Sonderdruck aus European Journal of Forest Pathology,
Band 16 (1986), Heft 1, S. 22–37

VERLAG PAUL PAREY · SPITALERSTRASSE 12 · HAMBURG 1

Department of Phytopathology, Institut Français pour la Recherche en Coopération (ORSTOM), Abidjan, Côte d'Ivoire

Root rot diseases of Hevea brasiliensis

I. Physiological and biochemical aspects of host aggression[1]

By J.-P. Geiger, M. Nicole, D. Nandris and B. Rio

U. S. Copyright Clearance Center Code Statement: 0300-1237/86/1601-0022/$ 02.50/0
Eur. J. For. Path. 16 (1986) 22–37
© 1986 Verlag Paul Parey, Hamburg und Berlin
ISSN 0300-1237

Abstract

A study on biochemical mechanisms of the rubber tree aggression by two root rotting fungi *Rigidoporus lignosus* and *Phellinus noxius* was carried out. The activity of eight enzymes and their spatial distribution in adult tap root tissues were measured. Finally their origin, host or parasite, and physiological signification in the pathogenic process were discussed.

1 Introduction

Rigidoporus lignosus and *Phellinus noxius*, Polyporaceae, are widespread white rot parasites in equatorial and humid tropical zones. Even though they attack a large number of shrubby species (CHEVAUGEON 1959), they exist in equilibrium in the natural forest. They are of practical importance, at least in Africa, since they are the most dangerous root parasites of rubber trees. These fungi, especially *R. lignosus*, represent a menace for rubber plantations which is just as serious as that posed by *Heterobasidion annosum (Fomes annosus)* for conifer or spruce plantations in temperate climates.

The main purpose of this work was to elucidate the changes of host tissue (lignified roots) enzyme activities caused by parasite attack. The enzymes examined were chosen for their possible participation in the degradation of the lignocellulose complex and thus in the pathogenic process.

As mentioned by JOHANSSON and UNESTAM (1982), we should remember that there are practically no biochemical data concerning host-parasite interactions in lignified organs, while there is a large body of results in the case of non-lignified hosts. The rare data occasionally concern enzymatic activities (JOHANSSON and THEANDER 1974; SHAIN 1971) but most often deal with disturbances in the inorganic ion balance (WONG and PREECE 1978) or in that of organic substances (sugars, phenols, lignin, fungitoxic substances, etc.) (SHAIN and HILLIS 1971; PEEK et al. 1972 a, b; CERNY 1973; POPOFF et al. 1975; JOHANSSON et al. 1976; EKMAN and WEISSENBERG 1979; WORRALL and PARMETER 1982; JENG et al. 1983; etc.).

We personally decided not only to estimate the degree of biochemical disturbances caused by parasite attack, but also to verify the origin of the enzymes and the possibility of their participation in the degradation of root tissues.

Finally, we attempted to review all available information in order to schematically deduce the time course of biochemical events accompanying pathogenesis.

[1] Paper presented as a communication at the 27th Congress of the French Phytopathological Society, Paris, November 1984.

2 Materials and methods

2.1 Culture of fungi in vitro and preparation of culture filtrates

R. lignosus and *P. noxius* were grown in *Hevea* sawdust (10 g per 100 ml of water in 250 ml Erlenmeyer flasks) at 30 °C for 3 weeks. The cultures were then filtered through sintered glass No. 1 and the filtrate was centrifuged at 20000 g for 15 min. The resulting supernatant constituted the enzyme solution.

2.2 Analysis of plant material

Two types of plant material were examined:

– Taproots of rubber plantlets, 3 to 6 months old, either healthy or artificially infested as described elsewhere (NANDRIS et al. 1983).

– Tissues of plantation grown adult rubber trees, 4 to 8 years old, partially invaded by either parasite (natural infestations).

In all the cases, only the lignified tissues of the xylem were used. They were removed with a wood chisel from adult taproots previously sectioned in two equal parts longitudinally, or from taproots of plantlets from which the bark had been stripped.

The wood chips from adult tissues were reduced to sawdust by a very rapid dry grinding (Gondard knife blade grinder). The resulting sawdust was then left to macerate overnight at 4 °C in sodium phosphate buffer (0.0125 M, pH 6) at 5 ml per g (fresh weight) of tissue.

Taproots from plantlets were cut into small fragments and ground (Ultraturax) in the presence of 0.0125 M phosphate buffer, pH 6, at 5 ml/g of tissue and were left to macerate overnight.

In both cases, the tissue extracts were recovered after maceration by centrifugation as before.

2.3 Sampling

Preliminary tests showed a considerable variability of enzyme activities from one tree to another, even for neighbors of the same age and which grew in identical climatic and pedological conditions. This variability probably resulted from the fact that taproots arise from seeds created by "illegitimate" fertilizations and so they have a different genetic stock. The same is true for the plantlets.

The problem of a "healthy control" thus became important. In the case of adult trees, we overcame this difficulty by analyzing taproots which were only partially invaded by the fungi and thus including a zone of healthy tissue and one of parasitized tissue. A prior study (GEIGER et al. 1976) of the distribution of laccase and peroxidase in taproots in fact showed that the tissues in the healthy part of the roots underwent no notable change at the level of their enzymatic equipment. This tissue can thus be considered as a control in each individual root against which the qualitative and quantitative changes in the parasitized tissues can be determined.

In the case of plantlets, each experiment involved several individuals (at least 3) of the same age, either healthy controls (not inoculated) or artificially infested. In the latter case, only the infested zone was sampled.

The spatial distribution of enzymes in roots was investigated only in adult trees. The following samples were analyzed in each root (Fig. 1 a):

- Healthy tissues (H), sampled much ahead of parasite progression front (control),
- Healthy front tissues (HF), sampled near the progression front,
- Parasitized front tissues (IF),
- Parasitized tissues (I) removed much behind the front ("old" colonized tissues),

