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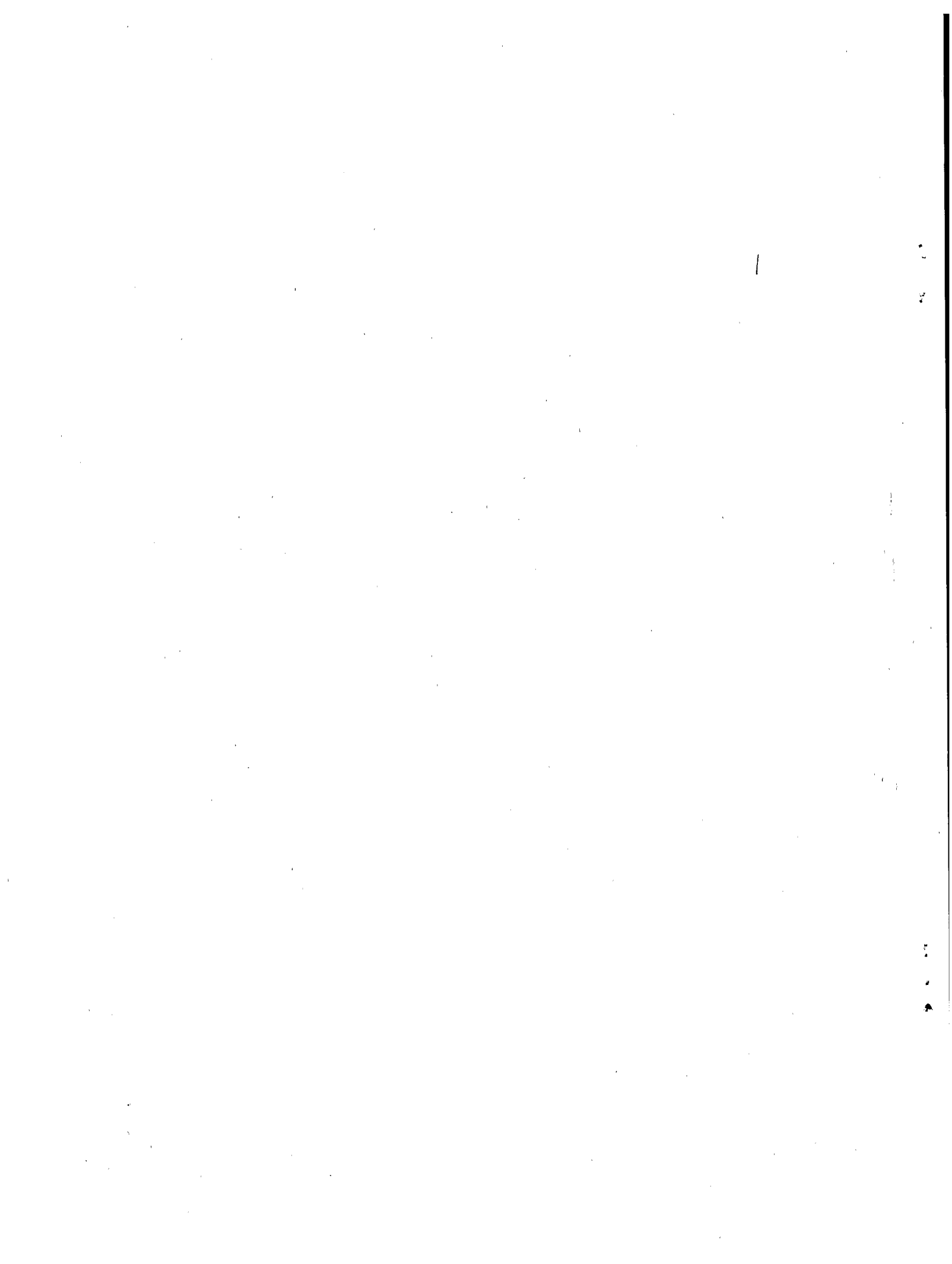
Defense responses of *Hevea brasiliensis* to elicitors from root rot fungi

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Elicitation of root defense responses of rubber tree (*Hevea brasiliensis*) to *Rigidoporus lignosus* has been performed by injection of fungal cell wall extracts into the root system of 1-month-old seedlings. A time course study showed that tissue lignification is induced at 3, 8, 15, and 45 days after elicitor treatment, as observed under a light microscope. The ethanol-soluble elicitor fractions stimulated cinnamyl-alcohol dehydrogenase activity, involved in lignin synthesis, as well as callose deposits 15 days and 30 days after elicitor treatment. A differential phellogen activity was observed 15 days after injection of roots with fungal extracts not treated with pronase. Stimulation of chitinase activity in leaves, cell hyperplasia, and suberization of root cell walls did not succeed. These data show that some defense mechanisms of tree roots can be triggered by elicitors from root-rotting fungi and result in the stengthening of structural barriers in host tissues.

