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Peroxidase production in tissues of the rubber tree following infection by root rot fungi

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On estates, the rubber-tree, *Hevea brasiliensis*, is subject to a variety of diseases. Among them, root rots caused by *Rigidoporus lignosus* and *Phellinus noxius* are responsible for severe losses. When these parasites infect the roots they induce an increase in the peroxidase activity in the host tissues. Similar increases are shown in reaction zone tissues developed in response to *Sphaerostilbe repens* injury and in similar zones sometimes developed in *R. lignosus* infection, but then always devoid of any parasite. Comparative biochemical studies of the peroxidases from different types of tissue, including chromatographic separation into different peroxidase fractions, purification of one of the isoperoxidases and determination of some of its physico-chemical characteristics, lead to the following conclusions: (a) one isoperoxidase is mainly responsible for the increase in enzyme activity; (b) this isoenzyme is synthesized, probably *de novo*, by the host, not by the parasites, and thus represents a host reaction to parasite infection; (c) the presence of the parasite causes the tissue to produce this particular isoenzyme in excess of the amount normally found in healthy tissue; (d) this isoperoxidase polymerizes coniferyl alcohol, therefore it may contribute to the lignification reaction which was previously shown to occur. Thus, the peroxidase reaction may be involved, among others, in the host defence mechanisms.

INTRODUCTION

Infection of the roots of the rubber tree, *Hevea brasiliensis* Kunth. Müll. Arg., by *Rigidoporus lignosus* (Kl.) Imazeki or by *Phellinus noxius* (Corner) G. H. Cunn., two lignin-degrading basidiomycetes causing a white rot of wood [*16*, *17*], induces in the host tissues considerable modification of several enzyme activities [*14*, *15*]; among them, a marked increase in peroxidase activity was observed in the tissues located at the progression line of the parasites as well as in the reaction zone tissues occasionally formed after *R. lignosus* infection. Furthermore, the rubber tree usually reacts to infection by *Sphaerostilbe repens* Berk & Br. (another root parasite decaying only the cortical tissues and causing *Hevea* root canker) by forming reaction zone tissues surrounding and overlapping the decayed tissue. These reaction zone tissues, which are not invaded by the parasite, also exhibit much higher peroxidase activity than do healthy tissues.

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Abbreviations used in text: EF, total effluent; EL, eluate; H, healthy tissue; HF, healthy tissue in advance of infection; IF, infected tissue from an advanced edge of lesion; I, infected tissue behind lesion edge; R, reaction tissue.

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Preliminary investigations [*15*] indicated that one type of isoperoxidase might be enhanced particularly.

Peroxidase activity enhancement is a well-known reaction of plants undergoing different kinds of stress such as wounding, heat or osmotic shocks and air pollution [*33*, *44*, *52*, *53*]. This response, however, has been most extensively studied in the case of pathogenic infection where reports frequently associate stimulation of peroxidase activity with resistance to infection [*8*, *24*, *35*, *37*, *38*, *39*, *48*, *51*]. In this context, the peroxidases are assumed to operate the final step of lignin biosynthesis by co-polymerizing the cinnamyl alcohols [*20*, *21*] leading to an abnormally high tissue lignification [*1*, *2*, *34*, *35*]. This lignification reaction may constitute an active defence mechanism against pathogenic infection and intra-tissue progression of the pathogens [for review see *45*, *50*]. A number of reports have proposed or demonstrated the efficiency of such a mechanism [*3*, *6*, *12*, *19*, *27*, *40*, *41*, *47*, *49*] even in the case of premunition [*23*, *46*].

Most of the research reported on the effect of infection on the quantitative and/or the qualitative changes in peroxidase, correlated or not with resistance, has been performed on leaves or on herbaceous plants grown under controlled conditions and artificially inoculated. In contrast, the work reported here has investigated the effects on the highly lignified tissues of the tap-roots of adult rubber trees growing on estates and which are subject to natural root rot disease. The aims were (a) to show the quantitative and qualitative effect of root infection on the peroxidase activity of the host tissues, (b) to determine whether any implicated enzymes originated in the host or the parasite, and (c) to outline some aspects of the regulation of the synthesis and its potential significance in resistance.

MATERIALS AND METHODS

Plant material and sampling

On modern estates rubber trees are grafted. Since rootstocks result from non-controlled cross pollination there exists considerable genetic variability within a population of tap roots. Therefore, we have analysed mainly tap roots which were only partly colonized by fungi, and thus showed both a zone of healthy tissues and a zone of infected tissues, located respectively ahead and behind the front of parasite progression. This allowed the peroxidase activities (and isoenzymes) from infected tissues or reaction zone tissues, which are sometimes developed by the roots, to be compared with those which were extracted from healthy controls sampled from the same tap root. Therefore, each tap root was individually identified throughout this work. In the case of trees infected by *S. repens*, however, only reaction zone tissues were collected. In practice, five kinds of tissues were sampled as previously described [*15*]:

healthy tissues (H) taken some distance ahead of the parasite progression line
healthy tissues sampled in advance of fungal progression (HF)
infected tissues gathered from the front of fungal progression (IF)
infected tissues sampled behind the parasite progression line (I)
reaction zone tissues (R) harvested either in *S. repens*- or *R. lignosus*-infected tap roots

The (HF), (IF) and (R) zones sampled were approximately 1·5 cm broad; the (H) and (I) zones were much broader, about 5 to 10 cm. In experiments where only two types of tissue were analysed the tissue called (I) included both (IF)- and (I)-type samples.

Enzyme extraction and assay

The studies were performed only on xylem tissues. These were removed with a wood chisel from tap roots previously sectioned longitudinally into two equal parts. The resulting wood chips were reduced to sawdust by very rapid dry grinding (Gondard knife blade grinder). The sawdust was left overnight at 4 °C in a 0·0125 M sodium phosphate buffer at pH 6 (5 ml g^{-1} fresh weight). This extraction process was shown to be the most efficient.

The peroxidase activity was assayed by measuring the variation in absorbance at 420 nm of a 5 ml (final volume) reaction medium containing 0·05 M sodium phosphate buffer at pH 6, 0·1% H_2O_2 and 0·2% guaïacol. One enzyme unit corresponded to the amount of enzyme which caused a variation of 1 A_{420} in 1 min. When specific activities were determined, the absorbance of the reaction medium was followed by coupling the spectrophotometer to a recorder; the enzyme activity was calculated using the linear part of the curve.

