Biochemical Basis for Cessation of Latex Flow and Occurrence of Physiological Bark Dryness

H. CHRESTIN
ORSTOM, Abidjan, Ivory Coast
J.L. JACOB
IRCA-CIRAD, Montpellier Cedex, France
AND
J. D'AUZAC
Universite des Sciences et Techniques due Languedoc, Montpellier Cedex, France

Abstract in Bahasa Malaysia

Paras-paras penghasilan *Hevea* yang rendah dan kejadian kulit kering kerap dikaitkan kepada had aliran lateks yang boleh disebabkan oleh indeks palam yang tinggi akibat dari indeks letusan lutoid yang tinggi, *in vitro* serta juga *in vivo*. Lagi pula, kulit kering kerap dikaitkan kepada penggumpalan lateks *in situ* berhubung dengan penyahstabilan lutoid-lutoid di dalam lateks dari pokok-pokok yang berpenyakit.

Lutoid-lutoid dari lateks *Hevea brasiliensis* terdiri dari lompang-lompang mikro dengan sifat lisosom biasa. Disahkan baru-baru ini bahawa lutoid mengandungi aktiviti NAD(P)H-oksidase enzim yang menjanakan spesies oksigen toksik. Ini dapat dikesan di dalam lateks pokok-pokok berpenghasilan rendah, dan aktif khususnya di dalam lateks dari pokok-pokok dengan simptom-simptom kulit kering.

Enzim ini telah separa dicirikan, dan didapati tidak peka terhadap perencat-perencat biasa rantaian-rantaian respiratori mitokondria dan bakteria, tetapi diaktifkan oleh perencat-perencat lintasan mitokondria selang-seli (asid-asid hidroksamik dan propil gallat). Enzim ini pasti telah ditemui di dalam fraksi lutoid melalui pengemparan isopiknik. Enzim ini juga masih berfungsi pada kepekatan oksigen yang sangat rendah, dan diaktifkan secara meluas oleh kepekatan-kepekatan fisiologi kation-kation logam (Fe^{2+} dan Cu^{2+}) dan oleh sebatian-sebatian seperti kuinon. Sistem redoks lutoid ini boleh dikenalpasti sebagai NAD(P)H-kuinon-reduktase, menjanakan spesies oksigen toksik dari pengoksidaan sendiri beberapa sebatian separa seperti kuinon yang dikeluarkan dalam pengerjaan enzim.

Berbagai spesies oksigen toksik yang dijanakan membawa kepada degradasi peroksidatif lipid-lipid tidak tepu struktur-struktur membran dan menyebabkan penyahstabilan dan lisis organeles lateks, di antaranya adalah lutoid-lutoid sendiri

Perlakuan-perlakuan intensif kulit *Hevea* dengan etefon meningkatkan aktiviti penjanaan $O_2^{\cdot-}$ yang bersandar kepada NAD(P)H, dan serentak dengannya mengurangkan paras penggarut-penggarut sitosol. Ini membawa kepada lisis organeles di dalam lateks, terutamanya lutoid, dan menghasilkan penguculan 'faktor-faktor menggumpal' yang biasa dikandunginya. Fenomenon ini membawa kepada penggumpalan lateks di dalam saluran-saluran lateks — apa yang dinamakan 'sindrom alur torehan kering'.

Analisis komputer membolehkan satu model dibuat untuk menghuraikan peristiwa-peristiwa biokimia yang menyebabkan proses-proses peroksidatif dan membawa kepada kepalaman awal (penghasilan rendah) dan kekeringan kulit fisiologi.

Abstract

Low yielding levels of Hevea *and appearance of bark-dryness are often correlated to a limitation of latex flow which can be caused by high plugging index resulting from a high bursting index of the lutoids,* in vitro *as well as* in vivo. *Moreover, dry bark is often linked to latex coagulation* in situ, *associated with the destabilisation of the lutoids in the latex from these diseased trees.*

The lutoids from the latex of Hevea brasiliensis *consist of micro-vacuoles with typical lysosomal characteristics. It was recently confirmed that the lutoids contain an enzymatic NAD(P)H-oxidase activity which generates species of toxic oxygen. This is detectable in the latex of very low yielding trees, and is particularly active in latex from trees with typical symptoms of bark dryness.*

This enzyme has been partially characterised, and was shown to be insensitive towards the classical inhibitors of mitochondrial and bacterial respiratory chains, but is activated by inhibitors of the alternate mitochondrial pathway (hydroxamic acids and propyl gallate). It has been definitively located in the lutoidic fraction by isopicnic centrifugation. The enzyme is still functional at very low oxygen concentration, and is widely activated by physiological concentrations of metallic cations (Fe^{2+} and Cu^{2+}) and by quinone-like compounds. This lutoidic redox system could be identified as a NAD(P)H-quinone-reductase, generating species of toxic oxygen from the selfoxidation of some semi-quinone-like compounds produced during the working of the enzyme.

The various species of toxic oxygen that are generated lead to the peroxidative degradation of the unsaturated lipids of the membrane structures and cause the destabilisation and lysis of the latex organelles, among which are the lutoids themselves.

Intensive treatments of Hevea *bark with ethephon increases the NAD(P)H-dependent $O_2^{\cdot-}$ generating activity, and simultaneously decreases the level of the cytosolic scavengers. These lead to the lysis of organelles in the latex, especially of the lutoids, and results in the liberation of the 'coagulating factors' which they*

normally compartmentalise. These phenomena lead to latex coagulation within the latex vessels the so-called 'dry-cuts syndrome'.

Computer analysis enabled a model to be drawn describing the biochemical events accounting for these peroxidative processes leading to early plugging (low yields) and to physiological bark dryness.

The latex from *Hevea brasiliensis* is a fluid cytoplasm expelled from wounded latex vessels[1]. It is a colloidal suspension of diverse organelles and particles which remain stable because of the repulsive electrostatic forces generated from the negative charges present on their surrounding phospholipid membranes (or pseudo-membrane of rubber particles).

Production of natural rubber by *Hevea* is essentially attributable to the duration of the latex flow[2], which is limited by the coagulation phenomena at the tapping cut. Nowadays, it is well established that the latex itself contains its own coagulating factors which are essentially compartmentalised within the lutoids[3] and the Frey-Wyssling complexes[4].

The lutoids are single membrane bound micro-vacuoles[5] with lysosomal characteristics. In accordance with their vacuolar function, the lutoids accumulate and compartmentalise *in vivo* numerous ions, and in particular cations (H^+, Mg^{++}, Ca^{++}, Cu^{++}, *etc.*), and large amounts of cationic proteins[6]. Their liberation into the cytosol of the latex brings about the neutralisation of the negative charges, resulting in the aggregation and co-precipitation of macro-molecules, organelles and rubber particles, leading to latex coagulation[3,4]. Further, as lysosomes, the lutoids compartmentalise a broad spectrum of latent hydrolases[6,8], able to digest most of the biological solutes and macro-molecules, in particular, membrane-components. Therefore, it can be expected that the colloidal stability of the latex will depend on the efficient compartmentation of all these 'coagulating factors', and on the integrity of the vacuo-lysosomal membrane of the lutoids. Supporting this assertion, rubber production by *Hevea,* as a consequence of the duration of the latex flow[2], has been shown to be highly correlated to the 'bursting index' of the lutoids which is a measure of the stability of the lutoid membrane[5,9,10] *(Figure 1).*

In other respects, over-exploitation of *Hevea*, and in particular over-stimulation[11,12], can lead to a definitive cessation of yield resulting from a physiological disorder known as 'bark-dryness'.

Histological features such as formation of cross-walls, invasion of latex vessels by thylosoids from neighbouring parenchymatous cells, flocculation of latex and partial or complete coagulation of the latex within the vessels, as well as adherence of rubber particles to more or less damaged lutoids and other membrane structures[13,15,16] suggest the disorganisation of membrane structures. This is especially so inside the latex vessels and is typical with the onset and development of bark dryness.

