

INVITED REVIEW

## South American Leaf Blight of the Rubber Tree (*Hevea* spp.): New Steps in Plant Domestication using Physiological Features and Molecular Markers

REINHARD LIEBEREI\*

*Biocenter Klein Flottbek and Botanical Garden, University of Hamburg, Unit of Applied Ecology and Crop Science, Ohnhorststr. 16, D-22609 Hamburg, Germany*

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- **Background** Rubber trees (*Hevea* spp.) are perennial crops of Amazonian origin that have been spread over the whole tropical belt to guarantee worldwide production of natural rubber. This crop plant has found its place in many national economies of producing countries, and although its domestication by selection of suitable genotypes was very slow, it contributes a lot to the welfare of small farmers worldwide. Its development is limited by severe diseases. In South America, the main fungal disease of rubber trees is the South American leaf blight (SALB) caused by the ascomycete *Microcyclus ulei*. This fungus inhibits natural rubber production on a commercial scale in South and Central America.

- **Scope** The disease is still restricted to its continent of origin, but its potential to be distributed around the world rises with every transcontinental airline connection that directly links tropical regions. The need to develop control measures against the disease is an urgent task and must be carried out on an international scale. All control efforts so far taken since 1910 have ended in a miserable failure. Even the use of modern systemic fungicides and use of greatly improved application techniques have failed to prevent large losses and dieback of trees. The results of research dealing with both the disease and the pathosystem over more than 50 years are summarized and placed into perspective.

- **Future Prospects** A detailed knowledge of this host–pathogen combination requires understanding of the dynamics of *Hevea* leaf development, the biochemical potential for cyanide liberation, and molecular data for several types of resistance factors. Resolution of the *Hevea*–SALB problem may serve as a model for future host–pathogen studies of perennial plants requiring a holistic approach.

**Key words:** *Hevea*, South American leaf blight, resistance factors, source–sink relationship, stage-specific resistance, cyanogenesis, defence, resistance screening, scopoletin, marker-assisted breeding.

### INTRODUCTION

Natural rubber (NR) produced from latex of the rubber tree *Hevea brasiliensis* nowadays accounts for about 40 % of the world's total rubber consumption; 60 % is delivered by synthetic processes. The annual world demand for natural rubber is constantly growing because of its typical physicochemical properties, which are still not achieved by synthetic products. Worldwide production reached 9.7 million tons in 2004 (FAO, 2006). Most plantation areas are located in South East Asia, especially in Thailand, Indonesia and Malaysia, with growing production areas in Vietnam and China. In contrast to this geographical distribution of successful production of natural rubber, the biological centre of origin of the rubber tree is the Amazon basin in South America. The annual production of natural rubber in this vast area of Brazil is only 97 000 t, which is about 1 % of world production and far below the national needs of Brazil for rubber. The reason for this discrepancy is the fact that natural rubber in Asia, India and Africa can be grown on plantation scale, while in Brazil natural rubber is almost completely produced in extractive production systems in the Amazon basin, i.e. in co-operatives that collect latex from wild-growing rubber trees (e.g. Dean, 1987). Plantations in Central and South America so far have never

reached full production because they are destroyed before the trees reach physiological maturity (Table 1).

The main reason for low production and failure of plantation growth is South American leaf blight (SALB), a rubber tree disease caused by the endemic fungus *Microcyclus ulei*, an ascomycete that together with its host plant originates from the Amazon area. Since the beginning of rubber exploitation in the region, all attempts to plant rubber trees at a plantation scale in South and Central America have failed completely (Table 1), and to date all control measures against this disease have been unsuccessful (Smith *et al.*, 1995). Neither chemical protection nor biological control measures have promoted the development of healthy and productive plantations (Chee, 1980). Breeding and selection (Gonçalves *et al.*, 1983; Gasparotto *et al.*, 1997) as well as agronomical measures have not led to successful production on a plantation scale. The last concentrated efforts were taken in the governmental programs PROBOR I, II and III of Brazil from 1966 to 1989 (Smith *et al.*, 1995).

At this point in time, the fungus has not spread to other continents on which rubber is cultivated, at least in part because of intensive control of international air traffic and freight shipments from South America to other tropical areas. At present, there are almost no direct intercontinental flights between Brazil and other humid tropical countries,

\* For correspondence. E-mail: reinhard.lieberei@botanik.uni-hamburg.de

TABLE 1. Impact of South American leaf blight on rubber tree cultivation in Central and South America

Surinam	1911	40 000 trees planted
	1918	Plantation destroyed, first reported epidemics by <i>M. ulei</i>
Brazil	1927	Fordlandia: 3 200 ha, 200 000 trees
	1933	Plantation destroyed by <i>M. ulei</i> attack
	1936	Belterra: 6 478 ha
	1943	Plantation destroyed by <i>M. ulei</i>
Panama	1935	Plantation created by Goodyear Comp.
	1941	Plantation destroyed
Colombia	1941	Epidemic development of <i>M. ulei</i>
Costa Rica	1942	Epidemic development of <i>M. ulei</i>
Brazil	1967	Start of SUDHEVEA, a three-step-programme for the development of rubber cultivation in Central Amazonia
	1972–1975	PROBOR I
	1978–1982	PROBOR II
	1984–1994	PROBOR III
	1986	Programme was stopped: about 100 000 ha of the 150 000 ha of plantation were already devastated
Brazil	2002	Scientific meeting about new efforts towards rubber cultivation in areas affected by SALB
Brazil	2005	Plantations only in areas free the fungal pressure: in all other areas latex is collected from wild-growing rubber
Colombia	2006	Programme on selection of rubber trees resistant to SALB

but future direct intercontinental flights are a probability. Disease control is becoming more complex, and because of these factors the probability of an uncontrolled spread of this pathogen is high.

It has been demonstrated that the majority of cultivated natural rubber tree genotypes outside Brazil are susceptible to the disease (Chee, 1976). This finding, from both intensive field tests and laboratory studies, is not surprising. Rather, if one considers the way rubber tree seeds were distributed historically, the fact that the pathogen has never been introduced into the Old World is remarkable. The complete genetic basis for rubber production outside Brazil can be ascribed to only some hundred genotypes, all based on the Wickham collection. Wickham brought more than 80 000 rubber tree seeds from the Amazon region to Kew Gardens. Only a small number of these recalcitrant seeds survived the transport and storage and germinated at Kew. These seedlings formed the basis of the 'oriental' clones on which the whole plantation culture outside the Americas has been based. Some new introductions to the South East Asian production areas were made late in the 1980s, as a result of the first international approach to dealing with the rubber tree germplasm problem.

The introduction of *Microcyclus ulei* into the South East Asian areas of production is a continuing threat; such an introduction would effect a drastic change in the national economies of the rubber-producing countries as well as the world market for all fine rubber products, especially for high quality tires for aeroplanes and automobiles (Chee and Holliday, 1986). The serious nature of this threat to the world economy has recently led to the decision to include South American leaf blight in a list of biological weapons (United Nations Office on Drugs and Crime,

[http://www.unodc.org/unodc/terrorism\\_weapons\\_mass\\_destruction\\_page005.html](http://www.unodc.org/unodc/terrorism_weapons_mass_destruction_page005.html)).

In this paper, the state of knowledge concerning the specific nature of the plant–fungus interaction is analysed with an intent to elucidate the bottlenecks that limit the spread of the pathogen and to highlight factors that need to be given special consideration for the successful future development of natural rubber germplasm. My goal in this review is to facilitate production of plant material tolerant or resistant to *M. ulei* and to initiate the development of an international management strategy for successful resistance.

#### DESCRIPTION OF THE HOST PLANT MATERIAL

The genus *Hevea*, family Euporiaceae, comprises eleven species (Gonçalves *et al.*, 1983). The main source for high quality natural rubber is *Hevea brasiliensis*, a tree that contains latex vessels in the inner bark and that can be tapped by cutting the bark. Latex flow begins immediately after injury to the bark and stops when internal reactions in the latex cause coagulation of this milky substance. Fused isoprene droplets form a plug in the laticifers. The quality of a rubber tree is correlated with the latex composition and production, most importantly the flow rate of latex after cutting. Selection of trees with superior latex quality has been successful, but almost all of these highly productive trees are also highly susceptible to attack by SALB. *Hevea brasiliensis* is a tall tree native to the Amazon basin, that may reach heights of more than 20 m within a forest, with a trunk of up to more than 50 cm in diameter at 1.5 m stem height. Since the beginning of the last century there have been numerous attempts to cultivate this tree in plantations throughout the tropics. This species has not been completely domesticated, but selection of useful parameters has been made with respect to latex quality, tapping suitability and stress resistance. Concentrated selection, such as that which has occurred in the domestication of main world crop plants like wheat, maize or potato, remains to be carried out with this technically useful plant.

The first major steps towards scientifically rational rubber plant development can be linked to the International Rubber Excursion for collection of germplasm material in 1984 (Allen, 1984; Bahia *et al.*, 1985). Many subsequent genetic improvement programs for natural rubber now pursue high latex quality, reduced panel dryness, different canopy forms, good root formation, annual leaf shedding pattern and refushing of the canopy, etc. (e.g. CIRAD, 2005). However, agricultural and agroforestry breeding research leading to understanding and control of SALB, or management of resistance to it, is still not a focus of research and development activities.

There is no mass production of latex in Brazil; rubber is only obtained from small plantations in areas on the margins of the ranges of the forests, such as in Mato Grosso del Norte, where environmental conditions are unfavourable for fungal development (Gasparotto *et al.*, 1997). In the Amazon region, latex is still extracted from

wild-grown trees in the same manner as it was 100 years ago. Rubber trees in the primary forest occur in low abundance, about two trees per hectare. For the related species *Hevea guianensis*, a density of 1.8 trees (>10 cm at 1.50 m stem height) per hectare has been reported (Degen, 1999). Rubber gatherers can normally collect latex from 100–120 trees per day. This number of trees constitutes a ‘seringal’, a tapping trail in the rain forest.

