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Assessing resistance of rubber tree clones to *Microcyclus ulei* in large-scale clone trials in Ecuador: a less time-consuming field method

Franck Rivano · Malena Martinez · Victor Cevallos · Christian Cilas

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Abstract Resistance of rubber tree clones to South American Leaf Blight (SALB) caused by the fungus *Microcyclus ulei* is normally assessed in specific large-scale clone trials, which in general entail a considerable amount of work. Four variables are observed monthly on each tree over many years: disease severity and conidial sporulation intensity on young leaves, and severity and stroma density on mature leaves. In order to simplify this field assessment method, we tested the resistance of eight rubber tree clones to *M. ulei* in Ecuador in a Fisher block design with four replicates per treatment. Three months after planting, monthly observations were made for a period of 12 months on the foliage focusing on the four variables, in order to quantify disease development. Given the correlations between the four variables, assessment of conidial sporulation intensity on young leaves and stroma density on mature leaves should be sufficient. The most suitable period to start the assessment was 6 months after planting, for a duration of six to nine months. As repeated observations on the same trees were autocorrelated, it was possible to reduce the assessment frequency to once every 2 months. To conclude, assessing the resistance of rubber tree clones to SALB in large-scale clone trials can be optimized to reduce the number of observation times by 50%.

Keywords *Hevea brasiliensis* · South American leaf blight · Resistance · Sporulation

F. Rivano (✉)
CIRAD, UPR Systèmes de pérennes,
Avenue Agropolis, TA B-34/02,
34 398 Montpellier Cedex 5, France
e-mail: franck.rivano@cirad.fr

M. Martinez · V. Cevallos
INIAP, Estación Experimental Santo Domingo,
Km 38 via Santo Domingo,
La Concordia, Ecuador

M. Martinez
e-mail: martinezmalena@yahoo.es

V. Cevallos
e-mail: vjcevallos75@yahoo.com

C. Cilas
CIRAD, UPR Bioagresseurs de pérennes,
Avenue Agropolis, TA A-31/02,
34 398 Montpellier Cedex 5, France
e-mail: christian.cilas@cirad.fr

Introduction

South American Leaf Blight (SALB) caused by the ascomycete fungus *Microcyclus ulei* (Henn.) Arx is the most destructive disease of the rubber tree (*Hevea brasiliensis* Muell.–Arg.) in South and Central America, where it is endemic and drastically limits natural rubber production (Guyot et al. 2008). It causes severe shedding of young leaves, which are successively attacked, thereby weakening or even killing the trees (Lieberei 2007).

Planting resistant cultivars is acknowledged to be the most promising avenue for controlling SALB (Darmono and Chee 1985; Junqueira et al. 1990; Rivano et al. 1989; Rivano 1992). SALB research programmes have largely focused on identifying resistance mechanisms in *Hevea* spp. (Garcia et al. 1995, 1999), and selecting resistant rubber tree clones, with a view to combining them with latex productivity (Chee 1976; Rivano 1997b). Two types of resistance have been found. The first is total resistance, characterized by an absence of conidial sporulation, despite the existence of lesions; the second is partial resistance for which there is a varying degree of conidial sporulation. Although both types of lesions can be found on the same clone, because it can be infected by different pathotypes of the fungus, preference has been given to the second type as it is quantifiable and easily measurable both in the field and under controlled conditions (Junqueira et al. 1988, 1990; Rivano 1992).

However, combining durable resistance to SALB with high rubber yields has yet to be achieved, as the fungus has a great ability to develop new races capable of overcoming a large number of selected resistances (Miller 1966; Chee and Holliday 1986; Junqueira et al. 1986; Gasparotto and Ferreira 1989; Hashim and de Almeida 1987; Rivano 1997a; Le Guen et al. 2008). In the State of Bahia, Brazil, Mattos et al. (2003) revealed the physiological variability of *M. ulei* by artificial inoculation of 12 rubber tree clones with 50 *M. ulei* isolates from Bahia, and differentiated 36 new races of the pathogen.

Resistance of rubber tree clones to SALB is usually assessed in large-scale clone trials, based on a specific statistical design recommended for testing new materials in a given area, on the usual planting density (510 trees ha^{-1}) with a large number of trees per treatment (Clément Demange et al. 1995; Rivano 1992). The four variables used characterise different stages of the infection cycle of the fungus; they enable classification of clones during the immature period of the trees, which is the first 3 years after planting (Chee 1976; Junqueira et al. 1990; Rivano 1992, 1997b; Le Guen et al. 2002). The four variables are attack severity on young leaves, attack severity on mature leaves, conidial sporulation intensity on young leaves, and stroma density on mature leaves. However, such trials entail a considerable amount of work, often in a multi-site network comprising several similar trials, as observations are carried out monthly, tree by tree and for at least 2 years, as long as leaves are accessible from the ground. Although essential, these large-scale clone trials are costly and take a long time to set up in rubber producing countries. It is necessary to produce the planting material required to set up each trial, train technicians in resistance assessment methods, and reserve the funds needed for maintaining and monitoring the experimental plots for 10 to 15 years.

In view of these difficulties, the purpose of this study was to simplify and optimize the method currently used to assess *Hevea* clonal resistance to SALB. In order to make it less costly and time-consuming, whilst maintaining the precision needed for efficient clone differentiation, several levels of simplification were considered. We first sought to reduce the number of variables observed, along with the observation frequency. Then we sought the best date to start observations, along with their optimum durations. This study was conducted in Ecuador, where eight rubber tree clones were studied in an area where the disease was rife in neighbouring plantations. Rubber has been grown for more than 40 years on the Pacific coast in Ecuador, where it is near its second planting cycle. SALB, which now exists in all plantations, has developed pathotypes that are able to overcome the resistance of clones selected for use in crown budding. Those clones have now mostly been abandoned as they are highly susceptible: there remains no other solution but to use durable resistance.

