J. Rubb. Res., **3(2)**, 115–128

Role of Cassiicolin, a Host-selective Toxin, in Pathogenicity of Corynespora Cassiicola, *Causal Agent of a Leaf Fall Disease of* Hevea

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Histological study confirmed rapid and direct penetration of Corynespora cassiicola *(12 h after inoculation) throughout the lower epidermis, observed also in previous work. The fungal colonisation of leaf tissues was intracellular, irrespective of clonal resistance. For the susceptible clone, fungus penetration led to an important collapse of abaxial epidermis and to cell disorganisation in front of the hyphal progression, characterised by nuclear degeneration and absence of starchy grains. This observation demonstrated that* C. cassiicola *behaved as a necrotrophic fungus. For the resistant clone,* C. cassiicola *invasion was restricted to a few cells leading to a hypersensitive-like reaction. The production of toxin in culture filtrate of the fungus, was confirmed. This toxin, named cassiicolin, reproduced similar leaf disease symptoms as observed after fungal inoculation. The role of the host-selective toxin in* C. cassiicola *pathogenesis was demonstrated using toxin antibodies and pure toxin. Results have shown that cassiicolin was essential for pathogenicity and can be considered as the primary determinant to* C. cassiicola *pathogenesis. A close correlation between sensitivity of* Hevea *clone to cassiicolin and their susceptibility to the fungus has been proven using an aggressive isolate from Philippines. Higher toxin concentrations could overcome the resistance of GT 1 which exhibited resistance to lower concentrations. Thus, clonal resistance to cassiicolin was not absolute and seemed to be strictly linked to the toxin concentration tested. The amount of toxin production by 11* C. cassiicola *isolates from different countries was positively correlated to their pathogenicity. On the basis of these results which must be confirmed with a wide range of isolates, the using of cassiicolin for assessing the level of resistance of* Hevea *clones to* C. cassiicola *is discussed.*

Corynespora cassiicola (Berk. & Curt.) Wei is a fungus of world-wide importance with a very wide host range. It has been reported on a great number of economically important crops from tropical to sub-tropical countries[1]. During the past decade, the pathogen has caused extensive damage to rubber tree plantations and may become a potential limiting factor in rubber yield

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in Asia. *Corynespora* leaf disease affects both mature and immature *Hevea brasiliensis* leaves, leading to brown necrotic lesions along the veins and finally to massive and repeated defoliation. These lead to die-back of shoot and death of affected trees. Since the first description of the disease, in 1958 in India on seedling nurseries[2], field observations report the existence of some resistant *Hevea* clones to *C. cassiicola*[3]. The host tissue invasion process and the resistant mechanisms remain unknown. Thus, biochemical knowledge of *C. cassiicola / H. brasiliensis* interaction appears necessary to perform genetic breeding of resistant clones. Several fundamental publications on this fungus covered sporulation, pathogenicity and epidemiology[4], symptomatology of photosynthetic intensity after infection[5], and the pathogenicity and polymorphism of different isolates[1,6–8].

A scanning electron microscopy study demonstrated the presence of an extracellular fibrillar sheath developed by the germ tubes of *C. cassiicola*[9]. This fibrillar sheath seemed to be involved in the mycelial adhesion to the leaf and also in fungal penetration. It appeared that *C. cassiicola* rapidly invaded host tissues by direct penetration of abaxial epidermis, rather than by stomatal penetration. Therefore, no difference in fungal penetration process was observed between resistant and susceptible *Hevea* clones. Previous studies, indicated that non-specific defense mechanisms of resistance (such as phytoalexin, PR-proteins, active oxygen species and lignification) were induced too late or were inefficient to contribute to the rapid inhibition of fungus invasion in the leaves of resistant clone[9,10].

The characterisation of pathogenicity factors that account for the ability of *C. cassiicola* to cause *Hevea* leaf fall disease are essential to understand the molecular events that determine the host-pathogen interaction and also, to identify the mechanism involved in clonal resistance.

It is well established that many plant pathogenic bacteria and fungi produce phytotoxic compounds that play important roles in plant pathogenesis[11]. The majority of these toxins are non-selective toxins. These compounds affect a broad range of hosts than the producing organism infects. On the other hand, non-selective toxins do not reproduce the pattern of resistance and susceptibility of the host to the pathogen[12]. Non-selective toxins contribute to virulence or symptom development of the disease in which they occur[13], but they are not primary determinants of pathogenicity[14]. Host-selective toxins (HSTs), however act positively in virulence or pathogenicity. HSTs, produced by at least 16 species of fungi, are unique metabolites inferred to be involved in pathogenesis. They are toxic only to hosts susceptible to the fungus but not to resistant or non-host plants; and toxin production appears strictly correlated with pathogenicity. The precise specificity of these compounds and conventional genetic analyses of toxin production and pathogenicity, suggest that HSTs play a causal role in plant pathogenesis[15]. The study of HSTs and the diseases in which they occur contributes to fundamental knowledge about the process and regulation of disease susceptibility and resistance[14]. The first evidence of a toxic substance produced by *C. cassiicola* was reported by Onesirosan *et al.* (1975)[16]. Authors demonstrated that a culture filtrate of this fungus was toxic to excised tomato leaves. Similar results were obtained by Liyanage and Liyanage (1986)[17] and Purwantara (1987)[18] on *Hevea* leaves. A host-selective toxin produced by *C. cassiicola*, named cassiicolin, was purified and biochemically characterised for the first time[10].