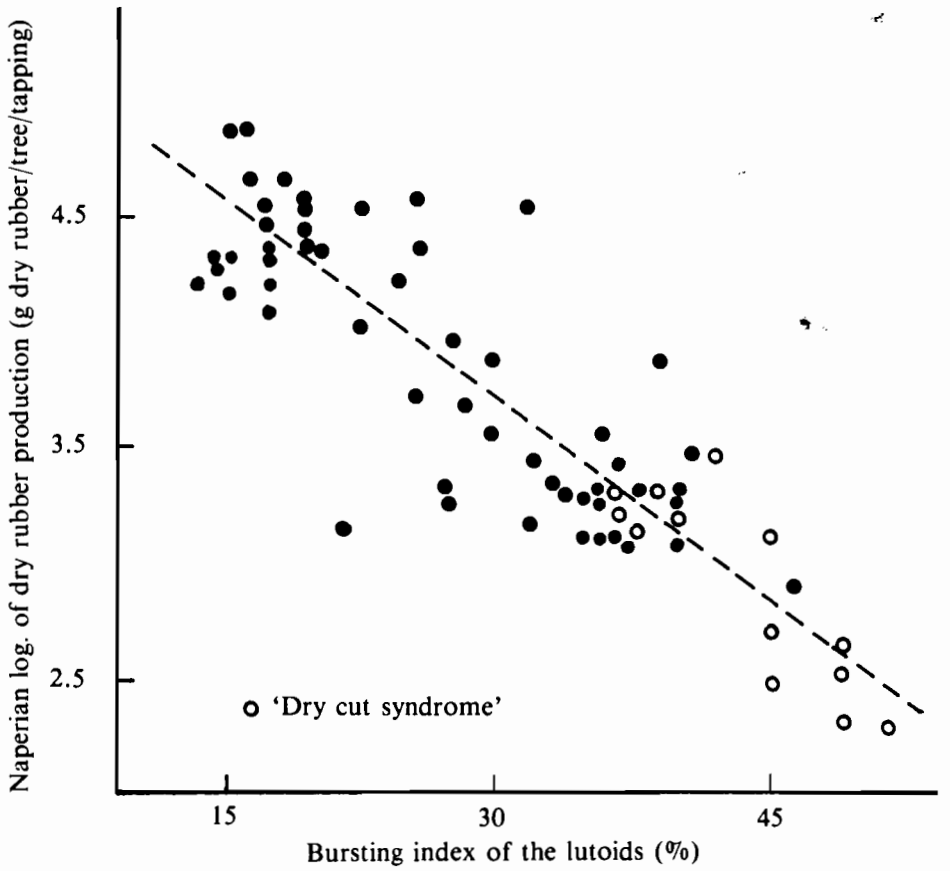

Figure 1. Correlation between latex yield and the bursting index of the lutoids.

Moreover, ultracentrifugation of the latex from partially dry trees showed virtual disappearance of the Frey-Wyssling complexes, flocculation and considerable reduction of the bottom fraction[11-15,17]. Further, isopicnic centrifugation of the bottom fraction from diseased trees often revealed considerable lightening of the residual lutoids[17,18]. Finally, abnormal instability of the latex organelles from partially dry bark was definitively evidenced from the demonstration of a relation linking the onset and development of dryness to abnormally high bursting index of the lutoids *(Figure 1),* with abnormal release of *o*-diphenol-oxidase from the Frey-Wyssling complexes in the latex cytosol from diseased trees[9,14,17].

All these observations taken together, strongly suggest that deterioration in the stability of all membrane structures, among which the lutoids and the Frey-Wyssling complexes which normally compartmentalise the quasi-totality of the latex coagulating factors, leads to latex destabilisation, resulting in latex coagulation *in situ.* Such phenomena, developing and spreading along the latex vessels could be regarded as

one of the primary causes for the onset and development of bark dryness. In order to explain these phenomena. attempts were made to identify and induce biochemical disorders within the latex vessels likely to cause membrane damage and subsequent degeneration of the latex cells.

By analogy with the NAD(P)H-dependent emission of superoxides by mammalian microsomes and granulocytes, which have been shown to induce lethal membrane damage and lysis of organelles[19,20,21], evidence for the occurrence of such peroxidative pathways, and their eventual scavenging activities within the latex of healthy and diseased trees were sought.

MATERIALS AND METHODS

Latex Collection and Centrifugation

First experiment. The latex were from fifteen- to seventeen-year-old GT 1 trees (½S d/3 6d/7). The tapping cut was localised on the mid-low panel. The trees were selected for their growth homogeneity and classified into apparently 'healthy trees' with high, medium or low yields, and 'diseased trees' with severe symptoms of dryness (>33% of the tapping cut definitively dry), but without apparent brown necrosis, bark disorganisation or pathological disease (fungi, *etc*).

After the first 10 ml of latex were discarded, the later flow was collected in glass vessels held in melting ice. The fresh latex was then immediately centrifuged (70 000 × g, 40 min, 5°C). The rubber phase was discarded and the clearest cytosol was sucked off and kept cold for eventual analysis. The pellet re-suspended in five volumes of an Hepes-Tris (50 mM), mannitol (0.32 M), pH 7.4 buffer, formed the crude lutoid fraction. When needed, the latter was washed two or three times with the same buffer, by successive centrifugations (30 000 × g, 10 min, 5°C), in order to remove contaminants.

Second experiment. Sixteen-year-old *Hevea* trees (GT 1) were selected for growth and yield (62 ± 7 g dry rubber per tree per tapping) homogeneity, as well as for the absence of any dry cut symptom or any apparent disease. They were tapped S d/3 6d/7 for eighteen months and were not stimulated for more than four months. After selection, a batch of twelve trees served as unstimulated control. Another batch of twelve trees was over-stimulated (ET 12.5% Ba 2/2 12Y) on scraped bark. All trees were tapped in a full spiral (S d/3 6d/7). The latex from these trees were collected and analysed individually, after every tapping, until the ninth tapping after the seventh stimulation.

Among the twelve over-stimulated trees, eight kept apparently healthy all along the experiment, but, as early as the second tapping following the fifth stimulation, four trees exhibited typical (more or less pronounced) symptoms of dryness (ranging from pronounced difficulties of the latex flow to the occurrence of partially dry cut). After the next stimulation treatment, the symptoms of dryness of these four trees became more severe (mean of 32% dry cut).

The results of the latex analysis for the eight over-stimulated trees which remained healthy, were re-grouped and plotted together, as well as those of the four trees which exhibited dryness symptoms after the fifth stimulation. Results were expressed in percentage compared with the twelve reference unstimulated trees.

The analysis performed on these individual latices, collected as described above, consisted of: the bursting index of lutoids (performed on fresh latex), and after centrifugation, activities of superoxide dismutase, catalase, peroxidase, NADH-oxidase and reduced thiol content, in the cytosol and in the washed bottom fraction of each latex.

Biochemical Tests

NAD(P)H-oxidase activities were monitored either polarographically, following oxygen consumption with a Clark electrode, or spectrophotometrically, following NAD(P)H oxidation, at pH 7.4. Emission of superoxide anions ($O_2^{-}\cdot$) was assayed spectrophotometrically, following the superoxide dismutase (SOD) inhibitable reduction of nitroblue tetrazolium[22]. Unsaturated lipid peroxidation was estimated by following the appearance of malondialdehyde (MDA) by the thiobarbituric acid method[23]. Activities of *o*-diphenol-oxidase were assayed following the dihydroxyphenylalanine dependent consumption of O_2, using a Clark electrode, in the same conditions as described by Hanower *et al*[4]. Catalase activities were measured polarographically, peroxidase spectrophotometrically at 470 nm, using guayacol as substrate. The bursting index of the lutoids were estimated as described by Ribailler[5].

