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In vitro characterization and *in vivo* detection of *Rigidoporus lignosus*, the causal agent of white root disease in *Hevea brasiliensis*, by ELISA techniques

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Abstract

The aim of these studies is to develop a method for early detection of *Rigidoporus lignosus* (Basidiomycete, Polyporaceae), the causative agent of white root disease of rubber tree. Two polyclonal sera were produced against soluble mycelial proteins of two *R. lignosus* isolates, one from Africa (FCI2), the other from Asia (FID2). The specificity of the antisera was tested using isoelectric focusing (IEF)/Western-blot and DAS-ELISA. The two sera recognized all 20 *R. lignosus* isolates from various geographical origins. The banding patterns obtained by Western-blot enabled a distinction to be made between isolates from Africa and those from Asia. In DAS-ELISA and Western-blot analyses, strong cross reactions were observed with *R. ulmarius*. Only slight reactions were observed in Western-blot analysis to *R. lineatus* and *P. noxius*, both causative agents of root rot in *Hevea*. These cross reactions were not observed under our DAS-ELISA analysis conditions. Finally, no cross reactions were obtained with 9 other *Polyporaceae* or *Hevea* root pathogen species. The sensitivity threshold of the DAS-ELISA method was 5 ng ml^{-1} of *R. lignosus* protein. An initial approach to using the DAS-ELISA test for the detection of *R. lignosus* in infected plants was carried out on artificially inoculated root samples. The DAS-ELISA protocol enabled detection of *R. lignosus* in the root systems of diseased plants. Moreover, no cross reaction was observed with healthy plant extracts.

Introduction

Rigidoporus lignosus (Klotsch) Imaz. is a telluric fungus found throughout the wet equatorial and tropical zones of the world. It is highly polyphagous and is considered to be one of the main pathogens in rubber tree plantations (*Hevea brasiliensis*, (Will, ex de Juss.) Muell.-Arg) [Compagnon, 1986]. The control methods currently recommended are chemical treatments, by which epidemics can be restrained [Tran Van Cahn, 1986; Gohet *et al.*, 1991].

Treatments are only carried out on diseased trees and their effectiveness depends on reliable and early detection of the pathogen. Leaf symptoms were examined to detect diseased trees [Liyanage and Peries, 1973]. However leaf symptoms are linked to severe deterioration of the roots which prevents an adequate water supply, and they appear too late for subsequent treatment to be effective. Other detection methods are based on looking for the existence or absence of typical white rhizomorphs. Before planting, the wood-trap technique can be used in which a piece of sterile wood is buried in the soil and later checked for the presence or absence of the fungus [Declert, 1960]. Several techniques are usual for existing plantation. One is designed to favour surface development of the fungus by placing mulch around the foot of the threes and thereby maintaining humidity favourable to rhizomorph growth around the collar [Martin, 1964]. Another method consists of removing the soil around the tree so that rhizomorphs in contact with the roots can be seen [Martin and Du Plessix, 1965]. This involves digging a 10 cm depression around the tree and examining the root bulb and the beginning of the tap root and lateral roots. From practical and economic points of view, it is difficult to envisage deeper and

more complete soil clearance. All of these methods are arduous and costly on a large scale. They are also inaccurate insofar as a large portion of the root system may escape from observation during inspection rounds and there is a risk of detecting other fungi with a similar mycelial morphology to that of *R. lignosus*.

Sensitive, reliable and early detection method for *R. lignosus* is thus needed to improve control methods. Immunoassays are useful tools for the detection of microorganisms and have been used for the study of many plant-fungus interactions [for a review, see Werres and Steffens, 1994]. In the case of tree pathogens, these techniques are hampered by difficulties in processing woody samples. Nevertheless, serological methods have been successfully adopted for the diagnosis of *Ceratocystius ulmi*, the causative agent of Dutch elm disease [Svircer *et al.*, 1988] and *Ceratocystis fimbriata* f. sp. *platani*, the causative agent of canker stain of plane trees [Diop-Bruckler, 1991].

In the present article, we describe the production of polyclonal antisera to *R. lignosus* mycelial proteins and we assess the usefulness of DAS-ELISA for the detection of *R. lignosus* in *Hevea brasiliensis* roots.

Materials and methods

Fungal isolates and culture conditions. Rigidoporus lignosus isolates were obtained from the roots of infected rubber plants. Twenty isolates from different geographical origins were analyzed (Table 1). Nine isolates belonging to related polyporaceae species and 5 others hevea roots pathogens isolates were also included in the study (Table 1).

The collection was preserved in tubes on a malt (2%) agar (2%) medium, at 28 °C for *R. lignosus* and at 25 °C for the isolates of the other species. Mycelium from still cultures was used for the preparation of antigens and for protein extractions. Five mycelial plugs (diameter = 8 mm) taken from a 7-day old culture were inoculated in a Roux bottle containing 100 ml of 2% malt medium and the cultures were then kept at 28 °C in the dark for 10 days. The mycelium was collected and rinsed with deionized water under sterile conditions and subsequently freeze-dried or used immediately for extraction.

Plant material and inoculation method. Inoculations were carried out on young rubber plants from two different origins: seedlings from illegitimate seeds of the clone GT1 from the Ivory Coast, and *in vitro* plantlets (Clone No. 60) obtained from microcuttings produced in the BIOTROP/CIRAD laboratory (Carron, Montpellier). A method of culture in a root growth chamber ('rhizotron'), developed during a study of the *Hevea* root system (Pagès, INRA, Avignon) was used. The 'rhizotrons' consisted of two vertical plates (30×50 cm), one in polyvinyl chloride (PVC), the other in transparent plastic (Makrolon). A 5 mm space between the plates was maintained using PVC strips bonded to the opaque plate. The two plates were held together with clamps. The 'rhizotrons' were filled with vermiculite. The inoculum consisted of pieces of *Hevea* wood (10×1×0.5 cm) colonized by *R. lignosus*. The pieces of wood were first sterilized in Roux bottles with 100 ml of water for 45 min at 120 °C. Thereafter, each flask received 5 mycelial plugs as described above, and was then kept in the dark at 28 °C for 8 months. Inoculation was carried out by placing a piece of wood diagonally in each 'rhizotron' containing a young *Hevea* plant. Inoculations were made just after germination in the case of seeds, or just after hardening off in the case of *in vitro* plantlets. The five isolates FCI2, FGA2, FCA1, FID2 and FML2 were used to inoculate seedlings. The *in vitro* plantlets were inoculated using the isolates FGA2, FID2 and FML2. Five plants were inoculated with each isolate. The controls were seedlings or *in vitro* plantlets grown in a 'rhizotron' without pieces of wood. The 'rhizotrons' were then stored away from light, in a closed wooden box with slits in the lid through which the aerial parts of the plants passed. The root systems of the seedlings and the *in vitro* plantlets were sampled 3 months later.