Key words: root rot diseases, elicitation, lignification, cambium stimulation, callose.

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L'élicitation de quelques réactions de défense du système racinaire de l'Hévéa (*Hevea brasiliensis*) aux attaques du *Rigidoporus lignosus* a été réalisée en injectant des extraits de parois fongiques dans les racines de jeunes plantes agées de 1 mois. Une étude cinétique a révélé, au microscope optique, une induction de la lignification 3, 8, 15 et 45 jours après l'injection d'éliciteurs. Les fractions pariétales solubles dans l'éthanol ont permis de stimuler l'activité de la cinnamyl alcool deshydrogénase, une enzyme intervenant dans la biosynthèse des monomères de la lignine et d'induire la synthèse de callose, 15 et 30 jours après l'injection d'éliciteurs. De même, les fractions de parois non traitées à la pronase stimulent significativement l'activité du cambium subéro-phellodermique. L'élicitation d'autres réponses comme l'induction d'une activité chitinase dans les feuilles, la subérification des parois cellulaires et l'hyperplasie des cellules des racines a également été tentée, mais sans succès. Ces résultats montrent que certaines réactions de défense des racines des plantes ligneuses peuvent être stimulées par des éliciteurs fongiques dans le but de renforcer les barrières structurales des tissus hôtes.

Mots clés : pourridiés, élicitation, lignification, stimulation du cambium, callose.

Introduction

Responses of trees to root rot diseases have seldom been investigated. Rishbeth (1972) published the first review on resistance of tree roots to fungal pathogens, with major references to gymnosperms. Anatomical changes and accumulation of chemical compounds were then reported on some woody plant species (*Abies* sp., *Eucalyptus* sp., *Picea* sp., *Pinus* sp., *Quercus* sp.) infected with root rotting fungi (*Armillaria mellea* (Vahl:Fr.) P. Kum., *Ganoderma* sp., *Heterobasidion annosum* (Fr.:Fr.) Bref.) (Pearce and Rutherford 1981; Tippet and Shigo 1981; Shigo 1984). Recently, Blanchette and

Biggs (1991) presented a review on defense mechanisms of woody plants against fungi in which several aspects of root responses to infection were described.

Attempts to understand how rubber trees (*Hevea brasiliensis* (Willd. ex Adr. Juss.) Müll. Arg.) defend against root rot diseases was first reported by Sharples (1936) following an infection with *Ganoderma pseudoferreum* (Wakef.) Overh. & Steinm. More precise studies were performed by Geiger and Goujon (1977) who pointed out an *Hevea* root isoperoxidase reaction to *Rigidoporus lignosus* (Kl.) Imazeki and *Phellinus noxius* (Corner) G. H. Cunn, two basidiomycetes widespread in tropical countries (Nandris *et al.* 1987). This enzyme is involved in the lignification of cells within the reaction zone and barrier zone (Nicole *et al.* 1986; Geiger *et al.* 1989). Other active responses were also initiated both at the cell and the

tissue levels in the same system. Among them, it was found that differentiation of new roots, cell wall thickening related to suberization or lignification, wall appositions, cell hyperplasia, and stimulation of phellogen and of vascular cambium activities were associated with delay of fungal colonization (Geiger et al. 1986; Nicole et al. 1983, 1986, 1991).

Despite these data, attempts to trigger defense mechanisms of tree roots to vascular and root rot fungal elicitors is poorly documented compared with studies involving changes triggered by elicitors of stem and foliar pathogens in herbaceous plants (Toppan and Esquerré-Tugayé 1984; Mazau et al. 1987; Roby et al. 1986; Hahn et al. 1989). So far, only a few papers dealing with gossypol elicitation by conidia cell walls of Verticillium sp. in Gossypium sp. (Bell 1981), isoperoxidases elicitation of cotton bolls by conidia of V. flavus Link (Melon and Lee 1985) and chitosan-induced monoterpene in stem phloem of lodgepole pine (Pinus contorta Dougl.) (Miller et al. 1986) have been published.