The effect of calcium on enzyme activity was measured in reaction media buffered with Tris–maleate (0·05 M in Tris) at pH 6·8, with or without 0·005 M calcium chloride. The effect of temperature on enzyme activity was measured using the reaction media previously heated at the desired temperature. The thermostability of the peroxidases was tested by assaying the enzymes after previous heating (in 1 ml buffer) for 10 min at the desired temperature.

Chromatographic separation of the peroxidases into two characteristic fractions

The peroxidases from crude extracts were separated into two fractions using a column (h = 12 cm; d = 2 cm) of DEAE-cellulose equilibrated with a 0·0125 M sodium phosphate buffer at pH 6. In order to carry out this separation a known volume of extract, with a known peroxidase activity, was loaded onto the column. After adsorption was achieved, the column was washed with the same buffer until no more peroxidase activity could be detected in the effluent (EF). The total effluent was recovered; it constituted the (EF)-peroxidase fraction. The column was then eluted with the same buffer containing 0·25 M sodium chloride. The eluate (EL) was recovered until no more enzyme activity was detected; the total eluate constituted the (EL)-peroxidase fraction. This separation procedure demonstrates that the isoperoxidases of the (EF)-fraction differ from those of the (EL)-fraction. The activity of the (EF)- and (EL)-fractions were assayed both in the presence and in the absence of 0·005 M calcium chloride.

Chromatographic separation of the (EL)-peroxidases by gel permeation on Sephadex G200

The isoperoxidases of the (EL) fractions were further fractionated by gel permeation on a Sephadex G200 column (h = 100 cm; d = 1·8 cm) under the following experimental conditions: eluting buffer: 0·05 M Tris–HCl at pH 8·4, 0·1 M KCl; flow rate: 10 ml h^{-1}; fraction volume: 1·1 ml. In order to control the reproducibility of the

runs, the (EL) peroxidase samples were co-chromatographed with the following molecular weight markers: blue dextran (determination of the void volume of the column), alcohol dehydrogenase (125000 D), and cytochrome *c* (12500 D). The different peaks (P2a and P2b) showing peroxidase activity were recovered. The effect of calcium on both peroxidase fractions was measured, and the percentage of P2a and P2b content of the (EL)-fraction was calculated.

Isolation of the P2a isoperoxidases from infected and reaction zone tissues

The P2a isoperoxidases were purified from tissue extracts in a three-step process.

1. "Batch system": a small amount of DEAE-cellulose (Whatman DE-52) in a 0·0125 M sodium phosphate buffer at pH 6 was added to the crude extract. After a 30-min contact with gentle stirring, the DEAE-cellulose was removed by filtration on a sintered glass funnel. This process was repeated three times in order to adsorb most of the proteins (including the isoperoxidases corresponding to the (EL)-type; the (EF)-type enzymes are not adsorbed). The DEAE-cellulose batches were mixed, washed with the extraction buffer, and the peroxidases were eluted with the same buffer containing 0·3 M NaCl. The elution step was repeated three times. This batch system allows rapid recovery of the enzymes from the large volume of crude extract (several litres) into 200 to 400 ml of eluate; in addition, most of the brownish colour of the crude extract, probably due to oxidized phenolics, was discarded. The eluates were mixed, centrifuged, and concentrated to about 10 ml in an Amicon ultrafiltration cell using a PM10 ultrafilter membrane (normal cut-off: 10000 D). The concentrate was diluted ten-fold by the addition of 90 ml of 0·025 M Tris–HCl at pH 8·4, and concentrated again. This process was repeated three times—allowing rapid desalting and buffer change of the initial eluate without loss in enzyme activity.

2. DEAE-cellulose chromatography at pH 8·4. The proteins from the concentrated solution were separated on a DEAE-cellulose (Whatman DE 52) column (h = 15 cm; d = 2 cm) using the following eluting conditions. Linear gradient: 0·025 M Tris–HCl at pH 8·4 to 0·025 M Tris–HCl at pH 8·4, 0·35 M NaCl; total volume: 500 ml; flow rate: 50 ml h^{-1}; fraction volume: 4·5 ml. The most peroxidase-active fractions were collected and concentrated as described above, including desalting and buffer change (Tris–HCl + NaCl to 0·0125 M sodium phosphate at pH 5).

3. DEAE-cellulose chromatography at pH 5. The purification of the P2a isoperoxidase was achieved using DEAE-cellulose column chromatography with the following elution gradient: 0·0125 M sodium phosphate buffer at pH 5 to 0·0125 M sodium phosphate buffer at pH 5, 0·3 M NaCl. Only the fractions showing the superposition of the peaks of peroxidase activity, absorbance at 280 and 400 nm, and the lowest A_{280}/A_{400} ratio were recovered. These fractions were mixed, concentrated and desalted. A portion of this concentrate was used to measure specific activity, pH and temperature effect, and to determine the molecular weight of the proteins. Another portion was diluted in 0·05% acetic acid, concentrated again, and finally lyophilized; this fraction was used to determine the amino acid composition of the peroxidase protein and its finger-print after trypsin digestion as indicated below.

Molecular weight determinations

The molecular weight of the native enzymes were determined by electrophoresis on polyacrylamide slabs containing a polyacrylamide gradient from 5 to 30%. Protein

markers of different molecular weight were run on the same gel: tyroglobulin (670000 D), catalase (240000 D); ceruloplasmin (150000 D) and bovine serum albumin (68000 D). The molecular weight of the denatured peroxidase was determined according to Weber and Osborn [*54*] using the following markers: cytochrome *c* (12500 D), carbonic anhydrase (30000 D), egg ovalbumin (45000 D), and bovine serum albumin (68000 D).

Amino acid composition

The amino acid composition of the peroxidase was determined after degradation of the protein by chlorhydric acid or methanesulphonic acid hydrolysis or performic acid oxidation. The amino acids of the resulting solutions were determined using a Durrum D500 amino acid analyser.

Trypsic finger-prints

The peroxidase finger-prints were performed after a 4-h trypsic digestion of the protein [peroxidase: trypsin 20:1 (w/w)]. The resulting oligopeptides were subject to a bidimensional separation on a thin-layer cellulose plate; first dimension: electrophoresis [90 min under 400 V in a mixture of pyridine, acetic acid, acetone and water (10:20:75:395 v/v)]; second dimension: chromatographic run in a mixture of pyridine, acetic acid, *n*-butanol and water [100:30:150:120 (v/v)].

