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Assessment of resistance to *Ceratocystis cacaofunesta* in cacao genotypes

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Abstract Ceratocystis wilt of cacao (caused by *Ceratocystis cacaofunesta*) is a dangerous disease and results in the death of the plant. This fungus was recently identified in the major cacao-producing regions of Brazil, and was observed to be more aggressive than isolates from other geographical locations. The objective of this study was to develop and test a consistent method to assess cacao genotype response to *C. cacaofunesta*, based on young plants (seedlings or cuttings). The fungus was inoculated by the deposition of propagule suspensions on cut stems. The parameters to assess disease progress were (a) disease incidence, (b) differences in mortality between the most contrasting cacao genotypes for resistance and susceptibility, (c) disease index, (d) consistency of response over time and (e) relative lesion heights. When seedlings were used for the analyses, the ICS-1 and TSH-1188 genotypes proved to be useful as genetic standards for susceptibility and resistance to *C. cacaofunesta*, respectively. Inoculum concentrations between 10^4 and 10^5 propagules ml^{-1} and the moment at which the disease incidence stabilized provided appropriate conditions for genotypic comparison. When ten cacao genotypes propagated by cuttings (clones) were assessed, the results confirmed TSH-1188 as the reference genotype for

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resistance to *C. cacaofunesta*, while the remaining clones could be grouped as resistant (CEPEC-2008), moderately resistant (CEPEC-2002, CEPEC-2007) and susceptible (CEPEC-2009, CCN-10, CCN-51, HW-25, PH-16, SJ-02). The analytical concepts and results were discussed in terms of their application in breeding programmes aimed at developing genetic resistance to Ceratocystis wilt of cacao.

Keywords Ceratocystis wilt · *Ceratocystis fimbriata* · *Theobroma cacao* · Plant breeding · Resistance assessment

Introduction

Ceratocystis wilt of cacao is a lethal disease that has been causing economic losses to this crop in Brazil since the end of the 1970s (Bastos and Evans 1978). Described for the first time on sweet potato in 1890 (Halsted 1890), the etiological agent of this disease was named *Ceratocystis fimbriata*. However, a large genetic variability (Barnes et al. 2001; Marin et al. 2003) associated with a wide geographical and host distribution (Harrington 2004; Zauza et al. 2004; Silveira et al. 2006) suggests that this is a complex of cryptic species showing host specialization, with many species yet to be described (Baker et al. 2003; Engelbrecht and Harrington 2005). Despite the already demonstrated possibility of worldwide human-mediated dispersal of the fungus (Thorpe et al. 2005), the infection of plants under field conditions occurs through wounds caused mainly by tools of common agricultural practices, such as prunings and cuttings. Most of the time, the infection is also caused in association with insects from the Coleoptera—Scolytidae taxa, e.g. *Xyleborus* spp. (Coitía and Rosales 2001; Marin et al. 2003; Delgado and Suárez 2003). Therefore, long-distance dispersals tend to be inefficient, leading to geographical isolation of fungal populations, host specialization and speciation (Baker et al. 2003; Engelbrecht and Harrington 2005). Molecular techniques and morphological differences among isolates from cacao (*Theobroma cacao*), sweet potato (*Ipomoea batatas*) and sycamore (*Platanus* spp.) allowed the cacao-specific species to be re-classified as *Ceratocystis cacaofunesta* (Engelbrecht and Harrington 2005).

When penetrating the plant, the fungus causes a necrosis of the ray parenchyma cells, compromising the xylem; such lesions advances in the direction of the plant apex, although the cross-section is also thoroughly colonized (Harrington 2004). Hence, the disease symptoms are chlorosis, darkening of stems and branches, and wilt of leaves, which dry and die in 2 to 4 weeks, remaining attached to the plant (Delgado and Suárez 2003; Silva et al. 2004). At the end of the 1990s, this disease was recognized for the first time in Brazil's major cacao-producing region, comprised of the northern part of the state of Espírito Santo and the southeastern part of the state of Bahia, both in grafted young plants (Bezerra 1997) as well as in adult plants (Bezerra et al. 1998). This introduction was possibly due to infected cacao cuttings (Harrington 2000). Currently, the disease is well disseminated in the southeastern Bahia, with the plants derived from the ICS-1 clone (such as the 'Theobahia' variety) showing a highly susceptible response *C. cacaofunesta* (Silva and Luz 2000; Ram et al. 2004). This is especially problematic, since breeding programmes in this region have focused on the selection of cacao genotypes resistant to *Moniliophthora perniciosa*, the causal agent of the witches' broom disease (Aime and Phillips-Mora 2005), with most genotypes and clones that have been used in this area coming from the cross between 'Scavina-6' *and* ICS-1 (Luz et al. 1997; Ram et al. 2004).

As in a diverse array of diseases of economically important crops worldwide, the most efficient method to control Ceratocystis wilt is with the use of genetically resistant genotypes (Harrington 2004; Silva et al. 2004). Geographical differences in virulence have been verified for isolates of *Ceratocystis* spp. (Zauza et al. 2004; Silva 2005). In the cacao-growing region of Bahia, *C. cacaofunesta* has been shown to be more aggressive than other isolates from Latin America, since standard genotypes for resistance in neighbouring countries were susceptible to them (Delgado and Suárez 2003; Silva et al. 2007). Besides, breeding programmes need to assess and develop genotypes of cacao that are resistant to both witches' broom and Ceratocystis wilt of cacao.

