1 and 2 (left) and 2 and 3 (right), as defined by the biochemical characteristics mentioned in the text. Axis 1: high toxic peroxidatic activities; Axis 2: high catalase activities; Axis 3: high SOD activities. (●) high yield; (○) middle yield; (♦) low yield "A"; (◇) low yield "B"; (⊙) dry barks.[22,25]

The second factor (Axis 2) essentially represented high catalase activities in the cytosol and in the bottom fraction, associated with low peroxidase activities in the lutoids. This factor was therefore regarded as "the axis of catalase activities".

The third factor (Axis 3) was shown to contrast low SOD activities and somewhat high toxic oxidative activities in latex. This factor was identified as "the Axis of the superoxide dismutase activities".

The "projection" of each individual rubber tree in planes 1-2 (Figure 4A) and 2-3 (Figure 4B) as defined by the biochemical characteristics of its latex considered in the analysis revealed that in the plane defined by Axes 1 and 2, the "toxic oxidase activities" of the indivisible "cloud" of the high- and medium-yielding trees contrasted clearly with the low-yielding or dry-bark trees" (Figure 4A).

The axis of catalase activities (Axis 2) made it possible to differentiate dry-bark trees in a special group of low-yielding trees (called "B"), the latter being characterized by high catalase but low SOD activities (Figure 4A and 4B). Finally, the SOD activities (Axis 3) differentiated another type of low-yielding trees (type "A") from two distinct groups corresponding to the partially dry trees and "B" type low-yielding trees.

Thus, such analysis makes it possible to identify four distinct groups of rubber trees, defined in an almost disjunctive manner by the various possibilities of orientation of the chemical and enzymatic pathways involved in the stabilization and destabilization process in membrane structures in latex. It was thus shown that in such an analysis the biochemical variables alone were sufficient to account for the wide variation in latex production and the physiological dryness disease. It was then possible to define groups of *Hevea* characterized either by "hyper" or "hypo" enzymatic activities.

This multivariate analysis carried out on computer was used to draw up a model describing the biochemical events leading to the bark dryness syndrome (Figure 5). It was seen that even if healthy, high-yielding trees are not especially characterized by intense detoxifying activities, they display above all very low (if detectable) superoxide-generating NAD(P)H oxidase activities. Furthermore, their unsaturated structures are protected effectively from casual activation of peroxidative activities by very high levels of scavenging "anti-oxygen" substances in the cytosol.

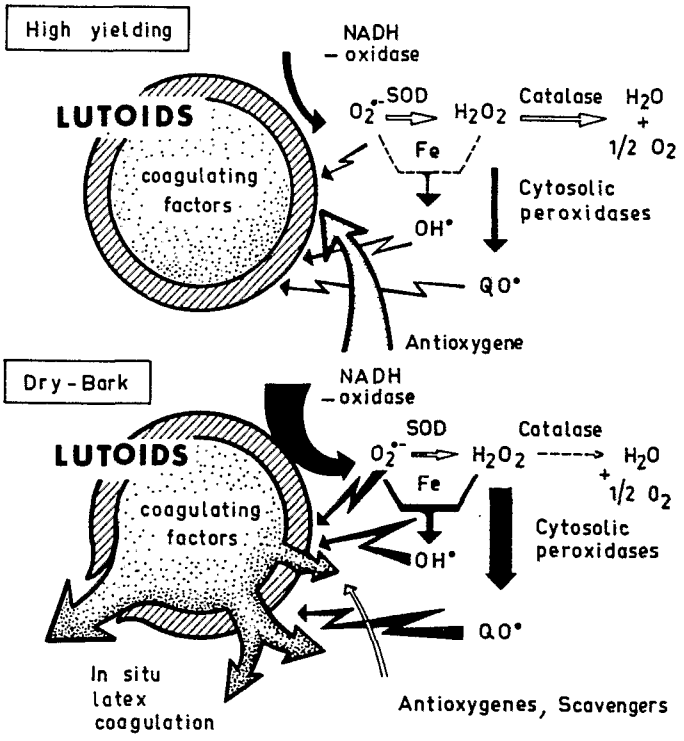


FIGURE 5. Schematic interpretation of peroxidative metabolism of latex from normal and more or less dry bark trees. Interpretation of the peroxidative degradation of the lutoidic tonoplast, leading to the release of the latex "coagulating factors" and lysosomal enzymes *in situ*, related to dry-bark disease. The size of the arrows indicates the relative activity of each pathway (black arrows, peroxidative pathways; open arrows, protecting and scavenging pathways).