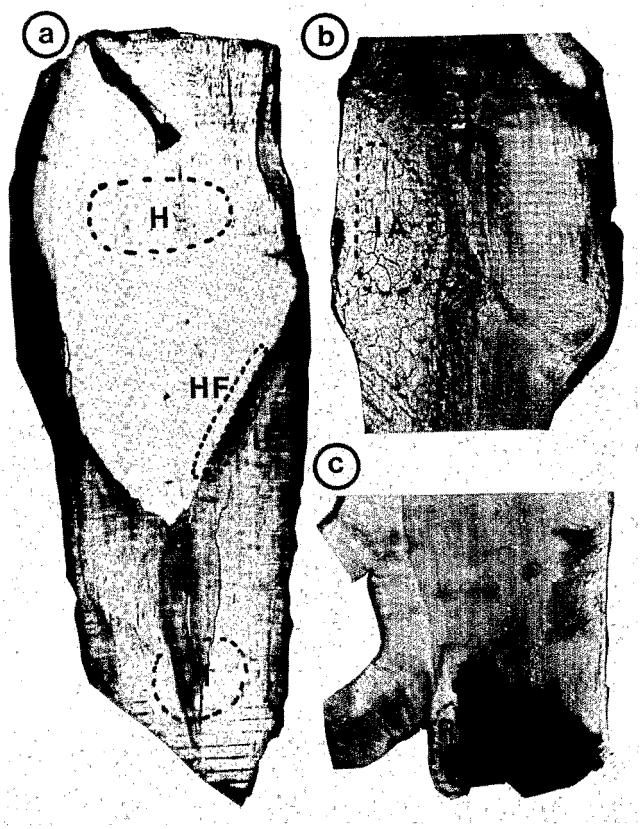

Fig. 1. a. Partially *R. lignosus* infected tap root: localization of the different tissue types (H: healthy; HF: healthy, near of the front of the parasite progression; IF: infected at the front; I: infected far from the front. b. IA: *P. noxius* infected tissue of alveolar type. c. R: reactional tissue surrounding *R. lignosus* infected tissue

– Parasitized alveolar type tissues (IA), removed from zones in which the tissues were highly degraded and which present this characteristic appearance (Fig. 1 b) (infestation only by *P. noxius*).

In some experiments we also analyzed reaction tissues (R) (Fig. 1 c) observed occasionally in taproots attacked by *R. lignosus* and very often following a cortical attack by *Sphaerostyble repens*.

2.4 Enzyme assays, expression and presentation of the results

2.4.1 Enzyme assays and expression of the results

The following enzymes were assayed at pH 4.5 in 0.05 M acetate buffer: acid phosphatase (Pase) (E. C. 3.1.3.2.), β-glucosidase (β-glu) (E.C. 3.2.1.20), α- and β-galactosidase (α- and β-gal) (E.C. 3.2.1.22 and E.C. 3.2.1.23). Activities are expressed as nmoles of substrate transformed per minute and per gram fresh tissue weight (or per ml of culture filtrate).

CM-cellulase (E.C. 3.2.1.4.) and pectinase (E.C. 3.2.1.11.5) were assayed at pH 4.6 in 0.025 M citrate buffer using the technique of viscosimetry (Ostwald viscosimeter). The

results are expressed as RA/g fresh tissue or per ml of culture filtrate, where RA (relative activity) = 1000/T50, T50 being the time in min required for the viscosity of the reaction medium to decrease by 50 %.

Peroxidase (E.C. 1.11.1.7) was assayed at pH 6 in 0.05 M sodium phosphate buffer, as was laccase from *R. lignosus*. Laccase from *P. noxius* was assayed at pH 4.5 in 0.05 M acetate buffer and activity is expressed as $A_{420} \times 1000$/min/g fresh tissue weight (or per ml of filtrate).

The reaction media for these assays have been described elsewhere (GEIGER 1975).

2.4.2 Presentation of the results

The following principles were adopted for the presentation of the results in the form of histograms:

For each enzyme, the height of "columns" corresponding to the different types of tissue is proportional to the activity noted in the extract of the tissue in question. This height is calculated with reference to the maximum activity recorded (regardless of the nature of the tissue) to which the index of 100 is attributed. This representation enables the tissues to be "positioned" with reference to each other and for each enzyme. In addition and for each enzyme, we indicate the true value of the activity (expressed in the units defined above) which corresponds to the index 100. This enables the activities of the different enzymes to be compared (when these activities are expressed in the same units: Pase, β-glu, α- and β-gal; CM-cellulase and pectinase; laccase and peroxidase).

This type of presentation is shown in Figs. 2 a, b and 4 a, b.

Finally, a similar presentation was used for comparing among themselves and by type of tissue and culture filtrate, the four enzymes Pase, β-glu, α- and β-gal whose activities are expressed in the same units.

2.5 Electrophoresis and chromatography

2.5.1 Electrophoresis

Some enzymes were electrophoretically separated by starch gel electrophoresis as described by SMITHIES (1955): 14 % Connaught starch gel in 0.03 M Tris-maleate buffer, pH 6.5.

2.5.2 Chromatography

Lignocellulose was broken down by *R. lignosus* and *P. noxius* filtrates in a reaction medium consisting of 1 % lignocellulose suspended in 0.0125 M citrate buffer, pH 4.6 + variable quantities of culture filtrate. The sugars resulting from this breakdown were separated by silica gel thin layer chromatography on a 0.25 mm thick gel [Silica gel 60 precoated plates (Merck)]. Deposits at the origin were 10 μl of reaction medium and 5 μl of each reference sugar as a 10^{-2} M solution in distilled water. The migration solvent used is butanol/acetic acid/water (4/2/3, v/v/v). Chromatograms were developed by spraying with a mixture of 100 ml of 1 % diphenylamine in acetone, 1 ml of aniline and 10 ml of 85 % orthophosphoric acid. The plates were heated at 105 °C for 5–10 min and the sugars were seen as dark blue spots on a white background.

3 Results and discussion

3.1 Type and extent of disturbances of enzyme systems caused by parasite aggression

The histogram in Fig. 2 a shows the principal results we obtained. Several basic characteristics are seen, enabling us to distinguish healthy from parasitized tissues. In addition, we can distinguish parasitized tissue resulting from *R. lignosus* aggression from that caused by *P. noxius*. The differences exist on both the quantitative and qualitative levels.

– Qualitatively: three enzymes are present only in parasitized tissues. They are CM-cellulase, pectinase and laccase, for which their major participation in the pathogenic process may be presumed.

– Quantitatively: with the exception of phosphatase and partially of β-glucosidase, enzyme activities in infected tissues are much higher than those in healthy or reaction tissues.

Finally, infestation by *R. lignosus* and *P. noxius* can be distinguished by several characteristics. Thus, the activities of hydrolytic enzymes (except for phosphatase) in tissues colonized by *P. noxius* are always much higher than that of the homologous enzymes extracted from tissues invaded by *R. lignosus*. The reverse situation is shown in the case of laccase and peroxidase.

A similar comparison was carried out between healthy and artificially infected plantlets. In general, the results were similar to those mentioned above. Differences involved the generally higher levels of hydrolases, except for CM-cellulase and pectinase, in the plantlet extracts in comparison to extracts from adult taproots. In addition, the β-glucosidase activity in tissues parasitized by *R. lignosus* was higher than that in extracts from tissues invaded by *P. noxius*.

It is thus clear that parasite aggression disturbs the enzyme equipment of rubber trees both quantitatively and qualitatively. The amplitudes of these disturbances vary with the enzyme and also depend on the causative parasite.