Soluble protein extraction for immunization. One gram of freeze-dried mycelium or 10 g of fresh mycelium were placed in a flask with 15 ml of phosphate buffer (PBS; 1.5mM KH_2PO_4, 10 mM Na_2HPO_4, 3 mM KCl, 140 mM NACl, pH 7.4) and 50 g of 0.4 mm glass beads and ground at 4 °C on a Braun MSK homogenizer for 1 min. After filtration, the extract was centrifuged at 25,000 g at 4 °C for 45 min. The supernatant was recovered and dialyzed against PBS for 24 h. The protein concentration was evaluated by the Bradford method (1976) using Bovine Serum Albumin (BSA) as the reference protein. The extracts were adjusted to 1 mg ml^{-1}, aliquoted into 1 ml fractions and stored at – 80 °C. All these steps were carried out under sterile conditions.

Preparation of fungal extracts for enzyme immunoassays. 200 mg of freeze-dried mycelium were ground

Table 1. Isolates used in this study: geographic origin, plant pecies from which the isolates were obtained and source

Isolate	Geographic origin	Host	Date & source
Rigidoporus lignosus			
FCI2	Ivory Coast (South East)	*H. brasiliensis*	1978, Botton
FCI3	Ivory Coast (South East)	*H. brasiliensis*	1989, Van Cahn
FCI7	Ivory Coast (South West)	*H. brasiliensis*	1989, Van Cahn
FCI9	Ivory Coast (South West)	*H. brasiliensis*	1989, Van Cahn
RP1	Ivory Coast (South East)	*H. brasiliensis*	1978, Galliano
FCA1	Cameroon (South West)	*H. brasiliensis*	1987, Van Cahn
FCA3	Cameroon (South West)	*H. brasiliensis*	1989, Despréaux
FGA2	Gabon (North)	*H. brasiliensis*	1989, Despréaux
FGA3	Gabon (North)	*H. brasiliensis*	1991, Louanchi
FGA4	Gabon (North)	*H. brasiliensis*	1991, Louanchi
FGA6	Gabon (North)	*H. brasiliensis*	1991, Louanchi
FGA7	Gabon (South)	*H. brasiliensis*	1991, Louanchi
FGA8	Gabon (South)	*H. brasiliensis*	1991, Louanchi
FID1	Indonesia (Sumatra)	*H. brasiliensis*	1988, Despréaux
FID2	Indonesia (Sumatra)	*H. brasiliensis*	1989, Despréaux
FID3	Indonesia (Kalimantan)	*H. brasilensis*	1989, Despréaux
FML1	Malaysia (South Peninsula)	*H. brasilensis*	–, Hashim
FML2	Malaysia (South Peninsula)	*H. brasilensis*	–, Hashim
FML3	Malaysia (South Peninsula)	*H. brasilensis*	–, Hashim
FML4	Malaysia (South Peninsula)	*H. brasilensis*	–, Hashim
Other *Polyporaceae* species[a]			
Rigidoporus lineatus (RL)	USA (CBS 167.67)	Carpophore	1965, Nobles
R. ulmarius (RU)	India (CBS 443.76)	*Picea smithiana*	–, Bakshi
R. vinctus (RV)	Costa Rica (CBS 174.71)	angiosperme wood	1973, Lowe
Heterobasidion annosum (Fa4)	France	*Picea abies*	1978, Delatour
H. annosum (Fa64)	France	*Picea abies*	1978, Delatour
Fomes fomentarius (FF)	Yougoslavia (CBS 311.82)	*Fagus sylvatica*	–, –
Polyporus squamosus (PS)	– (CBS 426.48)	*Ulmus sp*	–, Nobles
Polyporus tuberaster (PT)	Bulgaria (CBS 442.85)	*Quercus sp*	–, Szemerdzieva
Leptoporus chioneus (LC)	Belgium (CBS 171.79)	*Betula sp*	–, Demoulin
Other *Hevea* roots pathogens[a]			
Phellinus noxius (PN1)	Malaysia (IMI 108700)	*H. brasilensis*	1964, –
P. noxius (PNS)	Malaysia (IMI 327416)	Cacao	1988, Hiong
Ganoderma philippii (GP)	Malaysia (IMI 108700)	*H. brasilensis*	1964, –
Sphaerostilbe repens (SR)	Ivory Coast (IMI 135503)	*H. brasilensis*	1968, Guillaumin
Armillaria heimii (AH)	Gabon	*H. brasiliensis*	1988, Michels

[a] RL, RU, RV, FF, PS, PT and LC were obtained from the Centraalbureau Voor Schimmelcultures (CBS), Baarn, The Netherlands, PN1, PN2, GP and SR were obtained from the International Mycological Institute (IMI), U. K. (–) origin or date unknown.

with a pestle and mortar in liquid nitrogen. One ml of TE (Tris 10 mM, EDTA 1 mM, pH 7) was added to the resulting powder. The suspension was centrifuged at 25,000 g at 4 °C for 30 min. The supernatant was recovered and stored at −80 °C. The protein concentration was evaluated as described above.

Preparation of plant extracts for the ELISA test. Samples were taken from lateral roots and from main roots.

They were washed carefully in water. The roots were examined closely under a binocular microscope to ensure the absence of surface mycelium, then freeze-dried. Freeze-dried root portions (1g) were ground with 500 mg of Fontainebleau sand (Prolabo) in 20 ml PBS-T-PVP buffer (0.05% v/v Tween 20, 2% w/v Polyvinylpyrrolidone in PBS) using a pestle and mortar. The extracts were frozen at −20 °C. For the enzyme immunoassays, the extracts were stirred and allowed to stand for one hour before being deposited on microtitration plates.