Materials and methods

Site

The trial, set up in February 2006, was located at the Santo Domingo Station of the National Institute for Agricultural and Livestock Research (00°01′ N, 79° 22′ W, at an elevation of 300 m above sea level), in the coastal humid tropical zone, Esmeraldas province, Ecuador. The meteorological data came from the National Meteorology and Hydrology Institute station, located at the Santo Domingo Station. This region typically has a mean annual precipitation of 2,800 mm in a mono-modal wet season (approximately January to June) with an average aboveground

temperature of 24.6°C, an average relative humidity of 88%, and 700 h of sunshine per year. However, wet spells can occur during the dry season (July to December) as in 2006–2007. Figure 1 gives the monthly climatic data for Santo Domingo, from February 2006 to April 2007. Rainfall for the January to April period varied substantially between 2006 (1,880 mm) and 2007 (2,590 mm). There was also an increase in maximum temperatures in the wet season between 2006 and 2007, whilst the average relative humidity remained unchanged.

Previously, since 1963, the Santo Domingo Station had been exclusively devoted to oil palm research. The present trial was the first plot of rubber trees to be planted after a former oil palm plot, which was felled the same year as the rubber trees were planted. The trial was planted in the middle of a large oil palm estate and the nearest rubber trees plantation was about 4.5 km away. The *M. ulei* primary inoculum was therefore probably transported to the trial site on the prevailing winds from distant plantations. The land was uniform, with a flat topography. A legume cover crop, *Pueraria phaseoloides*, which had already been established between the oil palms, was kept for the rubber trees, in order to limit weed invasion and facilitate row upkeep.

Experimental design

The eight rubber tree clones, all pure *H. brasiliensis* originating from Central and South America (Table 1), were chosen for their good vigour and production characteristics, mostly achieved on an experimental scale as they had recently been selected in Brazil (Le Guen et al. 2002; Garcia et al. 2004). Two of them, FX 3864 and FX 4098 have been grown for decades on a commercial scale in Brazil. Clone FX 3864 was chosen as a control because it has partial resistance to *M. ulei*. The other six clones, CDC 56, CDC 312, FDR 4575, FDR 5597, FDR 5788, and MDF 180 have ancestors originating from the Madre de Dios region in Peru, and have been planted for around 30 years in a plantation in Bahia State, Brazil, which is very severely infested by *M. ulei*, and where the fungal pathogenic diversity is the greatest (Mattos et al. 2003). The clones have been tested under natural and controlled conditions since 2001, with artificial inoculations using nine highly aggressive (poly) virulent strains selected from a collection of diverse strains. The encouraging results from those natural and artificial infections, along with the first production data, enabled the dissemination of the material throughout Brazil and in other Latin American countries. The purpose of such dissemination is to set up a network of large-scale clone trials to test their performance under different climatic and disease pressure conditions.

We set up a large-scale clone trial, which is routinely used to assess the field performance of new rubber tree clones in new areas (Clément Demange et al. 1995; Rivano 1992). The plot was arranged in a Fisher block design with 4 replicates

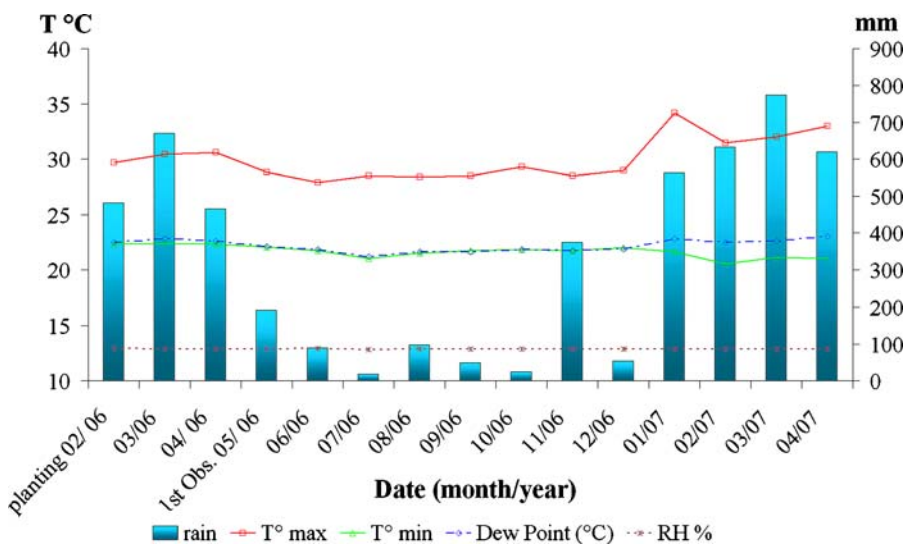

Fig. 1 Monthly climatic data for Santo Domingo Station (Ecuador), February 2006 to April 2007

Table 1 Plant material description: the eight rubber tree clones tested at Santo Domingo Station, Ecuador, 2006–2007

Clone	Parents (Female × Male)	Country of origin	Year of introduction into Ecuador
FX 3864 (control)	PB 86 × FB 38	Brazil	1964
FX 4098	PB 86 × B 110	Brazil	2004
CDC 56	MDX 91 × RRIM 614	Guatemala	2004
CDC 312	AVROS 308 × MDX 40	Guatemala	2004
FDR 4575	FDR 18 × FX 3032	Brazil	2004
FDR 5597	HAR 68 × TU 42–525	Brazil	2004
FDR 5788	HAR 8 × MDF 180	Brazil	2004
MDF 180	Primary clone of *H. brasiliensis*	Peru	2004

AVROS Algemene Vereniging Rubberplanters Oostkust Sumatra, *B* Belterra, Brazil, *CDC* Clavellinas Dothidella Cross, *FB* Ford Belem, *FDR* Firestone Dothidella Resistant, *FX* Ford Cross, *MDF* Madre de Dios Firestone, *HAR* Harbel Estate (Firestone), Liberia, *RRIM* Rubber Research Institute of Malaysia, *TU* Turrialba, Costa Rica

and 8 treatments, corresponding to the 8 rubber tree clones; each replicate consisting of 4 rows of 20 trees (80 trees), giving a total area of 5.69 ha (245.0 m × 232.4 m). The unit plot measured 1,568 m² (56 × 28 m). The rubber trees were planted in single rows 7 m apart, at a tree spacing of 2.8 m, with a planting density of 510 trees per hectare. Two border rows of clone GU 198 (a Guatemalan selection) surrounded the trial.