RESULTS

Occurrence of a Lutoidic NAD(P)H-Oxidase-generating Superoxides

Figure 2 shows that the addition of NADH to a suspension of washed lutoids from partially dry trees induced a significant consumption of oxygen in solution, and a parallel reduction of nitroblue tetrazolium into formazan. The two phenomena were powerfully inhibited by the addition of exogenous superoxide dismutase. This is a typical evidence for the emission of $O_2^{-}\cdot$ during the functioning of the NADH-oxidase. Further addition of exogenous catalase resulted in a significant release of molecular oxygen into the medium, indicating a previous accumulation of H_2O_2 as associated with the functioning of the lutoidic redox system.

Relations between NADH-Oxidase Activity and Yield or Physiological State of Trees

As seen in *Figure 3,* the activity of the lutoidic NADH-oxidase is highly correlated with the bursting index of the lutoids, which is in turn, itself very highly correlated with rubber production *(Figure 1).* Then, the lutoids from healthy high-yielding and medium-yielding trees exhibit only traces, when it exists, of NADH-oxidase activity. The lutoids from very low-yielding trees, but without evident symptoms of typical dryness, show poor but detectable NADH dependent consumption of oxygen. Only trees with evident symptoms of dryness exhibit fully efficient lutoidic NADH-oxidase

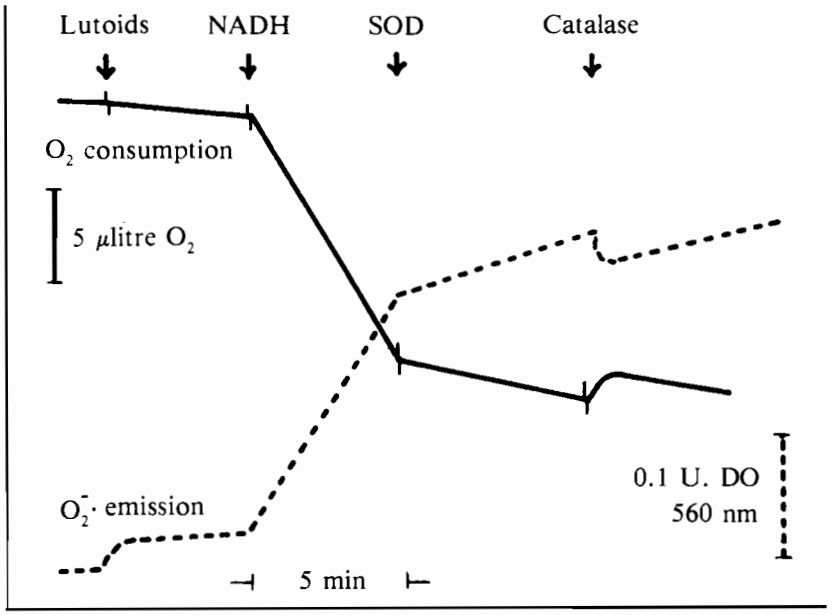

Figure 2. Parallel polarographic (Clark electrode) determination of the NAD(P)H-dependent consumption of O_2, and SOD inhibitable emission of O_2^-·, as followed by the direct reduction of nitroblue tetrazolium into formazan[22] at O.D.560 nm. Inhibition by exogenous SOD and catalase. The assays were performed with lutoids from partially dry trees, in the absence of exogenous iron chelate, at pH 7.4 and 26°C.

activity. So, only the latex from very low-yielding or partially dry trees were used for preliminary characterisation of the enzyme.

Subcellular Localisation of NAD(P)H-Oxidase

As seen from *(Figure 4)* isopicnic centrifugation (sucrose gradient) of the bottom fraction of latex from partially dry trees, the NADH-oxidase activity, followed either polarographically or spectrophotometrically, is strictly associated with the acid phosphatase activities: a typical marker of the lutoids[5,6] and absolutely not connected with the *o*-diphenol-oxidase activities, a marker of Frey-Wyssling complexes[4]. Further, the NAD(P)H-oxidase was insensitive towards the classical inhibitors of the mitochondrial and bacterial respiratory chains as shown below:

Addition of	O_2 consumption (μlitre O_2^-· min^{-1} ml^{-1} lutoid)
None	6.6
Antimycin	6.4
Rotenon	5.2
KCN	5.3
Hydroxamic acid	11.6

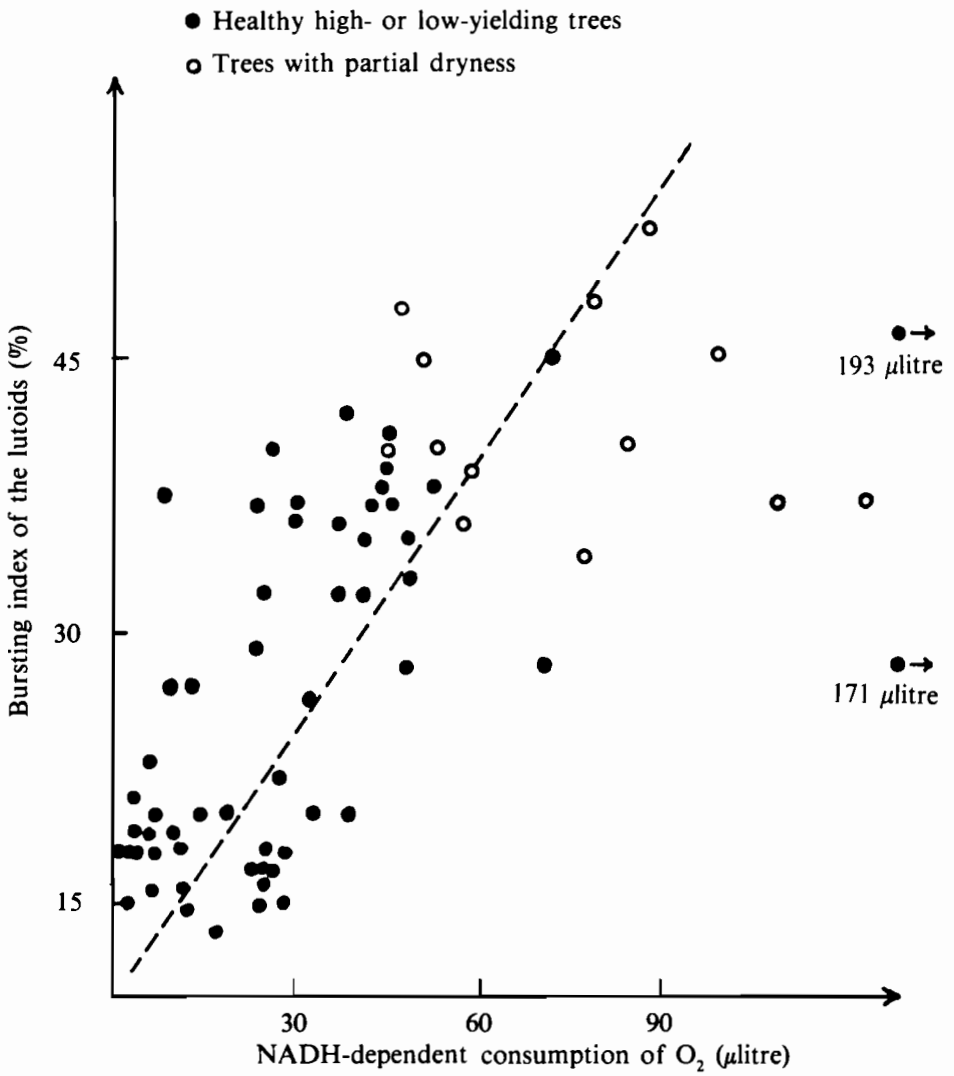

Figure 3. Correlation between the bursting index of the lutoids and their NAD(P)H-dependent consumption of oxygen.

All these results taken together do show that the NAD(P)H-oxidase is located inside (or on the membrane of) the lutoids. It cannot be attributed to mitochondrial or to bacterial contaminants.