Antisera production. Rabbits were immunized with soluble mycelial proteins from two isolates, FCI2 and FID2. The immunization protocol involved 5 intramuscular injections of the protein extract mixed v/v with incomplete Freund's adjuvant (Sigma, St Louis, MO), except for the first injection when the adjuvant was complete. The 0.25 ml injections (125 μg of proteins) were given in the upper leg of the rabbits every 5 days. After 7 weeks, the animal was given a 0.5 ml booster injection without adjuvant. Samples were taken from the ear at day 0 (T0, before immunization), day 68 (T68, at the time of the booster), day 72 (T72) and day 77 (T77). The sera, obtained by blood coagulation at 37 °C then centrifugation for 5 min at 3,000 g, were stored at −20 °C.

Immunoglobulin G purification and conjugate preparation. The γ-globulin (IgG) fractions were purified on protein-A-sepharose (Pharmacia, Uppsala, Sweden) according to Harlow and Lane [1988]. The conjugate was obtained by coupling the purified IgG with alkaline phosphatase as described previously [Balesdent *et al.*, 1995].

Enzyme linked immunosorbent assays (ELISA). Both the antigen-coated plate ELISA (ACP-ELISA) [Voller *et al.*, 1976] and the double antibody sandwich ELISA (DAS-ELISA) [Clark and Adams, 1977] were used in this study. In all cases, a volume of 100 μl per well was used at each step, and each sample was deposited in three randomly distributed wells per plates. The plates were washed three times with PBS-T (0.05% v/v Tween 20 in PBS) between the steps. ACP-ELISA was used to choose the best bleeding and to determine the titer of the sera. The first step consisted of depositing antigens diluted in carbonate buffer (0.05 M, pH 9.6) on the plates for 2 h at 37 °C. The raw serum diluted in PBS-T-PVP was added to the plates and incubated for 2 h at 25 °C with constant shaking. The goat anti-rabbit alkaline-phosphatase conjugate (Sigma) diluted 1:1000 (v/v) in PBS-T-PVP was added to the plates and kept overnight at 4 °C. DAS-ELISA was used to study the sensitivity and specificity of the method and to detect the fungus in root extracts. The plates were coated with 2 μg ml^{-1} of IgG in the carbonate buffer for two h at 37 °C. The fungal antigens or root extracts, diluted in PBS-T-PVP, were added and the plates were incubated for 2 hours at 25 °C with constant shaking. The conjugate, diluted in PBS-T-PVP at 1:1000 (v/v) for the assays on fungal extracts and 1:500 (v/v) for the assays on plant samples, was then added as above. For both techniques, the substrate (1 mg ml^{-1} p-nitrophenyl phosphate in diethanolamine buffer, pH 9.8) was added and the plates kept at room temperature. Four to 5 optical density (OD) readings were taken at 405 nm for approximately one hour after substrate deposition, using an Emax ELISA plate reader (Molecular Devices, Palo Alto, CA). The change in OD over time (d(OD)/dt) was calculated for each well. A standard range of a protein extract from isolate FCI2 was deposited on each plate for a comparison of values between plates. The analyses of variance of d(OD)/dt values were carried out using STATITCF software.

Isoelectric focusing (IEF) and Western blotting. IEF on servanet 3–10 geb, semi-dry transfert of the proteins to immobilon-P membranes (Millipore, Milford, MA) and blots treatments were performed according to Balesdent *et al.* [1995].

Results

Antiserum testing. The different serum samples were tested on a range of FCI2 and FID2 mycelial extract dilutions (Table 2). Preimmune sera (T0) did not react to the antigens, giving OD values not significantly different from the buffer control. With the other bleedings, the responses were positive for antigen concentrations ranging from 50 μg ml^{-1} to 5 ng ml^{-1}. In some cases, responses significantly different from the detection limit were observed for an antigen concentration of 0.5 ng ml^{-1}. The strongest responses were obtained with antisera sampled 4 days after the booster (T72). Only the T72 bleedings were used for western blotting, IgG purification and conjugate preparation.

Antiserum specificity in Western-blot analysis. IEF/Western-blot analysis revealed bands reacting with the two antisera for all the *R. lignosus* isolates. With the

Table 2. ACP-ELISA values obtained with different bleedings. Evaluation of the humoral immune responses of rabbits immunized with mycelial proteins of *L. lignosus* isolates FCI 2 or FID 2. Values given are the mean O, D of three replicate wells +/− standard deviation as determined by ACP-ELISA

Serum	Antigen dilution ($ng\ ml^{-1}$)	Bleeding (days after first immunization)			
		T0	T68	T72	T77
Anti-FCI2	50000	0.056 ± 0.004	1.912 ± 0.123	2.901 ± 0.071	2.574 ± 0.225
	5000	0.056 ± 0.002	1.649 ± 0.063	2.462 ± 0.200	2.020 ± 0.157
	500	0.060 ± 0.008	0.670 ± 0.031	1.131 ± 0.058	0.878 ± 0.133
	50	0.057 ± 0.005	0.175 ± 0.011	0.468 ± 0.015	0.197 ± 0.007
	5	0.073 ± 0.085	0.085 ± 0.011	0.145 ± 0.013	0.108 ± 0.015
	0.5	0.072 ± 0.022	0.067 ± 0.006	0.095 ± 0.007	0.089 ± 0.019
	Buffer	0.055 ± 0.008	0.064 ± 0.002	0.056 ± 0.009	0.064 ± 0.005
	Detection limit	0.079	0.070	0.083	0.079
Anti-FID2	50000	0.082 ± 0.003	1.398 ± 0.096	2.694 ± 0.019	1.927 ± 0.127
	5000	0.082 ± 0.005	1.372 ± 0.063	2.538 ± 0.060	1.378 ± 0.050
	500	0.079 ± 0.005	0.628 ± 0.009	1.176 ± 0.084	0.510 ± 0.016
	50	0.072 ± 0.001	0.183 ± 0.009	0.326 ± 0.011	0.169 ± 0.005
	5	0.079 ± 0.004	0.103 ± 0.012	0.141 ± 0.007	0.124 ± 0.011
	0.5	0.083 ± 0.005	0.088 ± 0.006	0.089 ± 0.007	0.108 ± 0.007
	Buffer	0.067 ± 0.004	0.063 ± 0.004	0.078 ± 0.003	0.079 ± 0.007
	Detection limit	0.079	0.082	0.087	0.100