In contrast, trees displaying the typical symptoms of dryness were shown to exhibit abnormal NAD(P)H oxidase and peroxidase "hyper activities", but very low SOD and virtual disappearance of catalase activities. This must result in accumulation of superoxide and perhydrol. It is highly probable that these two toxic forms of oxygen interact in a Fenton and Haber-Weiss-like reaction leading to the generating of the most toxic oxygen free radicals, namely, ($OH^{\cdot}$) and singlet oxygen, with highly deleterious effects on unsaturated lipids and then on membrane structures. These processes are thought to result in lysis of the lutoids themselves, with subsequent release of the latex "coagulating factors" that they normally compartmentalize in latex. This must lead naturally to coagulation of latex within the latex vessels, resulting in the stoppage of flow and then degeneration of the cells.

V. LINK BETWEEN INDUCTION OF DRYNESS BY OVERSTIMULATION AND ACTIVATION OF LUTOID NADH OXIDASE AND LOSS OF PROTECTIVE ACTIVITIES

As it has been clearly demonstrated that overstimulation with Ethrel® can lead to the appearance of typical bark dryness symptoms,[14] attempts were made to verify whether induction of bark dryness by deliberate overstimulation might be associated with the onset of imbalance between peroxidative activities and scavenging protective activities in latex.

The results (Figure 6) showed that whatever the tree the stimulatory treatments induced transitory activation of lutoid NADH oxidase as well as simultaneous classic increased yields.

At the same time, it was shown that enzymatic scavenging activities such as catalase and SOD were also transitorily activated in latex.[21,22,25]

It was suggested that this simultaneous increase in potentially toxic peroxidative activities and in scavenging activities accounted for the relative stability of membrane structures in latex in healthy trees, as shown by the slight but reproducible significant lowering of the lutoid BI after treatment with Ethrel® (Figure 6).

It was only when the first typical symptoms of dryness became evident at the tapping cut (after the second tapping following the fifth stimulation) that the biochemical parameters analyzed in the experiment could differentiate trees with typical symptoms of dryness from overstimulated trees which remained healthy throughout the experiment. These biochemical symptoms were shown to persist and even become more serious from the fifth to the seventh (final) stimulation. Symptoms consisted of: (Figure 6)

1. Rise and persistently high level of superoxide-generating lutoid NAD(P)H oxidase activity (× 12 compared to control)
2. Persistent rise in the BI of lutoids (over 60% more than control)
3. Dramatic fall in catalase and SOD activities[21,22,25]
4. Irreversible fall in cytosol reduced thiol content[16,30]
5. Correlative decrease in rubber production as a response to overstimulation with Ethrel®[24]

It was thus clearly confirmed that overstimulation could induce dryness. The biochemical symptoms seen as a whole suggested the establishing of severe imbalance between toxic peroxidative activities and scavenging activities in latex resulting in degradation of the lutoid membrane, which is the first deleterious event induced by successive overstimulation with Ethrel®.