RESULTS

Effect of infection on the level of intracellular peroxidase activity

Table 1 shows the peroxidase activity of extracts from different tissue types sampled from 29 tap roots of trees grown on rubber estates. These trees were either healthy or were infected by *R. lignosus*, *P. noxius* or *S. repens*.

Considering the average values, the peroxidase activity of *R. lignosus*-infected tissues and reaction zone tissues was much higher than the activity extracted from healthy roots or healthy zone tissues of partly infected tap roots. In *P. noxius* infection these values were lower than in healthy controls. These average values were not really conclusive since they included three different situations, particularly in the case of *R. lignosus* infection: namely, either an increase, or a decrease in peroxidase activity, or no effect. Furthermore, considerable variability was found within a population of tap roots.

Qualitative aspects of the modification in peroxidase activity induced by the infection

The existence of a qualitative disturbance in peroxidase activity was shown when crude extracts from different tissue types were subject to chromatographic separation on DEAE-cellulose. Two fractions were isolated: fraction (EF) corresponding to the column effluent, and fraction (EL) containing the enzymes which were eluted with a buffer containing 0·25 M NaCl.

The results (Table 2) of experiments performed on extracts of different tissue types taken from a variety of tap roots showed that the healthy tissues contained mainly peroxidase of (EF)-type, whereas the activity of infected and reaction zone tissues was mainly of (EL)-type. Every tap root showed the same result. Similar differences occurred even in the case of "atypical" tap roots (numbers 1, 2 and 19) where the total

TABLE 1

Peroxidase activity (units g^{-1} fresh wt) of crude extracts from different types of tissue sampled on 29 tap roots

Designation of the tap roots[a]	Tissue type			
	Healthy	Infected by *R. lignosus*	Infected by *P. noxius*	Reaction zone
1	23·0	21·0	—	—
2	12·2	6·7	—	—
3	5·7	15·9	—	—
4	13·3	23·7	—	—
5	14·2	10·2	—	—
6	—	48·9	—	—
7	9·9	—	—	—
8	27·3	—	—	—
9	18·7	156·5	—	18·6
10	10·8	91·2	—	—
11	17·2	114·6	—	—
12 (H & I)	6·4	17·9	—	—
12 (HF)	10·9	—	—	—
13	7·5	11·0	—	15·4
14	15·0	32·8	—	—
15	—	61·3	—	—
16 (H & I)	1·7	—	2·3	—
16 (HF & IF)	1·4	—	10·1	—
17 (H & I)	4·8	—	+	—
17 (IF)	—	—	0·4	—
18 (IF)	—	—	0·2	—
18 (I)	—	—	0	—
19	14·3	—	2·7	—
20	6·2	—	—	—
21 (*Sr*)	—	—	—	30·8
22 (*Sr*)	—	—	—	31·2
23 (*Sr*)	—	—	—	21·1
24 (*Sr*)	—	—	—	23·8
25	7·0	—	—	—
26 (*Sr*)	—	—	—	30·1
27 (*Sr*)	—	—	—	47·0
28 (*Sr*)	—	—	—	26·9
29 (*Sr*)	—	—	—	21·0
Average	11·4	47·1	2·2	26·6

Each tap root is identified by a number (the same throughout this work). Tap roots 8, 20 and 25 were healthy. Tap roots 1 to 7 and 9 to 15 were partly invaded by *R. lignosus*. Only healthy tissues were sampled on tap root 7. Tap roots 9 and 13 showed reaction zone tissues (R). Tap roots 16 to 19 were *P. noxius*-infected. Tap roots 21 to 24 and 26 to 29 were *S. repens*-infected; on these roots only reaction zone tissues were sampled. In general, on *R. lignosus*- and *P. noxius*-infected roots both healthy (H) and infected tissues (I) were sampled. However, on tap roots 12, 16, 17 and 18 additional tissues were selected: (HF), healthy tissues taken near the front of fungal progression, and (IF), infected tissues taken only at the front of fungal progression.

TABLE 2

Peroxidase activity of two characteristic fractions (EF and EL) isolated by chromatographic separation of the peroxidases of crude extracts from different types of tissue, and the effect of calcium on the peroxidase activity of the (EL)-fraction

Designation of the tap roots[a]	Tissue type: Healthy tissue extracts EF[b] % [c]	Healthy tissue extracts EL[b] % [c]	Healthy tissue extracts EL[b] (−Ca++)[d]	Infected tissue extracts EF[b] % [c]	Infected tissue extracts EL[b] % [c]	Infected tissue extracts EL[b] (−Ca++)[d]	Reaction zone tissue extracts EF[b] % [c]	Reaction zone tissue extracts EL[b] % [c]	Reaction zone tissue extracts EL[b] (−Ca++)[d]
1 (*Rl*)	89	11	19	1	99	110	—	—	—
2 (*Rl*)	67	33	18	0	100	—	—	—	—
3 (*Rl*)	65·6	34·4	5	0·2	99·8	96	—	—	—
7 (*Rl*)	75	25	24	—	—	—	—	—	—
8 (H)	97·3	2·7	9	—	—	—	—	—	—
9 (*Rl*)	52	48	49	1·2	98·8	104	18	82	70
10 (*Rl*)	72	28	19·4	0, 1	99·9	104	—	—	—
13 (*Rl*)	78	22	39	0	100	104	8·2	91·8	93
19 (*Pn*)	70·7	29·3	—	2·3	97·7	—	—	—	—
20 (H)	65	35	35	—	—	—	—	—	—
21 (*Sr*)	—	—	—	—	—	—	5	95	111
22 (*Sr*)	—	—	—	—	—	—	11	89	68
23 (*Sr*)	—	—	—	—	—	—	10	90	94
24 (*Sr*)	—	—	—	—	—	—	21	79	90
25 (H)	88·5	11·5	—	—	—	—	—	—	—
26–29 (*Sr*)	—	—	—	—	—	—	13·7	86·3	91

[a] Tap roots 8, 20 and 25 were healthy (H). Tap roots 1, 2, 3, 7, 9, 10 and 13 were *R. lignosus* (*Rl*)-infected. Tap root 19 was *P. noxius* (*Pn*)-infected. Tap roots 21–24 and 26–29 were *S. repens* (*Sr*)-infected. Different tissue types (healthy, and/or infected, and/or reaction zone tissues) were removed from these tap roots and their peroxidases were extracted (crude extracts); the crude extracts of tissues sampled on tap roots 26–29 were mixed before chromatographic separation.