The trial was planted with polybag seedlings budded at 12 months, then cut back and transferred to the field with a dormant eye, on 8–11 February 2006. Plant recovery was uniform and estimated at 92% 2 months after planting. Dead plants were replaced within 6 months after planting.

Data measurements

Monthly observations were carried out on the same plants, i.e. the 40 trees of the 2 central rows of each replicate (50% of the total number of plants of each replicate) during the immature period of the trees, beginning 3 months after the trial was planted (first observation date: date 1, 2 May 2006), so that the scions could start growing and develop a stem bearing one or two leaf stages, up to 15 months after planting (date 12, 18 April 2007). Susceptibility to *M. ulei* was assessed on young foliage (growth stage C), and on mature foliage (growth stage D), based on leaf stages (flushes) described by Hallé and Martin (1968). Attack severity on young leaves (ATYL) and attack severity on mature leaves (ATML) were recorded on the last and upper flush, on an adapted Chee and Holliday's scale (1986) from 0 to 4: 0=nil (<1% leaf area necrotized); 1=low (1–5% leaf area necrotized); 2=medium (6–15% leaf area necrotized); 3=high (16–30% leaf area necrotized); 4=very high (>30% leaf area necrotized). Conidial (asexual) sporulation intensity on lesions on young leaves, a variable which we have called "type of reaction" (TR), was observed with the naked eye and recorded on a scale of 1 to 6 derived from Junqueira et al. (1986): 1=necrotic lesions without spores; 2=chlorotic lesions without spores; 3=very low and heterogeneous sporulation on the underside of the leaf; 4=high, heterogeneous or partial sporulation on the underside of the leaf; 5=very high and uniform sporulation covering the entire lesion on the underside of the leaf; 6=very high sporulation covering the entire lesion on the underside and upper side of the leaf. If in doubt, a sample of three leaflets per tree was taken on the same day to examine sporulation intensity in the laboratory under a stereomicroscope (x 40). Stroma density (ST) on the upper side of mature leaves, linked to the sexual phase of the fungus, was recorded on a scale of 0 to 4: 0=no stromata; 1<5 stromata per leaflet; 2=5–10 stromata; 3=11–30 stromata; 4>30 stromata.

Observations focused on the final leaf stage of each plant: i, if the leaves of this stage were young and at stage C, the ATYL and TR scores were attributed; ii, if the leaves of this stage were mature and at stage D, the ATML and ST scores were attributed. The number of trees observed for young foliage therefore varied as it depended on the development status of the final leaf stage. For mature

leaves, it was always 40 trees per replicate that were observed. For the four variables, the most severely attacked leaves of the leaf stage were observed. For each measurement, and for each replicate of each clone, the mean of each variable was calculated.

In addition, the trunk diameter 1 m from the ground was measured 1 year after planting, on 7 March 2007, on all the trees in the plot. This measurement may reveal any differences in vigour between the clones that correlate with their susceptibility to SALB.

All these monthly assessments were very time-consuming: indeed, each evaluation occupied a technician for 5 days and an agricultural labourer for 3 days. Given that one technician-day was financially equivalent to four labourer-days, each monthly field assessment of the clones cost 23 agricultural labourer-days.

Data processing and statistics

The monthly observations carried out on the same plants for 12 consecutive months were, by nature, correlated and initially required longitudinal data analysis. In fact, longitudinal data analysis makes it possible to define a model of individual data autocorrelation over time. The different structures of the variance/covariance matrices tested corresponded to the models generally used for longitudinal data: the Compound Symmetric model (CS) with constant correlation between the different years and uniform variances, the Compound Symmetric model with heterogeneous variances (CSH), the first order autoregressive model with uniform variances (AR1), the first order autoregressive model with heterogeneous variances (ARH1), and the unstructured model (UN), i.e. the correlations between the different dates are independent from each other. The different models can be written as follows:

$$Y_{ijkl} = \mu + Ci + Dj + (CD)ij + bk + E_{ijkl}$$

where:

Y_{ijkl}	independent variable (ATYL, TR, ATML, ST)
μ	general mean
C_i	effect of clone i
D_j	effect of date j
$(CD)_{ij}$	clone x date interaction
b_k	effect of block k
E_{ijkl}	residual of tree l belonging to clone i block k for date j

and:

1- in the case of the first order autoregressive model, in which measurements on the same trees are dependent, with the dependency decreasing steadily when the time difference between assessment dates increases:

$$V(E_{ijk}) = \sigma^2 \text{ if variances are uniform between dates (AR1)}$$
$$= \sigma_j^2 \text{ otherwise (ARH1)}$$

and

$$corr(E_{ijkl},\ E_{i'j'k'l'}) = \rho^{|j-j'|} \ \text{if}\ i = i',\ k = k',\ l = l'$$
$$= 0 \text{ otherwise}$$

2- in the case of the Compound Symmetry model, the correlation between dates is stable, whatever the difference between observation dates:

$$V(E_{ijkl}) = \sigma^2 \text{ if variances are uniform between cycles (CS)}$$
$$= \sigma_j^2 \text{ otherwise(CSH)}$$

and

$$Corr(E_{ijkl}, E_{i'j'k'l'}) = \rho \ \text{if}\ i = i',\ k = k',\ l = l'$$
$$= 0 \text{ otherwise}$$

3- in the case of the Unstructured model, correlation between each pair of the dates is estimated:

$$V(E_{ijkl}) = \sigma_j^2$$

and

$$Corr(E_{ijkl}, E_{i'j'k'l'}) = \rho_{jj'} \ \text{if}\ i = i',\ k = k',\ l = l'$$
$$= 0 \text{ otherwise}$$

Different statistical criteria were proposed to select a most appropriate model: the log of likelihood, the Akaike criterion (AIC), or the Schwartz criterion (BIC) (Verbeke and Molenberghs 2000). Smaller values of these criteria indicate a better model. Longitudinal data analyses were performed with the

mixed model of the SAS statistical software package, version 9.1.3 for Windows (SAS Institute Inc. 2007; Littell et al. 1996).