This correlation between metabolic modifications identified in the tissues and the parasitic process supplies no precise indication on the mechanisms involved. Increased

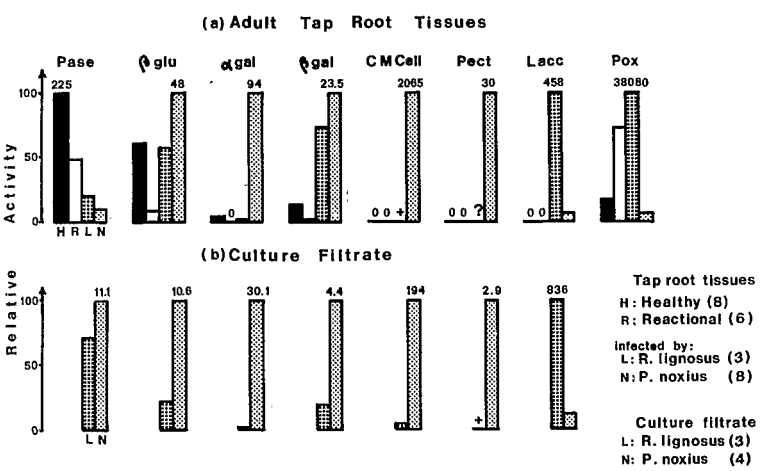

Fig. 2. Comparative enzymatic activities of acid phosphatase (Pase), β-glucosidase (β-glu), α- and β-galactosidase (α-gal, β-gal), carboxymethyl-cellulase (CM-Cell), pectinase (Pect), laccase (Lacc), and peroxidase (Pox), a. from the different tissue type extracts, b. from the *R. lignosus* and *P. noxius* culture filtrates

enzymatic activity in infested tissues my have two different origins with opposite implications in the pathogenic process.

- Host reaction: the increased enzymatic activities or "appearance" of new enzymes would result respectively from the stimulation of the biosynthesis of enzymes normally present in healthy tissues, or from the induced biosynthesis of enzymes normally repressed. The enzymes involved would participate in host defense reactions against the parasite by degrading structural polymers in the mycelial wall (WARGO 1975; PEGG 1977).
- Excretion of parasite-produced enzymes into the host tissues: these enzymes would participate in pathogenesis by degrading the polymers in the invaded tissues. This is the most frequently proposed and verified hypothesis (ALBERSHEIM et al. 1969).

The case of some β- $(1 \rightarrow 3)$ glucanases is a perfect illustration of this dichotomy. When they are synthesized by tomato plantlets, they degrade the β-$(1 \rightarrow 3)$ glucans of the invading parasite *Verticillium alboatrum* (PEGG and YOUNG 1981; YOUNG and PEGG 1981). If they are synthesized by *Colletotrichum lagenarium* they degrade glucans in melons tissues (RABENANTOANDRO et al. 1976), attacked by this parasite.

For these reasons, it was necessary to verify the origin of enzymes in parasitized tissues by direct or indirect means.

3.2 Origin of enzymes in parasitized tissues

This was conducted in several steps. The first two involved the comparison of the activities of enzymes in parasitized tissues and those in culture filtrates and particularly the spatial distribution of the enzymes in taproots. This enables a partial response to be given to the question. The third step, reserved for ambiguous cases, involved a direct electrophoretic comparison of enzymes extracted from parasitized tissues and those contained in fungal culture filtrates.

3.2.1 Comparison of enzyme activities in vivo (rubber tree taproot) and in vitro (culture filtrate)

As shown, the most significant findings coming from the analysis of root tissues is the fact that some enzymes (CM-cellulase, pectinase, laccase) are present only in parasitized tissues. The most probable hypothesis to explain this is that these enzymes are synthesized by the parasite and not by the host. It remains to be shown that the fungi are indeed capable of carrying out the biosyntheses of those enzymes. Figure 2 b shows that these enzymes are excreted *in vitro* by the parasites. In addition, the excretion capacities manifested by *R. lignosus* and *P. noxius*, respectively, reflect the activities of the three enzymes recorded in parasitized tissues. Thus, laccase activity is much higher in cases (culture filtrate and parasitized tissues) involving *R. lignosus* than in those involving *P. noxius*. The inverse situation occurs for CM-cellulase and pectinase.

These two observations, the absence of the three enzymes in healthy tissues and the homology between parasitized tissues and culture filtrates, are consistent with the increased activities of CM-cellulase, pectinase and laccase being due to enzymatic excretion by the parasites, rather than to a host reaction.

The case of phosphatase and the glycosidases (β-glucosidase, α- and β-galactosidase) is less obvious. Theses enzymes are in fact produced both by the host (in healthy tissues, Fig. 2 a) and by the parasites in pure culture (Fig. 2 b). The four enzymes of the parasitized tissues can thus originate from the host, from the parasite or both.

Nevertheless, the comparison of the relative activities of the four enzymes according to their origin (tissues parasitized by *R. lignosus* $\leftrightarrow$ culture filtrate of *R. lignosus*, or tissues parasitized by *P. noxius* $\leftrightarrow$ culture filtrate of *P. noxius*) shows a clear similarity between parasitized tissues and the homologous cultures. The histogram (Fig. 3) constructed from

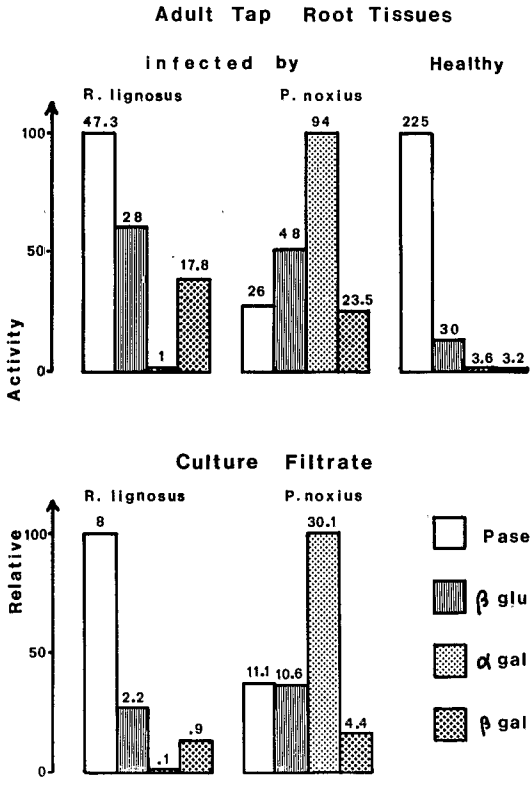

Fig. 3. Comparative enzymatic activities of phosphatase, β-glucosidase, α- and β-galactosidase in respectively: *R. lignosus* and *P. noxius* infected tissue extracts; healthy tissue extracts; *R. lignosus* and *P. noxius* culture filtrate

data gathered for the four enzymes tested with similar methods (activities expressed in the same units) clearly shows this homology. It should also be noted that as a first approximation, α-galactosidase constitutes a distinctive character of *P. noxius*, both in pure culture and in infested tissues.

More generally, we note that hydrolase activities dominate in *P. noxius* in comparison to oxidases. As already mentioned, this trend is reversed in *R. lignosus*.