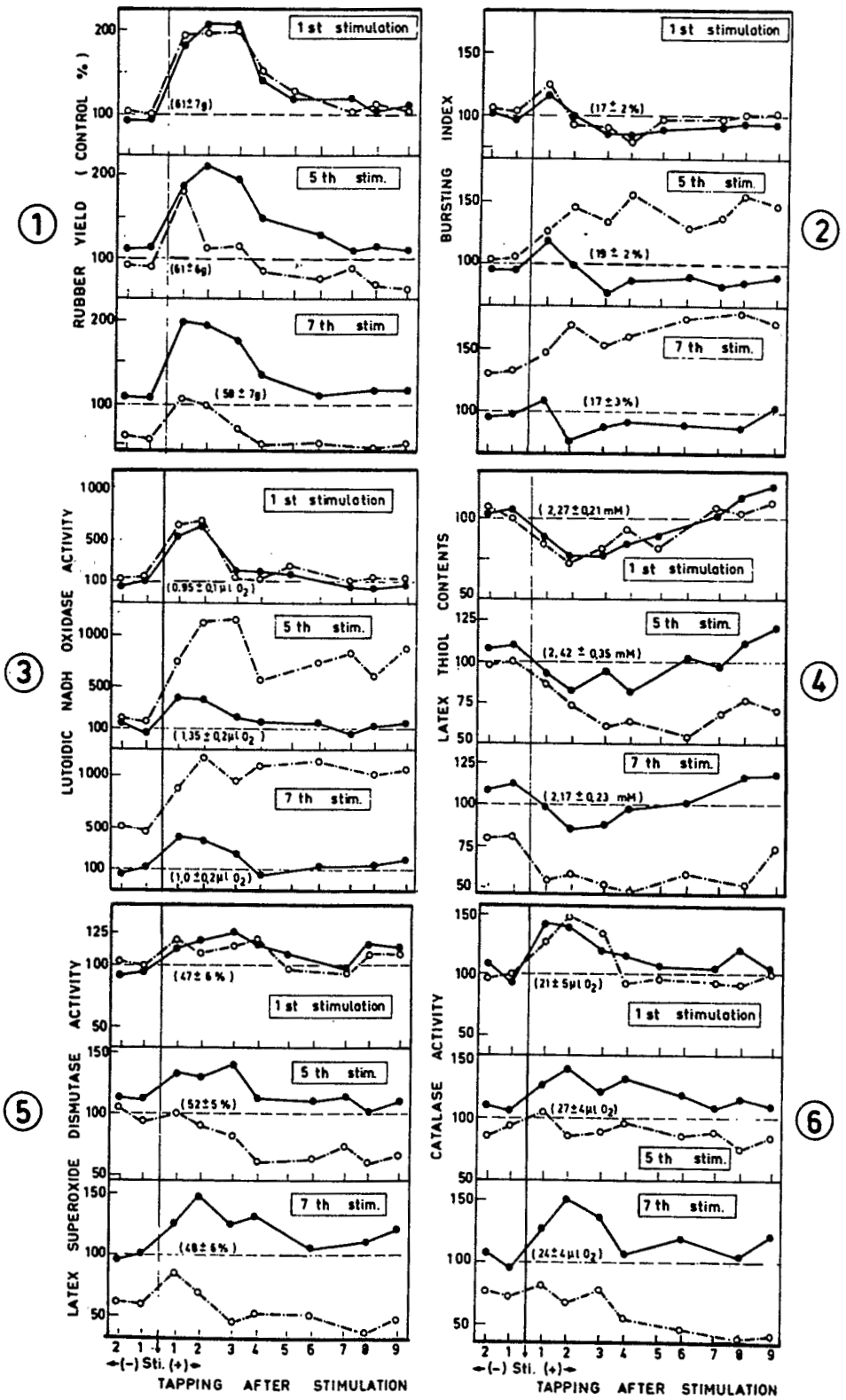
VI. CONCLUSION

All these data demonstrated that the latex from partially dry trees is characterized by abnormally high lutoid NAD(P)H oxidase activity, leading to a high level of release of superoxide anions, simultaneous decrease in concentrations of latex cytosol scavengers (reduced thiols and ascorbate), as well as virtual disappearance of scavenging enzyme activities (SOD and catalase).

The combination of increased peroxidative activities and considerably diminished quantities of scavengers in latex from diseased trees results in the destabilization and then lysis of lutoids; this leads to the release into latex of the coagulating factors that they normally compartmentalize. It can be considered that such destabilization and degradation of membranes does not affect only lutoids. Strong evidence has been found for the destabilization of Frey-Wyssling particles as well (*o*-diphenoloxidase released into the cytosol). It appears quite probable in fact that all the membrane structures in latex cells may be exposed to such peroxidative degradation, with a resulting impairment of nutrient supply and water exchanges at plasmalemma level as postulated elsewhere,[18,88] or impairment of protein synthesis regulation if the nuclear membrane is damaged.

It is therefore suggested that disorganization of membrane structures caused by peroxidative degradation of their unsaturated lipids might be a primary cause of the onset of bark dryness, at least as induced by overexploitation, and especially by overstimulation with Ethrel®, as demonstrated here.

Great care should be taken in order to avoid any type of overexploitation in *Hevea* plantations either through overstimulation (dose and frequency) with ethylene generators or through overtapping which could also lead to endogenous ethylene production because of wound reaction.[21,22,25]


1
2
3
4
5
6
RUBBER YIELD (CONTROL %)
BURSTING INDEX
LUTOIDIC NADH OXIDASE ACTIVITY
LATEX THIOL CONTENTS
LATEX SUPEROXIDE DISMUTASE ACTIVITY
CATALASE ACTIVITY
1 st stimulation
5 th stim.
7 th stim.
(61±7g)
(61±6g)
(58±7g)
(17±2%)
(19±2%)
(17±3%)
(0,95±0,1µl O2)
(1,35±0,2µl O2)
(1,0±0,2µl O2)
(2,27±0,21mM)
(2,42±0,35 mM)
(2,17±0,23 mM)
(47±6%)
(52±5%)
(48±6%)
(21±5µl O2)
(27±4µl O2)
(24±4µl O2)
2 1 ↓ 1 2 3 4 5 6 7 8 9
←(-) Sti. (+)→
TAPPING AFTER STIMULATION

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