The aim of this present work is to understand the fungus colonisation process, to determine more precisely the role of cassicolin in pathogenicity of *C cassicola* and to discuss the feasibility of using the toxin in screening *Hevea* clones for resistance to this fungus

MATERIALS AND METHODS

Fungal Culture and Leaflet Inoculation

The single conidium cultures of *C cassicola* were grown in the dark at 25°C on Potato Dextrose Agar (PDA) medium Sporulation was induced on a 7-day-old culture by exposing for three days at 28°C to continuous light The conidial suspension was titrated at 2.3×10^4 conidia mL^{-1} in sterile distilled water Inoculation was performed by positioning 10 µL of conidial suspension on the abaxial side of leaflet (C stage) placed in Petri dishes in moist conditions After incubation (72 h at 28°C photoperiod 12 h), resistance and susceptibility were estimated by the measure (mm^2) of necrosis size on the infected leaves The *Hevea* clones used in the study are listed in *Table 1*

Light Microscopy Technique

Necrotic zones from infected leaves previously fixed in paraformaldehyde 2%–glutaraldehyde 1% solution were dehydrated in gradual series of absolute ethanol Samples were embedded in Technovit 7100 resin for 12 h at room temperature The blocks were cut transversely on a Historange LKB microtome into 3 µm thick sections Tissue sections were double stained with Periodic Acid-Schiff reagent (PAS) and with Naphthol Blue Black reagent (NBB) and observed with a Leitz Laborlux microscope

Fungus Culture for Phytotoxin Production

A modified Czapeck liquid medium was used for cassicolin production[19] One hundred millilitres of this liquid medium was placed

TABLE 1 *HEVEA* CLONES USED IN THE SCREENING OF THEIR RESISTANCE/SUSCEPTIBILITY TO THE CCP *C CASSICOLA* ISOLATE CLONES WERE GRADED IN A DESCENDING ORDER OF RESISTANCE FROM IAN 6486 (THE MOST RESISTANT) TO PB 330 (THE MOST SUSCEPTIBLE)

IAN 6486	IRCA 18	IRCA 1159	RF 6
P 9	PB 235	PB 217	IRCA 111
GT 1	FRX 3864	RF 2	RO 38
IRCA 305	F 4506	IRCAGY 7	RRIM 805
BPM 24	IRCAGY 8	RRIC 100	IAN 873
AC 58	IRCA 144	PB 310	FX 4425
IRCA 41	RRIM 712	IRCA 408	PB 5/51
PUA 8	PB 49	IRCA 1262	F 4542
RF 5	IRCA 1232	IAN 710	CIRAD 3
RRIM 600	IRCA 331	IRCA 22	RRIM 901
IRCA 209	IAN 717	PB 260	IAN 710
RRIM 729	IRCA 122	IRCA 109	PB 330
IAN 2878	IRCA 416	IRCA 631	

in 500 mL flasks. Each flask was inoculated with three mycelial plugs (5 mm in diameter) from 7-day-old culture of *C. cassiicola*. Liquid cultures were incubated without agitation at 25°C (photoperiod: 12h) for 12 days and filtered under vacuum through a 0.22 μm Millipore membrane. The toxin was purified and biochemically characterised[10].

Toxin Activity Bioassay

Two assay procedures were used to determine toxin ability to cause host cell damage. In the leaf-wilting bioassay, petioles of *C. cassiicola* susceptible *Hevea* leaflets (PB 260) were excised from the stem under water and immediately transferred to 20 mL flasks containing toxin fraction previously diluted in 5 mL of distilled water. The toxic activity caused wilting of *Hevea* leaflets with early necrosis. The wilting intensity was quantified (water loss estimation) by the ratio of fresh weight / dry weight in percent of a control. In the leaf-puncture bioassay, detached leaflets were placed in Petri dishes in moist conditions and 20 μL drops of phytotoxin samples were placed on a needle-puncture wound on the abaxial leaf surface. The toxicity was estimated by measuring the size of necrotic lesions on the leaflets. In the two bioassays control leaflets were treated with distilled water or non-inoculated Czapeck liquid medium, and the leaflets were incubated at 25°C (photoperiod: 12 h) until symptoms developed (1 to 3 days).

Antibody Production and Serological Technique

Polyclonal antibodies were raised against purified cassiicolin in rabbits at the Altergen Society (France). The antisera were stored at –30°C. For dot-blot immunoassay, samples (10 μL) were applied under vacuum on immobilon-P^{sq} membrane (Millipore), which was incubated for 2 h at room temperature with 5% non-fat dry milk in Phosphate Buffer Saline (PBS). After rinsing four times (5 min) in PBS, the membrane was incubated in the same conditions with the anti-toxin antiserum (1:1000 dilution) in PBS containing 1% non-fat dry milk. Following incubation, the membrane was washed four times (5 min) with PBS and incubated for 1 h at room temperature with goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma, 1:30 000 dilution) in PBS-milk. The membrane was then rinsed three times (5 min) with PBS and twice with Alkaline Phosphate Buffer (APB), pH 9.6. Alkaline phosphatase activity was revealed after membrane incubation in the dark at room temperature in substrate buffer (Sigma).