Methods to study the response of cacao to *C. cacaofunesta* inoculation have been used, allowing a sufficient recognition of resistant genotypes under local conditions (Mata 1991; Delgado 2003). Most of that work employed a classical method developed by

Delgado and Echandi (1965) that is based upon application of propagule suspensions on sections of branches and evaluation of perithecial development. Other techniques based on inoculations and assessments *in planta* have also been used (Dominguez and Velasquez 1972; Alarcon 1994; Silva et al. 2006). The reproducibility of those methods on other combinations of cacao genotypes and *C. cacaofunesta* isolates from different regions has not been accomplished, mainly due to reaction specificities of the local pathogens to hosts (Delgado and Suárez 2003; Silva et al. 2006), to aspects related to the local genetic material (Delgado 2003; Silva et al. 2004), and to physiological differences among materials under testing, associated with the characteristics of the evaluation techniques (Espinoza and Delgado 1971; Mata 1991). Hence, the objective of this study was to develop a consistent methodology for evaluation of cacao genotype responses to inoculation with *C. cacaofunesta*, as well as to test this method on the comparison of cacao clones developed for resistance to witches' broom disease. The hypothesis was that the application of the method on nursery-derived young plants, either from seedlings or cuttings, would allow an appropriate assessment of the resistance levels of cacao genotypes, suitable for practical use in selection procedures.

Materials and methods

Inoculum treatments and evaluation period

The most suitable inoculum concentration and appropriate timing to differentiate susceptible plants from resistant were studied. Seedlings from open pollination of the ICS-1 and TSH-1188 genotypes were employed, which were previously designated as susceptible and resistant to *C. cacaofunesta*, respectively (Silva 2005). These plants were ~4 months-old, being cultivated in tube-like pots of 300 cm^3 of soil. The experiment was conducted in a screen-house with 50% sunlight cover at the research facilities of MARS Cacau (county of Itajuípe, Bahia, Brazil).

The *C. cacaofunesta* isolate ALF-78 from the fungal collection was used as it was observed to be the most aggressive in preliminary trials. This isolate was obtained from infected cacao trees in Bahia and preserved on 0.25 cm^2 filter paper stored in glass vials at 4°C (Dhingra and Sinclair 1985). The fungus was grown in Petri dishes containing acidified potato-dextrose-agar (PDAA, pH 3.0, adjusted by lactic acid) and incubated at room temperature (~25°C) for 8 days. Sterile distilled water was added to the plate, which was gently scraped with a glass slide for the initial propagule suspension, containing chlamydospores, ascospores, conidia and mycelial fragments; this suspension was filtered through sterile gauze to remove small pieces of culture medium.

The plants were inoculated by superficially cutting the seedling stem with a scalpel 4 to 5 cm from the base. A single longitudinal cut per plant (1 to 1.5 cm high by 0.3 to 0.5 cm deep) was made, into which a 20 μl-suspension was applied. The concentration treatments tested were 'zero' (conc. '0' = only distilled water), 5×10^3 (conc. '1'), 1×10^4 (conc. '2'), 5×10^4 (conc. '3'), 1×10^5 (conc. '4') and 5×10^5 (conc. '5') propagules ml^{-1}. Directly after inoculation, humid chambers were assembled, with the cut stems covered with cotton moistened with distilled water and wrapped with polyethylene film for 2 days. The seedlings were assessed daily until the first death was recorded, which occurred at the tenth *d*ay *a*fter *i*noculation (DAI); afterwards, 12 more evaluations were performed at the 15th, 20th, 25th, 30th, 35th, 40th, 45th, 50th, 55th, 60th, 65th and 70th DAI, when the experiment was ended. This experiment was performed twice.

Parameters for genotype assessment

In order to compare genotype resistance to *C. cacaofunesta*, disease incidence was used, by assessing the percentage of dead plants in each evaluation on experimental units of four plants per replicate for each treatment. In addition, two other parameters were also established for the comparisons. The first was the average disease incidence in each evaluation, defined as the 'disease index', which is here proposed as an internal reference for experimentation under distinct conditions. The second was the difference in mortality between the most contrasting genotypes, which was estimated by disease incidence on the resistant genotype TSH-1188 subtracted from that on the susceptible genotype ICS-1.

When death of plants was not observed, the total length of the lesions, above and below the inoculation point, and the height of the respective plants were

taken. The ratio between these measurements was defined as the *r*elative *l*esion *h*eight (RLH). In a set of independent experiments, ten cacao seedlings from TSH-1188 and ten from ICS-1, aged between 6 and 10 months for both genotypes, were inoculated and incubated under the same overall conditions described above. After the time previously indicated without death of plants, the RLH variable was evaluated and used to compare the genotypic responses to the pathogen. Control seedlings from both genotypes were inoculated with water. The experiment was repeated twice, with similar results.

Resistance assessment of cacao clones to *C. cacaofunesta*

Four month-old plants were obtained from plagiotropic cuttings (clones), grown in similar tube-like pots, which were kindly provided by the Instituto Biofábrica de Cacau (organization associated to the State Government, located in the county of Ilhéus, Bahia). Ten genotypes were chosen for comparison. These were selected among several other genotypes, due to their high levels of genetic resistance to witches' broom disease, based on the recommendations of the Cacao Research Centre (CEPEC-CEPLAC) for crop renewal in the cacao-growing region of southeastern Bahia. The CCN-10 and CCN-51 genotypes originated from Ecuador; CEPECs 2002, 2007, 2008 and 2009 were selected by the Centre's staff from local commercial crops; HW-25, PH-16 and SJ-02 were selected by local farmers; TSH-1188 was introduced from Trinidad–Tobago, and used as a positive control for resistance.

The *C. cacaofunesta* isolate and overall procedures were the same as described above. Inoculum was adjusted to 1×10^5 propagules ml^{-1} by a haemacytometer for application on all cacao genotypes. The same longitudinal cuts were employed, as well as the deposition of the 20 μl suspensions, followed by 48 h in the humid chambers. The control plants were inoculated with distilled water under the same conditions. After inoculation, the plants were observed daily until the 11th DAI, when the first death was recorded (evaluation no. 1); then, 11 more evaluations were performed after 15, 19, 23, 27, 31, 35, 39, 43, 47, 51 and 55 DAI, when the experiment ended.