[b] The peroxidases of the crude extracts were separated by DEAE-cellulose column fractionation into two characteristic fractions: (EF)-fraction containing the isoenzymes which were not adsorbed on the DEAE-cellulose, and were thus recovered in the column effluent (EF), and (EL)-fraction containing the isoenzymes which were eluted from the DEAE-cellulose with a buffer solution containing 0·25 M NaCl.

[c] The peroxidase activity content is expressed as the percentage of the activity of the fraction with reference to the total activity of the corresponding crude extract.

[d] The effect of calcium on the peroxidase activity is expressed as the percentage of the residual activity assayed in the absence of calcium with reference to the corresponding activity assayed in the presence of 0·005 M calcium.

peroxidase activity in infected tissues was lower than that of the corresponding healthy control (Table 1). Thus, the increase in the activity of the (EL)-fraction seems to be a general effect of the parasitic invasion.

The (EL)-peroxidase activity is calcium sensitive; however, the intensity of the calcium effect is dependent upon the nature of the tissue from which the enzymes were extracted. (EL)-peroxidase activities from healthy tissues are stimulated to different degrees by calcium, whereas (EL)-activities from infected and reaction zone tissues are either not affected or are inhibited by calcium. This suggests that the (EL)-fractions contain at least two peroxidases differing in their sensitivity to calcium.

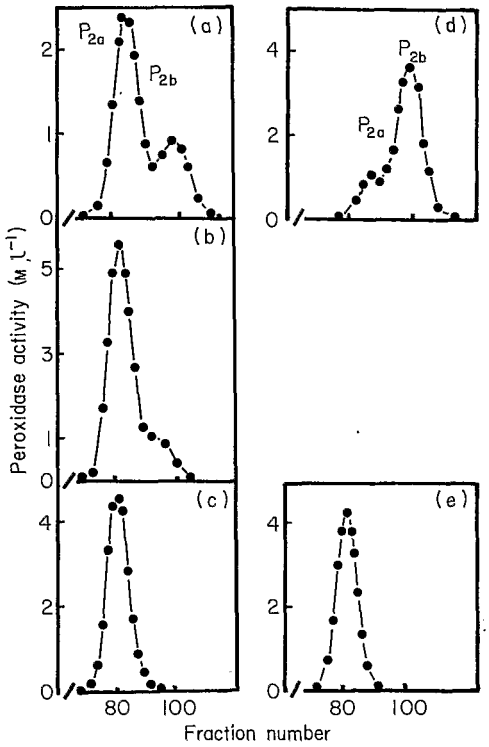

FIG. 1. Gel permeation profiles of the peroxidases from the (EL)-fractions collected after preliminary DEAE-cellulose fractionation of crude extracts from different types of tissue. In (a), (b) and (c), the (EL)-fractions of healthy (H), reaction zone (R), and infected (I) tissue extracts, respectively, were chromatographed; these tissues were sampled on tap root number 9 belonging to the TJ1 rubber tree family. The (d) and (e) profiles correspond, respectively, to (EL)-fractions isolated from healthy (H) and infected (I) tissue extracts; these tissues were sampled on tap root number 14 belonging to the GT1 family. This tap root did not differentiate any reaction zone tissues. Both tap roots were infected by *R. lignosus*. The gel permeation runs were performed under the following conditions. Sephadex G200 column (h = 100 cm; d = 1·8 cm); elution buffer: Tris–HCl 0·025 M at pH 8·4, KCl 0·1 M; flow rate: 10 ml h^{-1}; fraction volume: 1·1 ml. Note the separation of the peroxidases of the (EL)-fraction from healthy and reaction tissues into two fractions called P2a and P2b respectively [in (a), (b) and (d)]. The (EL)-fractions from infected tissues contain only the P2a peroxidase [in (c) and (e)].

Peroxidase content of the (EL)-fraction and effect of calcium on the peroxidase activity

In fact, gel permeation chromatography of the (EL)-fraction (Fig. 1) demonstrated the existence of two fractions, P2a and P2b, differing in molecular weight. In the healthy tissues the balance between P2a and P2b seems to vary considerably [Fig. 1 (a and d)], while there is a predominance of P2a in the reaction zone tissues [Fig. 1 (c)], and only P2a in infected tissues [Fig. 1 (d and e)]. Furthermore, Fig. 2 shows a very large difference in the calcium effect on P2a and P2b activities. The activity of the P2a peroxidase is slightly inhibited by calcium whether extracted from healthy or infected—or reaction zone—tissues; the activity of the P2b peroxidase increases approximately ten-fold in the presence of 5×10^{-3} M $CaCl_2$, whether it was extracted from healthy or from reaction zone tissues.

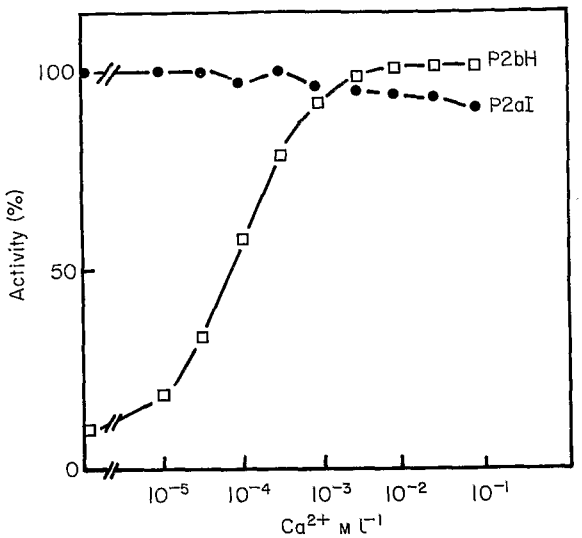

FIG. 2. Calcium effect on the activity of the P2a and P2b peroxidases.

Estimation of the variation of the P2a peroxidase pool induced by R. lignosus *infection*

The experimental data recorded on the residual activity (expressed as the percentage of the peroxidase activity measured in the absence of calcium, compared with the activity where calcium is present at 5×10^{-3} M), of purified P2a (105%) and P2b (10%) and of the crude (EL)-fraction enables the percentage of each P2a and P2b activity contributing to the total (EL)-peroxidase activity to be calculated, using the following equations:

$$\begin{cases} 105x + 10y = z \\ x + y = 1 \end{cases} \Rightarrow x = (z-10)/95$$

where:

x = that part of the (EL) activity due to P2a ($0 < x < 1$)
y = that part of the (EL) activity due to P2b ($0 < y < 1$)
z = the residual peroxidase activity of the (EL)-fraction in the absence of Ca^{++} (experimental data in %).