RESULTS AND DISCUSSION

Leaf Invasion Process by *C. cassiicola*

Microscopy study of resistant and susceptible leaves of *Hevea* inoculated with an aggressive isolate of *C. cassiicola* from Philippines (CCP) *(Figure 1)* revealed no difference between resistant and susceptible *Hevea* clone in the rapidity of the direct fungus penetration (12 h soon after infection) (*Figure 1 A* and *1 B*). The cellular progression of *C. cassiicola* in the two clones was intracellular *(Figure 1)*. An unidentified yellow brown substance accumulated in the necrotic zone of resistant and susceptible *Hevea* clone. We have shown that this substance does not play any important role in the resistance of *Hevea* to *C. cassiicola*[10]. A great difference in the efficiency of *C. cassiicola* to invade host tissues can be observed between resistant and susceptible *Hevea* clones. The colonisation by the fungus in the resistant clone was restricted to a few cells around the hyphae (hypersensitive like reaction) and fungal development seemed effectively stopped *(Figure 1 B–1 D)*. Around

this zone, the appearance of cell nuclei and the presence of starchy grains revealed the absence of infection Whereas, in the susceptible clone, the fungal invasion was accompanied by an important collapse of the epidermis cells *(Figure 1 A)* Moreover, majority of the cells, largely beyond the penetration zone, were empty, nucleus appearance revealed their degeneration and no starchy grains could be observed *(Figure 1 C)* Observations of cross sections of the susceptible infected leaves suggested the involvement of a phytotoxic compound rapidly secreted by *C cassiicola* during the initial invasion process

Phytotoxicity of Culture Filtrate and *Hevea* Clones Screening for Resistance

Toxin produced by *C cassiicola* was obtained from cultures grown in Czapeck liquid medium After 12 days, the culture filtrate was used for leaf bioassays The culture filtrate from CCP isolate was highly toxic only to susceptible *Hevea* leaves *(Figure 2)* The phytotoxicity led to wilting On the resistant clone, no leaf symptoms were observed The causal phytotoxic compound secreted by *C cassiicola* in the culture filtrate had been purified and biochemically characterised[10] The host-selective toxin was named cassiicolin and was also identified in conidial germination fluid Mass spectrometry showed the existence of only one molecular species in the pure fraction[10]

The correlation between tested *Hevea* clones *(Table 1)* susceptibility to the CCP isolate and their sensitivity to the toxin has been examined and proved to be very satisfactory *(Figure 3)* As a matter of fact, wilting was limited to 60%–65%, thus explaining the non-linear curve It appears that no *Hevea* clone was resistant to the fungus and sensitive to the toxin and, *vice versa*, no *Hevea* clone was susceptible to the fungus and resistant to the toxin These results demonstrated that the toxic fraction can be used in preliminary testing for assessing the level of resistance of *Hevea* clones to CCP isolate These results also suggested that cassiicolin play a primordial role in the *C cassiicola* (CCP) pathogenesis

Role of Cassiicolin in Pathogenesis and Level of Clonal Resistance to Cassiicolin

Cassiicolin antibodies and pure toxin could be used to confirm the important role of cassiicolin in *C cassiicola* pathogenesis Inoculation of susceptible *Hevea* leaves with a CCP conidial suspension containing antibodies against cassiicolin strongly decreased the severity of infection compared to a normal inoculation (without toxin antibodies) *(Figure 4)* Similarly, inoculation of susceptible *Hevea* leaves with a non-pathogenic isolate (BGA3) mixed with pure toxin (5 μg mL^{-1}) increased intensity of the leaf symptoms It was previously verified that the rate of conidial germination was not affected by cassiicolin antibodies and pure toxin These results clearly showed that cassiicolin could be considered as the primary determinant of the *C cassiicola* pathogenicity

Pure toxin reproduced similar disease symptoms as induced by the fungus, such as necrosis and brown discoloration of the veins giving a typical 'fish-bone' appearance *(Figure 5)* The resistance to cassiicolin *(Figure 3)* of resistant clones to *C cassiicola* (CCP isolate) was not absolute as it is dependant on the concentration of toxin tested *(Figure 5)* The application of 20 μL droplets containing 10 μg mL^{-1} of cassiicolin to GT 1 (resistant clone) induced leaf symptoms *(Figure 5 A)* but not at lower concentrations PB 260, a clone very susceptible to *C cassiicola*, was sensitive at all cassiicolin concentrations tested *(Figure 5 B)* These results suggested that the threshold of clonal resistance to *C cassiicola* isolates is linked to the quantity of toxin produced