Partial Characterisation of Lutoidic NAD(P)H-Oxidase

It was shown[25] that this lutoidic redox system could function with only traces of oxygen, and could accept electrons either from NADH or NADPH, apparently with a similar efficiency. Its *Km* for NADH was estimated to be 40-45 µM. The NAD(P)H-oxidase is strongly activated by physiological concentrations of metallic

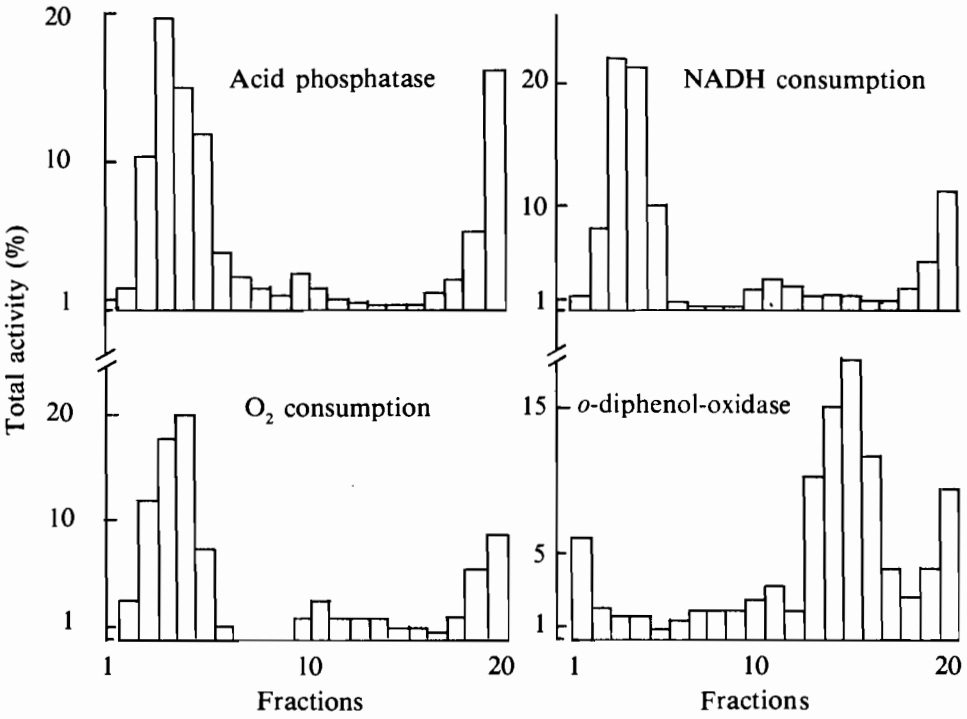

Figure 4. Localisation of the acid phosphatase activities, NADH-dependent consumption of O_2, oxidation of NADH and o-diphenol-oxidase, on a continuous sucrose gradient (0.6/1.7 M) (1 : dense bottom fraction / 20 : supernatant).

cations such as Fe^{3+} and Cu^{2+}, particularly in the presence of chelating agents (EDTA or ADP), which were shown to increase the affinity of the redox system for oxygen[25]. It is also greatly activated by some phenolics, quinones and quinoid compounds; this could explain the wide activation of the NAD(P)H-oxidase by salicylylhydroxamic acid (SHAM), the classical inhibitor of the mitochondrial alternate pathway[25], with a phenolic structure.

Recently, the enzyme has been partially purified[26], and its molecular weight estimated around 10^{+5} Da. Further characterisation showed that it could consist of an NAD(P)H-quinone-reductase, emitting toxic oxygen species, from the self-oxidation of the semiquinones produced. Indeed, this enzyme can transfer electrons from NAD(P)H to various quinones[26] with different affinities, and different efficiency on emitting superoxide anions ($O_2^{-}\cdot$).

Peroxidative Lysis of Organelles during Functioning of the Lutoidic NAD(P)H-Oxidase

It was shown that the functioning of the lutoidic NAD(P)H-oxidase induced a rapid peroxidation of exogenous, as well as endogenous, unsaturated lipids, especially in the presence of Cu^{2+} or iron chelates[25]. The peroxidative degradation of lipids was effi-

ciently inhibited in the presence of superoxide dismutase + catalase, and most efficiently in the presence of mannitol[24], a powerful scavenger of the highly toxic hydroxyl radical ($OH\cdot$)[20], which is known to result from the interaction of $O_2^-\cdot$ with H_2O_2, in the presence of traces of metallic cations[22,27].

Figure 5 shows the effect of an addition of NAD(P)H to a suspension of lutoids from partially dry trees or low-yielding trees, in the absence of exogenous scavenger. It induces in parallel an immediate consumption of oxygen and oxidation of NAD(P)H, a rapid peroxidation of the lutoidic unsaturated lipids (as seen from the appearance of malondialdehyde), followed by a significant increase of the lutoid bursting index. These latter degradative processes were significantly inhibited by exogenous scavengers, especially in the presence of the 'cocktail' superoxide dismutase + catalase + mannitol (not shown here). All these phenomena were shown to be scarcely detectable when using lutoids from healthy high- or medium-yielding trees, in the same conditions.

It was concluded that only the lutoids from very low-yielding trees (with abnormally high plugging index and high lutoid bursting index), and partially dry trees, contained a fully active NAD(P)H-oxidase, generating species of 'toxic oxygen' ($O_2^-\cdot$, H_2O_2, $OH\cdot$, *etc.*), able to induce the peroxidative degradation of the membrane unsaturated lipids, leading to the lysis of organelles, including the lutoids themselves.

Disequilibrium between Peroxidatic and Scavenging Activities in Latex, Responsible for 'Fragility' of the Lutoids

Thus, it is clear that the lutoids from very low-yielding or partially dry trees exhibit abnormally high lutoidic NAD(P)H activities, leading to abnormal release of toxic oxygen species. But, this would not have been thought to result in deleterious effects on membrane structures, if the scavenging chemicals and enzymatic activities had been fully efficient in the latex. Therefore, we analysed in the latex from high-, medium- and low-yielding trees (with no apparent symptom of dryness), as well as from partially dry trees, some biochemical parameters supposed to be involved in membrane damage or protection:

- *Factors likely to be involved in membrane degradation* — these consist essentially in the lutoidic NAD(P)H-oxidase activity which generates highly toxic species of oxygen, and in peroxidase activities which can generate quinonic free radicals and their condensation products with highly agglutinating abilities
- *Factors likely to protect membranes from the deleterious effects of oxygen* — this protection is essentially assumed both by scavenging enzymes such as superoxide dismutase and catalase which have been shown to be present in the latex[17,30], and by scavenging chemicals such as thiol compounds and ascorbic acid ('anti-oxygens').

All these parameters measured independently from seventy trees *(Experiment No. 1),* were computed with different methods of multivariate data analysis[30]. Principal Com-