*Hevea brasiliensis*, a perennial tree that can have 50 years of commercial life, has a distinctive juvenile stage in which the growth and developmental pattern is quite distinct from that of adult trees (Hallé and Martin, 1968). The growing rubber seedling is characterized by a rhythmic-flush growth pattern. Whorls of leaves in successive cycles are produced throughout the year. These whorls are separated from each other by pronounced elongation of the internodes following the formation of a new whorl. Each whorl consists of 9–15 leaves, inserted in a 3/8 spiral phyllotaxy pattern. This process leads to rapid development of thin, but elongated, stems. After 5 or 6 years the crown develops the first lateral branches, and successive whorl formation is replaced by annual leaf shedding. Each year, the entire canopy is shed at the beginning of the dry season. Refoliation occurs at the beginning of the new rainy season (Chee and Holliday, 1986). Like many other members of the Euphorbiaceae, rubber tree inflorescences are made up of male, female and bisexual flowers (Dornelas and Rodríguez, 2005). Pollination is primarily dependent on small insects such as midges and thrips, but self-fertilization and inbreeding occurs to various degrees. Self-incompatibility has been observed and established by experiment (Gonçalves, 1986), but male-sterile clones (such as GT-1) also occur (Gonçalves, 1986). In recent breeding projects, cross-pollination or outcrossing has been shown to lead to better fruit set (Hamzah *et al.*, 2002). Unfertilized pistillate flowers quickly wilt and die (Hamzah *et al.*, 2002). The seeds produced in the three-lobed fruit capsules are liberated by explosive dehiscence, a mechanical ‘explosion’ of the fruit shell. Seeds are short-lived and lose their potential to germinate within 2 months of storage under ambient conditions (Holliday, 1970).

#### THE FUNGUS: BIOLOGY AND RACE STRUCTURE

The fungus is known by several names. The teleomorph of this fungus is *Microcyclus ulei* (Henn.) von Arx, a member of the bitunicate ascomycetes. Synonyms are *Dothidella ulei* Henn. 1904 and *Melanopsammopsis ulei* (Henn.) Stahel 1917. The conidial state is *Fusicladium macrosporum* Kuyper 1912 and the pycnidial state is *Aposphaeria ulei* Henn. A thorough account of the fungal systematics is given by Chee and Holliday (1986). In 1962, Muller and von Arx revised the systematics of the fungus and transferred the genus *Dothidella* to *Microcyclus*. An examination of the morphology and an updated taxonomic description of this species, documented with excellent illustrations, has appeared in print (Chee and Holliday, 1986).

The first report of *M. ulei* on wild *Hevea* spp. was made by Ule (1905), who collected samples in 1900 and 1901 from Brazil near the river Jurua and in 1902 from Peru near Iquitos. In subsequent years, outbreaks of severe infection in densely planted plots of *H. brasiliensis* occurred in many places in South and Central America (Table 1). In all cases, the plantations were destroyed when the trees reached the maturation stage between 6 to 7 years and changed from rhythmic-flush growth to annual leaf-shedding patterns. Belgrave (1922) and Weir (1929) gave early warnings of the extreme danger of SALB to rubber plantations, especially those in South East Asia.

Mass production of conidiospores and the rapid build-up of inoculum is the causal step in the epidemiological development of fungal infection in flushing canopies. The conidia are short lived, whereas the ascospores produced in black stromatic structures survive for several months. These ascospores can initiate new infection cycles as soon as susceptible young leaf tissues are produced by the host (Chee and Holliday, 1986). It is important to note that the initial infection in flushing tree canopies often starts from conidia that are present throughout the year on young leaf stages of rhythmic flushes of the seedlings or young, freshly budded trees.

The pathogen can be grown on complex media (Chee, 1978; Junqueira, 1985). Sexual stages do not appear to be formed *in vitro*, but mass production of conidia can be induced by synchronization of spore formation using appropriate light conditions (e.g. Lieberei *et al.*, 1983; Junqueira *et al.*, 1988; Rivano, 1992). New research activities involving examination of fungal races and virulence variation have been undertaken.

Mattos *et al.* (2003) recently reported the results of studies based on 12 rubber plant clones. The differentiation set of rubber tree genotypes used by these authors for race identification was virtually based on the genotypes selected for the studies of Junqueira *et al.* (1988). Thirty-six profiles of virulence have been identified among fungal isolates. Host ranges and quantitative ranges of aggressiveness of the isolates varied strongly. None of the isolates was able to infect all host genotypes. These results were combined with previous screening data of Junqueira *et al.* (1988), Rivano (1997), Hashim and Almeida (1987) and Chee (1976) and, in summary, a total of 68 races with different host reaction spectra have now been identified. The susceptible host-genotype range is very large.

Furthermore, the physiological tolerance of the isolates to temperature and relative humidity, as well as to complex environmental factors (Gasparotto *et al.*, 1989a, b, 1991), suggests that the potential for adaptation of fungal isolates to new environments is high. This variability underscores the potential threat of this fungus to new rubber growing areas outside South and Central America.

Important steps for analysis of the diversity of the fungus and its genetic polymorphism have also been carried out using molecular approaches (Le Guen *et al.*, 2004). In fungal isolates from Brazil and from French Guiana, microsatellite markers were described and assessed with respect



to their variability within the isolates. This approach offers a way to group the highly variable complex isolate groups on a genetic information basis.

#### THE DISEASE: PLANT REACTIONS TO FUNGAL ATTACK

*Microcyclus ulei* attacks young leaves, stems or fruit tissue of five or more species within the genus *Hevea* (*H. brasiliensis*, *H. benthamiana*, *H. spruceana*, *H. guianensis*, *H. camporum*; Chee and Holliday, 1986). Early breeding concepts for resistance were based on the observation that, in addition to highly susceptible genotypes, plants also occurred that seemed to be immune or completely resistant to *M. ulei* (Bos and McIndoe, 1965; Gonçalves, 1968; Chee, 1976). These resistant plants were used in crossings with high-yielding individuals of the 'oriental' (Wickham) clones. Unfortunately, most of the offspring of these crossings were susceptible to at least some of the fungal strains, and with progressive identification of the fungal virulence spectrum it became clear that the concept of specific or complete resistance *sensu* Parlevliet (1975) was not applicable to the rubber tree. Subsequent research was redirected to identify factors that contribute to general resistance (Simmonds, 1989). These consist of many interactive factors in the pathogen–host plant relationship that minimize disease development. Important studies have addressed the physiological aspects of resistance with the aim of identifying processes involved in the plant–fungus interaction (Hashim *et al.*, 1980; Hashim and Pereira, 1989; Mevenkamp, 1992; García *et al.*, 1995a, b) and, finally, to develop biochemical markers for marker-assisted breeding programs. Based on a broad range of observations, a comprehensive view of many of the various stages of the plant–fungus interaction has been achieved. Junqueira *et al.* (1988) prepared detailed descriptions of lesion types produced by a diverse set of host genotypes. Combinations of various host genotypes with selected fungal isolates varying in virulence spectra led to the formation of a sequence of lesion types with graded differences in the occurrence of necrotic tissue, lesion size, number of spores formed, distribution of conidial layers in the lesion, and stroma formation as a late symptom (Figs 1 and 2). Five main developmental levels in the host–pathogen interaction, which together contribute to the formation of phenotype resistance, were identified (Giesemann, 1986; Junqueira *et al.*, 1988).

- (1) Germination and pre-penetration phase with early impact of the plant on the spores' infection potential.
- (2) Penetration and initial infection phase with induction of early resistance responses.
- (3) Leaf colonization, intercellular growth and ramification of hyphae; a phase that is considerably influenced by cell wall modifications and leaf maturation processes.
- (4) Formation of conidiophores, which leads to differences in the spore number and spore distribution in and around the lesion.
- (5) Stroma production and ascospore formation.

Quantitative variations of each of these were observed; they ultimately combine to produce the phenotype of incomplete or partial resistance. In addition to these highly variable pathosystem features, there exists a stage-specific resistance. Only young, developing leaves (Fig. 1A), immature fruits and young stems of this tree species are susceptible. When infection pressure is low and leaves are not infected during the young stages of ontogenetic development, they develop to highly resistant leaf stages. Only a few studies have addressed these various aspects of physiological resistance (Blasquez and Owen, 1957; Junqueira *et al.*, 1990).

#### *Germination and hyphae formation, pre-penetration phase*

When the conidiospores of *M. ulei* come into contact with young leaf tissues of susceptible genotypes, the spores germinate on the reticulate surface of the rubber tree leaves (Fig. 1B–E). Once in contact with a wetted leaf surface, the spores germinate and are not washed off by rain, as they are tightly affixed to the cuticle by an electron-dense substance (Fig. 1E) (Giesemann, 1986). Infection hyphae penetrate the epidermal layer of the lower leaf side (Fig. 1F) in the contact zone of three epidermal cells with or without formation of appressoria. The degree of formation of branched germ tubes and the formation of appressoria is dependent on interaction in the pre-penetration phase. (Hashim *et al.*, 1978; Giesemann, 1986).

Spore germination and germ-tube formation occur immediately after contact of the conidiospores with the leaf. The morphology of the germ tube varies with the reticulate leaf surface structure of the young rubber tree leaves. In Fig. 1D and F, the flexibility of the germ tube morphology is seen and in Fig. 1E the formation of dark, contrasting material between fungus and plant is shown. Germ-tube diameter, germ-tube length, branching and formation of appressoria are strongly influenced by leaf surface constituents, which modify the infection process (Table 2; Blasquez and Owen, 1963; Hashim *et al.*, 1978; Giesemann, 1986).