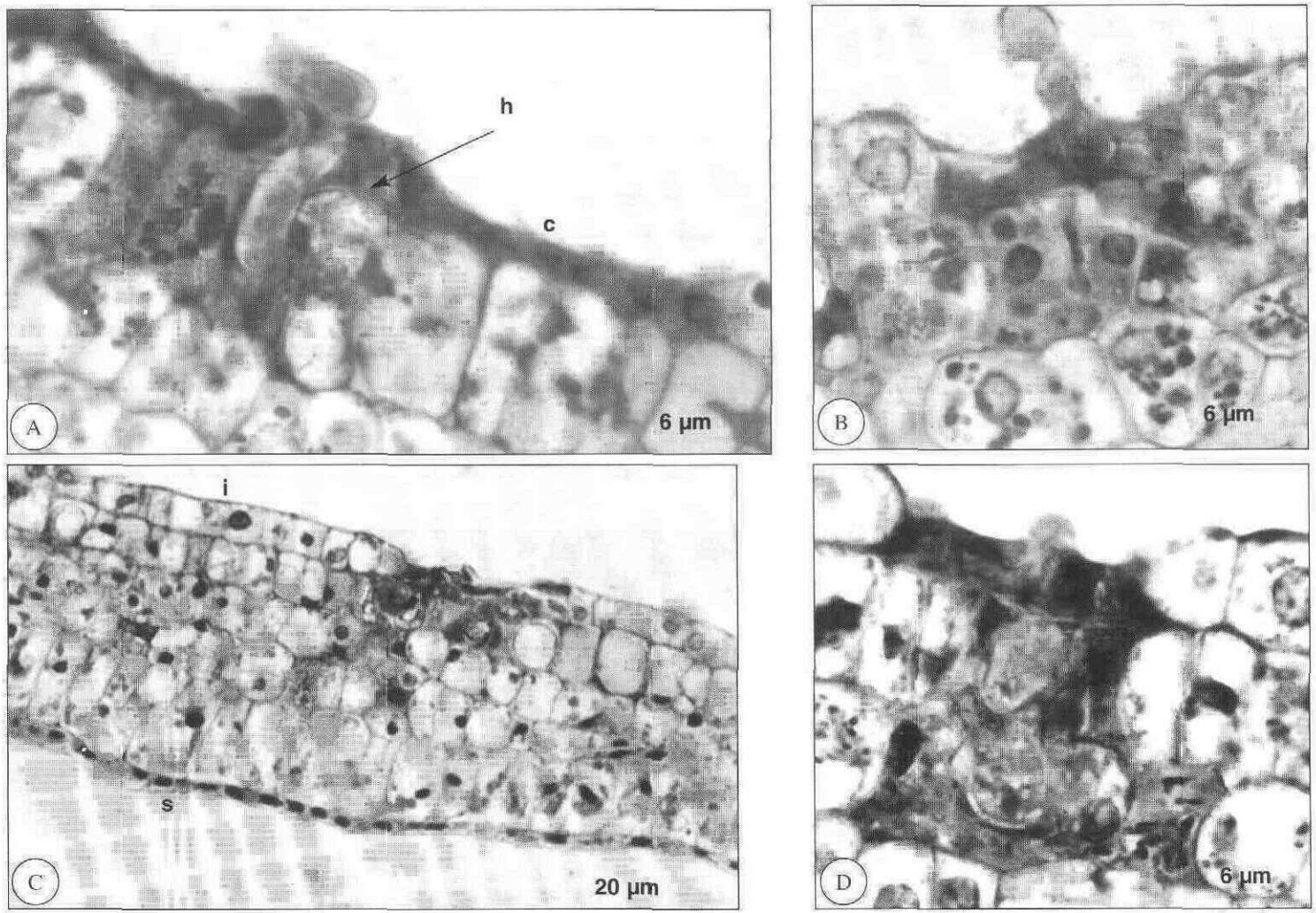

Figure 1. Light microscopy observations of cross sections of infected Hevea *leaves from susceptible (A-C) and resistant (B-D)* Hevea *clone inoculated with an aggressive isolate of* C. cassiicola *from Philippines (CCP). (A-B), 12 h after infection; (C-D), 24 h after inoculation. Collapse: c; hyphae: h; lower epidermis: i; upper epidermis: s.*

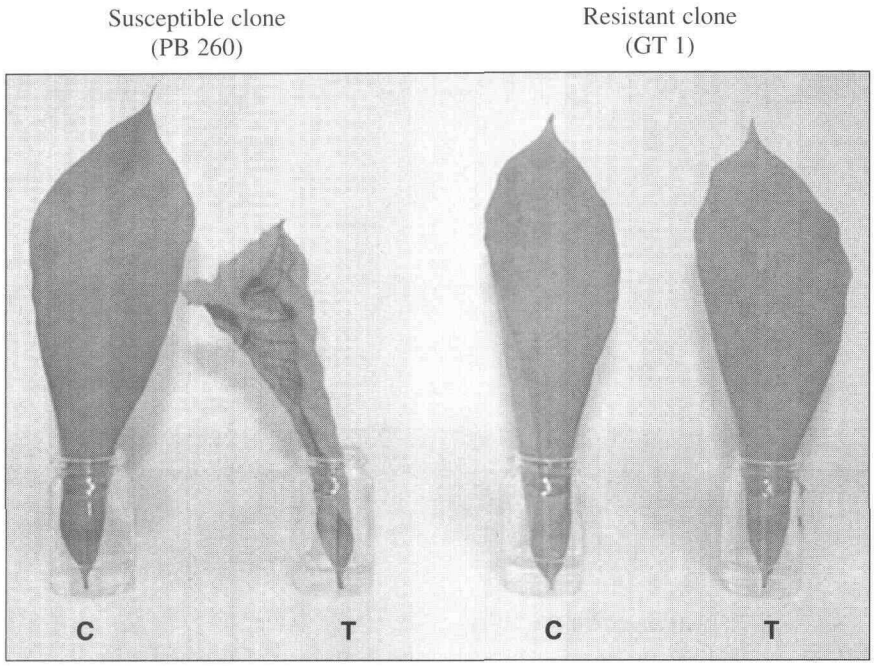

Figure 2. Effect of CCP culture filtrate (12-days-old) on resistant (GT 1) and susceptible (PB 260) Hevea *leaves, at 48 h after incubation (leaf-wilting bioassay). Control: C; treated: T.*