Elicitor-induced host responses generally result in the use of fungal cell wall extracts ranging from different crude cell wall preparations (Parker et al. 1988) to highly purified cell wall components (Sharp et al. 1984). In this study we used crude, ethanol soluble, and pronase-treated cell wall extracts from R. lignosus to trigger defense responses of H. brasiliensis. Cell wall modifications, phellogen activity, and cinnamyl-alcohol dehydrogenase (CAD, EC 1.1.1.195) in roots and chitinase activities (EC 1.1.1.3.14) in leaves were studied on seedlings grown in the green house after injection with fungal cell wall extracts into the tap root.

Materials and methods

Plant material

Rubber tree seedlings, clone GT 1, were collected in a plantation of the Institut de recherche du caoutchouc (IRCA) in the Ivory Coast. After germination in sand, the young seedlings were transplanted in tubs ($1 \times 1 \times 1$ m) filled with forest soil in which a high humidity level was maintained by watering to saturation. A neutronic moisture gauge (Solo 20, Nardeux, France) controlled the humidity level of the soil.

Artificial inoculations of rubber tree seedlings

Seedling inoculation was performed as previously described by Nandris et al. (1983). Five preinfected rubber wood fragments were applied against the tap root of each 1-month-old seedling, 20 cm deep in the soil. Root samples were collected at varying intervals following inoculation. Noninfected plants were used as controls.

Culture conditions of fungal strains

Isolates of R. lignosus were collected in rubber tree plantations in the Ivory Coast. The mycelium was obtained by growing R. lignosus, strain 1, on a malt (2%) agar (2%) medium at 28°C. Liquid shaken cultures (2% malt) were prepared by inoculation with agar-infected disks as inoculum (50/500 mL). One-month-old cultures were collected for elicitor extraction.

Controls

Four different controls were simultaneously performed to identify the induced reactions precisely as follows: (i) healthy plants without wounding and infection (control 1; C1), (ii) plants wounded by a needle without liquid injection (control 2; C2), (iii) roots of plants in which distilled water was injected (control 3; C3), and (iv) 1-month-old plants inoculated with R. lignosus as described above.

Elicitor preparation

Four different elicitor preparations, ELI 1, 2, 3, and 4, were prepared as described by Toppan and Esquerré-Tugayé (1984). ELI 1 was solubilized from the mycelium by autoclaving in water at 115°C for 2 h (10 g fresh mycelium/10 mL). The crude extract was filtered and concentrated by lyophylization. ELI 2 was obtained from ELI 1 after the pronase treatment (1 mg/mL overnight); this enzyme was then heat denatured (90°C, 15 min). ELI 3 was the ethanol-soluble fraction recovered from ELI 1 after precipitation by 2 volumes of 95% ethanol (overnight at -20°C). ELI 4 was prepared by the pronase treatment of ELI 3 under the same conditions as above. The amount of each elicitor fraction was expressed as micrograms of glucose (Glc) equivalents because it was found that their activity was correlated with their sugar portion (Roby et al. 1986). The different elicitors (200 µL corresponding to 240 µg Glc equiv.) were injected with a syringe into the tap root of 4-week-old seedlings, 2 cm below the collar.

Microscopic observations

Transverse sections of living roots, 10–20 µm thick, were made on a freezing microtome and used for lignin, suberin, and callose detection. The following histochemical tests were performed as described by Jensen (1962): phloroglucinol–hydrochloride, Maüle reaction (1% $KMnO_4$), and toluidine blue for lignin; sudan IIIB and IV for suberin; anilin blue (0.01%) for callose, observed under fluorescence conditions.

The sections were also illuminated with UV for autofluorescence examination of suberized and lignified cell walls and for periderm or barrier zone formation. Observations were made at 100, 250, and 1000× magnification under an Orthoplan Leitz Microscope (HBO 100 W mercury lamp) equipped with epifluorescence filters (block A).

Four plants were examined for each assay. At least 40 sections per plant were cut from 0.5 cm of root excised from each side of the injected site and treated for histochemical tests. Ten sections were used for lignin tests, 10 sections for suberin tests, and 10 sections for callose detection. In comparison with the controls, a scale (0–4) was established to evaluate the significance of each reaction. On these sections, hypertrophied cells and cell layers produced by the phellogen were also numbered.