#### *Penetration and initial infection stages*

After penetration of the leaf surface, the hyphae colonize the underlying tissue by intercellular growth. They often enter the tissue layers adjacent to the leaf vascular bundles and spread rapidly along the veins into the leaves (Fig. 1G, H; Giesemann, 1986). In this biotrophic phase, compatible combinations do not show cell death. However, in resistant clones, the cells in direct contact with the penetration hyphae collapse. Hashim *et al.* (1978) ascribed this to a hypersensitive reaction and to pre-formed resistance factors (Blasquez and Owen, 1957; Figari, 1965), and proof was also given for induced defence compounds such as scopoletin (Tan and Low, 1975; Giesemann *et al.*, 1986; García *et al.*, 1995b). This early detection process of the fungal presence in the attacked tissue of resistant plants leading to a hypersensitive response is a typical defensive reaction (Breton *et al.*, 1997). This reaction is

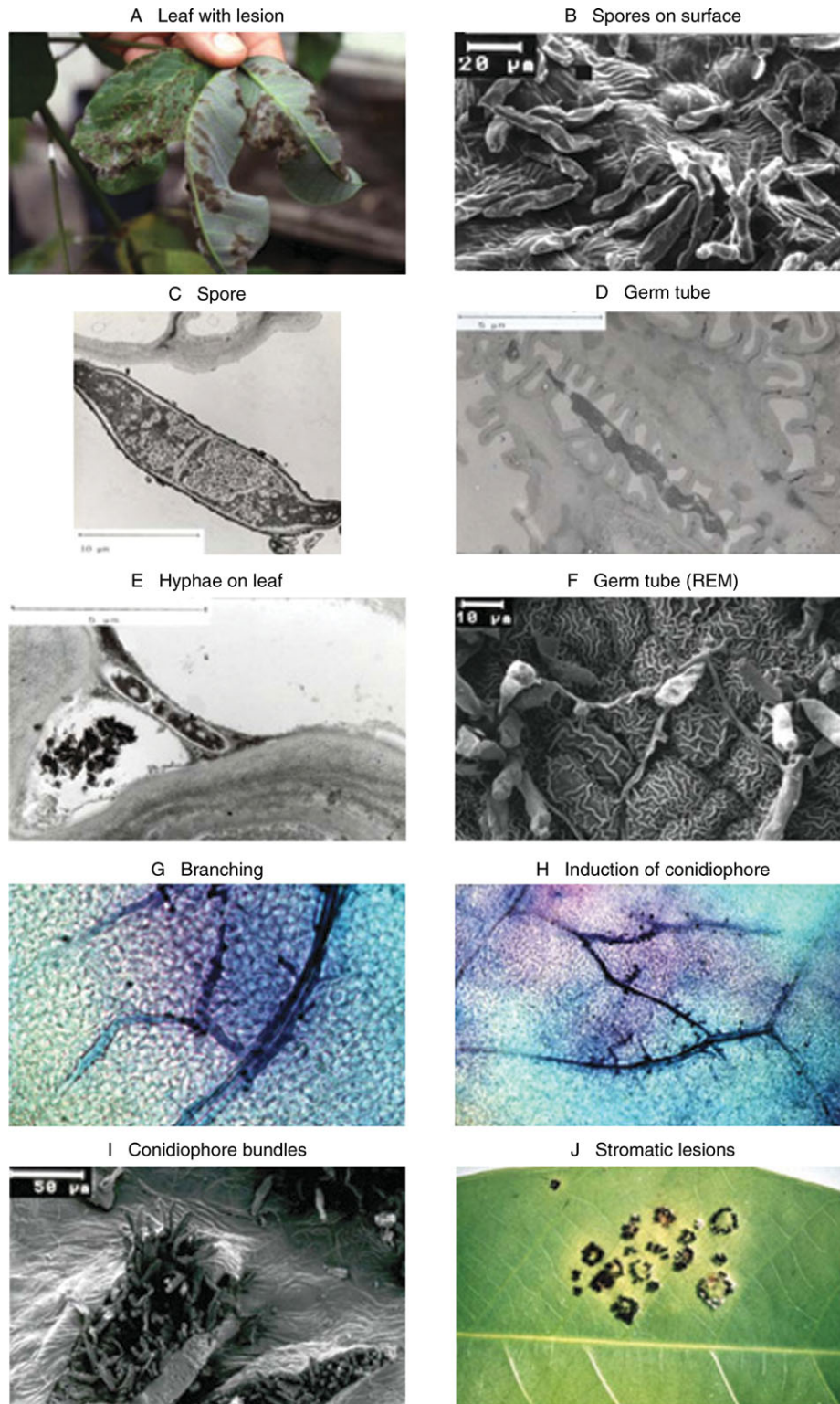


FIG. 1. Anatomical and morphological aspects of *M. uliei* attack. (A) Heavily infected leaflets of *H. brasiliensis* with greyish conidiospore layers on upper and lower leaf surfaces. (B) Dried conidiospores on lower leaf surface with reticulate surface structure. (C) Germinating conidiospore, section. (D) Germ tube adjacent to the wax layers on the reticulate surface structures of epidermal cells. The dark grey contrasted germ tube is very flexible and polymorphic. (E) Section through a germ tube that is fixed to epidermal cells by a slimy and sticky compound, which is set free from the conidiospore surface after intensive washing. (F) Germinating conidiospores forming penetration structures: a penetration hyphae is entering the leaf at the junction of three adjacent cells (middle). (G) Blue-stained hyphae are spreading through the leaf tissue, often parallel to the vessels (24–36 h post-infection). (H) Blue-stained hyphae; branching hyphae growing back to the surface form dark-blue points (60–72 h post-infection). (I) Conidiospores break through the lower leaf surface. (J) Black pigmented globose structures form ring-like stromata.

Evaluation scheme of *M. ulei* lesions

Number	Lesion size [mm]	Occurrence of spores	Necrotic reaction	Stroma formation
0	<<1	—	(+) chlorosis	—
1	=1	—	+	—
2	1–2	—	+	—
3	>2	—	+	—
4	<1–2.5	On the margins of the lesions	+	(+)
5	<1–2.5	Equally distributed in low amounts	(+)	(+)
6	2–2.5	Abundant abaxial/adaxial	—	+
7	=2	Abundant	—	+
8	2–2.5	Abundant	—	+
9	>2.5	Abundant	—	+
10	>2.5	Abundant abaxial/adaxial	—	+

Criteria: Lesion size  
Necrosis  
Spore production  
Stroma formation (late symptom)

*n* = 16 *M. ulei* isolates  
*n* = 33 rubber tree clones

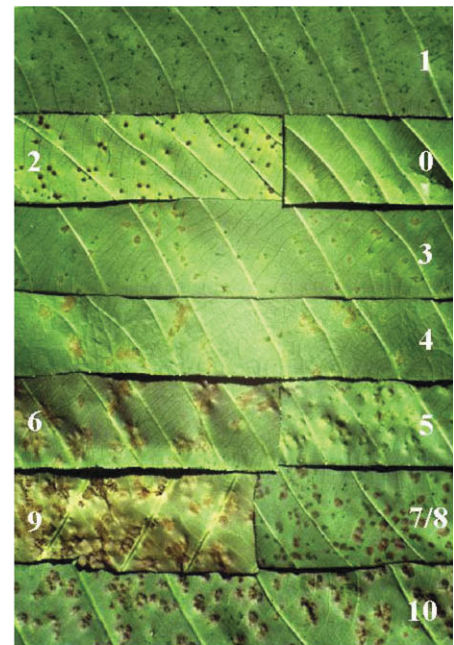


FIG. 2. Evaluation scheme of *M. ulei* lesions. The classification of lesions follows the description of Junqueira (1985). On leaf section '0' only very tiny necrotic spots become visible. The lesions develop within 3–4 d after infection and incubation in a humid chamber under controlled conditions.

regarded as an indicator for complete or vertical resistance, but this concept is applicable only to mature leaves of *H. brasiliensis* and occurs with most genotypes of the previously uninvestigated host species *Hevea pauciflora* (Junqueira *et al.*, 1988). At the biochemical level of host reactions, a hypersensitive response is often associated with well-described defence reactions such as formation of reactive oxygen-type compounds (García *et al.*, 1999), deposition of autofluorescent compounds in the cell wall (Mevenkamp, 1992), synthesis of callose, occurrence of scopoletin as phytoalexin (Giesemann *et al.*, 1986; García *et al.*, 1995*b*), and finally cell death in a restricted area surrounding the penetrating hyphae. Detailed and quantified descriptions have been given by García *et al.* (1999).

Fast hypersensitive response reactions typically lead to tiny necrotic lesions or to yellow microlesions in controlled

inoculation experiments (Fig. 2) and they have also been reported as field symptoms associated with both young tissues and with mature rubber tree leaves. Thus, mature leaves express the well-described stage-specific resistance. All adult leaves (Blasquez and Owen, 1963; Junqueira *et al.*, 1988), irrespective of the clonal origin, are highly resistant. This transient and stage-specific enhancement of biochemical resistance patterns needs to be considered in any new strategy of plant selection for broad-based resistance against *M. ulei*.

In the early phase of leaf penetration and colonization, cyanide liberation occurs as a consequence of cell breakdown (Lieberei, 1986). Cyanide liberation is due to the contact of vacuolar cyanogenic precursors with an apoplastic  $\beta$ -glucosidase. HCN, depending on the velocity of liberation and on precursor concentration, can be inhibitory to plant defence reactions or simply diluted out.

TABLE 2. Germination of *M. ulei* conidiospores on leaves of selected rubber tree clones: influence of host–pathogen combination in the pre-germination phase. On resistant-reacting leaves the germ tubes are shorter than on susceptible leaves, the germ tube diameter is bigger, and the amount of appressoria formed is higher on resistant leaf surfaces. On susceptible leaves, germ tubes are found to form branches

	Control	HS		R/S		R		
	H <sub>2</sub> O	GT 1	RRIM 600	IAN 573	Fx 25	F 4542	PuA 7	F 2261
Germination (% in 24 h)	96	95.5	95	95.8	76	95.5	95.8	95.5
Germ tube length ( $\mu$ m in 40 h)	124	114	127	56	62	70	66	73
Germ tube width ( $\mu$ m in 40 h)	4.2	3.6	3.4	6.0	6.0	5.8	6.1	6.2
Appressoria formed (% in 40 h)	5.2	5.8	5.5	19	17.5	14.5	22.2	20
Branched germ tubes (%)		22	n.t.	12	n.t.	n.t.	8	n.t.