The above data taken together are consistent with the enzymes of parasitized tissues having a fungal origin. In terms of the relative proportions of the different enzymatic activities, the extracts of these tissues resemble the distribution in the homologous culture filtrates.

3.2.2 Spatial distribution of enzyme activities

In order to refine this "image" and possibly support the hypothesis, we investigated the spatial distribution of the different enzyme activities in the taproots partially invaded by *R. lignosus* and *P. noxius*. This was done by analyzing different types of tissues sampled from characteristic zones of the taproots (Fig. 1 a).

3.2.2.1 Roots partially colonized by P. noxius

The histogram (Fig. 4 a) shows at least three types of situations depending on the enzyme in question.

Most often, there is a progressive increase of activities according to the sequence: H → HF → I → IA type tissues, i. e. healthy tissues → tissues invaded for the longest time. The enzymes falling into this scheme are β-galactosidase, CM-cellulase, pectinase and, to a lesser degree, α-galactosidase, β-glucosidase and laccase. Healthy tissues contain no CM-cellulase, pectinase or laccase, in agreement with results discussed above.

This type of distribution of enzyme activities favours a "relief" in parasitized tissues of enzymes originally synthesized by the host in healthy tissues. This relief is assured by the enzymes excreted by the parasite. This hypothesis becomes most probable when we note that the alveolar tissues, containing the highest activities, are brittle, dry and obviously dead. They are thus incapable of any type of reaction requiring the integrity of the protein synthesis system.

A second type of situation involves phosphatase. The activity gradient in this case is

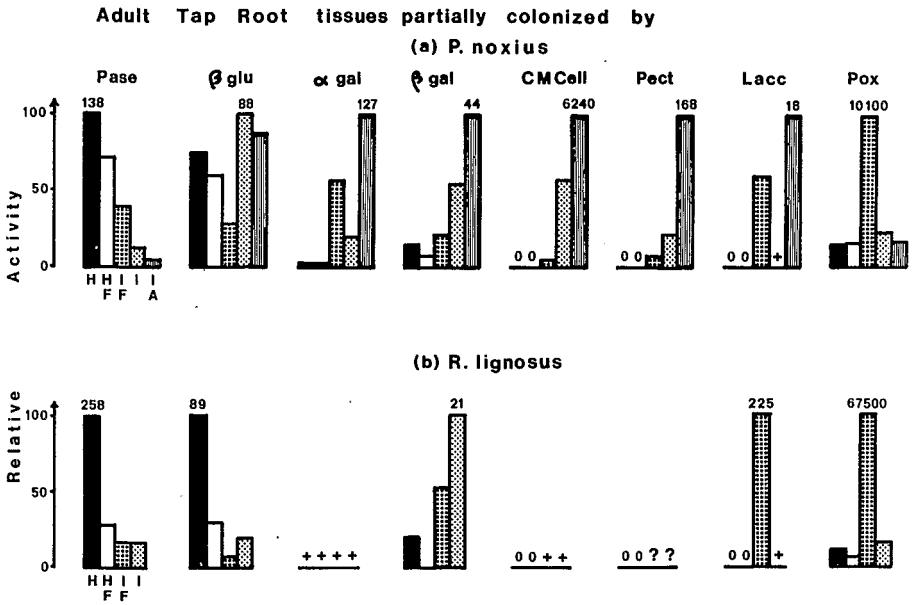

Fig. 4. Spatial distribution of the different enzymatic activities in tap roots partially infected, a. by *P. noxius*, b. by *R. lignosus*

reversed, i. e. activity decreases with the sequence H tissues → IA tissues. It is thus consistent to believe that phosphatase activity in parasitized tissues is simply a residual activity of the enzyme pool originally synthesized by the host and not renewed after parasite attack.

Finally, the case of peroxidase is particular, since in the H tissues → IA tissues sequence there is an abrupt increase of activity at the IF level, followed by a decrease just as rapid as we approach the IA zone. Two hypotheses may be invoked, each *a priori* as valid as the other, but in total opposition in terms of the biological consequences:

- Host reaction: in the initial moments of aggression, peroxidase biosynthesis is actively stimulated. At later stages, the peroxidase pool is no longer renewed as a result of tissue necrosis in zones of fungal colonization and so the activity decrease is a function of enzyme stability.
- Excretion of a peroxidase by the parasite: in this case, the secretion is merely transitory, in contrast to the situation for enzymes such as cellulase or pectinase.

In addition, it remains possible that both phenomena may coexist, since *P. noxius* (in contrast to *P. lignosus*) excretes a peroxidase *in vitro*.

3.2.2.2 Taproots partially colonized by R. lignosus (Fig. 4 b)

The three situations described above for roots attacked by *P. noxius* are also encountered here. The observed differences are quantitative, which does not change the general scheme. The different hydrolases (except for phosphatase) in the parasitized tissues are of fungal origin.

Increased peroxidase activity probably results from a host reaction, since *R. lignosus* does not excrete this enzyme *in vitro* (GEIGER 1975).

Laccase is probably of fungal origin. The abrupt decrease of its activity in type I tissues could be attributed to inhibition of the enzyme or to repression of its synthesis by degradation products of the tissues. This hypothesis remains to be verified.

3.2.3 *Electrophoretic verification of the origin of some enzymes in parasitized tissues*

This ultimate verification was carried out in the case of three enzymes [phosphatase, laccase (*R. lignosus*) and peroxidase] because of ambiguities concerning their origins.

3.2.3.1 *Phosphatase*

Results of electrophoresis (Fig. 5 a) show that the hypothesis we proposed is not tenable. Phosphatase activity in tissues parasitized (by *R. lignosus* or *P. noxius*) is not a residual activity of a preexisting enzyme pool in these tissues while they were in stage H or HF. In