The coefficients of correlation between the different parameters measuring attack intensity on young and old leaves were estimated for each of the dates in order to determine which variable(s) would be the most relevant for assessing susceptibility in the field.

Analyses of variance (ANOVA) for each variable studied and for each of the dates were then used to differentiate between the eight rubber tree clones based on their susceptibility to *Microcyclus* for each of the observation dates.

Results

Rubber tree clone susceptibility to *Microcyclus ulei*

The disease was low from date 1 (02 May 2006) to date 5 (18 September 2006). The attack levels on young and mature leaves did not exceed 1.0 over this first period (Fig. 2a and c). Attack severity then greatly increased

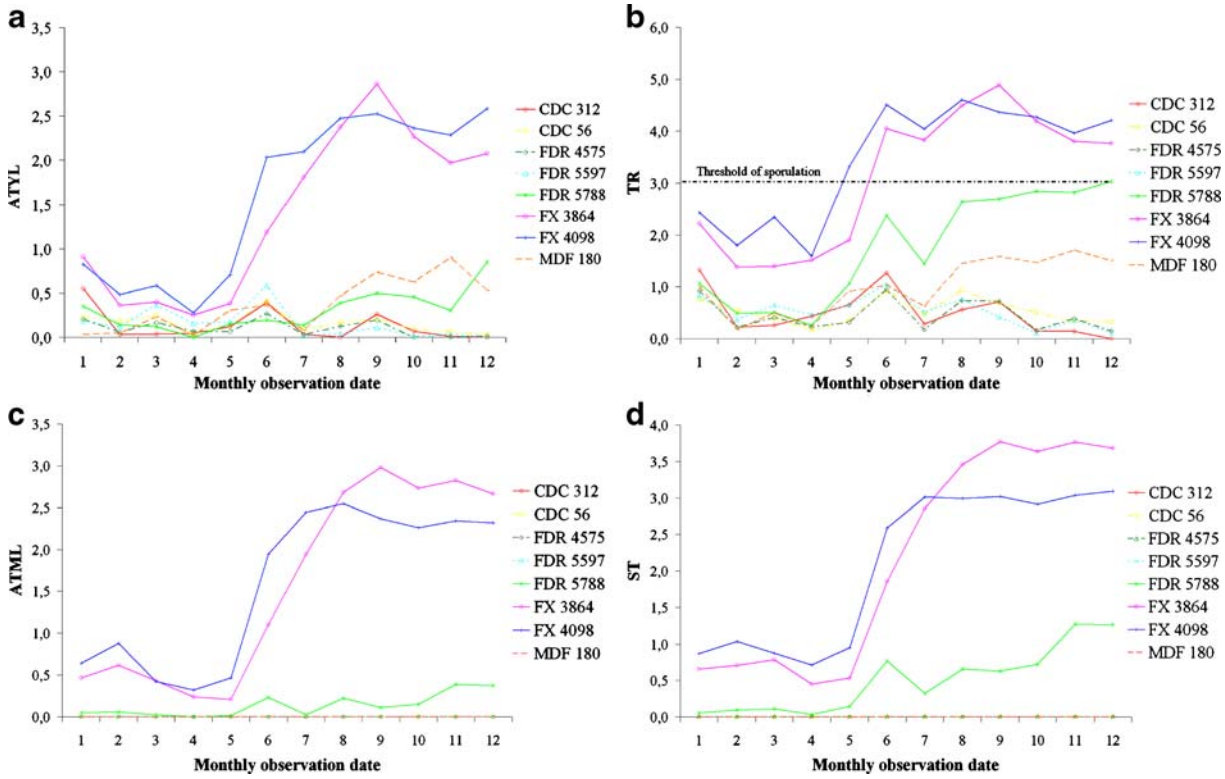

Fig. 2 Susceptibility to *M. ulei* of the eight rubber tree clones, measured on young and mature leaves at Santo Domingo Station, Ecuador, 2006–2007. **a** Attack severity on young leaves (ATYL). Attack severity on young leaves recorded on the last and upper flush, on an adapted Chee and Holliday's scale (1986) from 0 to 4: 0=nil (<1% leaf area necrotized); 1=low (1–5% leaf area necrotized); 2=medium (6–15% leaf area necrotized); 3=high (16–30% leaf area necrotized); 4=very high (>30% leaf area necrotized). **b** Type of Reaction on young leaves: conidial sporulation intensity (TR). 1=necrotic lesions without spores; 2=chlorotic lesions without spores; 3=very low and heterogeneous sporulation on the underside of the leaf; 4=high, heterogeneous or partial sporulation on the underside of the leaf; 5=very high and uniform sporulation covering the entire lesion on the underside of the leaf; 6=very high sporulation covering the entire lesion on the underside and upper side of the leaf. **c** Attack severity on mature leaves (ATML). Attack severity on mature leaves recorded on the last and upper flush, on an adapted Chee and Holliday's scale (1986) from 0 to 4: 0=nil (<1% leaf area necrotized); 1=low (1–5% leaf area necrotized); 2=medium (6–15% leaf area necrotized); 3=high (16–30% leaf area necrotized); 4=very high (>30% leaf area necrotized). **d** Stroma density on mature leaves (ST). 0=no stromata; 1<5 stromata per leaflet; 2=5–10 stromata; 3=11–30 stromata; 4>30 stromata