Data taken from Giesemann (1986), n.t.: not tested.



### Leaf colonization, intercellular hyphal growth and ramification

In susceptible host–pathogen combinations, the intercellular spread of the fungal hyphae takes place along the leaf vein (Fig. 1G) and freely into the apoplastic regions of the tissue between the veins (Fig. 1H; Giesemann, 1986). This phase ends after 3–5 d with a remarkable morphogenetic change in hyphal growth: the hyphae then form side hyphae, which grow perpendicular to the original hyphae. This new type of hyphae can be seen as coloured points adjacent to the leaf-colonizing hyphae (Fig. 1H). The external or internal factors that regulate these morphogenetic changes remain to be studied. These factors appear to be linked to the leaf maturation process. In the intercellular spaces around the growing hyphae, lignin-like material is deposited that can lead to reduction of the lesion size.

### Formation of conidiophores

The branching hyphae are directed towards the lower epidermis and within 24 h after formation they break through the lower leaf surface and form conidiophores and conidia (García *et al.*, 1995a). Small lesions that can fuse laterally to form ring-like structures often lead to large lesions (Fig. 2). The fact that the lesion formation patterns occur in defined types and in a reproducible manner indicates that intercellular growth and ramification of the hyphae underlies a plant genotype–fungal race interdependence (Junqueira *et al.*, 1988).

### Stroma production and ascospore formation

Stromatic structures with pseudothecia and ascospores are formed on infected leaves with low infection incidence. Leaves with low levels of infection remain on the trees, whereas heavily infected leaves fall from the tree after conidiospore formation and degrade on the ground. When less-infected leaves remain in the crown 2 months or more after infection, stomata on the upper leaf side are formed in small globose structures that may fuse to form rings (Fig. 1J). In these structures, the sexual stages of fungal development are completed with formation of ascospores. In many plant–fungus combinations, the formation of stomata does not occur. Junqueira *et al.* (1990) reported that conidial formation in lesions is dependent on the leaf age when infection takes place. The older the developing leaves, the smaller the number of spores formed. Subsequently, formation of stomata is delayed.

Description of the plant–pathogen interaction between *Hevea* species and SALB and the occurrence of host-specific reactions to various fungal pathogen races establishes that the large set of biochemical defence events known to occur in many other plants also occur in rubber trees and, furthermore, knowledge of these steps underscores the fact that a genetic precondition for biochemical defence is present in all rubber tree genotypes (Hashim *et al.*, 1978, 1980; Mevenkamp, 1992; García *et al.*, 1995a, b). Initiation and function of host defence processes depends on plant/pathogen signalling between host

genotypes and fungal isolates. These processes are linked to successful and rapid detection of signals and to activation of the complete biochemical signalling chain for both vertical and complete resistance systems. In addition to this general defence based on major resistance genes, many other variables and patterns in the formation of lesion size and of spore formation are expressed (see Figs 2 and 3). Some host plant genotypes are susceptible to all fungal isolates that have been examined. These variations in expression of partial or horizontal resistance must be based on genetic factors, but can be modified by transient factors on the physiological level of plant hosts (Lieberei *et al.*, 1989).

### PHYSIOLOGICAL LEAF PROPERTIES

Rubber tree leaves are formed in a flush growth pattern. Directly after bud burst, the leaves are thin, have a high respiration rate, no net photosynthesis (Table 3; Lieberei, 1984; Lieberei *et al.*, 1996) and are devoid of any resistance against the virulent isolates of *M. ulei*. In the course of maturation, rubber tree leaves change from susceptible to completely resistant (Blasquez and Owen, 1963; Holliday, 1970; Chee, 1980). This maturation requires 12–20 d after bud burst to occur; the maturation time is genotype dependent. This parameter is very important for disease management. Based on morphological criteria seen from bud burst until hardening of leaflets or leaves, Dijkman (1951) placed the developing leaves in four groups designated A to D (Fig. 4). Blasquez and Owen (1963), Hallé and Martin (1968) and Chee (1976) used leaf colour and the angle formed between the petiole and stem to place these leaves into groups, whereas Samsuddin *et al.* (1978) correlated the developmental groups with the angle formed between the leaflets and the petiole. In contrast, Lieberei (1984) used physiological data for the description of leaf developmental stages. Voß (2001) used leaf length increase (Fig. 4), and Lieberei (1984) chlorophyll per leaf area, the chl *a*:*b* ratio, the fresh weight per leaf area, and selected data on gas exchange to describe the sink status (Table 3).

Based on these physiological data, leaf stages A, B and C are completely sink leaves with no positive net energy balance (Lieberei *et al.*, 1996). Leaf size increase is almost exponential in the early stages of leaf development. In this phase of rapid cell wall growth and cell enlargement, cell wall hardening processes are retarded; leaves in stages A, B and C are almost free of lignin (Fig. 5A–C; Voß, 2001).

The amount of UV-absorbing compounds in 1-mm leaf sections were analysed with a UV-microspectrophotometer (UMSP 80, Zeiss; Fig. 5). This microspectrophotometric analysis permitted us to prove the occurrence of phenolic compounds in the middle lamellae and primary cell walls. Leaves at stages B and C contain some aromatic compounds, but do not contain lignin. UV-photographs of the leaf sections, taken with UV 280 nm light, show that in developmental stages B and C only lignification in the tracheids of conductive vessels occurs. Other cell walls are devoid of lignin (Voß, 2001). The typical UV absorption pattern of lignin with three peaks near 280 nm (see

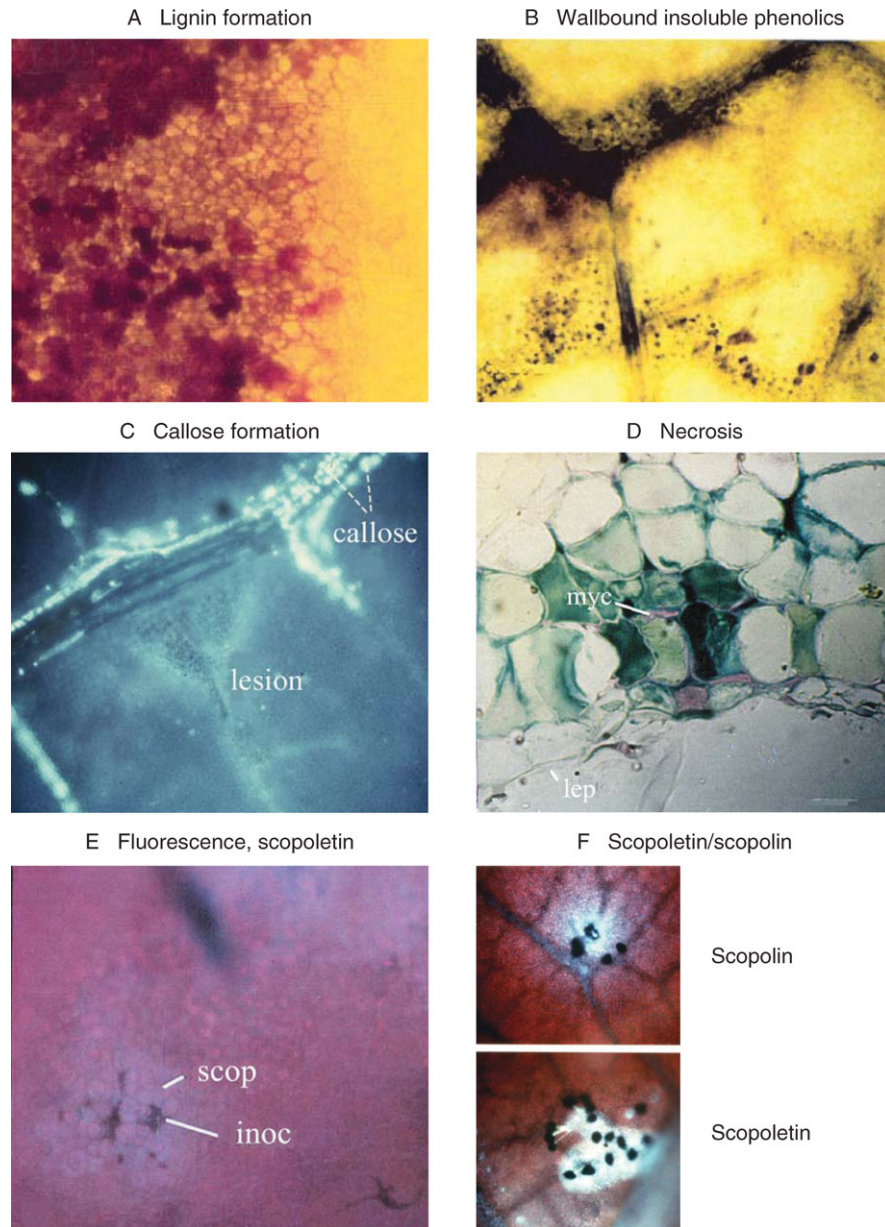


FIG. 3. Biochemical resistance reactions in rubber tree leaves. (A) Formation of lignin in cell walls around lesions of intermediately resistant leaves infected by *M. ulei*. There is a progressing impregnation of the cell walls with lignin (Vance *et al.*, 1980). (B) Resistance reactions are accompanied by the formation of wallbound insoluble phenolics, which become visible after treatment of leaves with Toluidin blue (Feder and O'Brien, 1968). (C) Callose formation around localized lesions (triangular zone in centre of image), detected with Aniline blue (Eschrich and Currier, 1964). (D) Necrotic spot in a hypersensitively reacting leaf adjacent to mycelium (myc); lep = lower epidermis. (E) View of an infected leaf surface under UV-irradiation, red chlorophyll fluorescence in the background and faint blue fluorescence of scopoletin (scop); inoc = inoculation site. (F) Scopoletin in a reticulate distribution under UV irradiation. The blue fluorescing field surrounds black germinating spores of *Periconia manihoticola*; leaf incubation 24 h under humid conditions in the dark in Petri dishes. Scopolin is formed when the leaves are left attached to the plant and are covered by black plastic bags. The fluorescent compound is located in the vacuoles.