Samples were collected at 3, 5, 8, 15, 30, and 45 days after elicitor treatment to perform a time course study.

Enzyme activity assays

Cinnamyl-alcohol dehydrogenase (CAD)

Root fragments (2 g fresh matter) were homogenized at 4°C in 10 mL 0.1 M Tris–HCl buffer (pH 7.5). After filtration through cheese cloth, the suspension was centrifuged at 15 000 × g for 20 min, and the supernatant was concentrated on an Amicon (PM10) ultrafilter. The CAD activity was assayed on the supernatant by measuring the formation of coniferaldehyde from the coniferyl alcohol by a spectrophotometric procedure under the conditions given by Grand et al. (1987). CAD activity was recorded 3, 15, and 30 days after the elicitor treatment and is expressed in OD/g fresh matter. Each assay consisted of root tissue (0.5 cm long) taken up from each side of the injected site of five seedlings for measuring the activity.

Chitinase

Leaves from seedlings the roots of which were examined for histological investigation and CAD activity assay were homogenized in acetate buffer (0.2 M; pH 4.5); chitin was used as a substrate for recording the chitinase activity. The released N-acetylglucosamine residues were assessed as described by Toppan and Roby (1982). Chitinase activity was measured at 5, 8, 15, and 30 days after elicitor treatment.

For both enzyme assays, enzyme and substrate blanks were carried out.

Results

Histological study

The cambium activity in roots was estimated by numbering the extra cell layers the phellogen produced near the injection
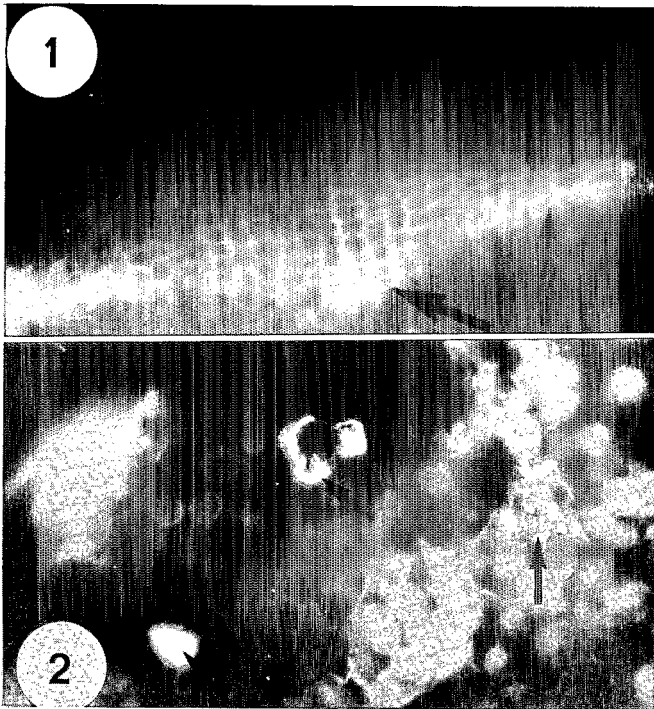
FIGS. 1 and 2. Photomicrographs of cross sections of *Hevea brasiliensis* roots observed under UV epi-illumination 15 days after *R. lignosus* elicitor treatment. Fig. 1. Stimulation of phellogen activity after ELI 3 treatment. Autofluorescence observation of the section showing nine additional cell layers produced by the stimulated phellogen (large arrow) compared with cell layers produced by a normal phellogen activity (small arrow). ×375. Fig. 2. Callose deposition (arrows) in medullar parenchyma cells after ELI 4 treatment. Aniline blue staining. ×940.