for two clones, FX 3864 and FX 4098, and remained greater than 2.0 until date 12 (18 April 2007). On the other clones, ATYL never exceeded 1, either over the first or second period. This effect was even stronger for ATML, since positive scores were recorded for only three clones, FX 3864, FX 4098 and FDR 5788. Sporulation (TR ≥ 3) was only detected on the same three clones (Fig. 2b). On date 5 (18 September 2006) the first sporulation was detected on clone FX 4098, then on FX 3864 15 days later, finally on FDR 5788 on date 12 (18 April 2007). Stromata were also only detected on the same three clones (Fig. 2d). The clones differed among themselves on date 5. Date 7 (23 November 2006) also corresponded with the beginning of the wet season.

Three groups of clones stood out for their resistance to *Microcyclus ulei*. Clones FX 3864 (control) and FX 4098 stood out from the others as they displayed low partial resistance to *M. ulei*: medium and high attack rates were observed on young and mature leaves, with ATYL between 3 and 5 and ATML > 2, high sporulation with TR > 4 during the wet season, and high stroma density, ST > 3. The susceptibility of the two FX clones was reversed on date 8. Conversely, clones CDC 56, CDC 312, FDR 4575, FDR 5597 and MDF 180 showed total resistance, with low attacks on young leaves (ATYL < 1), an absence of sporulation (TR < 3), and an absence of stromata on mature leaves (ST=0). Finally, FDR 5788 was in an intermediate position with good partial resistance: ATYL < 1, a low sporulation intensity (TR≤3), low attacks on mature leaves (ATML < 0.5) and low stroma density (max. ST=1.3).

The growth results, measured by the trunk diameter 1 m from the ground 1 year after planting (7 March 2007), showed significant differences among the eight clones (Table 2). Clones CDC 312 and FX 3864 (control) had the largest trunk diameter, corresponding to a circumference exceeding 10 cm, which is satisfactory for trees of that age. For the two most susceptible clones, FX 3864 and FX 4098, the attacks did not affect tree growth in the first year of the trial, since FX 3864 (control) and FX 4098 had the second and fourth largest tree diameter on 7 March 2007, respectively. Conversely, some resistant clones, such as FDR 5597 et FDR 4575, had the smallest diameters. At this very young stage of the plantation, the impact of SALB on tree development should still be insufficient to alter genetic growth characteristics.

Table 2 Rubber tree diameter measured at 1 year (April 2007) 1 m from the ground at Santo Domingo Station, Ecuador

Clone	Average Diameter (cm)	Uniform groups
CDC 312	3.37	a
FX 3864 (control)	3.22	b
FDR 5788	3.17	b c
FX 4098	3.10	c d
CDC 56	3.03	d e
MDF 180	2.99	e
FDR 5597	2.98	e
FDR 4575	2.89	f

Means separated by Student-Newman- Keuls test at $P<0.05$

Clone-Date interactions: choice of statistical model for data analysis

The overall data analysis was based on a longitudinal data analysis of variance model. The choice of a model that best accounted for the structure of the correlations over time was based on the AIC and BIC criteria (Table 3).

The best model was the first order autoregressive model with heterogeneous variances (ARH1). Indeed, for all the variables measured, the criteria (AIC and BIC) were minimum for this model, except the variable ATYL for which the UN model was better for the AIC criteria (Table 3). The ARH1 model indicated that the closer the dates were, the more the measurements of the trait in question were correlated. The variances were not stable and generally increased with the means. The ARH1 was therefore used for statistical inference (Table 4), particularly in testing the significance of Clone x Date interactions.

ANOVA with the ARH1 error model indicated significant Clone x Date interactions (Table 4). Clone classification therefore varied with time, particularly for the variables on young leaves, ATYL and TR (Fig. 2a and b). However, this interaction did not affect differentiation of susceptible clones from resistant ones. For the variables ATML and ST, only clones FX 4098 and FX 3864 were involved, their classification was reversed between 23 November 2006 (date 7) and 15 December 2006 (date 8), and clone FX 3864 ultimately displayed the greatest susceptibility. It is therefore very important to choose observation dates carefully when classifying planting

Table 3 Choice criteria for the longitudinal data analysis model for the monthly assessment of rubber tree clone susceptibility to *M. ulei*. The first order autoregressive model with heterogeneous variances (ARHI) is the best model

Variable	Attack severity on young leaves ATYL			Conidial sporulation intensity on young leaves, TR			Attack severity on mature leaves, ATML			Stroma density on mature leaves, ST		
Model	-2 Log Lik.	AIC	BIC	-2 Log Lik.	AIC	BIC	-2 Log Lik.	AIC	BIC	-2 Log Lik.	AIC	BIC
AR1	159	163	166	435	439	442	−344	−340	−337	−147	−143	−140
ARH1	**89**	115	**134**	**379**	**405**	**425**	**−397**	**−371**	**−352**	**−201**	**−175**	**−156**
CS	169	173	176	443	447	450	−233	−229	−226	−92	−89	−86
CSH	92	118	137	388	414	433	−303	−277	−257	−163	−137	−118
UN	−109	**47**	161	257	413	528	–	–	–	–	–	–

-2 Log Lik.: Log of likelihood; AIC: Akaike criterion; BIC: Schwartz criterion (Verbeke and Molenberg, 2000)

AR1: first order autoregressive model with uniform variances; ARH1: first order autoregressive model with heterogeneous variances; CS: compound symmetric model with constant correlation between the different dates and uniform variances; CSH: compound symmetric model with heterogeneous variances; UN: unstructured model

material for its resistance. In that perspective, we carried out ANOVA of each variable for each of the twelve dates (Table 5).