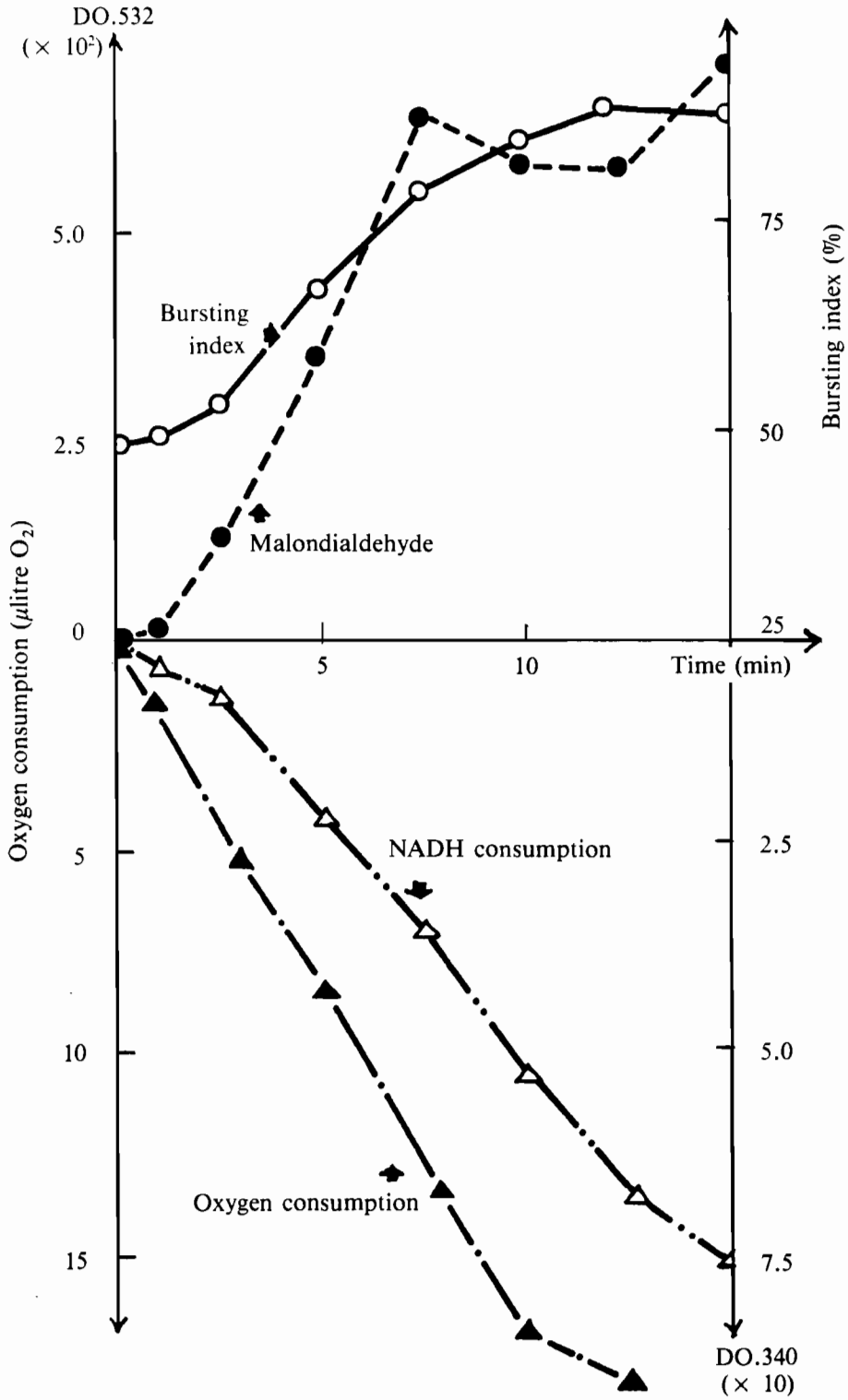

Figure 5. Evidence for the degradation of the lutoidic membrane in relation with the functioning of the lutoidic NADH-oxidase activity. Lipid peroxidation was monitored following the appearance of malondialdehyde[23] (532 nm). The bursting index of the lutoids was measured as described by Ribaillier et al[5].

ponents Analysis, Factorial Correspondence Analysis and Hierarchical Ascending Classification (Cluster Analysis). The results reported here are obtained from a Principal Components Analysis, where only the biochemical parameters were taken into consideration as active parameters, excluding the production data. Yield parameters were superimposed on the diagrams *(Figure 6),* as passive variables.

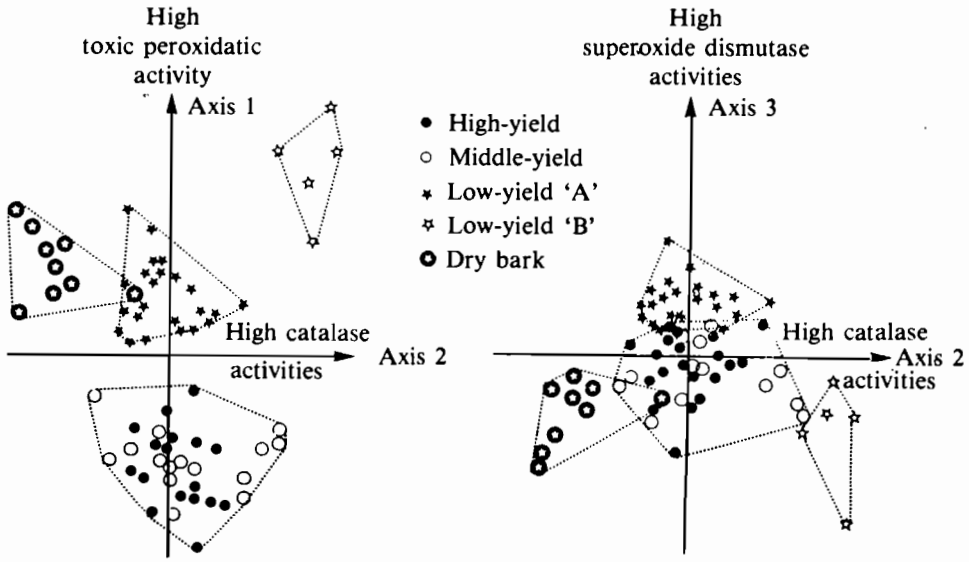

Figure 6. Diagrams presenting the projection of each individual Hevea *tree in the plans defined by the Axes 1 and 2 (left) and 2 and 3 (right) as defined by the biochemical characteristics of the latex studied in Experiment No. 1.*

It was shown[24,30] that the first factor (Axis 1) associated high peroxidative activities [NAD(P)H-oxidase and peroxidase activities] with low concentrations of reducing scavenging chemicals in the cytosol (reduced thiols and ascorbate). These parameters were also associated with high bursting index of the lutoids. This first factor which accounted for about 45% of the total variance introduced for the analysis did not take into account the classical scavenging enzyme activities (superoxide dismutase and catalase). This factor accounted essentially for the oxidative character (or opposite: the reducing character) of the latex, and could therefore be interpreted as, and called, 'the Axis of the toxic oxidative activities'.

The second factor (Axis 2) represented essentially high catalase activities in the cytosol and in the bottom fraction, associated with low peroxidase activities in the lutoids. This factor, which accounted for 20% of the total variance, could be regarded as 'the Axis of catalases'.

The third factor (Axis 3) opposed low superoxide dismutase activities to rather high toxic oxidative activities in the latex [NAD(P)H-oxidase and peroxidase activities]. This factor which accounted for about 12.5% of the total variance, could be identified as 'the Axis of superoxide dismutase'.

Figure 6 represents the projection of each individual tree in *Plan 1–2* and *Plan 2–3* as defined by the biochemical characteristics of its latex considered in this analysis. Each tree is represented by a specific symbol according to its yield characteristics, namely high yielding, medium or low yielding, or its physiological state: partial dryness. *Figure 6* reveals that, in the plan defined by the Axes 1 and 2, the 'toxic' oxidase activities' factor (Axis 1) clearly opposes the indivisible 'cloud' of the high- and medium-yielding trees, against the low-yielding or diseased trees. ***Figure 6*** shows that the catalase activities (Axis 2) allow discrimination of the diseased trees from a special group of low-yielding trees (called 'B'); the latter being characterised by high catalase but low superoxide dismutase activities.

Figure 6 also points out that the superoxide dismutase activities (Axis 3) discriminate the 'A type' low-yielding trees, from two distinct 'clouds' corresponding to the partially dry trees and the 'B type' yielding trees. Thus, this analysis separates four distinct groups of trees, defined in a ***quasi*** disjunctive manner, from the diverse possibilities of orientations of the chemical and enzymatic pathways involved in the stabilisation/destabilisation processes of the membrane structures within the latex. It is worth pointing out that the groups of high-yielding and medium-yielding trees remain perfectly indivisible. Then it can be assumed that the parameters considered in this analysis can account for only the extreme variations of yield characteristics, but can efficiently discriminate the healthy high- and medium-yielding trees from the very low-yielding or partially dry trees. Moreover, one can notice that the diseased trees with typical symptoms of dryness constitute a special, but distinct, case of low-yielding trees. Hence, it is shown that the biochemical variables alone, taken into consideration in such an analysis, are sufficient to account for the extreme variations of latex production and the physiological disease of dryness. Then, it was possible to define groups of trees characterised either by Enzymatic 'Hyper Activities' or 'Hypo Activities'.