The percentage of dead plants (incidence) was the parameter used to follow the disease progress, recorded at each evaluation. As the environmental reference, i.e. the specific experimental conditions, the disease index parameter was employed (the average disease incidence of all clones together) at every evaluation. Moreover, this parameter served as the threshold to consider a resistant or susceptible response to the fungus. In such a context, in order to refine the output of the comparison procedure, the consistency of response over time was assessed, following the method proposed by Finlay and Wilkinson (1963) and Eberhart and Russel (1966). This parameter was based on linear regression procedures between the disease incidence for each clone and the average incidence for all clones together (disease index), with each pair of data corresponding to each evaluation, from the first (11 days after inoculation) to the seventh (35 DAI), i.e. the period prior to the stabilization of the disease progress. The constancy of response for each clone was then estimated by the corresponding linear coefficient *b* obtained from the regression equation.

Experimental design and statistics

The experiments used to assess inoculum concentrations and evaluation time, which used seedlings of the contrasting genotypes TSH-1888 and ICS-1, were conducted on a randomised block design, with four replications. Each replication (experimental unit) consisted of four plants, in a total of 16 plants per genotype treatment. The results were statistically assessed by ANOVA and the *F* test ($P<0.01$), using the statistical software SAEG v. 5.0 (Federal University of Viçosa-MG, Brazil). Inoculum concentrations, genotypes and evaluation periods were the three sources of variation considered in the analysis.

For the experiment comparing the ten cacao genotypes (treatments), the same randomised block design was used, with three replications and five plants per experimental unit (replication). The results were statistically assessed by ANOVA and the *F* test ($P<0.01$), considering two sources of variation individually and their interaction: genotypes (clones) and evaluation time. The parameters used to distinguish the resistance levels among the clones were both the disease incidence (tested by Tukey at $P<0.05$) and the constancy of response over time (see above).

Results

Conditions for assessment of cacao genotype response to *C. cacaofunesta*

The effect of different inoculum concentrations was assessed on the cacao genotypes TSH-1188 and ICS-1, previously indicated as possible standards for resistance and susceptibility to *C. cacaofunesta*, respectively, under local farming conditions (Silva 2005; Silva et al. 2007). The genotype responses were assessed by disease incidence (percentage of dead plants) on each of the 13 evaluations performed, with the disease index for the experiment used as an internal reference for the analyses. The isolate used for the experiments was regarded as more aggressive than others from the collection accessed. The percentage of dead plants for the susceptible ICS-1 genotype was above the disease index for all inoculum concentrations tested, whereas the opposite was observed for TSH-1188 (Fig. 1). The death increase stabilized around 50 DAI, with minor changes until the end of the experiment (70 DAI, 13th evaluation). The increase in the disease incidence for both genotypes was roughly proportional to the inoculum concentration, although concentration

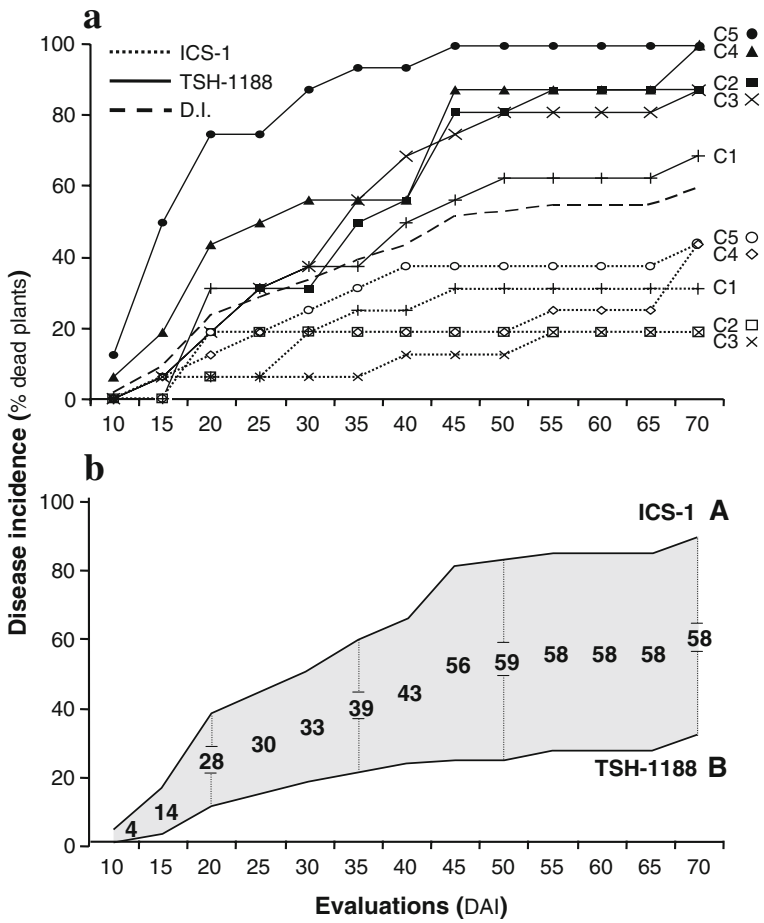

Fig. 1 Progress of disease incidence for seedlings of ICS-1 and TSH-1188 genotypes, with five *C. cacaofunesta* propagule concentrations (C1 to C5) and 13 evaluations up to 70 DAI. **a** The disease index (*D.I.*) was the average disease incidence, considering all plants and concentrations of the experiment, except C0 (control with water only), which showed no death of plants for both genotypes. **b** Average disease incidence for the six inoculum concentrations for ICS-1 and TSH-1188. The *numbers in the shaded area* are the difference in mortality between the two averages. *Letters at the side* of genotype names indicate statistical differences for all evaluations, except the first. This experiment was performed twice under the same conditions, with similar results

no. 1 killed more TSH-1188 plants than no. 2 and 3 (Fig. 1a). The highest concentration tested (no. 5=5×10^5 propagules ml^{-1}) was the most aggressive to the plants, causing death (6%) from the first evaluation. This concentration also gave the highest disease incidence on all evaluations for both genotypes, whereas the remaining concentrations were similar in their effects on plant death. None of the plants died from the control treatment (no. 0 = distilled water). The results from the ANOVA and F test ($P<0.01$) showed a statistical significance for the variables under test (inoculum concentration, genotype and evaluation period), considering their independent effects as well as their interaction (Table 1).