Analysis of variance per date for each variable

ANOVA on each date (Table 5) confirmed the observations illustrated by Fig. 2, and revealed a change in clone classification. At the outset, as the disease was very low, the classifications were much less discriminatory and it took 5 or 6 months of observations, up to date 5 (September 2006) or date 6 (October 2006), to achieve better differentiation between clones, as shown in Fig. 2a to d.

For young leaves, clone classification remained similar whatever the date chosen with ATYL (attack severity) and TR (conidial sporulation). However, on date 6 (October 2006), three clone groups formed, making it possible to distinguish the group of most susceptible clones, FX 3864 (control) and FX 4098, followed by an intermediate group of clones FDR 5788 and MDF 180, and the final group of totally resistant clones, FDR 5597, CDC 56, CDC 312 and FDR 4575.

For mature leaves, ANOVA for variables ATML (attack severity) and ST (stroma density) on each date showed stability in the performance of the clones over time, and three clone groups could easily be distinguished. First, the two susceptible clones, FX 3864 (control) and FX 4098, with severe attack levels and a large number of stromata; second, the intermediate group with only one clone, FDR 5788, which had a high level of partial resistance (ST maxima=1.28); finally, the group of five clones with no stromata (ST=0) FDR 5597, CDC 56, MDF 180, FDR 4575 and CDC 312.

Table 4 Analysis of variance based on the ARH1 model, first order autoregressive model with heterogeneous variances

Effect	DF	ATYL	TR	ATML	STR
Clone	7	168.60 (<0.0001)	281.00 (<0.0001)	259.75 (<0.0001)	374.10 (<0.0001)
Block	3	6.80 (0.0022)	1.39 (0.2730)	0.77 (0.5239)	1.01 (0.4082)
Date	11	45.74 (<0.0001)	56.83 (<0.0001)	83.67 (<0.0001)	90.98 (<0.0001)
Clone x Date	77	12.63 (<0.0001)	9.49 (<0.0001)	33.41 (<0.0001)	30.24 (<0.0001)

ATYL attack severity on young leaves, *TR* conidial sporulation intensity, young leaves, *ATML* attack severity on mature leaves, *ST* stroma density, mature leaves

Table 5 Susceptibility of eight rubber tree clones to *M. ulei*, over 12 months. Means obtained for four variables

Date Clone	1	2	3	4	5	6	7	8	9	10	11	12
Attack on Young Leaves (ATYL)												
FX 4098	a 0.82	a 0.49	a 0.58	a 0.28	a 0.70	a 2.04	a 2.10	a 2.47	a 2.53	a 2.37	a 2.29	a 2.58
FX 3864	a 0.91	a 0.37	a 0.40	a 0.25	b 0.39	b 1.19	a 1.81	a 2.37	a 2.86	a 2.27	a 1.97	b 2.08
FDR 5597	a 0.18	b 0.12	a 0.37	b 0.16	b 0.17	c 0.58	b 0.02	b 0.04	c 0.11	c 0.02	c 0.04	d 0.0
CDC 56	a 0.22	b 0.18	a 0.24	b 0.03	b c 0.13	c 0.41	b 0.08	b 0.17	b c 0.21	c 0.09	c 0.06	d 0.03
MDF 180	a 0.03	b 0.05	a 0.23	b 0.02	b c 0.30	c 0.36	b 0.08	b 0.47	b c 0.74	b 0.62	b 0.90	c d 0.52
FDR 4575	a 0.21	b 0.06	b 0.17	b 0.07	c 0.07	c 0.27	b 0.03	b 0.12	b c 0.19	c 0.0	c 0.0	d 0.02
CDC 312	a 0.55	b 0.03	b 0.04	b 0.05	b c 0.12	c 0.39	b 0.03	b 0.0	b c 0.26	c 0.07	c 0.01	d 0.0
FDR 5788	a 0.35	b 0.14	b 0.12	b 0.0	b c 0.15	c 0.14	b 0.14	b 0.38	b c 0.50	b c 0.45	c 0.3	c 0.85
Type of Reaction (TR)												
FX 4098	a 2.43	a 1.80	a 2.35	a 1.59	a 3.31	a 4.51	a 4.05	a 4.6	b 4.37	a 4.27	a 3.96	a 4.21
FX 3864	a 2.22	b 1.38	b 1.39	a 1.51	b 1.90	a 4.05	a 3.82	a 4.49	a 4.89	a 4.19	a 3.81	b 3.76
FDR 5788	b 1.06	c 0.49	c 0.51	b 0.26	c 1.06	b 2.38	b 1.44	b 2.64	c 2.69	b 2.84	b 2.82	c 3.03
FDR 5597	b 0.84	c 0.38	c 0.65	b 0.47	c d 0.65	c 1.05	b 0.52	c d 0.76	e 0.42	d 0.11	d 0.36	e 0.12
CDC 56	b 0.75	c 0.54	c 0.40	b 0.17	d 0.36	c 0.93	b 0.48	c d 0.94	e 0.69	d 0.51	d 0.34	e 0.32
MDF 180	b 1.00	c 0.17	c 0.50	b 0.23	c 0.91	c 1.03	b 0.63	c 1.45	d 1.59	c 1.46	c 1.70	d 1.50
FDR 4575	b 0.92	c 0.23	c 0.41	b 0.23	d 0.31	c 0.94	b 0.18	c d 0.74	e 0.72	d 0.17	d 0.39	e 0.15
CDC 312	b 1.32	c 0.22	c 0.26	b 0.43	c d 0.65	c 1.26	b 0.28	d 0.56	e 0.71	d 0.15	d 0.14	e 0.0
Attack on Mature Leaves (ATML)												
FX 4098	a 0.64	a 0.88	a 0.42	a 0.32	a 0.47	a 1.95	a 2.45	a 2.55	b 2.37	b 2.26	b 2.34	b 2.32
FX 3864	b 0.47	b 0.62	a 0.44	b 0.24	b 0.21	b 1.11	b 1.94	a 2.68	a 2.98	a 2.73	a 2.83	a 2.66
FDR 5788	c 0.05	c 0.06	b 0.03	c 0.0	c 0.02	c 0.23	c 0.02	b 0.23	c 0.11	c 0.15	c 0.39	c 0.38
FDR 5597	c 0.0	c 0.0	b 0.0	c 0.0	c 0.0	c 0.0	c 0.0	b 0.0	c 0.0	c 0.0	d 0.0	c 0.0
CDC 56	c 0.0	c 0.0	b 0.0	c 0.0	c 0.0	c 0.0	c 0.0	b 0.0	c 0.0	c 0.0	d 0.0	c 0.0
MDF 180	c 0.0	c 0.0	b 0.0	c 0.0	c 0.0	c 0.0	c 0.0	b 0.0	c 0.0	c 0.0	d 0.0	c 0.0
FDR 4575	c 0.0	c 0.0	b 0.0	c 0.0	c 0.0	c 0.0	c 0.0	b 0.0	c 0.0	c 0.0	d 0.0	c 0.0
CDC 312	c 0.0	c 0.0	b 0.0	c 0.0	c 0.0	c 0.0	c 0.0	b 0.0	c 0.0	c 0.0	d 0.0	c 0.0
Stromata (ST)												
FX 4098	a 0.87	a 1.04	a 0.87	a 0.72	a 0.95	a 2.59	a 3.01	b 2.99	b 3.02	b 2.91	b 3.04	b 3.09
FX 3864	b 0.66	b 0.71	a 0.79	b 0.45	b 0.53	b 1.85	a 2.86	a 3.46	a 3.77	a 3.64	a 3.77	a 3.68
FDR 5788	c 0.06	c 0.09	b 0.11	c 0.04	c 0.15	c 0.77	b 0.33	c 0.66	c 0.63	c 0.73	c 1.28	c 1.26
FDR 5597	c 0.0	c 0.0	b 0.0	c 0.0	c 0.0	d 0.0	b 0.0	d 0.0	d 0.0	d 0.0	d 0.0	d 0.0
CDC 56	c 0.0	c 0.0	b 0.0	c 0.0	c 0.0	d 0.0	b 0.0	d 0.0	d 0.0	d 0.0	d 0.0	d 0.0
MDF 180	c 0.0	c 0.0	b 0.0	c 0.0	c 0.0	d 0.0	b 0.0	d 0.0	d 0.0	d 0.0	d 0.0	d 0.0
FDR 4575	c 0.0	c 0.0	b 0.0	c 0.0	c 0.0	d 0.0	b 0.0	d 0.0	d 0.0	d 0.0	d 0.0	d 0.0
CDC 312	c 0.0	c 0.0	b 0.0	c 0.0	c 0.0	d 0.0	b 0.0	d 0.0	d 0.0	d 0.0	d 0.0	d 0.0