Figure 7 summarises the two extreme cases considered in our experiment (the healthy high-yielding trees and partially dry trees), associating the biochemical parameters involved in the stabilisation or destabilisation of the lutoid membrane (as a model) with the physiological state of the laticiferous tissues. From this, it can be seen that even if the healthy high-yielding trees are not especially characterised by very high detoxicating enzymatic activities, they are, above all, characterised by very low (if detectable) O_2^{-} generating NAD(P)H-oxidase activities. Further, their unsaturated structures are efficiently protected from any casual activation of the peroxidatic activities by very high levels of scavenging 'anti-oxygen' chemicals in the cytosol.

On the contrary, trees with typical dryness symptoms exhibit abnormal NAD(P)H-oxidase and peroxidase 'hyper activities', but very low superoxide dismutase and virtual disappearance of catalase activities. This must result in the

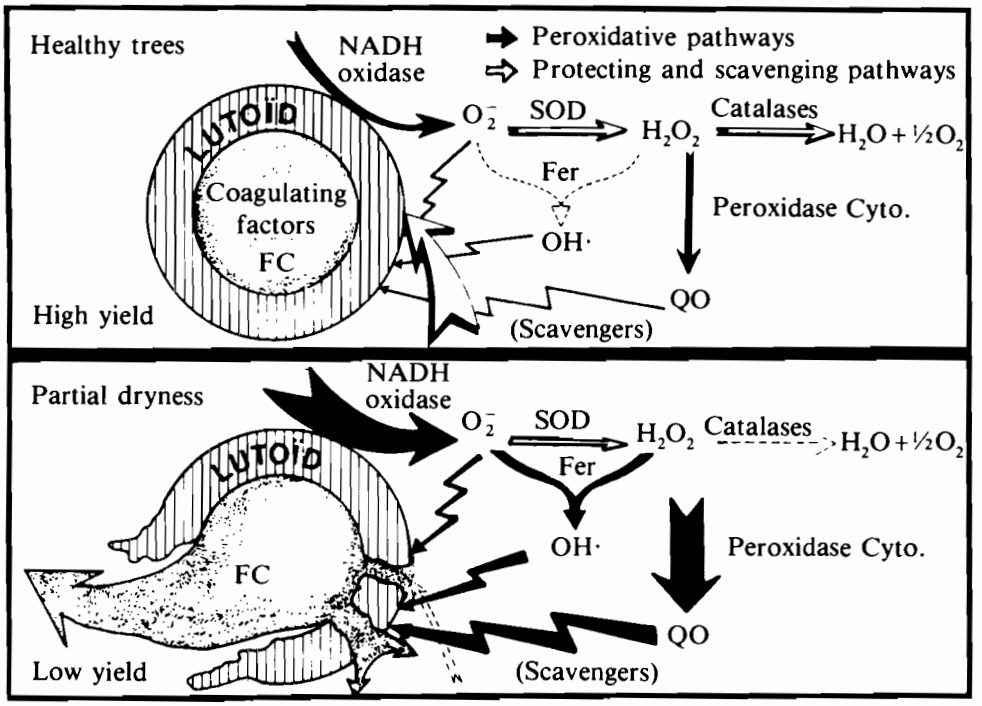

Figure 7. Interpretation of the peroxidative degradation of the lutoidic membrane, leading to the release of the latex 'coagulating factors', and lysosomal enzymes in situ, *related to dry-bark disease. The size of the arrows indicate the relative activity of each pathway.*

accumulation of superoxide anions which can only spontaneously dismutate (low superoxide dismutase) into H_2O_2, which also accumulates in the latex (low catalase). These two toxic species of oxygen can interact with high probability, according to Fenton and Haber-Weiss-like reactions, leading to the generation of the most toxic oxygen free radicals, namely (OH·) and singlet oxygen (O_2^1), with highly deleterious effects on membrane structures. The unsaturated lipids are then largely exposed to intensive peroxidatic degradations. Further, in the absence of sufficient amounts of scavenging reducing chemicals in the cytosol, the abnormally high peroxidase activities contribute to the degradative processes because of the resulting generation of toxic quinoids (activators of the lutoidic NAD(P)H-oxidase) and their agglutinating condensation products. Then the trees with severe symptoms of dryness exhibit complete destabilisation of their membrane structures. These processes are supposed to result in the lysis of the lutoids themselves, with subsequent liberation of the latent 'coagulating factors' that they normally compartmentalise in the latex. This must naturally lead to the coagulation of the latex within the latex vessels, resulting in the cessation of yield and then degeneration of cells.

Induction of Dryness by Over-stimulation is Linked with Activation of Lutoidic NAD(P)H-Oxidase

It was previously shown that over-stimulation with ethephon could lead to the appearance of typical symptoms of dryness[11,12]. We therefore tried to see if the onset of dryness, by deliberate over-stimulation, could be associated with the onset of a disequilibrium between the peroxidative activities with deleterious effects on membrane structures, and the scavenging protective activities within the latex. We report here the results of an experiment *(Experiment No. 2)* attempting to induce dryness by deliberate over-stimulation. The most significant results obtained, reported in *Figures 8* and *9,* are expressed as percentage variations compared with the non-stimulated control trees. They show that, whatever the trees, the stimulating treatments induced a transient activation of the lutoidic NAD(P)H-oxidase *(Figure 8)*, superimposable with the classical increase of yield. It was previously reported that, at the same time, the enzymatic scavenging activities (catalase and superoxide dismutase) in the latex were transiently activated as well[24]; this could explain the relative stabilisation of the membrane structures within the latex, as evidenced by the slight but reproducible and significant lowering of the lutoid bursting index after the stimulating treatments *(Figure 9).*

It was only when the first typical symptoms of dryness became evident at the level of the tapping cut (after the second tapping following the fifth stimulation) that the biochemical parameters considered could differentiate the four trees with symptoms of dryness, with the eight over-stimulated trees which remained healthy all along this experiment. These biochemical symptoms persisted and grew worse from the fifth to the last seventh stimulation. These symptoms consisted of:

- A persistent rise of the lutoidic bursting index (60% over the control) *(Figure 9)*
- A persistent rise (large amplitude) of the $O_2^{\cdot-}$ generating lutoidic NAD(P)H-oxidase activity ($\times$ 12 compared with the control) *(Figure 8)*
- An irreversible decline of the reduced thiols in the cytosol[24,31] (more than 50% below the control)
- A dramatic decline of the superoxide dismutase (70% below the control) and catalase activities[24,31]
- A correlative decrease of the rubber production as a response to over-stimulation[24,31].

This experiment clearly confirms that over-stimulation can induce dryness. All the biochemical symptoms considered together suggest the installation of severe disequilibrium between the toxic peroxidative activities and the scavenging activities within the latex, resulting in the degradation of the lutoid membrane, as early deleterious events induced by successive over-stimulation with ethephon.