anti-FCI2 serum, the banding patterns differed from one isolate to another. Clearly distinct patterns were obtained between the isolates from Africa and those from Asia (Fig. 1). The African isolates were characterized by a major band coded pr 4 and by an intensely coloured band coded pr 1. The Asian isolates were all characterized by a common band (pr 3). Another band, pr 2, was common to all the isolates except FML3 and FML4, for which coloration intensity was very low. For the other Polyporaceae species, only the three species of the *Rigidiporus* genus were recognized. A low intensity band was seen for *R. lineatus* (pr 1) and *R. vinctus* (pr 6) (Fig. 2a). In *R. ulmarius*, four bands were stained by the antiserum (pr 2, 3, 4 and 5) (Fig. 2a). *F. fomentarius, P. squamosus, P. tuberaster, H. annosum* and *L. chioneus* did not show any reaction to the serum (Fig. 2a). Of *Hevea* pathogen species, cross reactions were only seen for the PN2 isolate of *Phellinus noxius*, for which three bands of very low intensity could be distinguished (pr 9, 10 and 11). For the other *P. noxius* isolate and the isolates of the other species, no band formed with this antiserum (Fig. 2b).

With the anti-FID2 serum, identical patterns to those seen with the anti-FCI2 serum were obtained for all the *R. lignosus* isolates (data not shown). Likewise, only species of the *Rigidoporus* genus were recognized. In this case, the immunostaining was more intense and involved the same bands as with the anti-FCI2 serum (Fig. 2c). There were two additional strongly stained bands for *R. lineatus* (pr 7 and 8). For the other *Hevea* pathogens, a band (pr 12) of very low intensity was common to *P. noxius* (PN2), *G. philippii* and *S. repens*.

IgG specificity in DAS-ELISA analysis. Irrespective of the IgG used, the 20 *R. lignosus* isolates were detected by DAS-ELISA (Fig. 3), but quantitative differences were seen among the isolates. The mean ELISA response varied depending on the antiserum and the isolate tested. With the anti-FCI2 IgGs, the OD means varied form 0.515 (FCI3) to 0.122 (FML3) (Fig. 3a). Variance analysis revealed significant differences between the isolates, but no clearly distinct groups could be determined. Nevertheless, apart from a few exceptions, the African isolates responded more

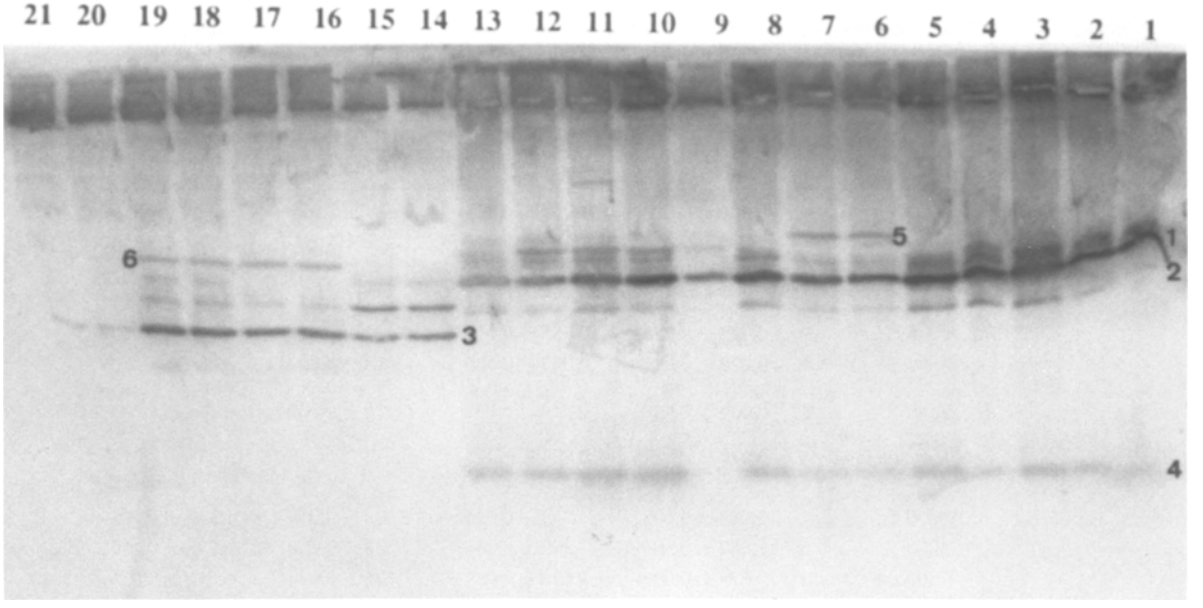

Fig. 1. Western blot analysis of *R. lognosus* isolates. Soluble mycelial proteins (6μg/lane) were separated on IEF gels and the blot was developed using an anti-FCI2 serum diluted to 1/1000. Isolates from Ivory Coast (1: FCI2, 2: FCI3, 3: FCI7, 4: FCI9, 5: RP1), Cameroon (6: FCA1, 7: FCA3), Gabon (8: FGA2, 9: FGA3, 10: FGA 4, 11: FGA6, 12: FGA7, 13: FGA8), Indonesia (14 and 15: FID1, 16: FID2, 17: FID3) and Malaysia (18: FML1, 19: FML2, 20: FML 3, 21: FML4).

strongly than the Asian isolates (Fig. 3a). The responses obtained with the anti-FID2 IgGs were lower than those obtained with the anti-FCI2 IgG (Fig. 3b). Variance analysis also revealed significant differences between isolates. The ELISA responses were very weak and not significantly different from the buffer control for the isolates of most of the Polyporaceae species studied (Fig. 4a). Only *R. ulmarius* revealed substantial cross reaction (Fig 4a). The ELISA response of *R. ulmarius* was similar to that obtained with certain *R. lignosus* isolates. Irrespective of the IgGs used, no cross reaction was seen with *R. lineatus* and *R. vinctus*. Likewise, no cross reactions were seen with the other *Hevea* root pathogens (Fig. 4b).

Sensitivity of the DAS-ELISA method. As with ACP-ELISA, the detection limit was between 0.5 and 5 ng ml^{-1} for both IgGs (Fig. 5). The OD means obtained with the anti-FCI2 IgGs were markedly higher than those obtained with the anti-FID2 IgGs.