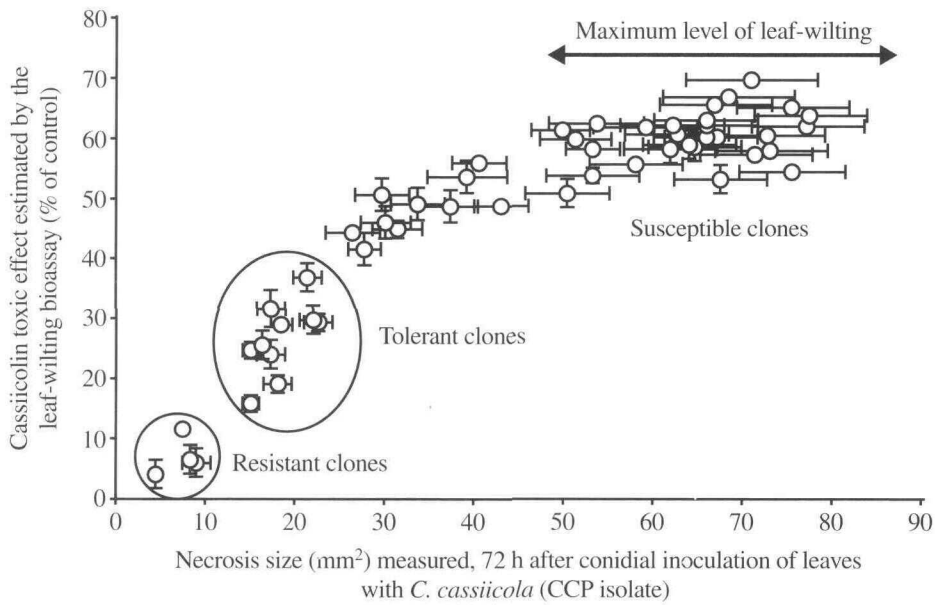

Figure 3. Correlation between the sensitivity of Hevea *clones to cassiicolin from CCP culture by the leaf-wilting bioassay and the susceptibility of* Hevea *clones estimated by the size of necrotic lesions following conidial inoculations (r^2 = 0.822). Each point represents a* Hevea *clone listed in* Table 1. *Significant level (range bar) corresponds to the variability obtained after thirty repetitions for each clone.*

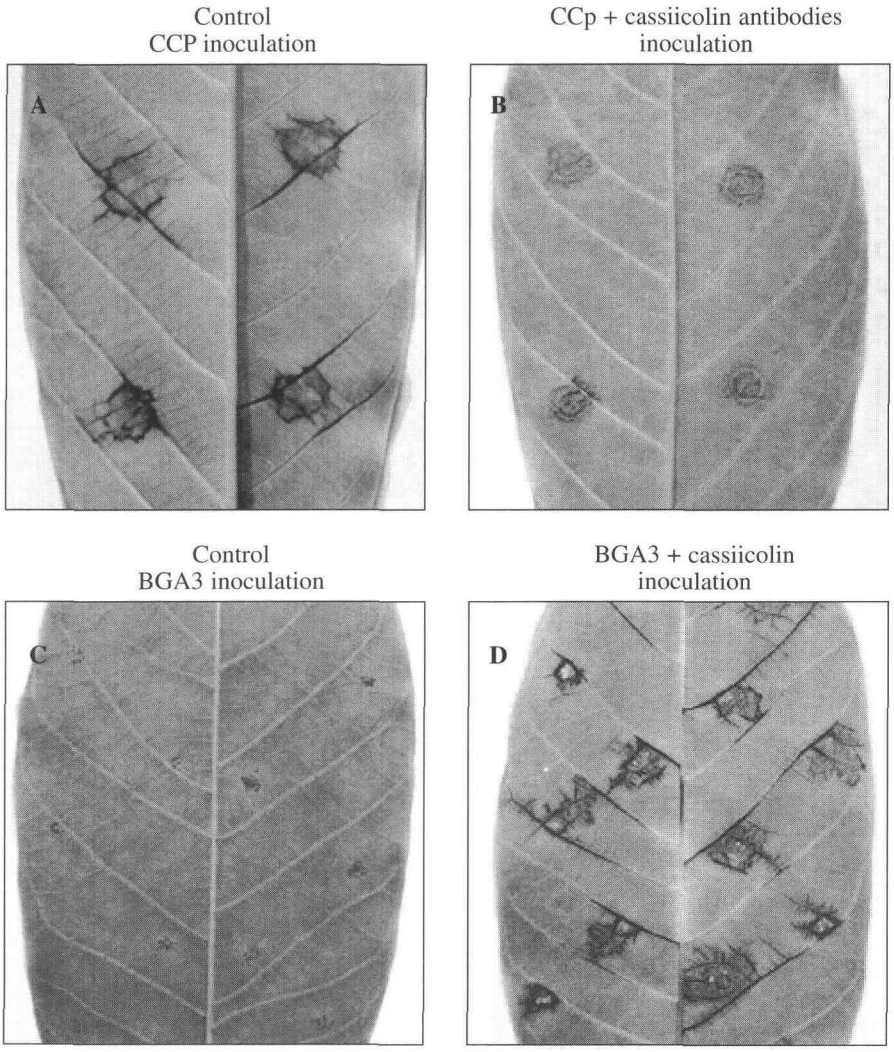

Figure 4. Effect of cassiicolin antibodies and pure toxin on the aggressiveness of a pathogenic (CCP) and a non-pathogenic (BGA3) isolates of C. cassiicola, *observed after conidial inoculation of leaves of susceptible* Hevea *clone (PB 260), 72 h after infection. A and C; controls inoculated with CCP and BGA3 isolates, respectively. B; CCP conidial suspension mixing with cassiicolin antibodies. D; BGA3 conidial suspension containing pure toxin at 5 $\mu g\ mL^{-1}$ concentration.*