CONCLUSIONS

All these results presented here demonstrate that the latex from very low-yielding trees (with high plugging index and high lutoid bursting index), and above all from the trees displaying typical symptoms of dryness, are characterised by:

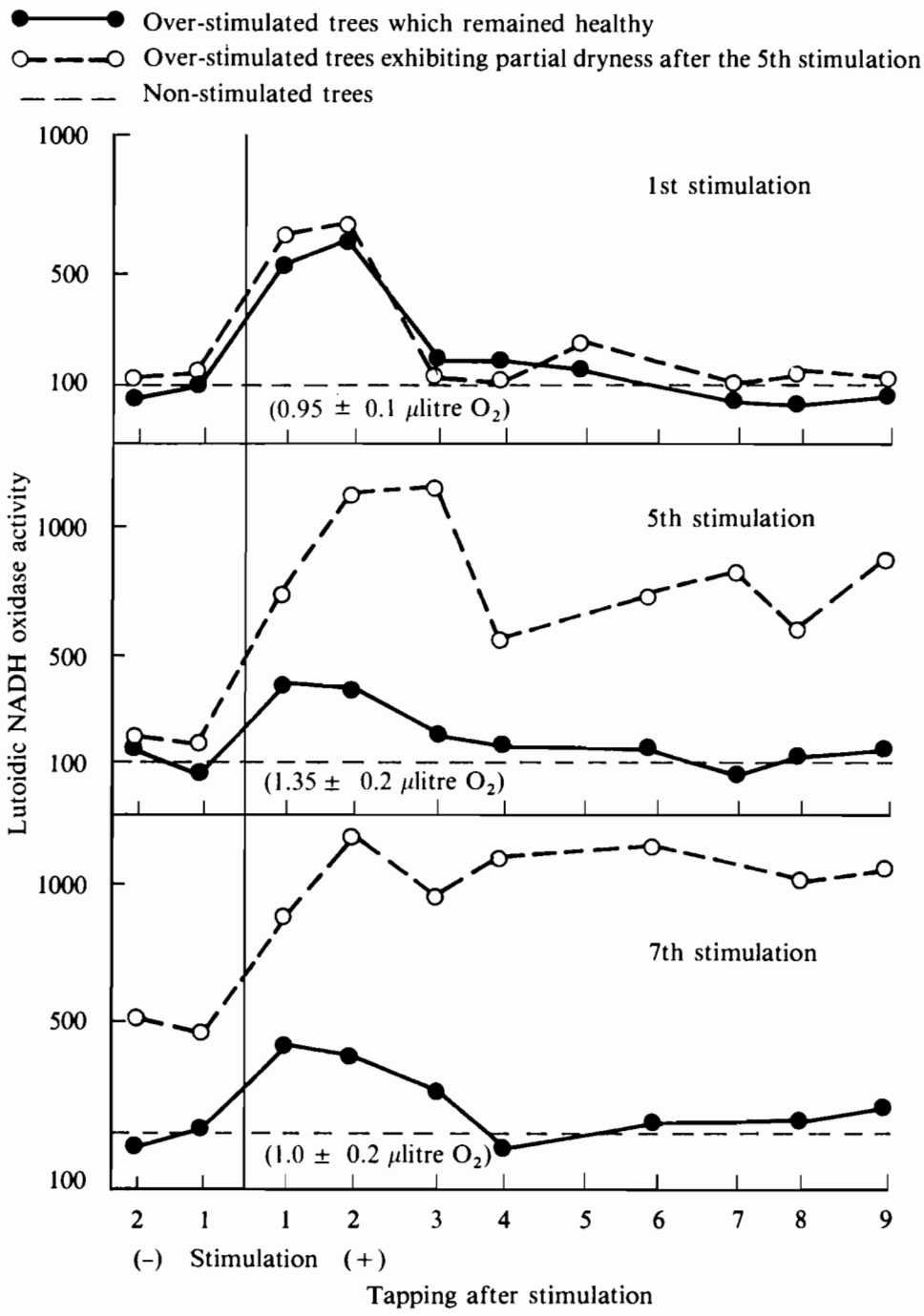

Figure 8. Influence of over-stimulation treatments on the activity of the lutoidic NAD(P)H oxidase.

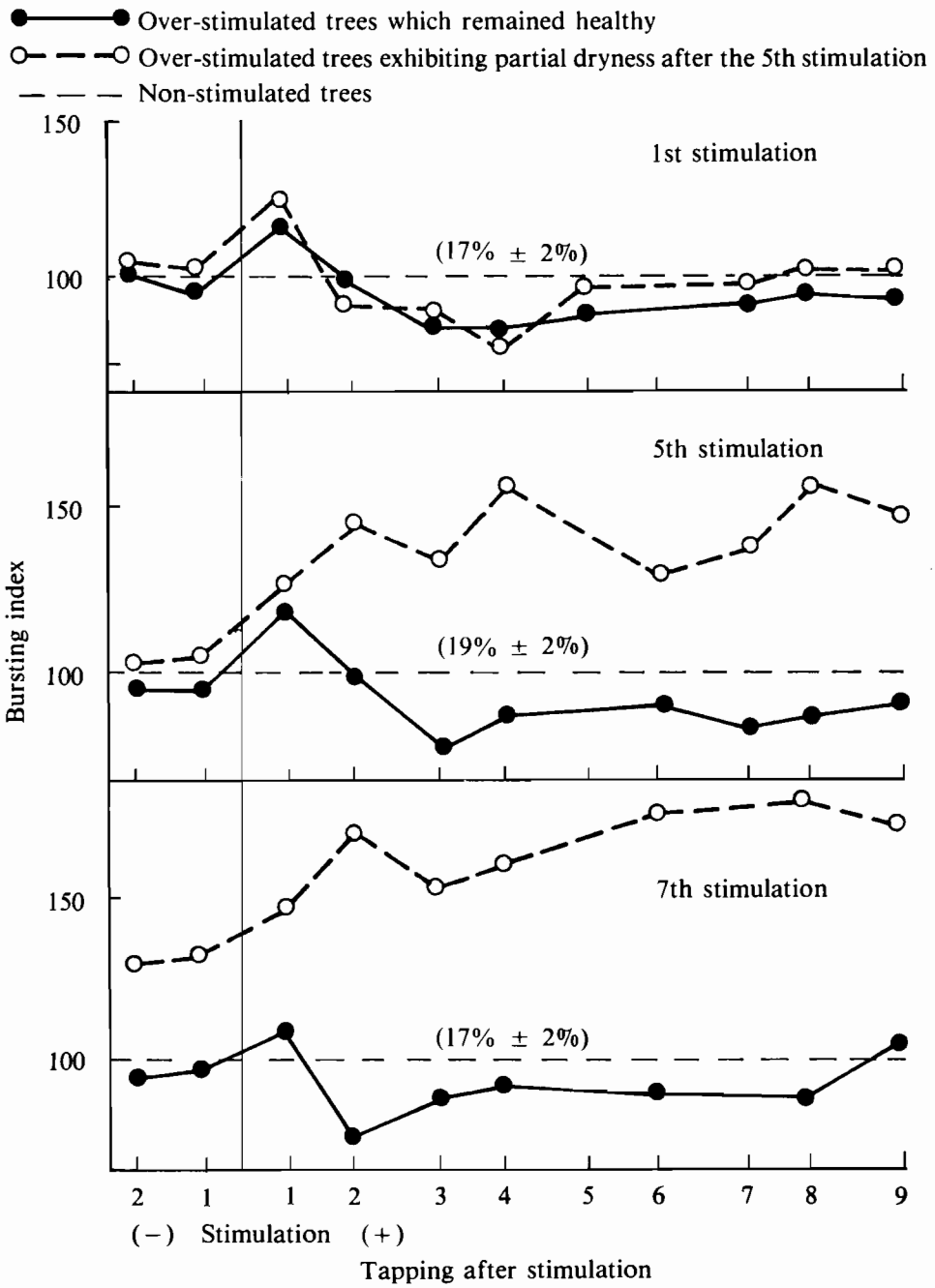

Figure 9. Influence of over-stimulation treatments on the bursting index of the lutoids.

- An abnormally high and persistent activity of the $O_2^{-}\cdot$ generating lutoidic NAD(P)H-oxidase *(Figure 9)*
- An abnormally high and persistent increase of the lutoidic bursting index *(Figure 9)*
- A parallel decrease in the concentrations of the cytosolic scavenging chemicals (ascorbate + reduced thiols)[31]
- A virtual disappearance of enzymatic scavenging activities (superoxide dismutase and catalase)[31].