This method was used to calculate the P2a peroxidase pool of the different tissue types from some tap roots and, subsequently, to estimate the stimulation factor corresponding to the increase in P2a activity following the parasite infection. The results (Table 3) show that:

1. The root colonization induces a marked increase in the P2a activity. With reference to the corresponding control, the stimulation may reach a factor as great as 170 (IF tissue of tap root number 10). In the infected tissues of tap roots 1 and 3 the stimulation factor, calculated from the data in Tables 1 and 2, is 87 and 84 respectively.

2. The infection most probably promotes a complex effect on the different peroxidase fractions. This hypothesis is supported by the results shown in Table 4 where tap root 13 was taken as an example. In this case, both an increase of the P2a activity and a decrease of the (EF) and P2b activities, at least in the (IF)- and (I)-type

TABLE 3

Variation in (EL) and P2a peroxidase activity, effect of calcium on the (EL) peroxidase activity, and estimation of the P2a activity stimulation in response to infection

Tissue types and peroxidase activity of the crude extracts ($U.g^{-1}$ f. wt)[a]		Activity of the EL fraction[b]		P2a peroxidase activity (calculated values)[c]			
		% relative to the activity of the crude extract	% residual activity ($-Ca^{++}$)	% relative to the activity of		Specific activity ($U.g^{-1}$ f. wt)	Stimulation factor
				EL	crude extract		
Tap root 9							
H	18·7	48	58	51	24	4·5	1
I	156·5	98·8	104	99	97·8	153·1	34
R	18·6	82	69	62	50·8	9·5	2
Tap root 10							
H	10·8	33	24	14·7	4·8	0·5	1
I	91·2	99·9	104	99	99	90·2	171
Tap root 13							
H	7·5	22	39	29	6·4	0·5	1
HF	3·9	75	57	49	36·7	1·4	3
IF	67·5	98·5	108	100	98·5	66·5	140
I	11·0	100	104	99	99	10·9	23
R	15·4	91·8	93	87	80	12·3	26

[a] Different types of tissue of three tap roots were extracted under standard conditions, and the peroxidase activity of the crude extracts was measured.

[b] The peroxidases of each extract were separated by DEAE-cellulose fractionation into (EF)- and (EL)-fractions. Their activity was measured and expressed as a percentage with reference to the activity of the corresponding crude extract; the table indicates the results corresponding to the (EL)-fractions only. The activity of the (EL)-fractions was assayed both in the absence and in the presence of 0·005 M $CaCl_2$, and the calcium effect is expressed as the percentage of activity in the absence of calcium versus the activity in the presence of calcium.

[c] The above results ([b]) were used to calculate the percentage of the activity of the (EL)-fraction and of the crude extract due to the P2a peroxidase. For each tissue, the specific activity of this isoenzyme was calculated as well as the stimulation factor with reference to the specific activity of the healthy tissue control of the corresponding tap root.

tissues, coexist. Furthermore, the stimulating effect on the P2a activity seems already to have developed in the HF-tissues, i.e., some distance ahead of the parasite progression line.

Origin of the P2a isoperoxidase of infected tissues: host or parasite?

Although *R. lignosus* does not secrete any peroxidase *in vitro*, even when grown on *Hevea* wood [*15*], there remains the possibility that the peroxidase is secreted by the fungus in infected tissues. Therefore, the P2a isoperoxidases isolated respectively from *R. lignosus*-infected tissues and reaction zone tissues (which are devoid of any parasite) were purified in order to compare a variety of their physico-chemical characteristics.

Polyacrylamide gel electrophoresis under denaturating and non-denaturing conditions has shown that the two isoperoxidases are dimers of the A2-type. Their subunits have a molecular weight of 54000 D. In addition, the following physico-chemical characteristics have shown such similarities that the two peroxidases can be considered

TABLE 4

Peroxidase activity (U g^{-1} fresh weight of tissue) of different fractions extracted and purified from five characteristic tissue zones sampled on R. lignosus-*infected tap root (number 13)*

	Tissue type				
	H	HF	IF	I	R
Crude extract	7·5	3·9	67·5	11·0	15·4
EF-fraction	5·8	1·0	1·0	0·1	1·3
EL-fraction	1·7	2·9	66·5	10·9	14·1
P2a	0·5	1·4	66·5	10·9	12·3
P2b	1·2	1·5	0	0	1·8

identical: (a) UV and visible spectra, (b) effect of pH and temperature on catalytic activity, (c) heat denaturation, (d) amino acid composition of the peroxidase proteins (Table 5), and (e) finger print after trypsic digestion of the proteins (Fig. 3). As a consequence, one can conclude that the P2a isoperoxidase that is present in the infected tissues is synthesized by the rubber tree, and that the increase in its activity in these tissues and in the reaction zone tissues is actually a host response to the infection process.

TABLE 5

Amino acid composition of the P2a peroxidases isolated from infected tissues (I) of R. lignosus-*infected tap roots and reaction zone tissues (R) of* S. repens-*infected tap roots respectively*

Amino acid	I	R
ASP	70	72
THR	47	43
SER	40	39
GLU	51	53
PRO	21	20
GLY	31	31
ALA	40	37
CYS	13	13
VAL	23	22
MET	7	6
ILEU	25	27
LEU	45	48
TYR	9	8
PHE	33	31
HIS	6	6
LYS	12	12
ARG	21	23
TRP	1	1
Number of amino acid residues:	495	492

The Pearson's coefficient of similarity [*36*] equals 0·99 (the identity corresponds to a coefficient of 1·0).