TABLE 1. Stimulation of phellogen activity in roots of *Hevea brasiliensis* at different times following treatments with *Rigidoporus lignosus* elicitors (ELI 1, 2, 3, and 4)

Assay	Days after elicitor treatment					
	3	5	8	15	30	45
ELI 1	0	0	0	6	2	2
ELI 2	2	0	1	1	2	0
ELI 3	0	0	2	11	0	0
ELI 4	2	0	0	0	2	0
C2	0	1	2	0	0	0
C3	0	1	0	0	0	1
nC1	4–5	5–6	7–9	9–11	11–13	13–16

NOTE: Data are expressed in number of additional cell layers compared with controls. Four plants were examined for each assay at each time. nC1, control 1, showing the number of cell layers produced by the phellogen in healthy plants; C2, control 2, plants wounded with a needle; C3, control 3, plants injected with water.

site. In healthy controls, the cell layers varied from 4 to 16, respectively, in 3- to 45-day-old plants (Table 1). ELI 1 and ELI 3 increased the phellogen activity by the formation of 6 and 11 additional cell layers (Fig. 1). On the contrary, in comparison with controls, no significant induction was observed 3, 5, 8, 30, and 45 days after injections in roots of the pronase-treated elicitors ELI 2 and ELI 4.

ELI 2 did not induce any significant modifications of host cell walls; data recorded after injection of this fungal cell wall preparation are not shown in Table 2. Elicitor-induced lignification was investigated on different root sections (Table 2).

TABLE 2. Time course study of lignin, callose, and autofluorescence induced in roots of *Hevea brasiliensis* in response to elicitation (ELI 1, 3, and 4)

Days after elicitor treatment	Lignin*			Callose†			Fluorescence‡		
	cc	phlo	mp	cc	phlo	mp	cc	phlo	mp
ELI 1									
3	2	2	**3**	0	0	2	0	0	3
5	0	1	1	0	0	1	2	0	2
8	1	1	2	0	1	2	1	2	3
15	1	1	3	0	0	1	3	2	3
30	1	1	3	0	0	0	1	2	3
45	2	1	2	0	0	1	0	1	2
ELI 3									
3	1	2	2	0	1	1	0	1	2
5	0	1	2	1	0	1	0	2	2
8	2	2	**3**	0	0	1	2	**3**	3
15	1	**2**	3	**2**	0	**1**	2	1	3
30	1	**2**	3	1	0	0	2	2	4
45	1	1	**4**	0	0	0	2	1	3
ELI 4									
3	1	2	2	0	1	1	0	**3**	1
5	0	1	1	0	2	2	0	2	2
8	1	1	**3**	2	0	2	3	**3**	3
15	2	1	**3**	0	0	1	2	2	3
30	0	1	3	**2**	**3**	**3**	2	3	2
45	1	0	**3**	0	0	1	2	1	3
C2									
3	1	1	1	0	0	1	2	0	2
5	1	1	2	0	0	1	1	2	2
8	2	1	0	0	1	1	2	1	2
15	0	0	1	0	0	0	2	1	1
30	1	1	3	0	0	0	2	2	3
45	1	0	1	0	0	1	0	1	2
C3									
3	2	2	1	0	0	1	0	2	0
5	0	2	1	1	2	1	2	1	1
8	0	2	1	1	0	1	2	1	1
15	1	0	2	0	0	0	2	0	2
30	1	1	2	0	0	0	2	2	3
45	1	0	1	0	0	1	1	0	2

NOTE: Data were estimated according to a scale intensity ranging from 0 to 4 where 0 means no reaction and 4 means very intense response. The most significant results compared with control data are boldface, e.g., 4 for ELI 3 lignin response in mp, 45 days after treatment. ELI 2 was not reported in this table because no significant results were recorded. Four plants were examined and histological tests were performed on 10 sections per plant. C2, control 2, plants wounded with a needle; C3, control 3, plants injected with water; cc, cortical cells in very young roots only; phlo, phloem; mp, medullar parenchyma.

*Phloroglucinol–HCl and KMnO₄ staining (red) under incandescent illumination.

†Aniline blue fluorescence (yellow) under UV epi-illumination.

‡Blue-yellow autofluorescence under UV epi-illumination.

In response to the ethanol-soluble elicitors ELI 3 and ELI 4, the cell walls of the medullar parenchyma reacted strongly to the Maüle test, suggesting an induction of the synthesis of material like guaïacyl-lignin. ELI 1 (3 days) also induced significant lignin synthesis that seemed to be an early response when compared with those induced by the two other fungal extracts. However, UV observations made on these lignin-positive sections only revealed a slight fluorescence.