Fig. 5C) can only be detected in leaf structures of stage D. This presence of lignin coincides with the onset of leaf hardening in early stage D.

Leaves of developmental stage D possess the physiological and structural parameters of mature leaves (Table 3, Fig. 5C). These leaves are completely resistant to *M. ulei*. The penetrating hyphae of all strains and isolates of *M. ulei* tested are stopped by hypersensitive responses

(Fig. 3: necrosis as described by Blasquez and Owen 1957, 1963).

With young leaves (stage B), fungal infection leads to incomplete defence reactions. In the course of these reactions, the soluble coumarin scopoletin is formed (Tan and Low, 1975; Giesemann *et al.*, 1986; García *et al.*, 1999). Scopoletin is a fungitoxic compound, but the concentrations reached in *Hevea* leaf tissue are not very high. According to



TABLE 3. Quantitative characteristics of developmental leaf stages of rubber trees

	Leaf stage			
	A	B	C	D
Chlorophyll ( $\mu\text{g cm}^{-2}$ leaf area)	26	33–38	25–33	43–51
Chl <i>a/b</i> ratio	1.2–1.6	1.8–2.2	2.2–2.9	3.3–3.6
Photosynthetic $\text{CO}_2$ fixation ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ )		–0.72	–0.24	5.1–11.6
Photosynthetic $\text{CO}_2$ fixation per unit chlorophyll ( $\mu\text{mol CO}_2 \text{ mg}^{-1} \text{ Chl } a \text{ s}^{-1}$ )		16.1	15.3	12.0
Respiration ( $\mu\text{mol CO}_2 \text{ mg}^{-1} \text{ Chl } a \text{ s}^{-1}$ )		–5.03	–4.48	–2.01
Fresh weight ( $\text{mg cm}^{-2}$ )	19	16	9	11
Dry weight (% of fresh weight)		18.9	19.6	40.7
Leaf soluble protein ( $\mu\text{g cm}^{-2}$ )	1150	865	365	740
HCN-p ( $\mu\text{mol CN g}^{-1}$ fresh weight)	20	30	37	20

García *et al.* (1999), the amount of scopoletin is not directly correlated to resistance.

#### DYNAMICS OF LEAF RESISTANCE: PERSPECTIVES OF LEAF FLUSH GROWTH IN PLANT DEVELOPMENT

Rubber tree leaves from susceptible *Hevea* trees are highly susceptible at leaf stage A, but become completely resistant at leaf stage D. The susceptibility of leaves of all *Hevea*

clones to infection with *M. ulei* generally declines with leaf maturation. This conversion of leaves is a transient and progressive process. The older the leaves are at the time of inoculation, the more distinct is the defence reaction (Junqueira *et al.*, 1990). Within the pathogen isolates, groups have been identified that vary with respect to infection intensity and with respect to their host range (Junqueira *et al.*, 1988; Fig. 2). The variation of resistance of *Hevea* clones at different stages of leaf development is presented in Figs 6 and 7. Leaves inoculated with virulent isolates

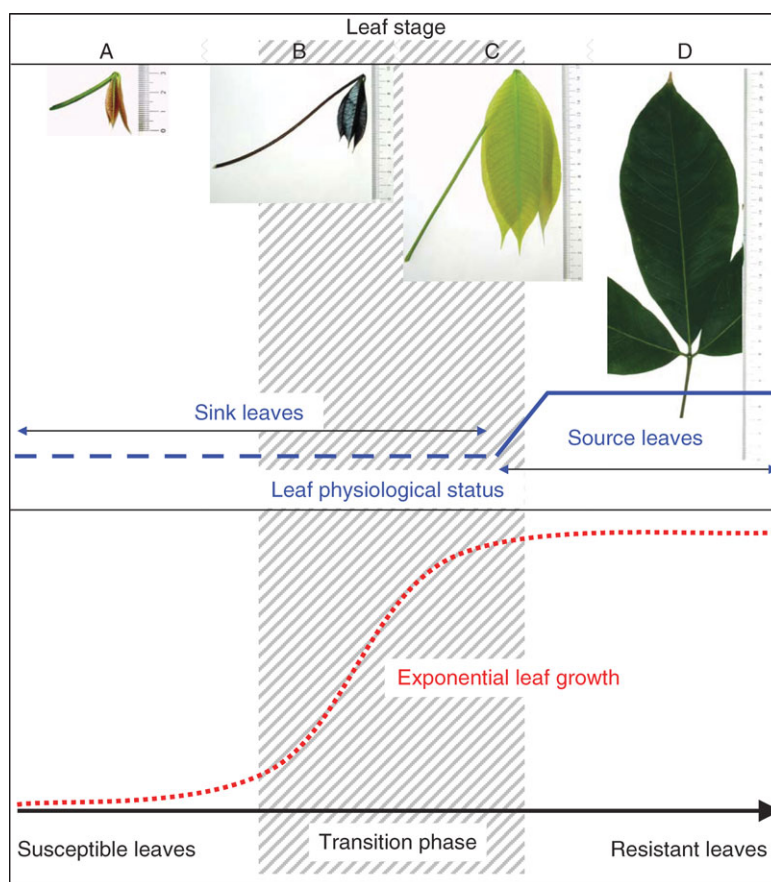


FIG. 4. Relationship between exponential leaf growth, transition phase from completely susceptible leaf stage to completely resistant leaf stage, and the short physiological step from sink to source leaves. This qualitative comparison attempts to visualize the different developmental processes that turn a susceptible rubber tree leaf into a completely age-resistant organ. In the hatched area the resistance factors interact in various ways and do not allow the correlation of the QTLs developed with the leaf properties in transition.

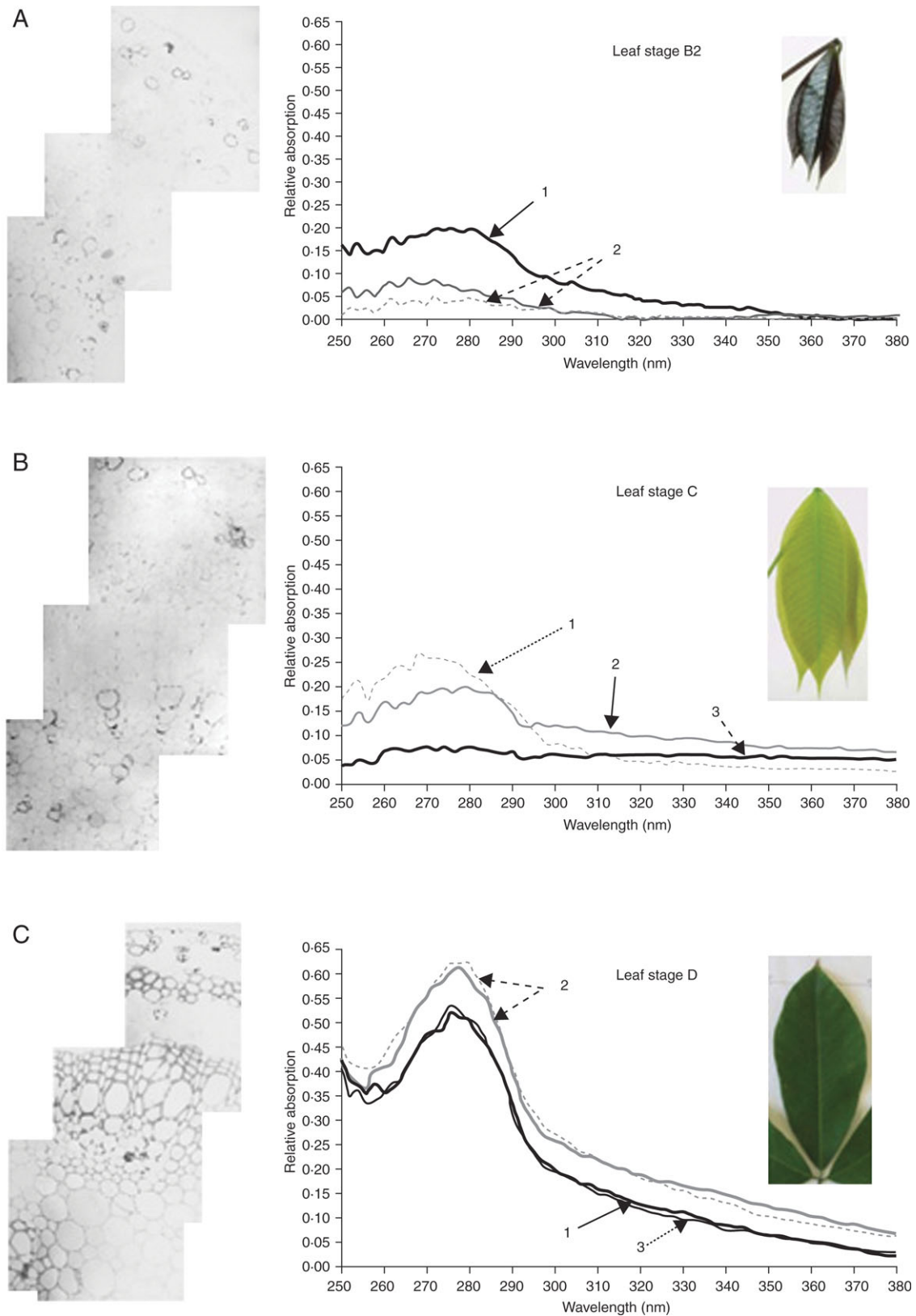


FIG. 5. UV photographs and UMSP 80 scans of rubber tree leaves at different developmental stages. Lignin formation was analysed with an ultraviolet (UV) spectrometric device (UMSP 80, Zeiss). Semi-thin sections of acetone-extracted leaves of  $1\ \mu\text{m}$  were mounted on quartzglass trays. Spectra were taken in the range of 250–280 nm. Leaves of stages B2, C and D were tested. 1, Middle lamellae and added material; 2, contact layer between three adjacent cells 3; secondary cell wall. The typical absorption spectra for lignin with three combined absorption peaks between 275 nm and 280 nm were detectable in leaf stage D, not before (Vo $\beta$ , 2001).