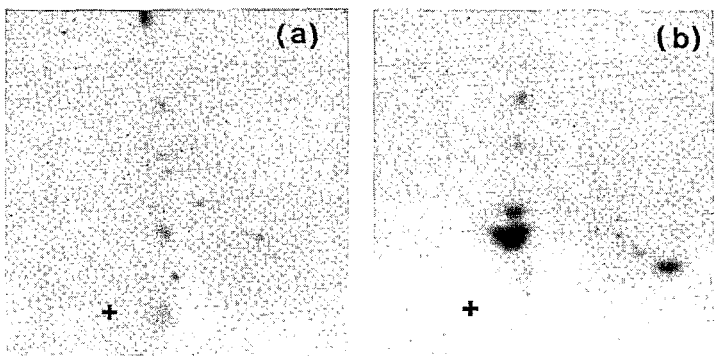

FIG. 3. Trypsic map (finger-prints) of the P2a purified from infected tissues of (a) *R. lignosus*-infected tap roots, and reaction zone tissues, and (b) *S. repens*-infected tap roots.

Regulation of the peroxidase activity increase and its potential significance

The P2a of healthy tissues was partly purified, and the specific activity of the three enzyme preparations (from healthy, *R. lignosus*-infected and reaction zone tissues respectively) were expressed with reference to their absorbance at 402 nm corresponding to the absorbance peak of the prosthetic group of the peroxidase, the haeme; reference to the A_{402} was found to be more adequate than reference to the protein content because of the presence of contaminants in the P2a solution of the healthy tissue.

The recorded specific activities were: 453, 510, and 650 (units/A_{402}) for the P2a isoperoxidases from healthy, *R. lignosus*-infected and reaction zone tissues respectively. These small differences cannot explain the very high stimulation factor observed in the infected tissues (up to 17000%) as well as in the reaction zone tissues. Therefore, it can be suggested that the increase in the P2a peroxidase activity results from a *de novo* synthesis of the enzyme.

Finally, P2a enzymes from the different tissue-types were shown to polymerize coniferyl alcohol *in vitro*. This indicates that they are able to perform (or at least to participate in) the ultimate step of lignin biosynthesis *in vivo*.

DISCUSSION AND CONCLUSION

The present paper reports both qualitative and quantitative results on the peroxidase content of tissues subject to parasitic infection in the field, i.e., under uncontrolled conditions. This particular situation could not allow kinetic studies to be performed. Nevertheless, the IF-type and I-type tissues can be considered as the initial and the late stage of infection respectively [*15*], although the time spent between these two stages cannot be properly measured (several months up to several years). On the other hand, our work provides information on some plant–pathogen interactions occurring under environmental conditions which have rather rarely been taken into account.

In general, when compared with healthy tissues, *R. lignosus*-infected tissues showed a four-fold increase in peroxidase activity. However, considerable variability was found which may reflect the genetic variability of the trees as well as environmental effects. The increase in peroxidase activity was not a result of a more efficient extraction of the

peroxidases from infected tissues disrupted by the lytic enzymes of the pathogen. Similar enhancement was found, indeed, in the non-degraded reaction zone tissues. Furthermore, the qualitative changes in the isoenzyme content of the reaction zone tissues were similar to changes in infected tissues and not to changes in healthy tissues [*32*].

In fact, one of the most significant effects of infection was qualitative: among the different isoenzymes mainly one type, P2a, appeared to be stimulated both in the infected and in the reaction zone tissues. In addition, although it was probably undervalued because of our very crude sampling system, this stimulation was quantitatively considerable: up to 170-fold. More generally, it appeared that (a) while the peroxidase reaction within a population of roots is quantitatively variable it is always identical qualitatively, and (b) the (IF)-zone tissues are still alive since they are able to react. As reported previously [*14*, *15*], the peroxidase activity in the (I)-type tissues was much lower than it was in the (IF)-type; in both tissues, however, the major isoperoxidase was P2a. The (I)-tissues were always brittle and already dead; therefore, it can be assumed that their enzyme activity was only a "residual" activity remaining after progressive denaturation or degradation of the P2a pool previously present in these tissues at the time they were at the (IF)-stage.

The P2a isoperoxidases from infected and reaction zone tissues are identical; thus, they are produced by the host, and their activity enhancement in both types of tissue corresponds to a reaction of the rubber tree against fungal attack.

The increased P2a activity most probably results in the stimulation of a *de novo* synthesis of the enzyme. Thus, the mode of *Hevea* reaction might be similar to that of other plants such as tobacco infected with tobacco mosaic virus (TMV) where the neosynthesis of the phenylalanine ammonia lyase (PAL) and *o*-methyltransferases (OMTs) is much enhanced [*9*, *10*, *13*]. Nevertheless, in the case of the peroxidase, an alternative explanation has to be considered, given that only the biosynthesis of the haeme may be stimulated. Subsequently, the haeme has to "couple" to the pre-existing apoenzyme to produce an active peroxidase. In fact, even if present in large quantities in the healthy tissues, the apoenzyme may not be detected during the purification process because of the lack of both catalytic activity and absorption at 402 nm which characterize the enzyme.

When compared with other plants, *Hevea* appears to be exceptional in both the specificity and the intensity of its peroxidase reaction. Most often the reaction of plants is much less intense (stimulation factor: 1·5 to 3) and much less specific: the activity of all the pre-existing isoperoxidases, or at least of some of them, being increased [*4*, *5*, *7*, *11*, *26*, *28*, *34*, *35*, *37*, *42*].

More generally, this work demonstrates that the peroxidase reaction of a lignified tissue is quite similar to that of a non-lignified one. Moreover, previous studies [*32*] have shown that *Hevea* reacts qualitatively in the same way whether the infection is caused by *R. lignosus*, *P. noxius* or *S. repens*, although the pathogenic processes induced by these parasites differ greatly from each other. This observation suggests that, while the host reaction is highly specific in terms of its "expression", it is non-specific with regard to the parasites causing it. This raises the question of the significance of the rubber tree peroxidase reaction.

As reported in the Introduction, many authors have demonstrated a close correlation

between peroxidase activity and/or lignification enhancement and host resistance. For some host–parasite couples however, such a close correlation has not been established [*22*, *25*, *28*, *29*, *43*]. In the rubber tree, besides the peroxidase reaction, several other kinds of reaction to infection by pathogens have been characterized [*32*]; these reactions include an increased lignification of mature xylem, an abnormal cell wall lignification in some phloem cells, and the formation of lignified wall appositions. Such responses, also observed in other host–parasite couples, take place in those tissues where the P2a activity increases considerably. This would indicate, at least, a close relationship between the lignification reaction and the P2a activity enhancement, but it does not demonstrate that the newly formed lignin is a result of the activity of this particular isoperoxidase activity, although it was shown to perform the last step of lignin polymerization *in vitro*. Moreover, whether or not the lignification itself would be efficient in limiting the spread of the fungi, remains open to question given that an infected tree generally dies. Nevertheless, it has been shown previously that, within a population of trees, some individuals, especially those actively developing tissue neogenesis (containing a high level of P2a) and rhizogenesis, survived [*30*]. Possibly these trees had been infected with hypovirulent strains of the pathogens [*18*, *31*]. This observation also suggests that: (a) the rubber tree is able to develop defence mechanisms which are efficient enough to overcome infection, and (b) resistance may involve a variety of defence reactions acting together with those leading to the re-inforcing of physical barriers.