Light microscopy observations (Table 2) also revealed that ELI 1 never or rarely induced significant callose deposition along walls of parenchyma and phloem cells. However, ELI 3 and especially ELI 4 elicited callose papillae in both parenchyma and phloem cells (Fig. 2). A strong positive reaction was observed mainly on the cell walls after 30 days, after ELI 4
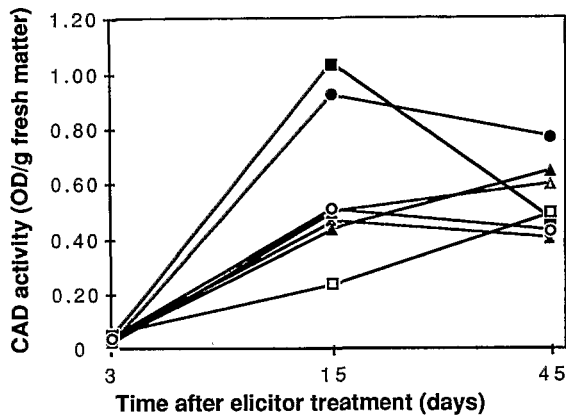
FIG. 3. Cinnamyl-alcohol dehydrogenase activity (CAD) in roots after treatment with elicitors (ELI 1, 2, 3, 4). □, control 1, healthy plants; O, control 2, plants wounded with a needle; △, control 3, plants injected with water; ×, ELI 1, ▲, ELI 2; ■, ELI 3; ●, ELI 4.
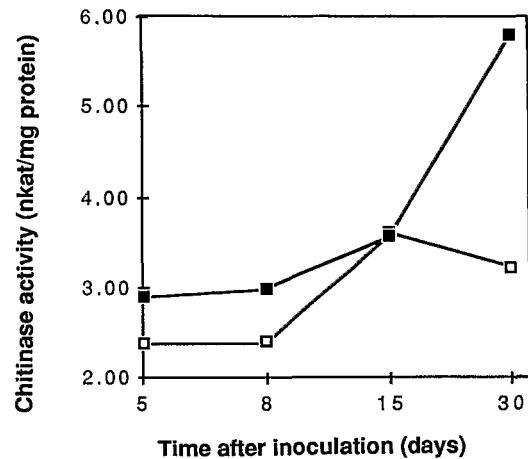

FIG. 4. Stimulation of chitinase activity in leaves of *Hevea* the roots of which were inoculated with *Rigidoporus lignosus* (■), compared with healthy plants (□).

treatment. No cell wall suberization was detected after elicitor treatment. Thus, autofluorescence observed in the phloem (e.g., ELI 4, 8 days) probably indicated the presence of phenolic substances since lignification also produced a low natural illumination of cells.

Cell hypertrophy and hyperplasia were frequently observed in treated roots mainly in cortical tissues near the injection sites, although at a low frequency. Moreover, there were no significant differences between elicitor-treated roots and controls. Thus, this reaction probably resulted from wounding rather than elicitation.

Biochemical study

The CAD activity in roots was enhanced 15 days after ELI 3 or ELI 4 injections. The stimulation factor obtained in these cases was twice as high as in the case of the two other extracts (ELI 1 and ELI 2) and of the controls (C2 and C3), and 5 times higher than in the case of healthy plants (control C1). Three and 45 days after treatment results were similar for all assays and controls (Fig. 3).

The elicitor treatment did not induce any significant chitinase activities in leaves when compared with the activity in leaves of healthy rubber tree (data not shown). However, chitinase activities in leaves of artificially infected plants were stimulated more than twice as much as those of leaves of healthy plants (Fig. 4).

Discussion

Elicitation of lignification, callose synthesis, and phellogen activity do occur in the roots of *H. brasiliensis* in response to elicitors prepared from *R. lignosus* cell walls. The same responses were previously reported on *H. brasiliensis* roots infected with *R. lignosus* and (or) *P. noxius* (Nicole *et al.* 1986; Geiger *et al.* 1989). In *Hevea* roots, lignification occurs early (4 days after inoculation) when cortical cells are resistant to *R. lignosus* penetration owing to wall thickening (Nicole *et al.* 1986). From the present data, one may assume that lignification mainly results from the interaction of root cells with ethanol-soluble fungal cell wall components. The fact that the activity of CAD, a key enzyme in lignin biosynthesis, is significantly increased in this system by elicitors 3 and 4 is in

good agreement with our previous finding. Intense lignification occurs in new roots differentiated by resistant trees; these roots have been shown to be resistant to pathogen invasion (Nicole *et al.* 1986). Moreover, root isoperoxidase activity, strongly enhanced following infection with *R. lignosus*, is capable of polymerizing lignin monomers (Geiger *et al.* 1989) and may contribute to defense lignification. It would be interesting to know if the increasing of CAD activity following elicitation is an early response of rubber tree roots, as in the case of elicitor-treated wheat leaves (Moerschbacher *et al.* 1989) and bean cells (Grand *et al.* 1987). As discussed by Walter (1990), it is possible that a particular defense CAD gene has been activated in defense responses and plays a role in regulation of lignin monomer biosynthesis.