Application of the DAS-ELISA technique to the detection of R. lignosus *in plants.* The IgGs chosen for this study were those directed against the soluble mycelial proteins of isolate FCI2, as the responses obtained were more intense and more specific than those obtained with the anti-FID2 serum. The OD values obtained for all the samples of the different infected root systems were significantly higher ($p = 0.001$) than those obtained with the healthy controls (Fig. 6). For seedlings, the OD values for the infected root samples varied from 0.101 to 0.310. ELISA responses differed not only between isolates, but also between plants infected with the same isolate (Fig. 6a). The root samples inoculated with isolate FID2 gave the lowest ELISA responses. For inoculated *in vitro* plantlets, the mean OD values ranged from 0.162 to 0.621 for the main root samples and from 0.175 to 0.768 for the lateral root samples (Fig. 6b). In contrast, healthy controls gave OD values ranging from 0.069 to 0.089. Apart from the strong responses obtained with the root system of one plant inoculated with FML2 which had severe root colonization, relatively uniform responses were obtained between the root samples inoculated with a given isolate. As with seedlings, isolate FID2 gave the weakest responses.

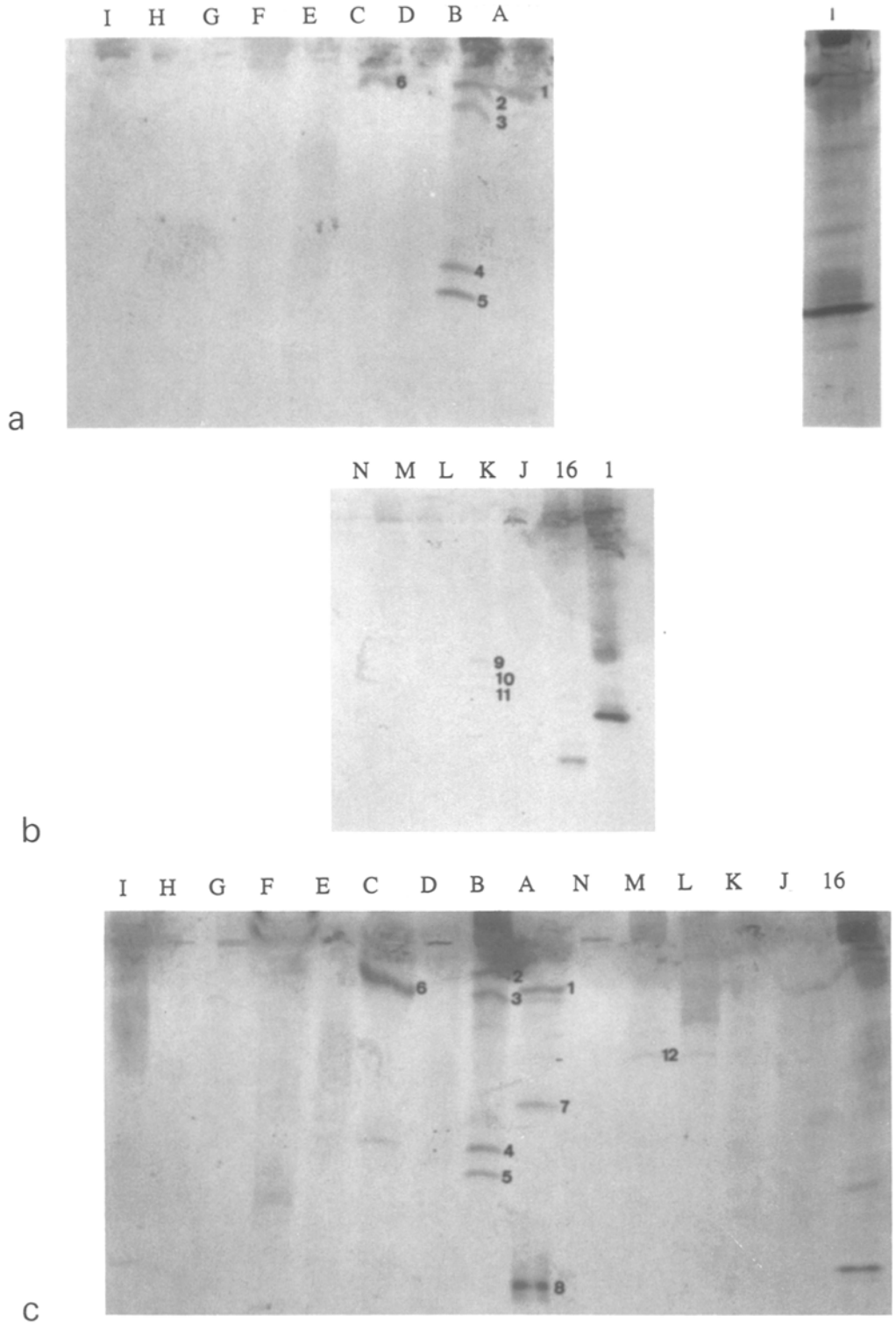

Fig. 2. IEF/Western blots of *Polyporaceae* species and Hevea's pathogens. Soluble mycelial proteins (6μg/lane) were separated on IEF gels and blots were developed using Anti-serum FCI2 (*a* and *b*), and *c* FID2 diluted to 1/1000. *Rigidoporus lignosus* (1: FCI2, 16: FID2), *R. lineatus* (A), *R. ulmarius* (B), *R. vinctus* (C), *H. annosum* (D: Fa4, E: Fa64), *F. fomentarius* (F), *P. squamosus* (G), *P. tuberaster* (H), *L. chioneus* (I), *P. noxius* (J: PN1, K: PN2), *G. philippii* (L), *S. repens* (M), *A. heimii* (N).