In order to define the DAI required for appropriate disease expression suitable for comparative assessment, the parameter 'difference of mortality' between the susceptible and resistant genotypes was established. Statistically significant differences between the two genotypes were obtained for all evaluations, with exception of the first (Fig. 1b). The differences in mortality observed varied from 4% to 58%, as the disease incidences were 89% for the ICS-1 and 31% for the TSH-1188 at the end of the experiment. The maximum phenotypic contrast between the two genotypes was observed after the eighth evaluation at 45 DAI, with the stabilization of this mortality difference around 58% until the end of evaluations (Fig. 1b). A comparison of the effects of inoculum concentration on each genotype specifically at the moment of disease stabilization at the 9th evaluation (50 DAI) was assessed; the differences in mortality between ICS-1 and TSH-1188 were 39% for concentration no. 1, 63% for no. 2, 69% for no. 3 and 4, and 63% for no. 5.

In order to define the inoculum concentration to use, a linear regression analysis was performed in which the number of propagules ml^{-1} were considered as a function of the average disease incidence between ICS-1 and TSH-1188, for each concentration (independent variable X). The equation obtained was $Y = -1,057 + 2,240X (R^2 = 0.96)$. Taking into account that the results have indicated 50 DAI as an appropriate time for disease assessment and that an average of ~50% of the plants in the experiment have died at this evaluation time (Fig. 1), solving the equation with this value of mortality for X provides a concentration (Y) of 1.109×10^5 propagules ml^{-1}. As concentration no. 4 (1×10^5) is the closest of the tested inocula, it was defined as the inoculum concentration of *C. cacaofunesta* for use in the experiment for cacao clone comparisons.

Table 1 Analysis of Variance (ANOVA) for disease incidence (% death of plants) of TSH-1888 and ICS-1, with inoculum concentration, genotype and evaluation as the sources of variation

	DF	MS	*F*
Block	3	4,065.68	1.89
Concentration (*C*)	5	30,293.94	14.10*
Error a	15	2,149.02	1.00
Sub-total 1	23		
Genotype (*G*)	1	158,271.70	83.96*
G × *C*	5	8,921.70	4.73*
Error b	18	1,885.13	1.00
Sub-total 2	47		
Evaluation (*E*)	12	16,407.86	138.74*
E × *C*	60	831.97	7.03*
E × *G*	12	4,209.20	35.59*
E × *C* × *G*	60	368.13	3.11*
Residue c	409	118.26	1.00
Total	623		

*$P<0.01$

Comparative assessment of cacao clones resistance to *C. cacaofunesta*

To validate the method developed, an experiment under the same overall conditions was set for the comparison of ten cacao genotypes propagated by cuttings (clones) and regarded as resistant to witches' broom. With the sources of variation being clones and evaluations, the results from the ANOVA for the incidence parameter were statistically significant by the F test (Table 2). In terms of plant death after pathogen inoculation, the results demonstrated that the clones behaved differently (Fig. 2), confirmed by a Tukey test at 5% significance. Similarly to the previous experiments, the disease progress stabilized around the seventh and eighth evaluations, corresponding to 35 to 39 DAI. In these conditions, this period proved to be ideal, as the differences in disease incidence among genotypes were most clearly visible (Fig. 2).

Considering the average incidence of all clones of the experiment at this evaluation time (disease index= 63%; Fig. 2) as reference, it was possible to note that

Table 2 Analysis of Variance (ANOVA) for disease incidence (% death of plants) of the ten cacao genotypes, with clone and evaluation as the sources of variation

	DF	MS	*F*
Block	2	10,830	
Clone (*C*)	9	19,970	15.3*
Error a	18	1,302	
Evaluation (*E*)	11	21,416	151.1*
E × *C*	99	582	4.1*
Residue b	220	142	
Total	359		

*$P<0.01$

the clones TSH-1188 and CEPECs 2008, 2007 and 2002 were classified as more resistant (incidence < disease index). According to their mortality, the former two were highly resistant, due to a higher difference in the disease index, whereas the latter two were moderate in resistance (incidence closer to disease index). The remaining clones CEPEC-2009, CCN-10, CCN-51, SJ-02, PH-16 and HW-25 were susceptible, as the percentage of dead plants was higher than the disease index (Fig. 2). The results obtained not only confirmed TSH-1188 as the reference genotype for resistance to *C. cacaofunesta*, but also revealed another possible standard for susceptibility, i.e. the SJ-02 genotype, which showed the highest incidence at the seventh evaluation (>90%) and 100% mortality after 47 DAI (tenth evaluation).

Despite these results showing resistance and susceptibility on the basis of the disease index and the reference genotype for resistance, we studied another parameter to refine the comparison among clones, that is the constancy of response over time, based on the method developed by Finlay and Wilkinson (1963) and Eberhart and Russel (1966). The disease incidence for each clone was subjected to a linear regression as a function of the disease index in the first seven evaluations (up to 35 DAI, a period of mostly linear progress of the disease—Fig. 2). For each clone, the linear coefficient *b* from the regression equation and the coefficient of determination R^2 were obtained. The R^2 was >0.9 for all clones, with the exception of the clone CEPEC-2008 (R^2=0.73). In this type of analysis, the linear regression coefficient *b* is an estimate of the constancy of response, i.e. higher slopes of the regression lines ($b>1$) indicate more variable clones, whose behaviour alter with time, above the average of the experiment (disease index). Smaller slopes ($b<1$) corresponded to more constant clones, showing less change with time; when $b=1$, the clone behaviour is similar to the disease index. Hence, the *b* values obtained for all clones were plotted in association with the corresponding disease incidence at the seventh evaluation, in order to provide a clearer view of their relationship (Fig. 3). The susceptible clones CEPEC-2009, CCN-10, CCN-51, SJ-02, PH-16 and HW-25 were characterized by a consistent increase in their percentage of dead plants up to the seventh evaluation (Fig. 2), thereby confirming their variability of response ($b>1$; Fig. 3). Out of this group of susceptible clones, the least variable was CEPEC-2009 (b=1.11). On the other hand, the clones regarded as resistant and