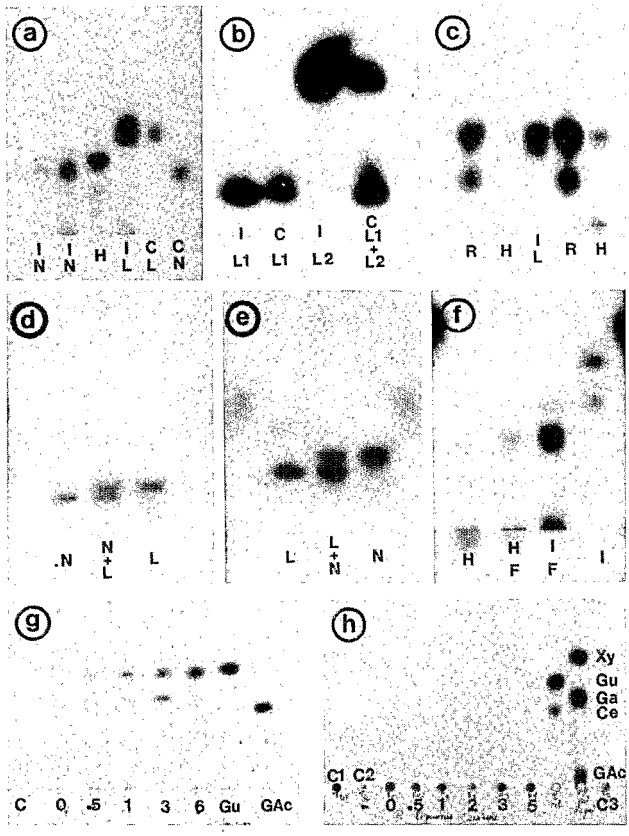

Fig. 5. a to f. Electrophoretical discrimination between enzymes from infected tissue extracts and culture filtrates. a. Phosphatases, H: healthy tissue; IN and IL: *P. noxius* and *R. lignosus* infected tissue; CL and CN: *R. lignosus* and *P. noxius* culture filtrates. b. Comparison of *R. lignosus* laccase isoenzymes L1 and L2 previously partially purified from infected tissue extracts (I) or culture filtrate (C). c. Peroxidase isoenzymes in different tissue type extracts (IL = *R. lignosus* infected; R = reactional tissue). d. Phosphatase isoenzymes from *R. lignosus* (L) and *P. noxius* (N) culture filtrate (L + N = mixture of both culture filtrates). e. as d but for laccase isoenzymes. f. peroxidase isoenzymes in different tissue types of a partially *P. noxius* infected tap root. g and h. silica gel TLC of sugars released from Hevea lignocellulose material during incubation with *P. noxius* (g) and *R. lignosus* (h) culture filtrate; c and c1 = control (culture filtrate); c2 and c3 control (lignocellulose after 0 and 4 hours of suspension in the buffer; 0 to 6: assay after 0 to 6 hours incubation in the presence of corresponding culture filtrate; GU = glucose, GAc = galacturonic acid, GA = galactose, Ce = cellobiose, Xy = xylose

fact the isoenzymes from infected tissues differ from those of healthy tissues. They respectively can be identified to the homologous isoenzymes of *R. lignosus* and *P. noxius* culture filtrates. In addition, they differ from each other as confirmed by electrophoretic profile (Fig. 5 d).

Thus, as is the case for all the other hydrolases, the phosphatase in parasitized tissues is of fungal origin.

3.2.3.2 Laccase

The results of the electrophoretic analysis (Fig. 5 b) are again formal. Tissues parasitized by *R. lignosus* contain two isoenzymes identical to those excreted by the fungus *in vitro*.

The hypothesis of a fungal origin of laccases in parasitized tissues is thus confirmed. We may note that in case of *Picea* attacked by *Fomes annosus* SHAIN (1971) described two kinds of laccase which differ in their origin; one of them, present in reaction tissues is synthetized by the tree, whereas the second, present in invaded tissues, is of fungal origin.

Finally, we may note that the two laccases excreted by *R. lignosus* are different from those produced by *P. noxius* (Fig. 5 e).

3.2.3.3 Peroxidase

The electrophoretic profile (Fig. 5 c) shows that there is a complex disturbance of peroxidase biosynthesis in tissues parasitized by *R. lignosus*, where apparently only one of the isoenzymes originally present in healthy tissues persists. There is no new isoenzyme, whose synthesis could possibly be attributed to the parasite. Thus, the considerable increase of peroxidase activity in type IF tissues results from the stimulation (biosynthesis or activity) of a single isoenzyme belonging to the host.

In light of the potential importance of this enzyme in defense phenomena, its origin was confirmed by other methods after exhaustive purification (GEIGER and HUGUENIN 1981 b). Here again, the hypothesis we formulated is verified. We may note that the activity increase observed in reaction tissues (Fig. 5 c) results from a more general induction of the isoenzymatic pool.

Infection by *P. noxius* presents an additional difficulty, since the parasite excretes a peroxidase *in vitro*. This situation is similar to that encountered with other enzymes, e. g. phosphatase and β-glucosidase.

Electrophoresis results (Fig. 5 f) furnish a clear picture of the events marking the crucial stage IF. Apparently, only one of the host isoenzymes is stimulated (this is the same isoenzyme whose activity increases after attack by *R. lignosus*). In addition, we note the presence of two additional isoperoxidases of fungal origin. In type I tissues, only the latter two persist.

In addition to identifying the peroxidases, this experiment shows that enzymes with a different origin and significance can coexist in the same tissue, at least temporarily.

In summary, electrophoretic analysis shows that phosphatase and laccase in parasitized tissues are indeed enzymes of fungal origin. The peroxidase activity in these same tissues, on the other hand, results exclusively from a host reaction in the case of attack by *R. lignosus* and from a mixture of host and parasite enzymes (at least during the initial stages of attack) when infestation is by *P. noxius*.

3.3 Role of enzymes excreted by the fungi in the degradation of lignified tissues

The effective participation of enzymes excreted by the parasites in the pathogenic process they cause *in vivo* has been demonstrated only on rare occasions. Thus, ENGLISH and ALBERSHEIM (1969) showed that the α-galactosidase produced *in vivo* and *in vitro* by *Colletotrichum lindemuthianum* governs the successful parasitic infestation of certain bean varieties.

Our purpose is not to demonstrate this same phenomenon, which presupposes extensive knowledge of host-parasite relations on the genetic level and the use of monogenic mutants of the fungi (cellulase-less ...). In the context of the present report, we limited ourselves to establishing the effective action of parasite secreted enzymes on lignocellulose prepared from adult taproot tissues. We will also discuss results previously obtained concerning the degradation of thioglycolic lignin.

3.3.1 Effect of crude culture filtrates on Hevea lignocellulose

The results we obtained (Fig. 6) clearly show that the enzymes contained in *R. lignosus* and *P. noxius* culture filtrates can degrade the lignocellulose complex as shown by the progressive release of sugars. Both kinetics are qualitatively comparable, but the quantitative rate of attack is much slower in the case of the *R. lignosus* filtrate than in that of the *P. noxius* filtrate. This difference is to be attributed to the differences in hydrolase activities, previously mentioned.