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REFERENCES

1. Asada, Y. & Matsumoto, I. (1969). Formation of lignin-like substance in the root tissues of Japanese radish (*Raphanus sativus*) root infected by *Peronospora parasitica*. *Physiological Plant Pathology* **1**, 377–383.
2. Asada, Y. & Matsumoto, I. (1972). The nature of lignin obtained from downy mildew infected Japanese radish root. *Phytopathologische Zeitschrift* **73**, 208–214.
3. Bird, P. M. & Ride, J. P. (1981). The resistance of wheat to *Septoria nodorum*: fungal development in relation to host lignification. *Physiological Plant Pathology* **19**, 289–299.
4. Birecka, H., Catalfamo, J. L. & Garraway, M. O. (1975). Cell wall and protoplast isoperoxidases of corn leaves in relation to cut injury and infection with *Helminthosporium maydis*. *Plant Physiology* **55**, 607–610.
5. Birecka, H., Catalfamo, J. L. & Urban, P. (1975). Cell wall and protoplast isoperoxidases in tobacco plants in relation to mechanical injury and infection with tobacco mosaic virus. *Plant Physiology* **55**, 611–619.
6. Cadena-Gomez, G. & Nicholson, R. L. (1987). Papilla formation and associated peroxidase activity: a non-specific response to attempted fungal penetration of maize. *Physiological and Molecular Plant Pathology* **31**, 51–67.
7. Catedral, F. & Daly, J. M. (1976). Partial characterization of peroxidase isoenzyme from rust affected wheat leaves. *Phytochemistry* **15**, 627–631.
8. Coffey, M. D. & Cassidy, D. S. M. (1984). Peroxidase activity and induced lignification in rusted flax interactions varying in their degree of incompatibility. *Canadian Journal of Botany* **62**, 134–141.

9. Collendavelloo, J., Legrand, M. & Fritig, B. (1982). Plant disease and regulation of enzymes involved in lignification. *De novo* synthesis controls *O*-methyltransferase activity in hypersensitive tobacco leaves infected by tobacco mosaic virus. *Physiological Plant Pathology* **21**, 271–281.
10. Collendavelloo, J., Legrand, M. & Fritig, B. (1983). Plant disease and the regulation of enzymes involved in lignification. Increased rate of *de novo* synthesis of the three tobacco *O*-methyltransferases during the hypersensitive response to infection by tobacco mosaic virus. *Plant Physiology* **73**, 550–554.
11. Daly, J. M., Ludden, P. & Seevers, P. (1971). Biochemical comparisons of resistance to wheat stem rust disease controlled by the Sr6 or Sr11 alleles. *Physiological Plant Pathology* **1**, 397–407.
12. Dean, R. A. & Kuc, J. (1987). Rapid lignification in response to wounding and infection as a mechanism for induced systemic protection in cucumber. *Physiological and Molecular Plant Pathology* **31**, 69–81.
13. Duchesne, M., Fritig, B. & Hirth, L. (1977). Phenylalanine ammonia-lyase in tobacco mosaic virus-infected hypersensitive tobacco. Density labelling evidence of *de novo* synthesis. *Biochimica et Biophysica Acta* **485**, 465–481.
14. Geiger, J. P., Nandris, D. & Goujon, M. (1976). Activité des laccases et des peroxydases au sein de racines d'Hévéa attaquées par le pourridié blanc (*Leptoporus lignosus* (Kl.) Heim). *Physiologie Végétale* **14**, 271–282.
15. Geiger, J. P., Nicole, M., Nandris, D. & Rio, B. (1986). Root rot diseases of *Hevea brasiliensis*. I. Physiological and biochemical aspects of root aggression. *European Journal of Forest Pathology* **16**, 22–36.
16. Geiger, J. P., Huguenin, B., Nicole, M. & Nandris, D. (1986). Laccases of *Rigidoporus lignosus* and *Phellinus noxius*. II. Effect of *R. lignosus* laccase L1 on thioglycolic lignin of Hevea. *Applied Biochemistry and Biotechnology* **13**, 97–111.
17. Geiger, J. P., Rio, B., Nicole, M. & Nandris, D. (1986). Biodegradation of *Hevea brasiliensis* wood by *Rigidoporus lignosus* and *Phellinus noxius*. *European Journal of Forest Pathology* **16**, 147–159.
18. Geiger, J. P., Rio, B., Nandris, D. & Nicole, M. (1986). Discrimination between isolates of *Rigidoporus lignosus* and *Phellinus noxius* on the basis of wood degradation and enzyme secretion. *Proceedings of the 18th World Congress of the IUFRO, Ljubljana*, **Division 2, Vol. 2,** 833 (Abstr.).
19. Glazener, J. A. (1982). Accumulation of phenolic compounds in cells and formation of lignin-like polymers in cell walls of young tomato fruits after inoculation with *Botrytis cinerea*. *Physiological Plant Pathology* **20**, 11–25.
20. Grisebach, H. (1977). Biochemistry of lignification. *Naturwissenschaften* **64**, 619–625.
21. Gross, G. G. (1977). Biosynthesis of lignin and related monomers. *Recent Advance in Phytochemistry* **11**, 141–184.
22. Grzelinska, A. (1970). Peroxidase isoenzymes in *Fusarium*-infected tomato plants. *Phytopathologische Zeitschrift* **69**, 212–222.
23. Hammerschmidt, R. & Kuc, J. (1982). Lignification as a mechanism for induced systemic resistance in cucumber. *Physiological Plant Pathology* **20**, 61–71.
24. Hammerschmidt, R., Nuckles, E. M. & Kuc, J. (1982). Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*. *Physiological Plant Pathology* **20**, 73–82.
25. Johansson, M. & Theander, T. (1974). Changes in sapwood of roots of Norway spruce, attacked by *Fomes annosus*. Part I. *Physiologia Plantarum* **30**, 218–225.
26. Johnson, L. B. & Lee, R. F. (1978). Peroxidase changes in wheat isolines with compatible and incompatible leaf rust infection. *Physiological Plant Pathology* **13**, 173–181.
27. Kimmins, W. C. & Wuddah, D. (1977). Hypersensitive resistance: determination of lignin in leaves with localized virus infections. *Phytopathology* **67**, 1012–1016.
28. Mellon, J. E. & Lee, L. S. (1985). Elicitation of cotton isoperoxidases by *Aspergillus flavus* and other fungi pathogenic to cotton. *Physiological Plant Pathology* **27**, 281–288.
29. Nadolny, L. & Sequeira, L. (1980). Increases in peroxidase activities are not directly involved in induced resistance in tobacco. *Physiological Plant Pathology* **16**, 1–8.
30. Nandris, D., Nicole, M. & Geiger, J. P. (1987). Root rot disease of rubber tree. *Plant Disease* **71**, 298–306.
31. Nicole, M., Nandris, D., Geiger, J. P. & Rio, B. (1985). Variability among African populations of *Rigidoporus lignosus* and *Phellinus noxius*. *European Journal of Forest Pathology* **15**, 293–300.
32. Nicole, M., Geiger, J. P. & Nandris, D. (1986). Root rot diseases of *Hevea brasiliensis*. II. Some host reactions. *European Journal of Forest pathology* **16**, 37–55.
33. Nyman, B. F. (1986). Industrial air pollution and peroxidase activity in Scots pine needles—two case studies. *European Journal of Forest Pathology* **16**, 139–147.