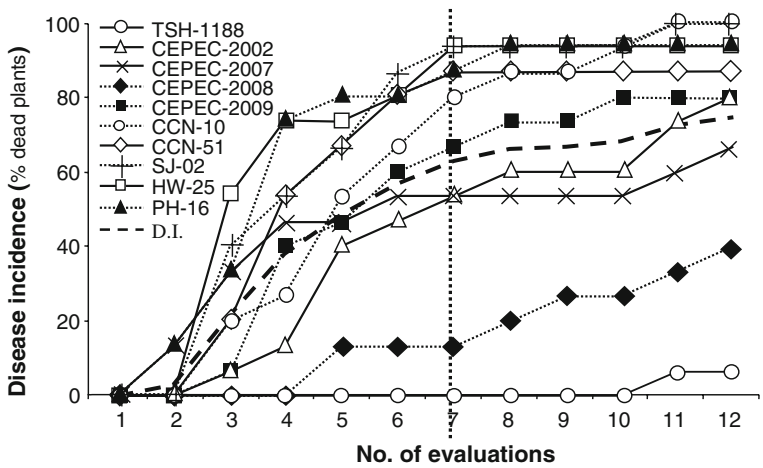

Fig. 2 Progress of disease incidence for the ten cacao clones inoculated with *C. cacaofunesta*. The clones identification are indicated on the *top left*; D.I. is the average incidence of the experiment (disease index)

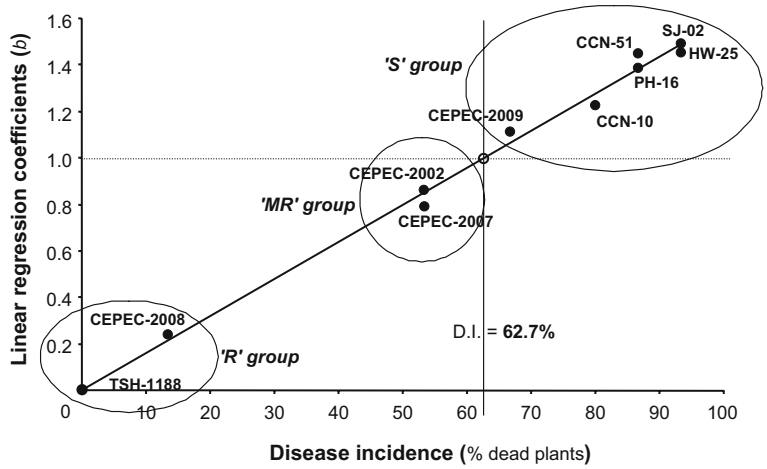

Fig. 3 The relationship between disease incidence for the clones at the seventh evaluation (35 DAI) and the corresponding linear regression coefficients *b*, used as an indicator of the stability of genotype response to *C. cacaofunesta* inoculation. The average incidence for the experiment (disease index) and the corresponding *b* is shown by the intersection between the *vertical* and *horizontal lines*. The *R* group indicates high resistance, *MR* indicates moderate resistance and *S* indicates susceptibility

moderately resistant showed a higher constancy for the trait, although not with the same magnitude. CEPECs 2007 and 2002 showed $0.4<b<1$, whereas $b<0.25$ for CEPEC-2008 and TSH-1188 clones (Fig. 3). The latter presented maximal constancy, as it showed no variation over time, independent from the disease index or evaluation (Figs. 2 and 3). The graphical view of the relationship between disease incidence and constancy of response for the clones allowed a clear identification of the three major groups of reactions to *C. cacaofunesta*. The 'R' group with incidence <20% and a highly constant resistance ($b<0.25$), the 'MR' group with a moderately constant resistance of 20% < incidence < 62.7% (disease index) and $0.75<b<1.0$, and the 'S' group of susceptible and inconsistent clones with their incidence > disease index and $b>1.0$ (Fig. 3).

Relative lesion height—alternative parameter for resistance assessment

In a set of other independent experiments performed in similar conditions, we observed that the cacao plants under study did not die after 90 DAI. Nevertheless, these plants showed the characteristic internal lesion caused by *C. cacaofunesta*, in a way similarly reported in other studies (e.g. Mata 1991; Baker et al. 2003). Under these circumstances, we hypothesised that a direct measure of the length of these lesions in association with the height of the plant would be likely to be useful to differentiate genotypes with respect to their resistance to the pathogen. The average RLH obtained after disease challenge of seedlings from the reference genotypes strictly followed the expected resistance and susceptible patterns of response observed when the disease incidence parameter was used (Fig. 4). The average RLH observed for TSH-1188 was approximately five times lower than that observed for ICS-1. The negative controls (water inoculation) did not show lesions of measurable sizes for both genotypes.