Callose synthesis and deposition in papillae is a typical defense reaction of many plants. It is induced early in response to elicitors (Table 2) as well as to infection in our system (Nicole *et al.* 1986). This suggests the involvement of a β-glucan synthase as reported by Valluri and Soltes (1990) in *Fusarium*-infected pines. It is likely that this enzyme exists in healthy rubber trees because of the presence of native callose in the phloem. Thus, callose deposition might result from the stimulation of an endogenous plasma membrane 1,3-β-glucan synthase rather than from *de novo* synthesis of the protein.

The formation of protective tissues has often been reported as a response of bark tissues to injuries or infections (Biggs *et al.* 1984). The resistance of older cotton roots to *Thielavopsis* sp. was attributed to the differentiation of a cork cambium (Mathre *et al.* 1966). In rubber tree infected roots, a new periderm was described as a mechanical barrier to hyphal spread of soil fungi such as *Armillaria* spp. (Rishbeth 1972) and *Ganoderma* spp. (Sharples 1936). Our previous results showed that phellogen stimulation occurred in *H. brasiliensis* upon infection with *R. lignosus*. Successful elicitation of the cork cambium supports the strong implication of this reaction in defense mechanisms of tree roots to fungal invasion. In addition, the resulting increase of cell layers was associated with new root formation, conferring the most vigorous trees with a tolerance to the disease.

Defense proteins such as chitinases and β-1,3-glucanases are known to be present in trees (Wargo 1975). In *H. brasiliensis*, chitinase activity is lower in leaves of unstressed trees

than in the latex of the trunk (Martin 1990). However, a rapid induction was often observed following treatment of these plants with abiotic or biotic elicitors (Martin 1990). The location in rubber tree root tissues of such defense proteins might account for the resistance of trees to root rot fungi. Indeed, partially purified preparations of chitinases and β-1,3-glucanases of roots of different tree species have been shown to lyse the hyphal walls of the root-rotting fungus *A. mellea* (Wargo 1975).

Because the chemical composition of the elicitors used in this study was not determined, it is not known whether the active fragments consisted of 1,3-β-glucan and 1,6-β-glucan, chitin oligomers, or proteins. Therefore, different components of *R. lignosus* cell walls probably elicit host responses. Table 1 shows that the nonpronase-digested elicitors, ELI 1 and ELI 3, have stimulated the phellogen activity, suggesting that proteinaceous compounds might be involved in the elicitation process. Such constituents have been demonstrated to be active eliciting compounds in parsley (Parker *et al.* 1988). Carbohydrates of *R. lignosus* cell walls, such as chitin and glucans, which are major components of basidiomycete cell walls (Bartnicki-Garcia 1968), may also be active elicitors. Chitin oligosaccharides (Barber *et al.* 1989) as well as glucan (Robertsen 1986) are known to induce the lignification process. Owing to chitinase activity already present in healthy rubber trees, it is possible that chitin fragments released from *R. lignosus* cell walls contribute to the induction of lignification. In contrast, chitin-related compounds did not increase chitinase activity significantly as reported in melon plants (Roby *et al.* 1987). However, they have been shown to stimulate callose synthesis via Ca^{2+} changes (Kauss 1989). Modifications of calcium level in host cell walls and plasma membranes are related to hormonal imbalances that control histopathological changes in the bark of trees (Hoque 1982). Whether these factors are involved in callose deposition and phellogen activity increase is not known in this system and deserves additional work.

In conclusion, defense responses of roots in woody plants can be elicited by cell wall extracts of root rot fungi. In this work, the elicitor-induced reactions described contribute to strengthening of the structural barriers of *H. brasiliensis* roots to slow the invasion of the fungus, thus preventing the decay extension.

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