Means separated by Student-Newman- Keuls test at $P<0.05$. Figures preceded by a same letter are not statistically different

Under the conditions in the coastal humid tropical zone of Ecuador, the most suitable dates for differentiating susceptibility between clones were thus between 18 September 2006 (date 5) and 18 April 2007 (date 12).

Variation in correlations between variables

High correlations were found between the different variables studied (Fig. 3). The correlation coefficients

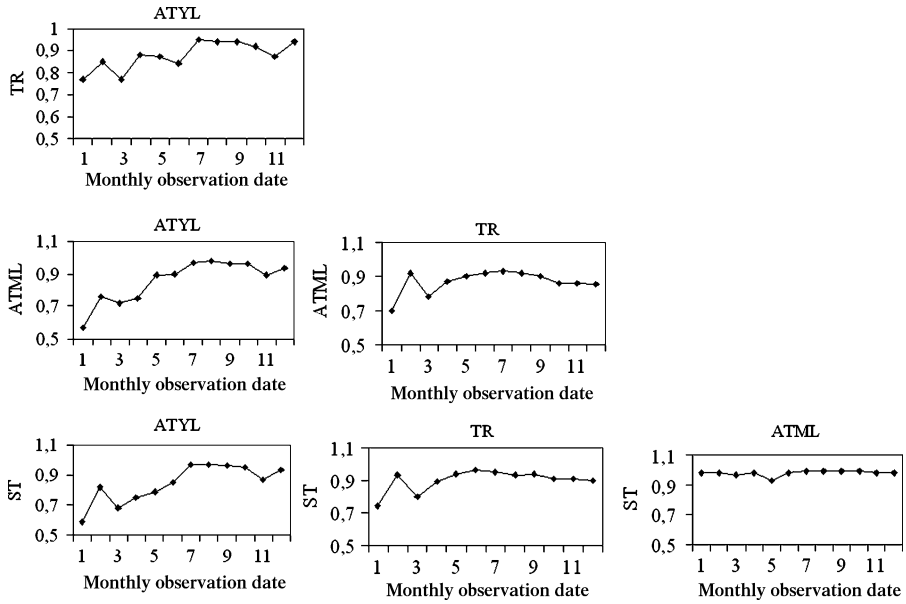

Fig. 3 Correlations among the four variables assessing susceptibility to *M. ulei* of the eight rubber tree clones over the observation dates (monthly observations, 12 months). ATYL: attack severity on young leaves; TR: conidial sporulation intensity on young leaves; ATML: attack severity on mature leaves; ST: stroma density on mature leaves

increased with time, with most ≥0.9 from date 4 onwards (date 4: 22 August 2006).

Discussion

The results confirmed that the four variables characterising different stages of the infection cycle of the fungus *M. ulei* enabled rapid classification of clones for their susceptibility to *M. ulei*. High correlations between the four variables suggested that clone susceptibility to *M. ulei* could be assessed with fewer variables; it would therefore be possible to make a choice of, for example, every other variable for each type of foliage. The choice then depended on the simplicity of observations in the field and on the precision of the information they provided.