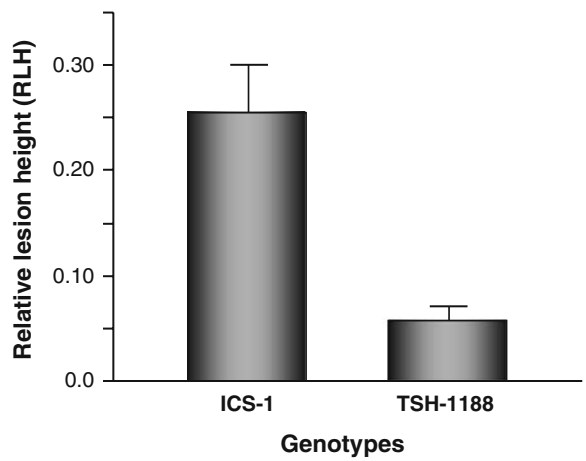

Fig. 4 Average *r*elative *l*esion *h*eights (RLH) for seedlings of reference genotypes for susceptibility (ICS-1) and resistance (TSH-1188) to *C. cacaofunesta*. *Error bars* correspond to ten replicates (seedlings) of each genotype. Control plants inoculated with water did not show measurable lesions

Discussion

The fact that Ceratocystis wilt of cacao causes the death of plants without the possibility of recovery, classifies it into a dangerous disease if widely disseminated, with a potentially high economic impact on cacao farming (Harrington 2004). Its prevention on the basis of genetic resistance is the main resource for disease management (Guerrero 1975). In this work, relevant information was presented for the definition of a consistent strategy for resistance evaluation of cacao genotypes.

Previous information (Silva 2005) indicated that the genotypes TSH-1188 and ICS-1 could serve as the standards for resistance and susceptibility to *C. cacaofunesta*, respectively, under the conditions of cacao farming in southeastern Bahia (Brazil). Their use in inoculation experiments with open pollinated seedlings (Fig. 1) increased the discriminating capacity of the test, as the higher genetic variability expected among the individuals provides a larger experimental variation. The results suggested that firstly, the percentage of dead plants (incidence) during disease progress is a proper parameter for genotypic comparison; secondly, these two genotypes worked well as contrasting standards of resistance and susceptibility to *C. cacaofunesta*; thirdly, higher inoculum concentrations tended to raise the mortality of a resistant genotype; and finally, the specific effects of *C. cacaofunesta* can be assessed up to the moment of mortality stabilization, which can be an adequate reference point to interrupt the evaluation procedure. The late increase in overall mortality observed at the last evaluations of the experiments (Figs. 1 and 2) was likely to be due to other factors, such as the attack of insects and other microorganisms, as well as the time interval that the plants stayed in pots with small volumes of soil, which probably reduced their vigour.

The disease index established for the analyses can be used as a stable threshold for resistance to *C. cacaofunesta*, as it relates proportionally to the disease incidences of the genotypes being compared. However, since such an index is an average of all treatments being tested, it is sensitive to the type of genetic material used in the experiments, tending to move towards the predominant phenotype (Fig. 2). Therefore, caution must be taken when analysing the results, which should be considered in a relative context; a wider distribution of genotypes between the extremes of resistance and susceptibility could provide the conditions for the establishment of a reference disease index, applicable as a more general, pre-defined standard for the evaluation of any genotype under test. The point at which the difference in mortality between the most contrasting genotypes reaches its maximum (Fig. 1b) can also be used as a reference to end an evaluation period. Although such a point will coincide mostly with the moment of disease stabilization, not all genotypes will display such stability after a certain number of DAI (Fig. 2). Moreover, this maximal difference between extreme phenotypes could also be a reference for the assessment of unknown genotypes. For instance, a scale of mortality could be set with '0' (zero) as the score for the resistance standard and '5' for the maximal susceptibility, with the other genotypes classified on the basis of such scale. It is worth mentioning that both the disease index and the maximal difference of mortality allow assessments under distinct experimental conditions, but only the latter is independent of the type of genetic material under test.

The detectable differences among genotypes tend to be of a lesser magnitude for inoculum amounts $<10^4$ propagules ml^{-1} (concentration no. 1), due to the lesser mortality observed for the susceptible standard (Fig. 1). On the other hand, similar effects observed for the remaining concentrations (no. 2 to 5), at least when using open-pollinated seedlings, indicated that any inoculum ranging from 10^4 to 5×10^5 propagules ml^{-1} appears to be suitable for the assessment of cacao genotypic response to *C. cacaofunesta*. These results were compatible with those from previous research indicating a lesser importance of the inoculum concentration on genotype comparison (Espinoza and Delgado 1971; Delgado and Suárez 2003). Since higher inoculum concentrations should cause more disease and death (Fig. 1; Silva et al. 2004), the linear regression associating the number of propagules with disease incidence provided a useful way of determining the initial amount of propagules as a function of the general level of mortality expected or achievable in the experiment. Other technical aspects should also be considered for defining the most appropriate inoculum concentration to use. The *C. cacaofunesta* propagules tend to be enclosed by an adherent gelatin-like mass that generates clumps of up to ten colony-forming units (CFU), difficult to break down even after addition of Tween 80 and thorough

homogenisation. Therefore, higher inoculum concentrations can lead to mistakes in haemocytometer counting (clumps viewed as single units), and therefore the resulting concentration of the propagule suspension. Another important argument is that the most useful inoculum concentration should be able to cause death of the greatest number of susceptible plants, whilst keeping the mortality of the resistant ones to a minimum. The 1×10^5 propagules ml^{-1} (concentration no. 4) was suggested by the regression equation as the most useful and satisfied the above conditions, justifying its use in the clones comparison experiment.

The tendency for higher aggressiveness of the Brazilian isolates of *C. cacaofunesta* (Delgado and Suárez 2003; Silva et al. 2004) indicates the need of establishing local cacao genotypes to serve as resistance standards, either for genotypic screening or for the development of new progenies and clones. The possibility of using TSH-1188 in this context was previously suggested (Silva et al. 2007) and clearly confirmed here, as its young plants from seedlings and cuttings showed the highest resistance (Figs. 1 and 2). Besides, this resistance was very constant during the evaluation period, suggesting the importance of taking into account both the mortality levels at 35 DAI and the constancy of response (Finlay and Wilkinson 1963; Eberhart and Russel 1966) for more efficient and informative criteria for classification (Fig. 3). For instance, in sharp contrast to TSH-1188 and CEPEC-2008, the clone CEPEC-2002 showed a less constant resistance response, as it grouped with the susceptible clones at the 12th evaluation (Fig. 2); the increase in plant death after the seventh evaluation rendered a *b* coefficient closer to 1.0 (Fig. 3). The assessment period of up to 35 to 39 DAI allowed adequate discrimination of clones, and occurred similarly in the seedling experiments (Fig. 1). Such an assessment time agreed with a previous report in which a comparison was made for cacao clones 30 DAI with *C. cacaofunesta* (Silva et al. 2007). The analytical approach of verifying the consistency of response over time has been used successfully for other cacao pathogens (Pinto et al. 1995; Yamada et al. 1999), as well as for diseases of other crops (e.g. Bradwaj and Sing 1983). The results also suggested that, besides the ICS-1 genotype, the clone SJ-02 can also be used as a reference for susceptibility to *C. cacaofunesta* (Figs. 2 and 3).