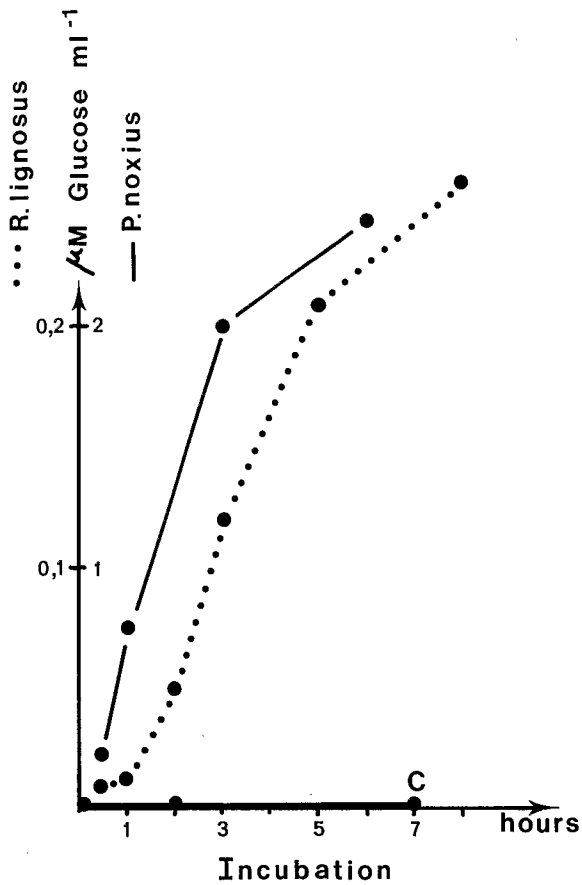

Fig. 6. Quantitative sugar release from lignocellulose during incubation with *R. lignosus* or *P. noxius* culture filtrate. Results of the dinitrosalicylic acid method are expressed as glucose

In parallel, we have used silica gel thin layer chromatography in an attempt to identify the sugars released into the reaction media (Fig. 5 g, h). Only glucose and cellobiose could be unequivocally identified. The other spots are either monomers or oligomers.

In the course of experiments using partially purified enzymes, we also have been able to characterize glacturonic acid but never xylose, in spite of the presence of a relatively active xylanase in *P. noxius* culture filtrates (GEIGER et al. unpublished observations). This is probably an endohydrolase type enzyme.

3.2.2 Effect on thioglycolic lignin

These experiments were carried out with laccase L1, purified from *R. lignosus* culture filtrates. This is the most actively excreted isoenzyme by the fungus.

The results obtained (GEIGER and HUGUENIN 1981 a; GEIGER et al. 1983) show that this enzyme can – at least partially – depolymerize lignin. This enzyme also polymerizes

low molecular weight oligomers, as well as coniferyl alcohol, one of the three monomers composing lignin. There is probably an equilibrium between these two reactions.

These results agree with the findings of ISHIHARA (1980) and support the data showing the basic importance of this enzyme in the biodegradation of lignin (KIRK 1971; ANDER and ERIKSON 1976; KERN 1983).

The laccase of *P. noxius* was not investigated in this context. Nevertheless, the fact that this enzyme is relatively similar to that of laccase L1 of *R. lignosus* (substrate specificity, spectral changes caused in the course of enzymatic oxidation of thioglycolic lignin) is consistent with it also acting on the degree of lignin polymerization.

These data show that the enzymes excreted by *R. lignosus* and *P. noxius* are active on natural substrates such as the lignocellulose of wood or substrates whose structure is similar to that of the natural polymer.

4 Conclusions

The purpose of the research we have undertaken was to define the biochemical events characterizing the installation of two parasites, *R. lignosus* and *P. noxius*, in the root tissues of rubber trees and their progression therein. Only the colonization of lignified tissues was investigated. The main results and conclusions are as follows.

- Infestation by either parasite causes considerable disturbances of some enzyme systems in host tissues.
- With the exception of peroxidase, the enzymes detected in the parasitized tissues are homologous with those excreted by the fungi *in vitro*.
- In addition, the study of the spatial distribution of these enzymes in the taproots and the electrophoretic characterization of phosphatase, laccase and peroxidase show that the hydrolases and laccase of parasitized tissues are of fungal origin. The increase in peroxidase activity in IF tissues, on the other hand, results from a host reaction.
- Finally, the enzymes excreted by the fungi are effectively capable of degrading polymers of lignified tissues *in vitro*.

These data, especially those obtained in the study of the spatial distribution of enzymes in taproots, enable us to propose a general scheme for the aggression process in lignified tissues. As the parasite progresses in the root, a given tissue passes through the following "states": healthy tissue (far from the parasite progression front) (H) → healthy tissue near the front (HF) → parasitized tissue at the front (IF) → parasitized tissue far from the front (I) → (possibly) alveolar tissue (IA). In the case of a healthy tissue in a given zone of the taproot, the time interval separating it from the IF stage depends on its distance from the front and the rate of intratissue parasite advance.

Thus, considering the histogram profiles and taking the origins of the enzymes into account, the different situations can be schematically represented as four models.

The first (Fig. 7a) is the "mean" course of phosphatase and glycosidase activities as a function of time (pre- and postinfection). Model 2 (Fig. 7 b) concerns CM-cellulase, pectinase and laccase. It differs from the preceding by the fact that the activity of these enzymes is null in healthy tissues. Model 3 (Fig. 7 c) involves the changes of peroxidase activity and shows that this enzyme is synthesized uniquely by the host. Model 4 (Fig. 7 d) is the particular situation in the case of infection by *P. noxius*.

In models 1 and 4 (Fig. 7 a, d), we have admitted the simultaneous presence of isoenzymes synthesized by the host and others by the parasite in IF tissues. This type of situation was in fact noted for peroxidase in tissues attacked by *P. noxius*. Nevertheless we cannot exclude that it is an artifact since the means used for sampling (with a chisel) is in reality quite crude. Only an immunocytochemical study could supply valuable information on this subject.

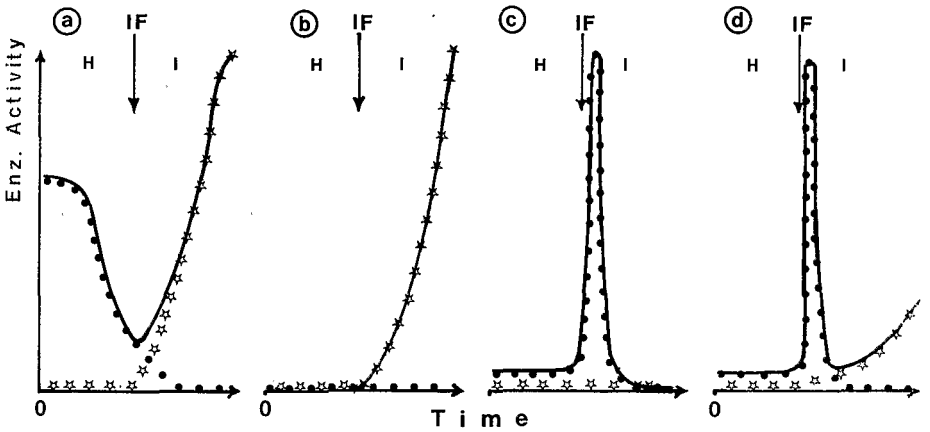

Fig. 7. Modelization of the enzymatic activity variation during the evolution of the tissue: from healthy to infected state. a. Pase, β-glu, α- and β-gal; b. CM-cell, Pect and Lacc; c. and d. Peroxidase, respectively in the case of *R. lignosus* and *P. noxius* infected root

At a more general level, it is worthwhile to remember that the curves presented are only models. The activity levels of the enzymes and the real values of "t" time, i. e. the intervals separating stages (H) from (I) or (IA) depend, on an individual enzyme basis, primarily on the synthetic capacities of healthy tissues, the excretion capacities of the parasite and, finally, on the reaction capacities of the host. It is clear that the phase HF → IF is crucial, since the fate of the tree – survival or death within a variable period – will depend on the equilibrium between the aggression capacity of the parasite and the host capacity to install an effective system of defense.