Pathogenicity of *C. cassiicola* Isolates in Relation to Toxin Production

Toxin production by pathogenic and non-pathogenic isolates was compared. The pathogenicity of 11 isolates of *C. cassiicola* was estimated by the size of necrotic lesions measured 72 h after conidial inoculation of *Hevea* leaves *(Figure 6)*. Amongst the tested isolates, SRI5, CCP, BCA5 and BCA3 were

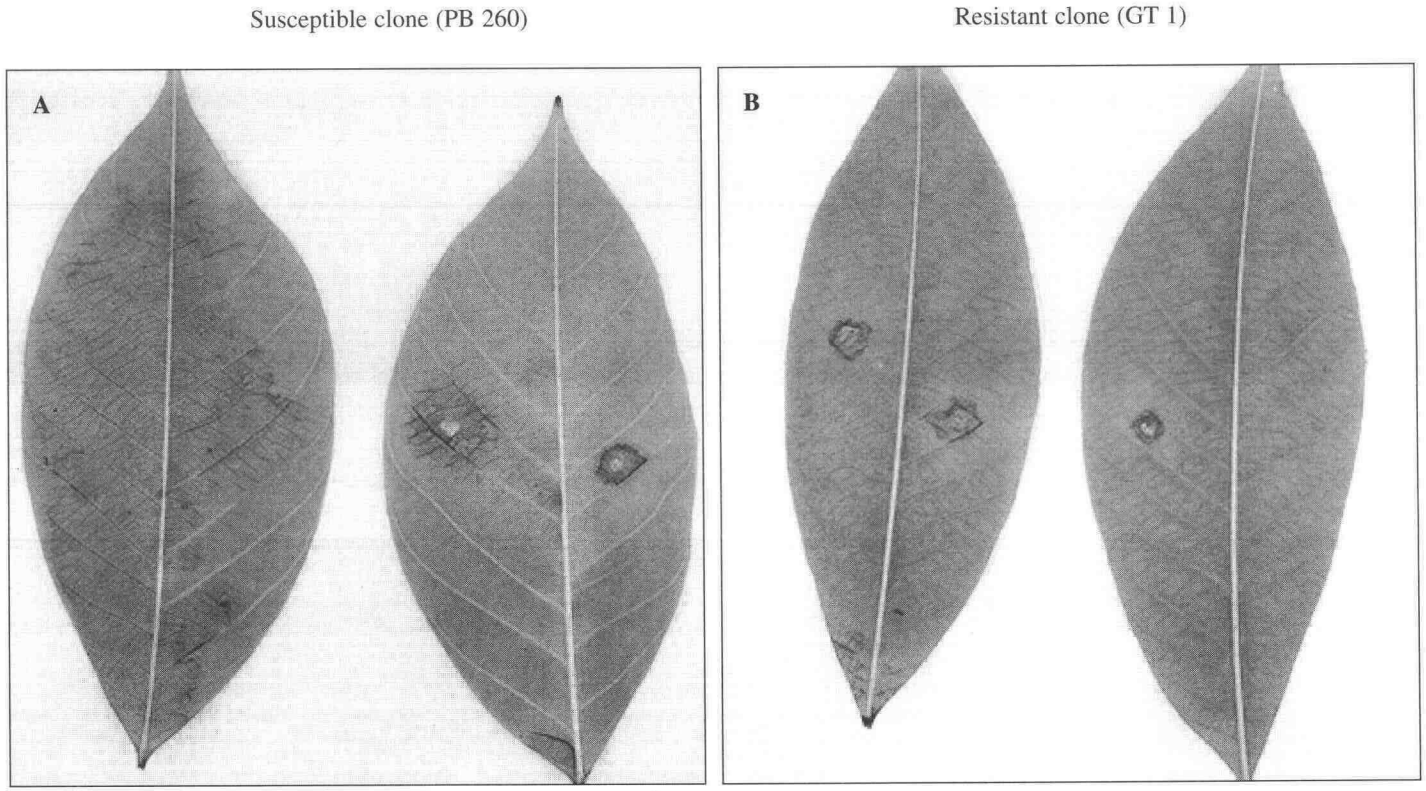

Figure 5. Effect of four cassiicolin concentrations, 5(a); 10(b); 15(c) and 20(d) μg mL^{-1}, on susceptible (PB 260) (A) and resistant (GT 1) (B) Hevea *clone to* C. cassiicola *(CCP isolate), 48 h after treatment. Biotests were realised according to the leaf-puncture bioassay.*

characterised as highly aggressive. Clone, GT 1 and IAN 6486 were resistant to all isolates except SRI5. The production of toxin by these isolates was estimated by the leaf-wilting bioassay using their culture filtrates *(Figure 7)*. The pathogenicity of isolates showed a positive correlation to the quantity of toxin production. Therefore, the difference in toxin production between SRI5, CCP and BCA5 was not significant and was not linked to their pathogenicity. These results were assigned to the physical limit of leaf wilting to 60%–65%. To overcome this fact, culture filtrate can be diluted or the antibody's colorimetric method can be used (Dot-blot) *(Figure 8)*. This method revealed a clear difference in quantity of toxin production between isolates. It appeared that the most pathogenic isolate, SRI5, produced a higher quantity of toxin than the other isolates. These results showed a close correlation between isolate pathogenicity and the quantity of toxin produced. Cassiicolin can be considered as the major factor in *C. cassiicola* pathogenicity. Only one toxin (cassiicolin produced by the *C. cassiicola* isolates) has been characterised (unpublished results).