The overall methodology discussed above is based on the mortality of young cacao plants after a specific time of challenging with *C. cacaofunesta*. However, there may be cases in which the plants do not die in a similar timeframe, depending upon the aggressiveness of the isolates available, the characteristics of inoculation protocols (propagule concentration; position, type and number of cuts on the plants), and the initial cultivation conditions (seedlings or cuttings, plant age, soil volume, fertilization, watering regime etc). Hence, the alternative available for the analyses is to observe the internal lesions caused by the fungus (e.g. Mata 1991; Baker et al. 2003). Where plants did not die, we noticed that the seedlings tested were cultivated in bags containing about three to five times more soil than the tube-like pots described for the mortality experiments. These conditions probably affected plant vigour, and so interfered with the pattern of response to the inoculated fungus. Considering that the rate of necrosis caused by *C. cacaofunesta* might be different from the rate of plant growth, and this could be associated with the genotype-specific response to the pathogen, we suggested that the resistance trait might be related to the ratio between lesion length and plant height, i.e. the relative lesion height (RLH). When comparing the contrasting cacao genotypes challenged by the fungus, assessment of RLH resulted in the same pattern of response observed with the mortality parameter, and was independent of the age of the plants under test. This suggests that RLH can be employed as an alternative for genotypic comparison, either as the single parameter of assessment or in conjunction with mortality. Despite its technical feasibility, the use of RLH is laborious, time-consuming and might be limiting in the assessment of many genotypes simultaneously where a great number of plants is examined.

Due to the characteristics of Ceratocystis wilt of cacao, genetically-based resistance is the most appropriate strategy for adequate control of this economic and environmental problem. The dissemination of *C. cacaofunesta* in the tropical cacao-producing region of southeastern Bahia is currently of major concern, requiring efforts to breed elite cultivars resistant to this fungus. Assessments of cacao germplasm are currently underway, and the information provided in this report will aid the consistent identification of resistant genotypes. It is hoped that the analytical

scheme discussed here will also be helpful for other plant–pathogen systems involving *Ceratocystis* spp.

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References

Alarcon, C. R. M. (1994). Determinación de resistencia de 250 clones de cacao de origen nacional al ataque de mal de machete. Graduation Thesis, Universidad Agraria del Ecuador.

Aime, M. C., & Phillips-Mora, W. (2005). The causal agents of witches' broom and frosty pod rot of cacao (chocolate, *Theobroma cacao*) form a new lineage of Marasmiaceae. *Mycologia*, *97*, 1012–1022.

Baker, C. J., Harrington, T. C., Kraus, U., & Alfenas, A. C. (2003). Genetic variability and host specialization in the Latin American clade of *Ceratocystis fimbriata*. *Phytopathology*, *93*, 1274–1284.

Barnes, I., Gaur, A., Burgess, T., Roux, J., Wingfield, B. D., & Wingfield, M. J. (2001). Microsatellite markers reflect intra-specific relationships between isolates of the vascular wilt pathogen *Ceratocystis fimbriata*. *Molecular Plant Pathology*, *2*, 319–325.

Bastos, C. N., & Evans, H. C. (1978). Ocorrência de *Ceratocystis fimbriata* Ell. and Halst. na Amazônia Brasileira. *Acta Amazônica*, *8*, 543–544.

Bezerra, J. L. (1997). *Ceratocystis fimbriata* causing death of budded cocoa seedlings in Bahia, Brazil. *Incoped Newsletter*, *1*, 6.

Bezerra, J. L., Almeida, O. C., Luz, E. D. M. N., & Silva, S. D. V. M. (1998). Ocorrência de *Ceratocystis fimbriata* em clones de cacau no estado da Bahia. *Fitopatologia Brasileira*, *23*, 228.

Bradwaj, C. L., & Sing, B. M. (1983). Interaction of minor host genes for resistance to *Puccinia striiformis* with changing temperature regimes. *Phytopathology*, *59*, 999–1001.

Coitía, W., & Rosales, C. J. (2001). Relación entre la incidencia de escolítidos y la necrosos del cacao em Aragua, Venezuela. *Manejo Integrado de Plagas*, *62*, 65–71.

Delgado, R. (2003). Fuentes de resistência al mal de machete em accesiones de cacao silvestre coletadas em la Amazonia Ecuatoriana. In *XII Seminário Nacional de Sanidad Vegetal*. Noviembre 19–21, 2003. Latacunga, Ecuador. 7p.

Delgado, A. J., & Echandi, E. (1965). Evaluación de la resistência de especies y clones de cacao al mal del machete provocado por *Ceratocystis fimbriata*. *Turrialba*, *15*, 86–89.

Delgado, R., & Suárez, C. (2003). Diferencias em agressividad entre aislamientos de *Ceratocystis fimbriata* de Ecuador y Brasil em cacao. In *XII Seminário Nacional de Sanidad Vegetal*, Noviembre 19–21, 2003. Latacunga, Ecuador. 8p.

Dhingra, O. D., & Sinclair, J. B. (1985). *Basic plant pathology methods*. Boca Raton, USA: CRC.