Recent work (NICOLE et al. 1983) has shown that some individuals in a population of artificially inoculated *Hevea* plantlets are capable of arresting the progression of *R. lignosus*. These same plantlets can be identified by the installation of a secondary reaction rhizogenesis. Work is in progress to verify if these individuals also present particular enzymatic characters in HF and IF tissues.

The last comment bears on the balance between hydrolases and laccase as a function of the fungus. We may draw two conclusions:

- *P. noxius* excretes a laccase and so is classified among the white rot agents, in contrast to opinions formulated earlier (PICHEL 1956; ROGER 1954).
- In light of their respective enzymatic equipment, *P. noxius* should preferentially degrade the polysaccharide fraction of wood, while *R. lignosus* would preferentially attack the more lignified tissues.

Lignin analysis (GEIGER et al. 1984) and light and electron microscopic observation (NICOLE et al. 1982 a, b) have supported these hypothesis. Finally, these results mean that the studied enzymes – or at least some of them – participate in the pathogenic process developed by *R. lignosus* and *P. noxius*.

Summary

R. lignosus and *P. noxius* are two Polyporaceae which attack the roots of *Hevea brasiliensis*, delimiting a zone of healthy tissues and one of parasitized tissues in partially invaded taproots.

Parasite aggression causes a considerable disturbance in the activities of certain enzymes. The host synthesizes phosphatase, and glycosidases in healthy tissues, but the homologous enzymes of parasitized tissues are of fungal origin. The CM-cellulases, pectinases and laccases are also of fungal origin, and their spatial distribution in the roots is strictly limited to the infested zone. Peroxidase activity increases considerably in infested tissues located at the front of parasite progression and is

a host reaction. A scheme to explain these various findings is presented. The fungal enzymes can degrade *Hevea* lignocellulose and so they are both white rot agents. Nevertheless, the characteristics of their enzyme equipments are such that *R. lignosus* preferentially degrades lignin while *P. noxius* would more easily attack polysaccharide structures.

Résumé

Les pourridies d'Hevea brasiliensis
I. Aspects physiologiques et biochimiques de l'agression parasitaire

R. lignosus et *P. noxius* sont deux Polyporacées qui attaquent les racines d'*Hevea brasiliensis* délimitant dans des pivots partiellement envahis une zone de tissus sains et une zone de tissus parasités.

L'agression parasitaire provoque une perturbation considérable au niveau de l'activité de certaines enzymes. Bien que l'hôte synthétise, dans les tissus sains, la phosphatase, la β-glucosidase et les α- et β-galactosidases, les enzymes homologues des tissus parasités sont d'origine fongique. CM-cellulase, pectinase et laccase sont, elles aussi, d'origine fongique et leur réparatition spatiale au sein des pivots est strictement limitée à la zone infestée. L'activité peroxydasique augmente dans des proportions considérables dans les tissus infestés situés au niveau front de progression des parasites; elle correspond à une réaction de l'hôte. Un schéma d'ensemble de ces évènements est proposé. Les enzymes des champignons sont capables de dégrader la lignocellulose d'Hévèa et sont donc tous deux des agents de pourriture blanche. Cependant, les caractéristiques de leur équipement enzymatique font de *R. lignosus* un champignon qui dégraderait préférentiellement la lignine, tandis que *P. noxius* s'attaquerait plus facilement aux structures polysaccharidiques.

Zusammenfassung

Wurzelfäule bei Hevea brasiliensis. I. Physiologische und biochemische Aspekte des Wirtsbefalls

Rigidoporus lignosus und *Phellinus noxius* sind Polyporaceen, die die Wurzeln von *Hevea brasiliensis* befallen, wodurch es zu einer Abgrenzung zwischen gesunden und befallenen Geweben in den Wurzeln kommt. Die Parasiten rufen eine deutliche Störung der Aktivitäten einiger Enzyme hervor. Der Wirt bildet Phosphatase und Glycosidase in gesunden Geweben, die homologen Enzyme in parasitierten Geweben stammen aber von den Pilzen. Die CM-Cellulase, Pektinase und Laccase sind von den Pilzen gebildet und ihr Vorkommen in den Wurzeln ist streng auf die besiedelten Bereiche begrenzt. Die Peroxidase-Aktivität steigt in infizierten Geweben deutlich an, und zwar im Frontbereich des vordringenden Parasiten, und ist eine Reaktion des Wirtes. Es wird ein Schema vorgestellt, das die verschiedenen Beobachtungen erklären soll. Die Pilzenzyme können die Lignozellulose von *Hevea* abbauen, d. h. beide Pilze sind Weißfäuleerreger. Trotzdem ist die Enzymausstattung der beiden Pilze dergestalt, daß *R. lignosus* bevorzugt Lignin abbaut, während *P. noxius* leichter Polysaccharide angreifen kann.

References

ALBERSHEIM, P.; JONES, T. M.; ENGLISH, P. D., 1969: Biochemistry of the cell wall in relation to infective process. Ann. Rev. Phytopathology 7, 171–194.

ANDER, P.; ERIKSSON, K. E., 1976: The importance of Phenol oxidase activity in lignin degradation. Arch. Microbiol. 109, 1–8.

CERNY, G., 1973: Hemmung einiger Ektoenzyme von *Fomes annosus* (Fr.) Cke. durch Holz- und Bastextrakte aus *Picea abies* (L.) Karst. Eur. J. For. Path. 3, 214–220.

CHEVEAUGEON, J., 1950: Le problème des pourridiés en Côte d'Ivoire. Rev. Mycologie. T. XXIV, 39–58.

EKMAN, R.; WEISSEMBERG, K., 1979: Sapwod extractives in Norway spruce inoculated with *Fomes annosus*. Acta Acad. Turku, Ser. B, 39, 8 pp.

ENGLISH, P. D.; ALBERSHEIM, P., 1969: Host-pathogen interactions. I. A correlation between α-galactosidase production and virulence. Plant Physiol. 44, 217–224.

GEIGER, J. P., 1975: Aspects physiologiques et biochimiques de la spécialisation parasitaire. Cas des *Corticium rolfsii* (Sacc.) Curzi et *Leptoporus lignosus* (Kl.) Heim ex Pat. Etude *in vitro*. Physiol. Vég. 13, 307–330.

GEIGER, J. P.; HUGUENIN, B., 1981 a: Action *in vitro*, d'une laccase purifiée de *Rigidopous lignosus* sur la lignine d'*Hevea brasiliensis*. Proceed. Colloque International sur la Protection des Cultures Tropicales, Lyon.