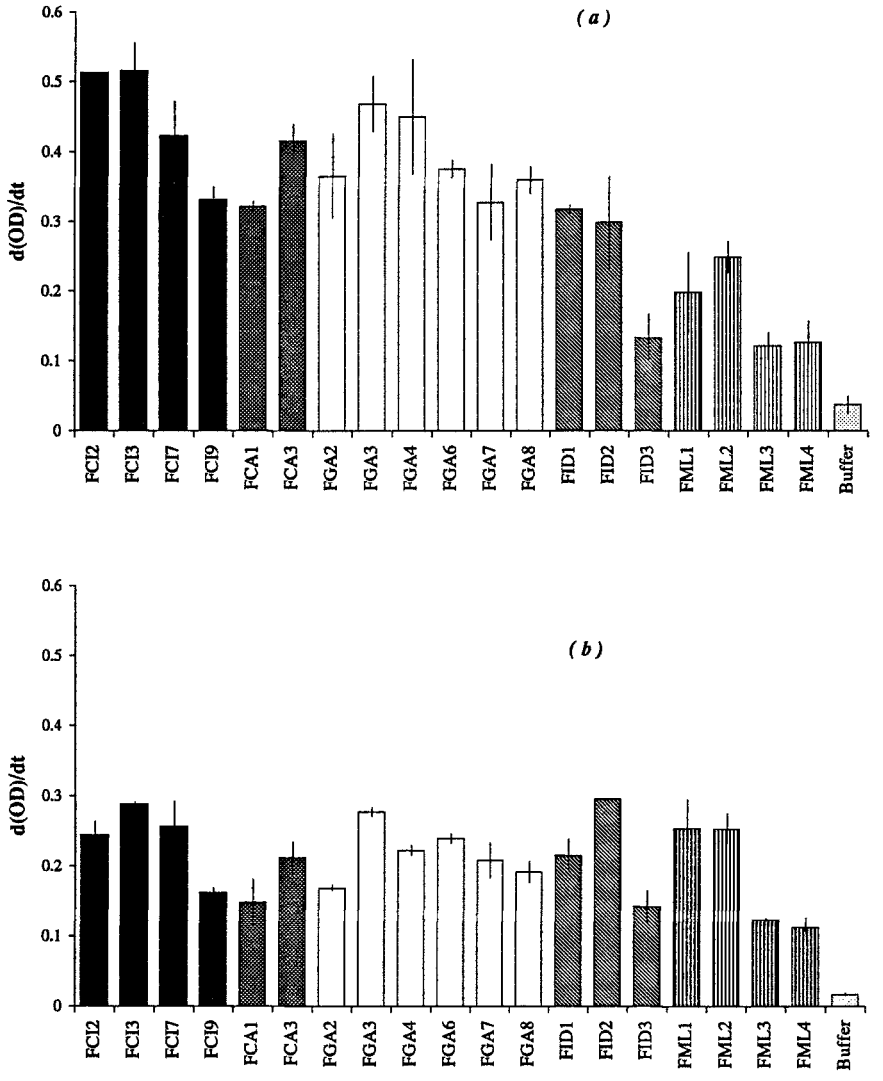

Fig. 3. DAS-ELISA analysis of *R. lignosus* isolates. IgG (2 μg ml^{-1}) were directed against FCI2 (*a*) or FID2 (*b*). Soluble mycelial proteins of isolates were adjusted to 2 μg ml^{-1}. The conjugate was diluted to 1:1000. Geographic origin of isolates: ■: Ivory Coast; □: Cameroon; □: Gabon; \\\: Indonesia; ||: Malaysia. Vertical bars represent twice the standard error.

Discussion

Two polyclonal sera directed against *Rigidoporus lignosus* were produced after intramuscular injections of soluble mycelial proteins. An analysis of protein banding patterns had already revealed differences between *R. lignosus* and other taxonomically related species [Louanchi, 1993]. These differences led us to choose soluble mycelial proteins of *R. lignosus* as the immunogen. In addition, a protein and isoenzyme analysis of 20 *R. lignosus* isolates had already revealed substantial intraspecific polymorphism correlated to the geographical origins of the isolates [Fofana *et al.*, 1992]. The two isolates chosen for immunization in this study, i.e. FCI2 and FID2, were representative of this diversity.

Western Blot analysis confirmed this polymorphism since banding pattern differences were seen between isolates, allowing a discrimination of african and asian isolates. These two groups were also differentiated by the restriction patterns of the intergenic region of the r-DNA genes [Louanchi, 1993]. IEF/western blotting therefore appears to be an additional characterization technique that reveals the genetic diversity of *R. lignosus* populations. Despite the protein differences observed between isolates FCI2

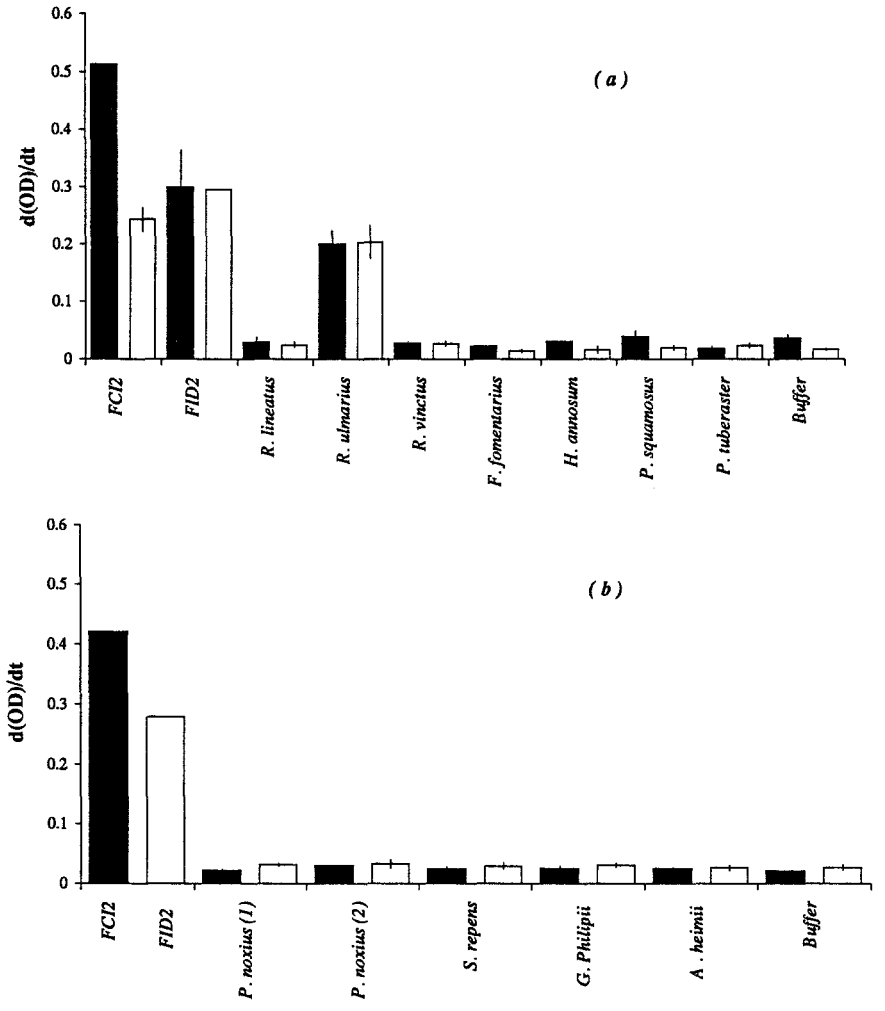

Fig. 4. Specificity of DAS-ELISA. Cross reactions were assessed using 2 μg ml^{-1} soluble mycelial proteins of isolates of *Polyporaceae* species (*a*) and Hevea's pathogens (*b*) IgG (2 μg ml^{-1}) were directed against FCI2 (■), or FID2 (□)and the conjugate was diluted to 1:1000. Vertical bars represent twice the standard error.

and FID2, the antibodies produced against one or the other of these isolates were not qualitatively different, since the banding patterns obtained following Western blotting did not depend on the antiserum used.