Dominguez, R. P. F., & Velásquez, F. (1972). Seleccion de plantas de cacao (*Theobroma cacao* L.) por resistência al hongo *Ceratocystis fimbriata*. *Revista de la Facultad de Agronomia (University of Venezuela)*, *6*, 57–73.

Eberhart, S. A., & Russel, W. A. (1966). Stability parameters for comparing varieties. *Crop Science*, *6*, 36–40.

Engelbrecht, C. J. B., & Harrington, T. C. (2005). Intersterility, morphology and taxonomy of *Ceratocystis fimbriata* on sweet potato, cacao and sycamore. *Mycologia*, *97*, 57–69.

Espinoza, A. S., & Delgado, J. C. (1971). Factores intrínsecos que influyen en la eficácia de la prueba de laboratório usada para evaluar la resistência a *Ceratocystis fimbriata* en cacao. *Turrialba*, *21*, 13–17.

Finlay, K. W., & Wilkinson, G. N. (1963). The analysis of adaptation in a plant-breeding programme. *Australian Journal of Agricultural Research*, *14*, 742–754.

Guerrero, H. A. M. (1975). Estúdio de la resistência a *Ceratocystis fimbriata* Ellis y Halst., em híbridos y clones de cacao (*Theobroma cacao* L.) utilizando dos metodos de evaluacion. M.Sc. Thesis, Instituto Interamericano de Ciências Agrícolas de la OEA (Costa Rica), 62 p.

Halsted, B. D. (1890). Some fungous diseases of the sweet potato. The black rot. *New Jersey Agriculture Experiment Station Bulletin*, *76*, 7–14.

Harrington, T. C. (2000). Host specialization and speciation in the American wilt pathogen *Ceratocystis fimbriata*. *Fitopatologia Brasileira*, *25*(Suppl.), 262–263.

Harrington, T. C. (2004). *Ceratocystis fimbriata* [update on the original text prepared by C.J. Baker and T.C. Harrington]. In: CABI Crop Protection Compendium. CAB International: Wallingford, UK. Retrieved March 15th, 2006, from http://www.public.iastate.edu/~tcharrin/CABIinfo.html.

Luz, E. D. M. N., Bezerra, J. L., Resende, M. L. V., & Oliveira, M. L. (1997). Cacau (*Theobroma cacao* L.) Controle de Doenças. In F. X. R. Vale, & L. Zambolim (Eds.), *Controle de Doenças de Plantas: Grandes Culturas, vol. 2* (pp. 611–649). Viçosa-MG, Brazil: Universidade Federal de Viçosa.

Marin, M., Castro, B., Gaitan, A., Preisig, O., Wingfield, B. D., & Wingfield, M. J. (2003). Relationships of *Ceratocystis fimbriata* isolates from Colombian coffee-growing regions based on molecular data and pathogenicity. *Journal of Phytopathology*, *151*, 395–405.

Mata, S. N. (1991). *Métodos de inoculacion de* Ceratocystis fimbriata *e Evaluacion de Resistencia en Cacao*. Graduation Thesis, Universidade de Costa Rica. 85 p.

Pinto, L. R. M., Silva, S. D. V. M., & Yamada, M. M. (1995). Estabilidade fenotípica e previsibilidade da resitência de cacaueiro a *Phytophthora* spp. *Fitopatologia Brasileira*, *20*, 416–421.

Ram, A., Valle, R. R. M., & Freitas, D. B. (2004). Controle de cancro ou murcha de *Ceratocystis* do cacaueiro na Bahia, Brasil. *Agrotropica*, *16*, 111–114.

Silva, S. D. V. M. (2005). Ensaios para avaliação do cacaueiro à murcha-de-Ceratocystis na Bahia, Brasil. In *Proceedings of the 14th International Cocoa Research Conference, vol. 2* (pp 1341–1347). Accra, Ghana: Cocoa Producer's Alliance.

Silva, S. D. V. M., Gomes, A. R. S., Mandarino, E. P., dos Santos-Filho, L. P., & Damaceno, V. O. (2007). Indicações de resistência à murcha-de-ceratocystis em genótipos de cacaueiros no sul da Bahia, Brasil. In *Proceedings of the 15th International Cocoa Research Conference, vol. 2* (p 967). San José, Costa Rica: Cocoa Producer's Alliance.

Silva, S. D. V. M., & Luz, E. D. M. N. (2000). *Ceratocystis fimbriata* em cacaueiros das variedades cultivadas na Bahia. *Fitopatologia Brasileira*, *25*(Suppl), 424.

Silva, S. D. V. M., Paim, M. C., & Castro, W. M. (2004). Cacau "Jaca" Resistente a *Ceratocystis fimbriata* na Região Cacaueira da Bahia, Brasil. *Fitopatologia Brasileira*, *29*, 538–540.

Silveira, S. F., Harrington, T. C., Mussi-Dias, V., Engelbrecht, C. J. B., Alfenas, A. C., & Silva, C. R. (2006). *Annona squamosa*, a new host of *Ceratocystis fimbriata*. *Fitopatologia Brasileira*, *31*, 394–397.

Thorpe, D. J., Harrington, T. C., & Uchida, J. Y. (2005). Pathogenicity, internal transcribed spacer-rDNA variation, and human dispersal of *Ceratocystis fimbriata* on the family Araceae. *Phytopathology*, *95*, 316–323.

Yamada, M. M., Silva, S. D. V. M., & Pinto, L. R. M. (1999). Evaluation of phenotypic stability of resistance to *Phytophthora* spp. in cocoa clones. In *12th International Cocoa Research Conference* (pp. 509–514). London, England: Cocoa Producers' Alliance.

Zauza, E. A. V., Alfenas, A. C., Harrington, T. C., Mizubuti, E. S., & Silva, J. F. (2004). Resistance of *Eucalyptus* clones to *Ceratocystis fimbriata*. *Plant Disease*, *88*, 758–760.