CONCLUSION

This study is a part of our investigations to have a better understanding of *C. cassiicola / H. brasiliensis* interaction. This paper reports new observations on the infection process of C. cassiicola, namely the rapid necrotrophic colonisation of *Hevea* leaves. The occurrence of cell death at a distance from the hyphae during early necrosis demonstrated probably the presence and diffusion of a toxin produced by *C. cassiicola*. This toxin seems to play a key role in establishing the initial necrotrophic colonisation only in susceptible clones[20,21]. This HST, named cassiicolin, was previously purified and biochemicaly characterised in our laboratory[10]. HSTs have been critical factors in two other major diseases of crops in the United States in the 20th century (Southern corn leaf blight for example) and are also important factors in several other economically significant diseases throughout the world[14,22]. All known HSTs play a causal role in plant pathogenesis[14,15], the current results demonstrated: (i) a close correlation between sensitivity of the clones to the toxin and their susceptibility to the fungus; (ii) a positive correlation between toxin productivity and pathogenicity of the fungus isolates; (iii) the colonisation of the susceptible clone by the pathogen only occured when the toxin is present. The data indicated that the toxin produced by *C. cassiicola* plays a significant role as a disease determinant of *C. cassiicola*. This finding suggested that disease resistance was due to insensitivity to the cassiicolin[15,23,24]. The resistant mechanism to the cassiicolin was unknown but a primary study suggested the presence of a detoxification process similar to the resistance of HC-toxin and albicidin[22,25,26].

Resistance based on insensitivity to toxins is expected to be stable in cases that can only be overcome by a gain of pathogenicity in the pathogen[22,27,28]. Phytotoxin resistance provides a paradigm indicating the need for greater emphasis on understanding of compatibility factors as a basis for engineering stable resistance to plant diseases[22]. As a matter of fact that results can be confirmed with a wide range of isolates, using cassiicolin to screen disease resistance to *C. cassiicola* has certain advantages over using fungal inoculations. Reactions to fungal infections are more sensitive to environments than reactions to toxin bioassays. Results of toxin reactions can be obtained in 24 h and more clones could be tested. Possible changes in pathogenicity of the fungus in culture are eliminated, as large quantities of cassiicolin can be made and