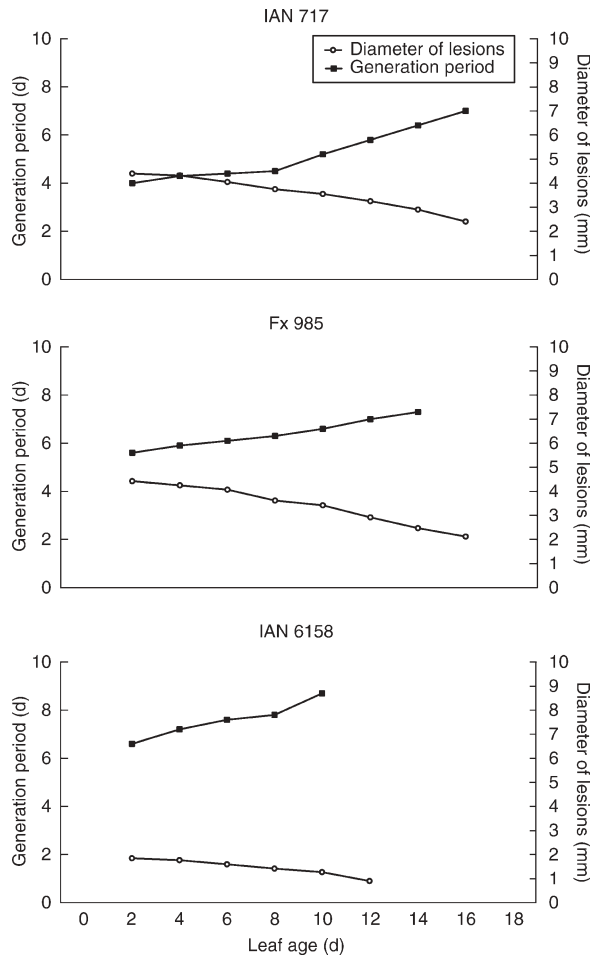


FIG. 6. Fungal generation period and lesion diameter on leaves inoculated at different days during the leaf flushing period. Leaves of selected clones were inoculated with *M. ulei* isolates highly virulent to the respective *Hevea* clones. The 'leaf age' corresponds to the day of inoculation after bud burst, the generation period is given in days from inoculation until spore formation of 50% of the lesions, and the diameter of lesions are given in mm. The data for the figure have been taken from Junqueira *et al.* (1990).

at an early leaf age generally form spore-producing lesions a short time after inoculation (Fig. 6). In addition to the short time of the generation period, these lesions are large in diameter. The older the leaves are at the time of inoculation, the smaller in diameter the lesions become. The expressed clonal characteristics of susceptibility are another important factor of disease variability. In clone IAN 717 (Fig. 7), the young leaves produce spores 5 d after inoculation and the lesions reach a size between 2–4 mm in diameter. In clone IAN 6158, spore-producing lesions are less than 2 mm when inoculation takes place 10 d after bud burst. When inoculation is carried out 12 d after bud burst, small necrotic lesions do occur, but no spores are formed in these lesions (Fig. 6). This stage of leaf maturation is characterized by a limited susceptibility to penetration and early fungal spread in the leaf tissue, but later resistance factors such as lignification inhibit the later developmental phases of the pathogen.

The susceptible leaf phases vary considerably with the clones studied (Fig. 7). Clones Fx 7 3925, IAN 873, and IAN 717 are susceptible until a leaf age of 16 d, whereas clones such as IAN 8 6158 or CNS-AM 7907 become resistant with 10 d. In addition to the clone-specific, leaf-age resistance there is also variation in pathogen development on the clones due to variation in maturation time. In leaves with a long leaf-maturation phase, fungal development is short, i.e. the fungal generation period, defined as the time from inoculation until the formation of new spores (Fig. 7), is shorter than on leaves with short leaf-maturation times. This relation of fungal development to leaf maturation is very important if one considers epidemiological aspects. If the first leaf of a flushing rubber tree canopy is infected and produces spores in the shortest time possible in clone Fx 3925, then by direct reinfection of young leaves at least three fungal generations will be produced, whereas in clones such as F 4542 or CNS AM 7907 the production of spores in infected leaves almost coincides with the onset of leaf-age resistance in the tree crown. Under these conditions reinfection by the newly produced spores in the canopy cannot occur.

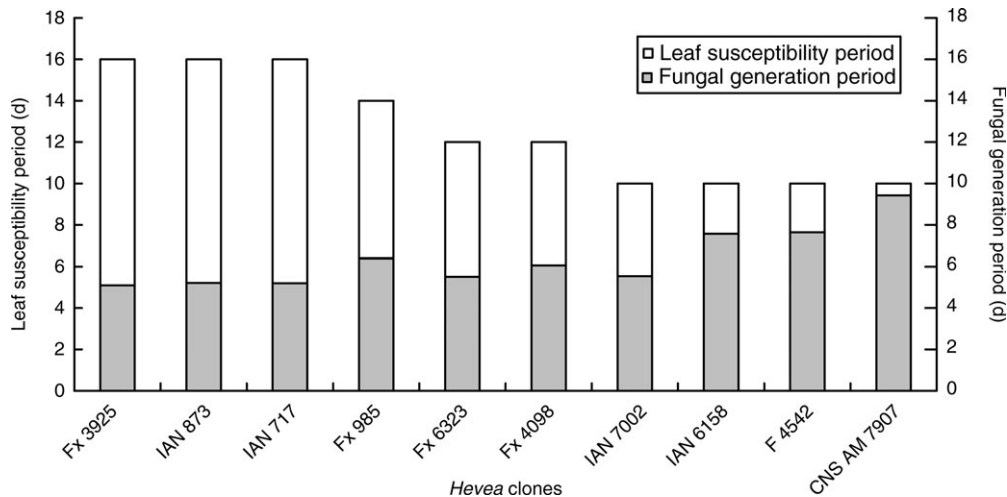


FIG. 7. Fungal generation period in relation to leaf susceptibility period.



The variation of lesion size on leaves at various stages of maturation represents a significant observation. The growth of the fungus through the host-leaf apoplastic space is reduced with increasing age of the leaf tissue. In a structural study of infection-induced defence reactions of rubber leaves against *M. ulei*, García *et al.* (1995a) reported scopoletin formation and accumulation of lignin-like substances around the infection site. These authors assumed that lignification may stop fungal development. Lignification is an energy-dependent process that is dependent on the availability of precursors and reductive equivalents. Under the assumption that only source leaves are able to carry out defensive reactions, it becomes important to conduct these studies under appropriate light conditions and with leaves attached to the mother plants. In the past many experiments have been carried out by incubation of detached leaves in humid chambers in the dark (e.g. Hashim *et al.*, 1980; Lieberei, 1986; García *et al.*, 1995b; Breton *et al.*, 1997).

#### PLANT CYANOGENESIS: LEAF INHERENT CONSTITUENTS WITH HIGH INTERACTION POTENTIAL WITH DEFENCE REACTIONS

In addition to the varying activities of phenolic metabolism and lignin formation, *Hevea* leaves are characterized by liberation of HCN upon infection and tissue injury. The rubber tree belongs to a large group of more than 3 000 species that are cyanogenic, i.e. they release cyanide on damage to the plant. These plants accumulate cyanogenic precursors in their vacuoles. Upon tissue injury the precursors, mostly  $\beta$ -glycosides, are set free from the vacuoles and are mixed with  $\beta$ -glycosidases. Generally, these  $\beta$ -glycosidases are stored separately from the cyanogenic precursors, mostly in the plant cell walls as active apoplastic constituents (Selmar *et al.*, 1988). The pre-formed enzymes cleave the liberated  $\beta$ -glycosides and give rise to a sugar moiety and an aglycone. The aglycone decays and forms HCN and a corresponding carbonyl compound (Seigler, 1998). The time course of HCN liberation from the aglycone depends on the leaf tissue, pH and on the occurrence of another enzyme, an  $\alpha$ -hydroxynitrile lyase, which accelerates HCN-liberation (Selmar *et al.*, 1989).

The cyanogenic precursors of the rubber tree are the glycosides linamarin and lotaustralin (Lieberei *et al.*, 1985). In the course of leaf infection by *M. ulei* or other leaf-attacking fungi, HCN is released from injured leaf tissue. The liberation of HCN follows a reproducible time course. In the case of leaf infection by *M. ulei*, there is a characteristic HCN peak in the early infection stage (Lieberei, 1986) about 24 h after penetration of the fungus into the tissue.

Cyanogenesis is an obligate feature of rubber trees. All living tissues, including seeds, are strongly cyanogenic and contain both the accumulated cyanogenic precursor and the respective  $\beta$ -glucosidase.

The amount of cyanogenic precursor accumulated per tissue weight permits calculation of the cyanide liberation potential of the tissue and is called the HCN-potential or HCN-p (Lloyd and Gray, 1970). The rate of HCN liberation (HCN-c) from an attacked tissue depends on the type of

tissue injury and the activity of the  $\beta$ -glucosidases in the given tissue. In many cases this is also linked to the presence and activity of an  $\alpha$ -hydroxynitrile lyase (Selmar *et al.*, 1989). Plants of *Hevea brasiliensis* as well as all other *Hevea* species studied are cyanogenic, although they vary considerably in their HCN-p and in their HCN-c (Lieberei, 1984). There is a clear relationship of HCN-c of the rubber tree to the susceptibility to *M. ulei*. The higher the HCN-c, the higher the susceptibility.