34. Ohguchi, T., Yamashita, Y. & Asada, Y. (1974). Isoperoxydase of Japanese radish root infected by downy mildew fungus. *Annals of the Phytopathological Society of Japan* **40**, 419–426.
35. Ohguchi, T. & Asada, Y. (1975). Dehydrogenation polymerization products of *p*-hydroxycinnamyl alcohols by isoperoxidases obtained from downy mildew infected root of Japanese radish (*Raphanus sativus*). *Physiological Plant Pathology* **5**, 183–192.
36. Pearson, K. (1926). On the coefficient of racial likeness. *Biometrika* **18**, 105–117.
37. Retig, N. (1974). Changes in peroxidase and polyphenol oxidase associated with natural and induced resistance of tomato to *Fusarium* wilt. *Physiological Plant Pathology* **4**, 145–150.
38. Reuveni, R. & Ferreira, J. F. (1985). The relationship between peroxidase activity and the resistance of tomatoes (*Lycopersicum esculentum*) to *Verticillium dahliae*. *Phytopathologische Zeitschrift* **112**, 193–197.
39. Reuveni, R. & Bothma, G. C. (1985). The relationship between peroxidase activity and resistance to *Sphaerotheca fuliginea* in melons. *Phytopathologische Zeitschrift* **114**, 260–267.
40. Ride, J. P. (1975). Lignification in wounded leaves in response to fungi and its possible role in resistance. *Physiological Plant Pathology* **5**, 125–134.
41. Ride, J. P. (1980). The effect of induced lignification on the resistance of wheat cell walls to fungal degradation. *Physiological Plant Pathology* **16**, 187–196.
42. Seevers, P. M. & Daly, J. M. (1970). Studies on wheat stem rust resitance controlled at the Sr6 locus. II. Peroxidase activities. *Phytopathology* **60**, 1642–1647.
43. Seevers, P. M., Daly, J. M. & Catedral, F. F. (1971). The role of peroxidase isoenzymes in resistance to wheat stem rust disease. *Plant Physiology* **48**, 353–360.
44. Stermer, B. A. & Hammerschmidt, R. (1984). Heat shock induces resistance to *Cladosporium cucumerinum* and enhances peroxidase activity in cucumbers. *Physiological Plant Pathology* **25**, 239–249.
45. Touze, A. & Esquerre-Tugaye, M. T. (1982). Defence mechanisms of plants against varietal non-specific pathogens. In *Active Defence Mechanisms in Plants*, Ed. by R. K. S. Wood, pp. 103–117. NATO Advanced Study Institutes Series. Serie A: Life Science.
46. Touze, A. & Rossignol, M. (1977). Lignification and the onset of premunition in musk melon plants. In *Cell Wall Biochemistry Related to Specificity in Host–Plant Pathogen Interactions*, Ed. by B. Solenheim & J. Raa, pp. 269–293. Universitetsforlaget, Oslo.
47. Vance, C. P. & Sherwood, R. T. (1976). Regulation of lignin formation in reed canary-grass in relation to disease resistance. *Plant Physiology* **57**, 915–919.
48. Vance, C. P., Anderson, J. O. & Sherwood, R. T. (1976). Soluble and cell wall peroxidases in reed canary-grass in relation to disease resistance and localized lignin formation. *Plant Physiology* **57**, 920–922.
49. Vance, C. P. & Sherwood, R. T. (1977). Lignified papillae formation as a mechanism for protection in reed canary-grass. *Physiological Plant Pathology* **10**, 247–256.
50. Vance, C. P., Kirk, T. K. & Sherwood, R. T. (1980). Lignification as a mechanism of disease resistance. *Annual Review of Phytopathology* **18**, 259–288.
51. Vegetti, G., Conti, G. G. & Pesci, P. (1975). Changes in phenylalanine ammonia-lyase, peroxidase and polyphenoloxidase during development of local necrotic lesions in Pinto bean leaves infected with Alfalfa Mosaic Virus. *Phytopathologische Zeitschrift* **84**, 153–171.
52. Wagih, E. E. & Coutts, R. H. A. (1982a). Peroxidase, polyphenoloxidase and ribonuclease in tobacco necrosis virus-infected or mannitol osmotically-stressed cowpea and cucumber tissue. 1. Quantitative alterations. *Phytopathologische Zeitschrift* **194**, 1–12.
53. Wagih, E. E. & Coutts, R. H. A. (1982b). Peroxidase, polyphenoloxidase and ribonuclease in tobacco necrosis virus-infected or mannitol osmotically-stressed cowpea and cucumber tissue. 2. Qualitative alterations. *Phytopathologische Zeitschrift* **104**, 124–137.
54. Weber, K. & Osborn, M. (1969). The reliability of molecular weight determination by dodecyl sulphate polyacrylamide gel electrophoresis. *Journal of Biological Chemistry* **244**, 4406–4412.