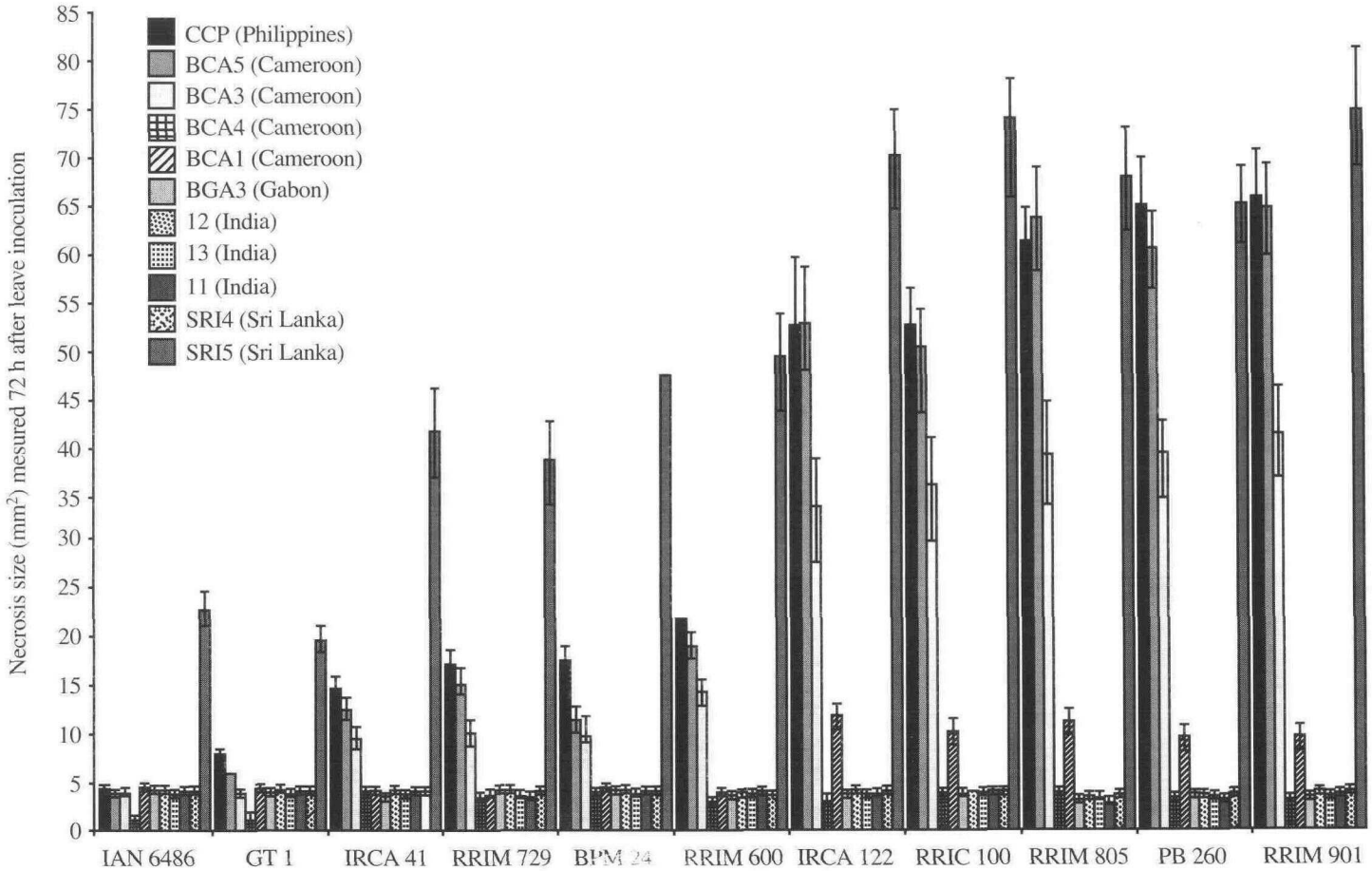

Figure 6. Pathogenicity of C. cassiicola *isolates estimated by the size of necrosis (mm²) measured 72 h after conidial infection of leaves from different* Hevea *clones. The range bar corresponds to the variability estimated after the measure of fifty necrotic lesions for each isolate.*

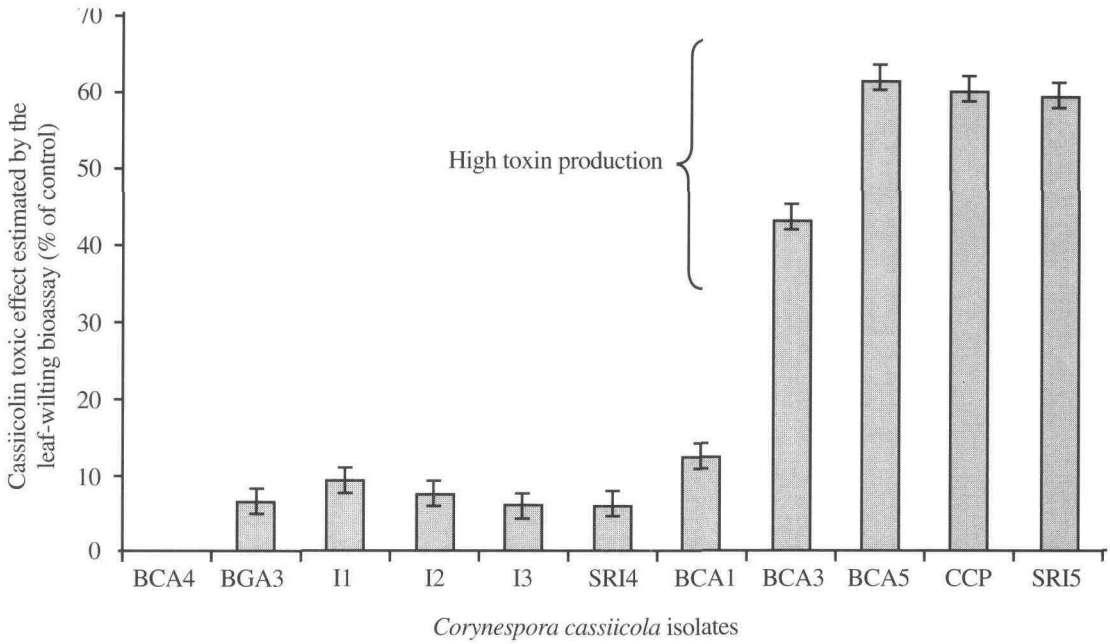

Figure 7. Estimation of the toxin production by various C. cassiicola *isolates using the leaf-wilting bioassay at 48 h after treatment.*

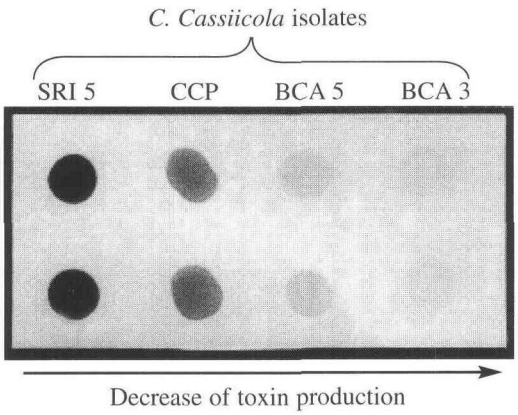

Figure 8. Colorimetric estimation of toxin production by C. cassiicola *isolates using antibodies raised against cassiicolin (dot-blot).*

stored for use over extended periods of time. Countries not affected by *C. cassiicola* can screen their clones to *C. cassiicola* disease without having to introduce the fungus. Differences in isolates could be studied through exchange of toxin preparation, eliminating the risk of introducing new strains of the fungus. Phytotoxins have proven useful as tools for screening in crop improvement programs[27,29,30].

On account of its host specificity and the similarity of its reaction to induce disease symptoms, cassiicolin appears to be an ideal model system for further study of host-parasite interactions. Thus, elucidating the basis for toxin resistance is one of our major goals to better understand *C. cassiicola* disease.

ACKNOWLEDGEMENTS

We are grateful to Dr. R. Kothandaraman of Rubber Research Institute of India for

isolates of *C cassiicola* The research conducted in our laboratory was supported by grants from the Institue Français du Caoutchouc (IFC)

Date of receipt December 1999
Date of acceptance May 2000

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