In plants with low HCN-c and HCN-p values, the resistance reactions are not impaired by HCN. These plants follow a well-described host-pathogen reaction pattern with the complete spectrum of lesion formation (Lieberei, 1988a, b). These plants have the classical race-specific resistance patterns. A more detailed examination of this plant-pathogen interaction has been conducted with selected pairs of rubber tree genotypes high in HCN-c and low in HCN-c and selected isolates of *M. ulei*, which were identified by their host range (Lieberei *et al.*, 1989).

When inoculated leaves were incubated in humid chambers, strongly cyanogenic leaves always developed severe lesions, whereas weakly cyanogenic leaves developed the defence patterns shown in Fig. 2. When HCN liberation was experimentally minimized in the incubation systems by resorption of HCN in alkali or by passing a flow of humid air through the incubation system, the highly susceptible, strongly cyanogenic leaves reacted with formation of smaller lesions and with good resistance responses (Lieberei *et al.*, 1989). These results proved that HCN liberation led to impairment of active defence in the leaves, and that leaves of high HCN-p plants after experimental lowering of HCN in the tissue produce the entire set of defence reactions that can be expressed after signal perception.

HCN liberation is a pre-formed trait in plant tissue, which may provide a very good defence against herbivores (e.g. Tattersal *et al.*, 2001; Ballhorn *et al.*, 2005), but which may render strongly cyanogenic plants more susceptible to microbial pathogens (e.g. flax to flax wilt, Trione, 1960). The theoretical background of this controversial reaction needs to be considered in greater detail as a complex phenomenon in quantitative ecology and evolution. For the rubber tree, this phenomenon must be taken into consideration for practical breeding and selection work. The fungal pathogen *M. ulei* is tolerant to HCN, at least in its vegetative phase (Lieberei *et al.*, 1983). In addition, the fungus produces a soluble extracellular  $\beta$ -glycosidase that has remarkably high affinity to the hosts' cyanogenic compound linamarin (Lieberei, 2006). The quantitative potential in the leaf material varies with leaf age. The youngest and generally most susceptible leaves have pronounced HCN-p and a high activity of  $\beta$ -glucosidase whereas, during maturation, the leaves (stage D) often reveal lower values (Table 3).

#### MOLECULAR ANALYSIS OF GENETIC DIVERSITY IN RUBBER TREES

In 1981, a germplasm collection excursion to the geographical area of the primary rubber tree germplasm in the Amazon was conducted by an international group, formed under the auspices of the International Rubber

Research and Development Board. The resulting IRRDB collection was the first systematic collection of *Hevea* germplasm in more than 100 years since the rubber seed collection of Wickham in the Amazon basin. This new rubber germplasm collection was carried out in order to broaden the genetic basis for worldwide rubber tree cultivation and domestication. Studies of the genetic and molecular diversity of the selected rubber tree germplasm subsequently began within the Biotrop program from CIRAD, France. Genome organization and molecular diversity were analysed, using 14 isozyme loci, cytoplasmic and nuclear RFLPs and, later, 17 microsatellite loci (Besse *et al.*, 1994; Le Guen *et al.*, 2003). The germplasm accessions could be separated into six genetic groups, which encompass populations of defined geographic origins, spread over the entire Amazon Basin and the western Andean region (1), the eastern region near the Tapajós river (2), the federal state regions of Acre (3,4), Rondonia (5), and Mato Grosso (6) (Besse *et al.*, 1994).

According to detailed studies by Seguin *et al.* (1996) and Lespinasse *et al.* (2000b), *Hevea brasiliensis* has a diploid genomic organization with rare duplicated loci. These studies led to identification of 18 basic linkage groups in a rubber genome of 2150 cM total map length. Using the 195 progenies of the population derived from the crossing of PB 260 × Fx 3899 and their response to six isolated strains of *M. ulei*, eight quantitative trait loci (QTL) with respect to resistance were identified on seven independent linkage groups.

Le Guen *et al.* (2003) and Lespinasse *et al.* (2000b), on the basis of their molecular data, prepared the mapping of genes conferring field resistance to South American leaf blight. For the first time, it was shown that both factors for partial resistance and for complete resistance were quantitatively expressed in the progeny and could be correlated with five loci. The molecular approach to this plant-pathogen combination has greatly enhanced the possibilities of proceeding with marker-assisted breeding and selection in a perennial tropical plant.

Nevertheless, some important questions remained to be answered. The authors debated why the defence mechanisms controlled by a locus termed M13-lbn differed strongly in behaviour between the field test and that within an inoculation chamber: the efficiency was best under field conditions, but weak or ineffective under controlled conditions. In susceptible progeny, the resistance conferred by M13-lbn was no longer detectable.

The same question arises for the QTLs that correspond to linkage groups g15 and g12. These factors are observable under controlled conditions but are not found in field experiments. For this reason, whether the lesion-diameter trait is a useful predictor for SALB resistance under field conditions is debated no matter what the environment. The hypothesis of a 'QTL with a major effect modulated by a few other minor QTLs' was strengthened by further analyses. In general, the state of development of genetic analysis in this tropical perennial plant is very promising and pursuing the next steps to marker-assisted breeding appears feasible.

In a parallel step, characterization of the variability of the ascomycete *M. ulei* is also proceeding well. Eleven

polymorphic microsatellite markers for *M. ulei* have been characterized and applied to six isolates from Brazil and four isolates from French Guiana.

Recent genetic studies of rubber tree resistance to *M. ulei* indicate that, in addition to the eight major resistance-conferring QTLs, there must be minor factors that modulate the action of the major components or that interfere with the phenotypic expression of these components. The major resistance factors, when active, result in total expression of defensive reactions and lead to complete resistance, i.e. to control of fungal development in the leaf; this ultimately means that no lesions or no spore-forming lesions occur (lesion types 0, 1 and 2, Fig.2). These lesion types do occur and give proof for the occurrence of major resistance factors.

But the conclusions drawn from the molecular studies also give clear evidence that in addition to the major genes for complete resistance, a set of minor components interfere with the performance of the major resistance factors.

From the results of previous studies on resistance physiology of rubber to *M. ulei*, the importance of various typical biochemical properties of the rubber tree that can actively interfere with resistance reactions on several levels is known. These reactions occur when the major gene resistance is overcome by pathogen isolates with their respective virulence genes. In these combinations of virulent pathogen and susceptible host, the minor genes or the polyfactorial resistance factors are expressed. Their action is expressed by quantitative variation in lesion size and spore abundance. Some of these polyfactorial reactions have been described with respect to scopoletin and lignin formation by García *et al.* (1999), but the studies have never been evaluated from the aspect of transient changes of leaf physiology in the course of the infection process. In the same context, resistance impairment by the process of cyanogenesis has never been regarded as interacting with the classical resistance processes. So far, the impact of cyanogenesis has never been considered to be important for the outcome of a disease response, though the studies of Lieberei (1986, 1988a) and Lieberei *et al.* (1989) clearly established that strongly cyanogenic *Hevea* genotypes have inefficient or no defence responses. Consequently, this raises the question as to what extent the two properties 'sink leaves' and 'cyanogenesis' interact with resistance such that they can explain some of the irregularities identified in studies on biochemical defence responses by García *et al.* (1999).

Leaf flush growth is a remarkable and special feature of tropical plants such as rubber trees (Hallé and Martin, 1968). Leaf stages A, B and C of rubber are metabolic sink leaves and their transition from a metabolic sink to a metabolic source organ is remarkably long. Some of the leaf parameters are summarized in Table 3, which is based on a study by Lieberei (1984). The amount of chlorophyll per leaf area increases from leaf stage A, with 26 mg cm<sup>-2</sup>, to leaf stage D with 47 mg cm<sup>-2</sup>, and the Chl *a/b* ratio shifts from a low value to the typical value found in photosynthetically active plants of about 3. Leaf stage C possesses a remarkably lower chlorophyll amount per area than either stage B or D. The fresh weight per

area is lowest in stage C, as is the amount of soluble protein per area. These data are a consequence of exponential leaf growth. The leaf area from stage B to stage C is enlarged mainly by leaf water uptake and cell enlargement. During this phase, the cell walls are very thin and flexible and cell wall hardening is minimal. Lignin is definitely absent from cell walls until stage D (Voß, 2001). On the other hand, it is remarkable that the vacuolar content of cyanogenic precursors per fresh weight rises from stage A to stage D and the activity of the apoplastic  $\beta$ -glucosidase/linamarase also is very high (Table 3, and Lieberei *et al.*, 1985). Cell wall changes and lignin formation obviously become a predominant reaction with the onset of leaf stage D. The ratio of dry weight to fresh weight doubles from stages B and C to D due to cell wall lignification, and this will also be the reason for the obligate restriction of fungal spread in the wall region of stage D leaves.

The leaves are metabolic sink tissues and dependent on the energy balance of the mother plant for a long time. Energy-dependent synthesis requires transport of assimilate into the leaves to occur. Under these conditions, it seems clear that resistance-screening experiments carried out with detached leaves will result in different results from those with leaves attached to the plants. Processes such as cinnamic acid synthesis or changes in pool sizes of amino acids, active synthesis of scopoletin, lignin and glycosides will be retarded or even stopped in detached leaves due to exhaustion of energy-delivering compounds.