The combination of increased peroxidatic activities together with a decreased level of scavengers in the latex from low-yielding or partially dry trees, results at least in the destabilisation of the lutoid membrane. Subsequently the vacuo-lysosomal compartment of the latex becomes less efficient in maintaining an adequate homeostasis, compatible with an optimum regenerating metabolism, within the latex cells[32]. This has been clearly evidenced by the lower efficiency of the lutoids to accumulate and compartmentalise H^+ and citrate, in the latex from low-yielding or partially dry trees[32,33]. Moreover, as far as the very low-yielding trees are concerned, the destabilisation of the lutoid membrane may be sufficiently severe to lead to an early bursting of the lutoids immediately after the opening of the latex vessels. This results in the early initiation of the coagulating processes and subsequent high plugging index, then low-yield[2,10]. As far as trees with typical symptoms of dryness are concerned, we conclude that the disequilibrium between the toxic peroxidative and scavenging activities in the latex may be so severe that it induces a lethal destabilisation of the lutoid membrane resulting in the lysis of the lutoids *in situ*. This leads to the liberation of the coagulating factors that they normally compartmentalise within the latex vessels, and results in the definitive cessation of yield of the plugged areas.

One can think that such a membrane destabilisation and degradation may not concern the lutoids alone. In this way, we obtained good evidence for the destabilisation of the Frey-Wyssling complexes in the latex from partially dry trees[9]. Moreover, it seems quite probable that all the membrane structures within the latex vessels could be exposed to such peroxidatic degradation, with consecutive impairment of nutrient and water exchanges at the level of the plasmalemma as postulated elsewhere[33-36], or in the regulation of protein synthesis, if the nuclear membrane is damaged.

We suggest therefore that the disorganisation of all the membrane structure resulting from a disequilibrium between the toxic peroxidative activities and the scavenging activities within the latex vessels, is a key event leading to early plugging (very low-yield) and, under a more severe form, to the onset and development of at least a kind of bark dryness (deliberate over-stimulation).

As demonstrated here, the latex from partially dry trees are characterised by abnormally high lutoidic NAD(P)H-oxidase activities, leading to the generation of $O_2^{-}\cdot$, and by subsequent interactions, to the appearance of free radicals ($OH\cdot$), with deleterious effects on membranes. We demonstrated that the stimulating treatments with ethephon induced a marked activation of this lutoidic redox system. This activation was shown to be persistent in the latex from diseased trees and only transient in the latex from trees which remained healthy.

It is now well documented that the $O_2^{-}\cdot$ and OH· radicals are involved in the synthesis of ethylene from its precursor l-aminocycloproprane-l-carboxylic acid (ACC) in plants[37,38]. Furthermore, the recent confirmations of an autocatalytic production of endogenous ethylene by various plant tissues, as a consequence of treatments with exogenous ethylene[39,40], lead to suggest the existence of some analogous phenomenon in the bark of the rubber tree. We therefore propose that any hormonal treatment of *Hevea* bark with stimulating agents generating exogenous ethylene, such as ethephon, will induce within the latex vessels, an autocatalytic biosynthesis of endogenous ethylene, as stimulated from the activation of the $O_2^{-}\cdot$ generating lutoidic NAD(P)H-oxidase. Moreover, it has been demonstrated that the amplitude of this production of endogenous ethylene is proportional to the dose of the exogenous ethylene applied to the plant tissues[40]. Thus it can be supposed that over-stimulation of the latex yield with ethephon, an ethylene generator, might bring about such an overproduction of endogenous ethylene, that the 'over dose' of ethylene constantly sustained within the latex vessels would result in the induction of irreversible processes leading to cellular degeneration and senescence[41]. Great care therefore needs to be taken in order to avoid any type of over-exploitation in *Hevea* plantations, either through over-stimulation (dose and frequency) with ethylene generators, or through over-tapping, which could lead, as well, to the production of endogenous ethylene, because of wound reactions.

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DISCUSSION

J.B. GOMEZ (Rubber Research Institute of Malaysia, Kuala Lumpur, Malaysia)

The proposed mechanism of toxic oxygen and the lysis of lutoid membranes are in agreement with our observations by electron microscopy reported in 1975 that there is a coagulation *in situ* in the latex vessels in cases of what we called incipient dryness. So I believe that this is probably a prelude to the development of dryness. I am very happy to note that a biochemical technique is now available to detect this phenomenon in latex tapped from the tree. The second comment I would like to make is that lutoids being lysosomes can be expected to lyse the unwanted products arising from degenerative processes. Have you found any evidence of such autophagy in the latex vessels by histochemical or other techniques?

H. CHRESTIN

Concerning the autophagy by lutoids, we have not looked for such a phenomenon. Dr Marin has shown the presence of degraded RNA inside the lutoid. Some ultracytologists have shown that lutoids may contain osmiophilic inclusions.

M.R. SETHURAJ (Rubber Research Institute of India, Kottayam, India)

This paper proposes the hypothesis that dryness commonly known as brown bast is related to lutoid instability caused by NAD(P) oxidase activity. Normally, the symptom of dryness is preceded by late dripping, that is, low plugging index and more flow. Thus, usually the dryness is preceded by a more stable latex. Therefore, the phenomenon you have observed appears to be a consequence of the symptom rather than the cost. Please comment.

H. CHRESTIN

Our experience showed that stimulation induced the superoxide generating NAD(P) activity in the lutoids. This caused destabilisation of lutoid membranes resulting in the release of coagulating factors. At the same time when the trees were not dry, their scavenger activities were increased. So, it is normal that when there is no problem of dryness the trees that remain healthy have stable lutoids and so no high plugging index because there is a contemporary increase in scavenging enzymes (catalase and SOD) and chemicals (anti-oxygen).

S.W. PAKIANATHAN (Rubber Research Institute of Malaysia, Kuala Lumpur, Malaysia)

I would just like to make a comment. French workers have shown that antioxidants act as scavengers. We have actually applied antioxidants on the cut and found that they help to promote flow to some extent, this agrees more or less with what you have suggested, but some of the antioxidants do not work. For example, propyl gallate was not effective, but when we added WSP which is a rubber antioxidant, it had some effect on the long cuts.

P.D. ABRAHAM (Rubber Research Institute of Malaysia, Kuala Lumpur, Malaysia)

What do you mean by over-stimulation? With puncture tapping, we are stimulating the trees with 10% ethephon monthly for a few years, even with young trees. I do not think there has been any report of dryness with puncture tapping. Please comment.

H. CHRESTIN

In our conditions, ½S d/3 6d/7, we did obtain typical dryness symptoms after the fifth stimulation (10% ET). After the seventh stimulation, the mean length of dryness of the diseased GT 1 trees increased to 35%. We do think that there is a clonal sensitivity towards dryness, which could explain your results.

It was shown that the length of the tapping cut has also an effect on the development of dryness. Total spiral induces more dryness than ½S or ¼S. In our case (conventional tapping), the addition of an ethylenic stress (10% ET) and injury stress (spiral or ½ spiral), may have favoured the onset and development of dryness. Dryness could also appear from the combination of excess of ethylene in the laticiferous tissues both from treatments with high level of exogenous ethylene and from endogenous ethylene from wound reactions.

CHANG AH KOW (Harrisons Malaysian Plantations Berhad, Kajang, Selangor, Malaysia)

Over-stimulation or under-stimulation would be in relation to the yield output from the trees; is the maximum or optimum yield level, on *Panels A* and *B* that can be extracted without encountering any problem of significant bark dryness?

H. CHRESTIN

We think that stimulation has to be carried out with low concentrations of ethephon ($\leqslant 5\%$) and the tapping intensity has to be regulated. Whether it is over-stimulation depends on the clones and certain clones are more subject to dryness than others. For example, PB 235 is very sensitive to over-stimulation, and it is a clone that needs stimulation particularly to obtain a good yield. Analysis of the biochemical and physiological parameters of latex can be useful to estimate over- and under-exploitation and to optimise production and avoid dryness.

LEONG TAT THIM (Guthrie Research Chemara, Seremban, Negri Sembilan Malaysia)

You have taken latex samples from partial dry trees. What is the degree of dryness of these partial dry trees?

H. CHRESTIN

Dryness was estimated as the percentage of the length of the tapping cut. In our experiment, the mean length of dryness was about 35% (21% to 73%).

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