In DAS-ELISA, the two antisera recognized all the *R. lignosus* isolates, but significant variations were observed between isolates. These quantitative differences were not correlated with the geographical origin of the isolates. The qualitative differences seen in Western blots were apparently not reflected in the DAS-ELISA responses.

No cross reaction was observed for most of the 12 Polyporaceae species or the other *Hevea* root rot agents used in the study. Only *R. ulmarius* revealed strong cross reactions with the two antisera in DAS-ELISA analysis and Western blotting. In fact, the comparisons of sequences in a conserved domain at the 5' end of RNA 285 showed that *R. ulmarius* is the species closest to *R. lignosus* [Louanchi, 1993]. These cross reactions are not a limiting factor for detection purposes, because *R. ulmarius* only exists in temperate regions and is therefore not found on *Hevea*. For *R. lineatus* and *P. noxius*, species taxonomically close to *R. lignosus* and likely to be found on *Hevea*, Western blotting revealed only slightly stained bands. The weak cross reactions could pose a problem for ELISA detection or diagnosis. However, these cross reactions are not detected when using DAS-ELISA, even with high

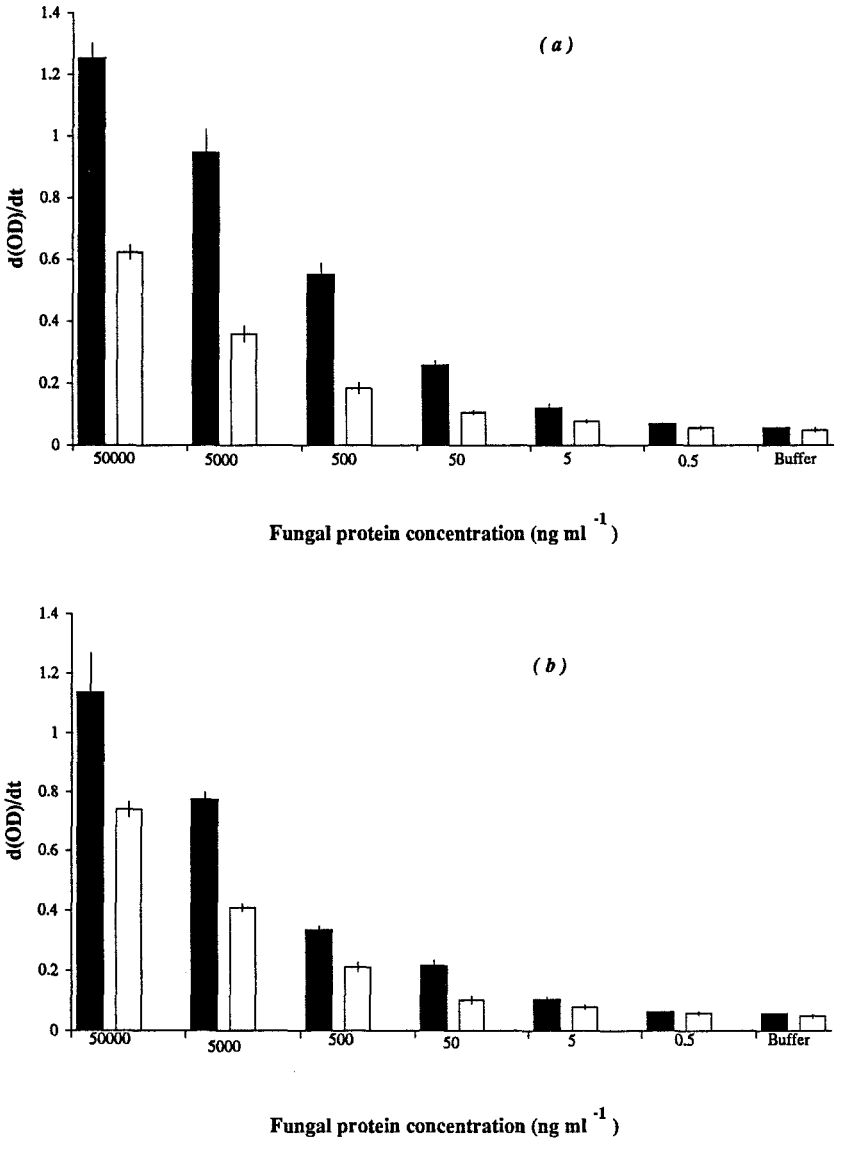

Fig. 5. Sensitivity of DAS-ELISA. IgG (2 μg ml^{-1}) were directed against FC12 (■) or FID2 (□) and the conjugate diulted to 1:1000. Soluble mycelial proteins of FCI2 (*a*) or FID2 (*b*) were serialy diluted in PBS-T-PVP (buffer). Vertical bars represent twice the standard error.

antigen concentrations. Be that as it may, the specificity could be improved, to avoid a wrong diagnosis, by cross-adsorption of the IgGs with antigens of the cross-reacting species, as has been proposed for *Serpula lacrimans* [Toft, 1993].

The sensitivity of the DAS-ELISA method using purified IgGs was checked against the homologous antigen. The sensitivity limit is below 5 ng of proteins per ml, which corresponds to the detection limit of the conventional DAS-ELISA method [Harlow and Lane, 1988]. However, differences in response intensity were observed depending on the IgGs used. For testing of inoculated plants, the antiserum giving the strongest responses was chosen, i.e., anti-FCI2 IgG. As an initial approach, the DAS-ELISA method was tested using root samples from seedlings and *in vitro* plantlets inoculated artificially. Under these conditions, the fungal proteins were detected in the plant. No reaction was observed with healthy plant samples.