——1981b: La peroxydase des tissus racinaires d'hévéa parasités par *R. lignosus*; origine et rôle physiologique potentiel. Proceed. Colloque International sur la Protection des Cultures Tropicales, Lyon.
Geiger, J. P.; Huguenin, B.; Nandris, D.; Nicole, M., 1983: Effect of an extracellular laccase of *Rigidoporus lignosus* on *Hevea brasiliensis* lignin. Proceed. Lignocellulose biodegradation Conference, Littlehampton. Appl. Biochem. Biotechnol. 1984, 9, 359–360.
Geiger, J. P.; Nandris, D.; Goujon, M., 1976: Activité des laccases et des peroxydases au sein des racines d'hévéa attaquées par le pourridié blanc [*Leptoporus lignosus* (Kl.) Heim]. Physiol. Vég. 14, 271–282.
Geiger, J. P.; Rio, B.; Nicole, M.; Nandris, D., 1984: Dégradation de la lignine d'*Hevea brasiliensis* par *Rigidoporus lignosus* et *Phellinus noxius*. Proceed. XXVII Colloque Société Française de Phytopathologie: Les pourridiés et autres maladies racinaires des espèces ligneuses.
Ishihara, T., 1980: The role of laccase in lignin biodegradation. In lignin Biodegradation: microbiology, chemistry and potential applications. Vol. II. Kirk, T. K.; Higuchi, T.; Chang, H. M. editors, CRC Press.
Jeng, R. S.; Alfenas, A. C.; Hubbes, M.; Dumas, M., 1983: Presence and accumulation of fungitoxic substances against *Ceratocystis ulmi* in *Ulmus americana*: possible relation to induced resistance. Eur. J. For. Path. 13, 239–244.
Johansson, M.; Theander, T., 1974: Changes in sapwood of roots of Norway spruce, attacked by *Fomes annosus*. Part. I. Physiol. Plant. 30, 218–225.
Johansson, M.; Unestam, T., 1982: The search for resistance to *Heterobasidion* root rot in Norway spruce – old and new approaches in studies of infection biology. Eur. J. For. Path. 12, 346–357.
Johansson, M.; Popoff, T.; Theander, O., 1976: Effects of spruce root constituents on extracellular enzymes of *Fomes annosus*. Physiol. Plant. 37, 275–282.
Kern, H., 1983: Action of the white-rot fungus *Sporotrichum pulverulentum* on lignosulfonates. Holzforschung 37, 287–292.
Kirk, T. K., 1971: Effects of microorganisms on lignin. Ann. Rev. Phytopathology 9, 185–210.
Nandris, D.; Nicole, M.; Geiger, J. P., 1983: Infections artificielles de jeunes plants d'*Hevea brasiliensis* par *Rigidoporus lignosus* (Kl.) Imaz. et *Phellinus noxius* (Corner) G. H. Cunn. Eur. J. For. Path. 13, 65–76.
Nicole, M.; Geiger, J. P.; Nandris, D., 1982 a: Interactions hôte-parasite entre *Hevea brasiliensis* et les agents de pourriture racinaire *Phellinus noxius* et *Rigidoporus lignosus*: Etude physio-pathologique comparée. Phytopath. Z. 105, 311–326.
Nicole, M.; Geiger, J. P.; Nandris, D., 1982 b: Aspects ultrastructuraux de la dégradation du phloème des racines d'*Hevea brasiliensis* parasité par *Rigidoporus lignosus*. C. R. Acad. Sci. Paris, série III, t. 295, 471–476.
Nicole, M.; Nandris, D. Geiger, J. P., 1983: Cinétique de l'infection de plants d'*Hevea brasiliensis* (Willd. ex. Adr. de Juss) Mull. Arg. par *Rigidoporus lignosus* (Kl.) Imazeki. Can. J. For. Res. 13, 359–364.
Peek, R. D.; Liese, W., Parameswaran, N., 1972 a: Infektion und Abbau der Wurzelrinde von Fichte durch *Fomes annosus*. Eur. J. For. Path. 2, 104–115.
——— 1972 b: Infektion und Abbau der Wurzelrinde von Fichte durch *Fomes annosus*. Eur. J. For. Path. 2, 237–248.
Pegg, G. F., 1977: Glucanolydrolases of higher plants: a possible defense mechanism against parasitic fungi. In: Cell Wall Biochemistry Related to Specificity in Host plant pathogen Interactions, ed. by B. Solheim and J. Rao. Oslo: Universitetsforlaget. 304–345.
Pegg, G. F.; Young, D. H., 1981: Changes in glycosidase activity and their relationship to fungal colonization during infection of tomato by *Verticillium alboatrum*. Physiological Plant Pathology 19, 351–362.
Pichel, R. J., 1956: Les pourridiés de l'Hévéa dans la cuvette congolaise. INEAC, Sér. Techn. 49, 480 pp.
Popoff, T.; Theander, O.; Johansson, M., 1975: Changes in sapwod of roots of Norway spruce, attacked by *Fomes annosus*. Part. II. Organic chemical constituents and their Biological effects. Physiol. Plant. 34, 347–356.
Rabenantoandro, Y.; Auriol, P.; Touze, A., 1976: Implication of β-(1$\rightarrow$3) glucanase in melon anthracnose. Physiological Plant Pathology 8, 313–324.
Roger, L., 1954: Phytopathologie des pays chauds. Tome III. P. Lechevalier, Ed. Paris.
Shain, L., 1971: The response of sapwood of Norway spruce to infection by *Fomes annosus*. Phytopathology 61, 301–307.
Shain, L.; Hillis, W. E., 1971: Phenolic extractives in Norway spruce and their effects on *Fomes annosus*. Phytopathology 61 841–845.
Smithies, O., 1955: Zone electrophoresis in starch gels. Group variations in the serum proteins of normal human adults. Biochem. J. 61, 629–641.

WARGO, P. M., 1975: Lysis of the cell wall of *Armillaria mellea* by enzymes from forest trees. Physiological Plant Pathology 5, 99–105.
WONG, W. C.; PREECE, T. F., 1978: *Erwinia salicis* in cricket batwillows: histology and histochemistry of infected wood. Physiological Plant Pathology 12, 321–332.
WORRALL, J. J.; PARMETER, J. R., 1982: Wetwood formation as a host response in white fir. Eur. J. For. Path. 12, 432–441.
YOUNG, D. H.; PEGG, G. F., 1981: Purification and characterization of 1,3-β-glucan hydrolases from healthy and *Verticillium alboatrum* infected tomato plants. Physiological Plant Pathology 19, 391–417.

Authors' address: ORSTOM – Laboratoire de Phytopathologie, B. P. V51, Abidjan (Côte d'Ivoire)

Receipt of Ms.: 12. 1. 1985.