In the case of young leaves, priority should be given to seeking sporulating lesions on which conidial sporulation intensity should be scored. In the case of mature leaves, it is the existence of stromata that should be sought, recorded for their density rather than lamina deformation.

To distinguish total or partial resistance, we can recommend conidial sporulation intensity on young leaves (TR) and stroma density on mature leaves (ST). The TR variable provides dual information on resistance: qualitative because it indicates whether the clone has total or partial resistance as well as quantitative as it indicates the intensity of sporulation (Junqueira et al. 1990; Rivano 1992, 1997b). The ST variable is a quantitative variable that also provides information on the degree of partial resistance. It can be considered reliable as the sum of attacks it represents is not likely to vary greatly and remain available on a leaf stage until the leaves fall through senescence. However, in the absence of stromata, it is not possible to conclude on total resistance, because high level partial resistance can be associated with an incomplete sexual reproduction cycle of *M. ulei* without stromata, despite the existence of conidial sporulation on young leaves. Such cases have already been described under other environmental conditions (Garcia et al. 1999; Junqueira et al. 1990; Le Guen et al. 2008).

Under the conditions at the Santo Domingo Experimental Station in Ecuador, the best period to begin observing the susceptibility of rubber tree clones was 6 months after planting. This prior duration enabled the plants to form new yet easily observable leaf stages, and enabled the disease to become established in the plot. The following period, lasting from 6 to 9 months, offered climatic conditions conducive to epidemic development and made it possible to detect significant differences between the clones. Indeed, in the present trial, little disease developed before September 2006 (date 5), except for a few non-specific symptoms with low scores (ATYL < 1): the first sporulation on susceptible clone FX 4098 (TR > 3) appeared in September 2006. Thereafter, the scores remained high throughout the wet season up to April 2007, the date of

the last observation (date 12). This second period corresponded to the active development of epidemics and made it possible to distinguish clones in terms of their susceptibility to the disease.

Statistical analysis of the longitudinal data results indicated that the closer the two observation dates were, the greater the correlation between the measurements. It is therefore not necessary to maintain a monthly frequency of assessing those traits. We therefore recommend a two-monthly observation frequency, which does not affect result reliability. This would lead to savings of 50 % in the resources devoted to such evaluations, which is appreciable when monitoring a network of rubber tree clone trials on a country or regional scale.

Although the large-scale trial is commonly used to study resistance to a leaf parasite, such as *M. ulei*, over many years under natural conditions, it could be useful to specify more precisely the number of years needed to assess clone resistance. Clones belonging to extreme categories of very susceptible (FX 3864, FX 4098) or very resistant (CDC 56, CDC 312, FDR 4575, and FDR 5597) can be detected very quickly, as indicated by the present results. Other clones, such as FDR 5788, and possibly MDF 180 are, in view of the results obtained by Le Guen et al. (2008), intermediate and require further observations for their degree of partial resistance to be fully expressed. Consequently, the susceptibility of these clones needs to be assessed over a few years, until the natural defoliation-refoliation process has become established. The role of phenology is well known for other leaf diseases, such as secondary leaf fall due to *Colletotrichum gloeosporioides* (Guyot et al. 2001).

We assumed that the primary inoculum came from distant rubber plantations, at least 4.5 km away, which was itself in the middle of an oil palm plantation. Primary infections of the rubber trees occurred first on the most susceptible clones (FX 3864 and FX 4098). A geospatial analysis might possibly determine the disease's point of entry and its subsequent spread over time and space, as shown by Mouen Bedimo et al. (2007) for coffee berry disease caused by *Colletotrichum kahawae*. Consequently, the presence of a susceptible control clone, such as FX 3864, is useful for generating inoculum rapidly in the trial, enabling the development of epidemics and the evaluation of resistance in all the clones tested.

The genetic diversity and pathogenicity of this natural *M. ulei* inoculum are unknown, and the risk of strains with unknown virulence appearing cannot be ruled out. The structure of the local *M. ulei* population will therefore have to be studied. Nevertheless, this type of trial offers the advantage of making it possible to monitor clone performance over time in relation to a pathogen population that can change, and thereby easily detect the appearance of any new virulence on clones with total resistance.

Le Guen et al. (2008) showed that high level and durable partial resistances might exist on *H. brasiliensis*, as in clone MDF 180. Five clones, CDC 56, CDC 312, FDR 4575, FDR 5597 and FDR 5788 in the present trial had ancestors belonging to the same genetic group as MDF 180 and might display the same type of resistance. Prior to being introduced into Ecuador, this material was successfully tested in Brazil. Consequently, the new germplasm introduced recently into Ecuador undeniably represents an interesting and promising source of resistance to SALB (Table 1).

To conclude, assessing the resistance of rubber tree clones to SALB in large-scale clone trials can be greatly optimized. First, the two important variables for determining clone susceptibility to *M. ulei* are conidial sporulation intensity on young leaves (TR), and stroma density on mature leaves (ST). Second, the most suitable period for assessing the susceptibility of rubber clones to *M. ulei* starts 6 months after planting, followed by a wet season, from November to June, under the conditions in the coastal humid tropical zone of Ecuador. Third, as repeated observations on the same trees were autocorrelated for dates close by, the observation frequency can be reduced to once every 2 months. Fourth, the clones recently introduced into Ecuador, except FX 4098, currently have a higher level of resistance than the clonal material used until now in that country, which is encouraging for Ecuadorian rubber growing, even though it will take a few years' observations before that resistance is confirmed. Finally, as SALB is the most destructive rubber tree disease in South and Central America, and as its accidental introduction into Asia and Africa could have devastating economic consequences, the proposal of a less time-consuming field assessment method could be useful to establish an international network of rubber tree clone trials.

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