The ELISA responses were stronger when the root portion was in direct contact with the piece of wood carrying the inoculum, probably reflecting severe root

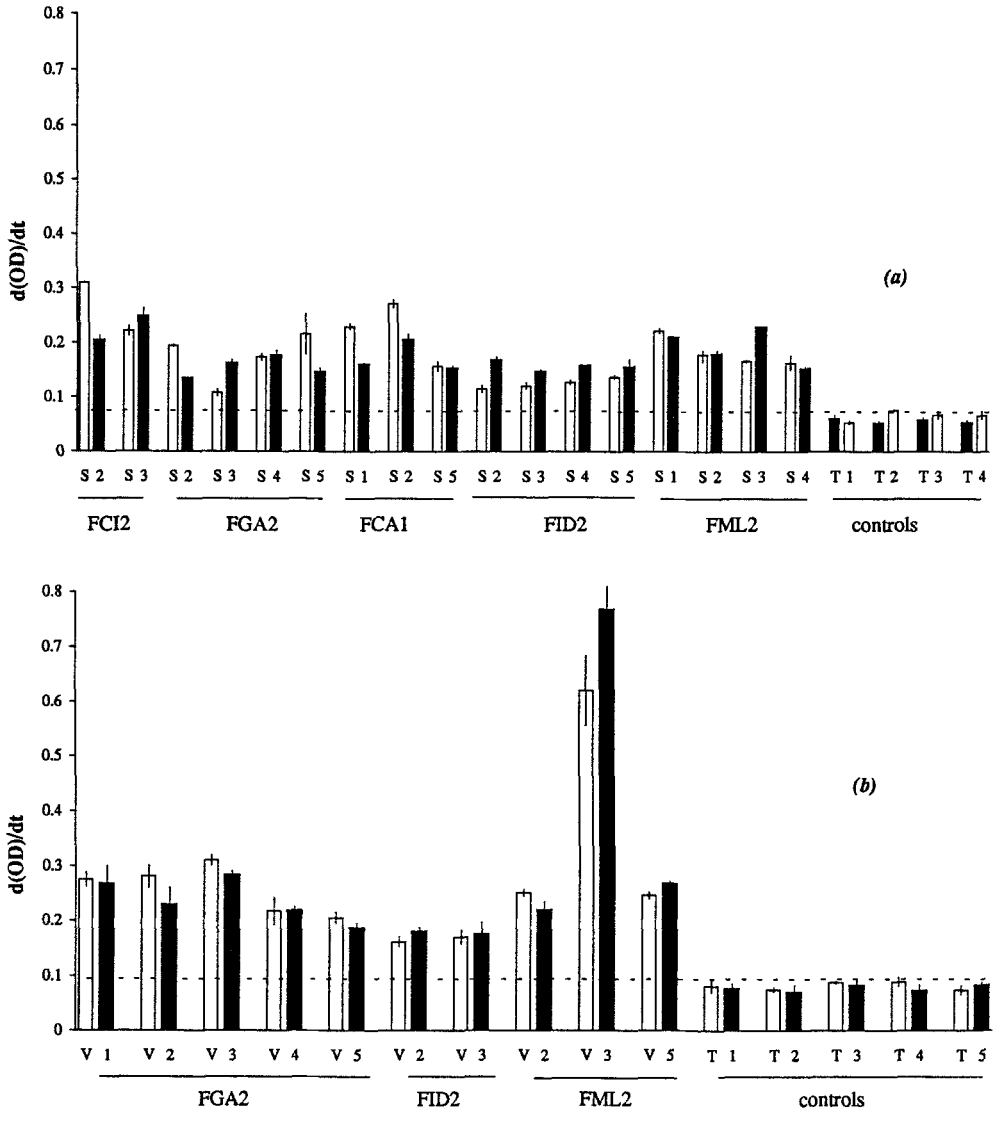

Fig. 6. Detection of *R. lignosus* in plants using DAS-ELISA. IgG (2 μg ml^{-1}) were directed against FCI2 and the conjugate was diluted to 1:500. Seedling (*a*) and vitroplants (*b*) were inoculated with isolates FCI2, FGA2, FCA1, FID2 or FML2 (see table 1). For each plant, primary roots (□) and secondary roots (■) were analyzed separately. Vertical bars represent twice the standard error. Dotted lines represent the detection limit (control mean + 3 times the standard deviation).

colonization. These results seem to confirm the role of woody debris colonized by the fungus in tree infection in plantations [Pichel, 1956; Fassi, 1964]. Positive but the weaker responses were obtained from samples having a ring of mycelium around the roots or where the mycelium was only in contact with the roots. DAS-ELISA responses were particularly weak for plants inoculated with FID2. This probably does not indicate a lesser development in the plants, since the ELISA responses obtained with FID2 proteins from *in vitro* cultures were also among the weakest. One of the plants contaminated with isolate FML2 gave very strong ELISA responses. The severe root system colonization seen in this particular case can be attributed to the plant's very poor physiological condition right from the time of inoculation. Apart from this sample, the responses obtained for a given isolate were more uniform with *in vitro* plantlets than with seedlings. This suggests that *in vitro* plantlets are more suitable for pathogenicity studies than seedlings which are not genetically fixed. This suggestion is backed up by various authors who have compared the pathogenicity of

several isolates from West Africa on GT1 seedlings [Nicole *et al.*, 1985; Nandris *et al.*, 1987]. Although substantial variations in pathogenicity were observed between different isolates, these authors do not rule out the possibility of within-family variability in GT1 seedlings.

The possibility of using specific antisera to detect pathogens in plants opens up a range of applications for the study of the host-parasite interaction. In particular, contamination in a 'rhizotron' combined with immunocytochemical studies could provide a clearer understanding of how infection occurs. Immunoassays could also lead on to applications aiding the search for resistant genotypes as has been proposed for the resistance of potato to *Phytophthora infestans* [Harrisson *et al.*, 1991]. In plantations, the potential for using the ELISA test to detect contaminated trees is still to be assessed, as regards both specificity and sampling technique. This would considerably enhance current detection methods by ensuring a more reliable delimitation of the infection foci. Finally, these methods could be used for more accurate monitoring of the disease in plantations and to answer certain epidemiological questions.

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