In tests with leaves attached to the plants under controlled conditions, pathogen development will perform differently because these leaves receive their metabolic supply from the adjacent source organs. The production of scopoletin, which is produced as a response to fungal attack in rubber tree leaves (Breton *et al.*, 1997; García *et al.*, 1999), is also dependent on the energy-delivering sources in the inoculated leaves. The amount of scopoletin accumulated in the inoculation droplets, as followed by García *et al.* (1995b, 1999) and by Breton *et al.* (1997) is the result of several reactions: the scopoletin formation as an induced response to pathogens and to tissue injury, the elicitor treatment, and other forms of stress to the plant tissue. Scopoletin is exuded from the living cell into the apoplast and can easily be detected under UV excitation as a blue fluorescent compound. Because of its weak antimicrobial action, scopoletin is regarded as a phytoalexin in *Hevea* species (Giesemann *et al.*, 1986). In the cell wall, apoplastic peroxidases convert scopoletin in the presence of  $H_2O_2$  to yellow/brown insoluble substances and the fluorescence is quenched (Breton *et al.*, 1997; Chong *et al.*, 1999). A second typical reaction lowering the scopoletin content is the glycosylation of scopoletin to yield the glucoside scopolin. Scopolin is formed by the action of UDP-glucosyl transferase (UGT); the glucoside is located in the vacuole. Recently Langlois-Meurinne *et al.* (2005) reported on the stress- and pathogen-related expression of UGT in plant-pathogen interactions and, in a general approach, Gachon *et al.* (2005) emphasized the importance of glycosylation for biosynthesis and storage of secondary compounds. In fact, in most plants that produce scopoletin in response to pathogen attack (e.g. potato, Hughes and Swain, 1960; tobacco, Sequeira, 1969;

sunflower, Tal and Robeson, 1986), scopolin co-occurs with scopoletin. In rubber tree experiments, García *et al.* (1995b, 1999) and Breton *et al.* (1997) did not detect scopolin. In contrast, Körner (1994) found both scopoletin and scopolin after infection of *H. brasiliensis* leaves with spores of the weak *Hevea* pathogen, *Periconia manihoticola*. Körner (1994) infected rubber tree leaves (stage B) with spores of *P. manihoticola* and incubated them as detached leaves in darkened Petri dishes on moistened filter paper at 22 °C and as leaves attached to the plant, covered by a black plastic bag that also contained a moistened filter paper. After 2 d, small necrotic lesions about 1 mm in diameter surrounded by a yellow halo had developed under both treatments. When analysed under UV-light, both treatments produced a blue fluorescence in the tissue. The fluorescence in detached leaves was found as a net-like distribution in the apoplast, whereas the fluorescence in the attached leaves was found to be concentrated in the vacuoles (Fig. 3). After methanolic extraction, these two compounds could be verified by TLC analysis as scopoletin in detached leaves and as scopolin with trace amounts of scopoletin in leaves attached to the plants. The general difference between the inoculations that produced scopoletin or scopolin was the fact that leaves attached to the plants appeared to receive sufficient assimilates from the mother plant to form the glucoside, whereas the detached leaves, as isolated sink organs, used reserve compounds to produce limited amounts of scopoletin, but did not possess enough reserve substances to provide energy and substrates for carrying out UDP-glucose formation. In this context, the experimental use of detached leaves for resistance screenings must be re-evaluated. The amount of scopoletin produced in detached leaf systems has also been demonstrated to be highly influenced by incubation conditions (Lieberei *et al.*, 1989). When leaves of strongly cyanogenic genotypes of *H. brasiliensis* are inoculated and incubated in closed Petri dishes, they form large lesions and large amounts of HCN ( $38.6 \pm 4.4$  mg HCN g<sup>-1</sup> d. wt) are liberated. The amount of scopoletin is small (0.48 mg Scop g<sup>-1</sup> d. wt).

When the incubation conditions are modified in such way that the HCN liberated from the leaves is immediately taken out of the system by absorption in alkali or by passing humid air through the incubation chamber, the lesions in the highly cyanogenic leaves do not develop to larger sizes but remain small, and the amount of scopoletin formed around these lesions is about five times higher than in the strongly cyanogenic condition. Thus, high cyanide liberation during pathogen development in the host tissue can lead to impairment of active defence, or in other words, the experiments indicate that strongly cyanogenic plants may contain all potential resistance factors in the same manner as resistant plants but these resistance factors are impaired by another plant factor, in this case cyanide liberation (Lieberei *et al.*, 1989).

#### *Effects of cyanogenesis: importance of threshold concentrations*

HCN liberation in plants can impair active defence (rubber tree, Lieberei, 1984, 1986; Lieberei *et al.*, 1989;



flax, Lüdtke and Hahn, 1953; Trione, 1960). This finding is hardly accepted by breeders, because it is counter-intuitive to the usual understanding of evolutionary mechanisms. It should be impossible to inherit susceptibility, but both qualitative and quantitative aspects of cyanogenesis are inherited.

A more in-depth look into cyanogenesis indicates that it is a phenomenon in the framework of polyfactorial evolutionary concepts. Cyanogenic plants can be generally divided into obligate cyanogenic plants and facultative cyanogenic species. In obligately cyanogenic plants, the genes for biosynthesis of cyanogenic precursors and their vacuolar storage and the genes for the synthesis of the  $\beta$ -glycosidases specific for each cyanogenic precursor are constitutively expressed. There are many quantitative variations in this phenotypic feature among the cyanogenic plants. Many genotypes contain small amounts of cyanogenic compounds in low micromolar ranges per tissue dry weight, whereas other genotypes of strongly cyanogenic plants such as *Phaseolus lunatus*, *Manihot esculenta*, *Hevea brasiliensis*, *Sorghum bicolor*, *Passiflora capsularis* and many others accumulate large amounts of cyanogenic precursors and the tissue content varies from less than  $1 \text{ mmol g}^{-1} \text{ d. wt}$  to several hundred  $\text{mmol}^{-1} \text{ d. wt}$  of tissue (e.g. Lieberei, 1988a for *Hevea* species; Busk and Møller, 2002, for *Sorghum bicolor*; Ballhorn *et al.*, 2005, for *Phaseolus lunatus*; Amelot *et al.*, 2006, for *Passiflora capsularis*).

With respect to plant–herbivore interactions, perennial woody plants are highly interesting models for the study of polyfactorial environmental pressure. During the life span of leaves ontogenetic changes in HCN-p and other metabolic patterns occur. This leads to variation of the biochemical scenarios presented to herbivores and leaf-attacking microbes (e.g. Gleadow and Woodrow, 2000a, b, 2002; Miller *et al.*, 2006).

Cyanogenic potential HCN-p (Loyd and Gray, 1970) and the capacity for HCN release per unit time (HCN-c) allow us to quantify cyanide liberation as a reaction to tissue injury. In addition to genotypic variability for HCN-p and HCN-c, tissues often reveal strong variability at different ontogenetic stages. Generally, young leaf stages have higher HCN-p and HCN-c than older leaves (e.g. Gleadow and Woodrow, 2000a, b).

Once liberated, HCN acts as a highly mobile component in the leaf tissue. Within seconds, this compound reaches the cells surrounding the locus of HCN-production. HCN is an inhibitor of many biochemical processes (Solomonson, 1981) and impairs active defence in cells surrounding the lesion (Lieberei, 1988a; Lieberei *et al.*, 1989, 1996).

#### NEW APPROACHES IN MARKER-ASSISTED BREEDING: HOW TO COMBINE COMPLEX PHYSIOLOGICAL PARAMETERS WITH MOLECULAR MARKERS

Identification of major components that contribute to QTLs permits identification of potentially resistant genotypes. The plants that contain these QTLs generally possess all

metabolic systems needed for active defence and, thus, these genotypes are highly interesting for further breeding. When characterized by QTLs for production of large amounts of high quality latex, these plant materials are a primary choice for breeding and selection. The most suitable plants for further development out of this group are those plants with a short ontogenetic transition from complete sink leaves to complete mature source leaves. It is necessary to check how long and to what extent young leaves are devoid of sufficient energy reserves for the processing of active, synthesis-dependent defence reactions. When significant formation of scopoletin and the onset of lignification are necessary for limitation of fungal development, a complete and successful response depends on both the presence of an active phenylalanine ammonia lyase (PAL) and a sizeable pool of aromatic amino acids, especially phenylalanine, for a successful resistance response.

The resistance-impairing effects of cyanogenesis have to be considered independently from the leaf development stage. Cyanide inhibits active defence by inhibition of photosynthetic  $\text{CO}_2$ -fixation and by inhibition of a number of other enzymes (Solomonson, 1981), among them the key enzyme for cinnamic acid production, PAL. Hanson and Havir (1972) reported in detail on the inhibition kinetics for PAL. Körner (1994) repeated these experiments and evaluated the same inhibitory response for PAL extracted from leaves of *H. brasiliensis*. This finding also explains the report of Lieberei *et al.* (1989), who showed better resistance when cyanide concentrations were experimentally lowered in inoculated leaves. Furthermore, unpredictable test responses for clones GT1, IAN 873 and other genotypes that are known to be strongly cyanogenic (García *et al.*, 1999) can be explained in this manner. Cyanide inhibition may also explain the discrepancy with some plants of greater susceptibility under *in vitro* conditions in comparison with field conditions (Lepinasse *et al.*, 2000a).

In general, this new approach for making selections of plant material for breeding studies can be simplified by avoiding use of strongly cyanogenic genotypes in experiments. Furthermore, only genotypes with short flushing periods and fast leaf maturation patterns should be used. Additionally, plants should be included that have irregular leaf shedding instead of regular and complete change of the canopy. The most promising genotypes are those with genes from *H. benthamiana* (Junqueira *et al.*, 1990).

#### MOLECULAR METHODS CAN BE USED TO LOWER PLANT CYANOGENESIS

Techniques for application of antisense technology to produce lower cyanogenesis levels have been successfully accomplished with respect to cassava. Both the groups of Møller (Kristensen *et al.*, 2005) and Sayre (Siritunga *et al.*, 2004) offer excellent molecular approaches to this field. Whenever weakly cyanogenic plants are selected, it should be verified to what extent a certain threshold level of cyanogenesis confers on a plant protection from pests and pathogens. Pathogens and pests employ different

strategies to respond to cyanogenesis and other plant defensive factors. Evolution is dynamic, and so are our problems.

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