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Resistance to Black Pod Disease in a Segregating Cacao Tree Population

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Abstract Black pod disease caused by *Phytophthora* spp. is the most important cacao tree disease worldwide. Genetic resistance has received much attention for the reduction of economic and environmental costs. In the present work, leaf disks from 282 individuals [$F_{1(TSH\text{-}1188xCCN\text{-}51)}$] were inoculated using suspensions of 3×10^5 zoospores of *Phytophthora palmivora* and *Phytophthora citrophthora*. Twenty disks of each genotype were distributed in four blocks with five disks per plot. After 5 days, each inoculated leaf disk was assessed for the infection level. It was verified statistically that the progeny's parents were intermediate between the control clones Catongo (susceptible) and Sca-6 (resistant). A highly positive linear relationship was observed between infection levels caused by both *Phytophthora* species in the F_1 progeny (r=0.9935**). However, the interaction was highly significant. It was also observed that the curve distribution is skewed towards higher resistance. This suggests that few genes are involved in controlling the resistance to both species, and that they maybe linked and species-specific. Although the parents did not differ statistically, the F_1 progeny segregated for resistance to each *Phytophthora* species and it is therefore an adequate population for breeding for resistance to black pod.

Keywords *Phytophthora* · *Theobroma cacao* · Genetic resistance · Pathogen · Polygenic

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1 Introduction

Black pod disease caused by *Phytophthora* spp. is the most important cacao tree disease and is responsible for an estimated 20 to 30 % in annual losses worldwide and tree deaths of up 10 % (Guest 2007). Although integrated management practices are efficient, genetic resistance has received much attention due to the reduced economic and environmental costs usually associated with fungicides.

Cocoa tree cultivation was introduced in Bahia, Brazil in 1746 and expanded rapidly and vigorously to represent up to 40 % of its exports (ABC et al. 2001). The incidence of fungus diseases such as witches' broom and black pod disease caused by *Moniliophthora perniciosa* and *Phytophthora* spp., respectively, has been a limiting factor for this crop in Bahia. Brazil is currently the sixth largest world producer with the fifth largest chocolate complex in the world with 230 t per annum (ICCO 2013), having ranked second in production in the past with 380 thousand tons per annum.

Germplasm collections are source of resistance genes for the genetic improvement of cacao. Out of 816 cacao accessions from the International Cocoa Genebank, Trinidad (ICG,T), 12.9 % were found to be resistant and 18.2 % moderately resistant to *Phytophthora* infections (Iwaro et al. 2006). In "Centro de Pesquisas do Cacau", Ilhéus, Brazil (CEPEC), among 82 genotypes of cocoa screened for

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resistance to *Phytophthora*, only 9 genotypes showed the same reaction of resistance (PA 30, PA 150, SIC 864 and SIAL 105) or susceptibility (AM 2, BE 3, BE 5, MA 11 and SIC 23) to infection by the three species *P. capsici*, *P. palmivora* and *P. citrophthora*, agents of black pod disease in Bahia state (Luz et al. 1996).

The use of resistant varieties is undoubtedly the most technically and economically viable alternative for producers to control the disease. Emphasis has been given to witches' broom studies at CEPEC, however, clones resistant to this disease are also being inoculated with the predominant species in the region today, *P. palmivora* and *P. citrophthora*, using different methods of inoculation and evaluation of symptoms (Luz et al. 1996, 2010; Pinto et al. 2007), including the leaf disc method (Santos et al. 2011). Obstacles such as the tree's long growth period of 3 to 4 years, the plant's size, varying between nine and 15 m (Dias 2001), and the time taken to produce fruits in sufficient quantity to identify resistant individuals, between 5 and 6 years, render resistance-seeking improvement an extremely slow process. For this reason, the technique of leaf disc inoculation is being used more frequently and for high correlation with black pod (Santos et al. 2011).

The objective of the present work was the quantitative characterization of an F_1 progeny from clones TSH-1188 and CCN-51 regarding its resistance to black pod disease, to understand the genetic patterns and to check the potential of this population for employment in the cocoa genetic improvement program.

2 Material and Methods

A segregating F_1 population consisting of 282 individuals resulting from crossing clones TSH-1188 and CCN-51 was used in this work. These parental clones originated from crosses among three different clones and have higher heterozygosity than clones involve in their genealogy (Bahia et al. 2013). Clone TSH-1188 (*Trinidad Selection Hybrid*) was selected in Trinidad and is derived from crossings involving clones IMC-67, ICS-1, P-18 and Sca-6, which is highly productive and moderately resistant to black pod disease. Another parent involved is clone CCN-51 (*Colección Castro Naranjal)* selected in Ecuador from the progeny resulting from crossing ICS-95, IMC-67 and Canelus. Sca-6 was used as a resistance standard and Catongo as susceptible control.

To assess resistance to black pod disease, five to six leaves were collected in the first hours of the day from each of the 282 progeny plants as well as from parents (TSH-1188 and CCN-51) and from resistant (Sca-6) and susceptible (Catongo) genotypes . The leaves were in good physiological conditions, at intermediate maturation phase and aged approximately 2 months.

The leaves were washed twice in sterile water and dried in sterile paper towels. After the wash, 20 disks measuring 2 cm in diameter were removed from each genotype using a puncher and placed abaxial surface up in plastic boxes measuring 32.5 cm×22.0 cm containing a humid sterilized sponge at the bottom. The disks were distributed in random blocks of four replications of five disks per box (Santos et al. 2009).

Two strains of *Phytophthora* were used as inocula, the isolates 62 (*P. citrophthora*) and 252 (*P. palmivora*) from CEPEC *Phytophthora* collection, both coming from southern Bahia cocoa pods. Ten Petri dishes were prepared for each pathogen in liquid carrot medium (200 g carrots/L) for isolate 62, and carrot agar (200 g carrot +20 g agar/L) for isolate 252. After transplanting the isolates, the plates were kept in incubator at 25 °C for 10 days (Luz and Silva 2001). Then, after verifying whether the colonies had undergone sporulation, 10 mL of sterile cold water was placed on each dish, which was transferred to a refrigerator where they were kept for 20 min. After this period the dishes were removed and placed at room temperature for another 25 min.

Suspension's concentration was determined with one drop of FAA (formaldehyde; alcohol; glacial acetic acid) that was added to the suspension of each species of *Phytophthora* to immobilize the zoospores released, thus allowing them to be counted to determine the suspension's concentration, with the use of a haemocytometer. The final concentration of both *Phytophthora* species zoospore suspensions was adjusted to 3.0×10^5 zoospores per mL.

Inoculation was performed by placing a drop of 0.02 mL suspension at the center of each leaf disk. By the end of the inoculation, the boxes were closed and kept in the dark at a temperature of 24±1 °C until the evaluation day.

After 5 days, the boxes were carefully opened and the symptoms were assessed on a 0–5 scale of infection level developed by Nyassé et al. (1995), where 0 = no symptoms; 1 = isolated small brown or dark brown spots; 2 = small brown spots with few interconnections between them; 3 = brown spots forming coalescing lesions of intermediate size; 4 = large coalescing lesions with light or dark brown points; and 5 = large necrotic lesion.

The mean for the 20 disks of each progeny individual was calculated. The assay was repeated twice, therefore the values obtained for each progeny individual was the mean of 40 leaf disks for each species inoculated.

The data obtained was assessed with the SAS package (SAS Institute 1989). The averages of the progeny plants were compared with the resistant (Sca-6) and susceptible (Catongo) controls using Dunnett's test. The parents' and the two control means were compared with the *t* test.

The genetic statistical model was used for data analysis, where effects are random except pathogen effect and the average:

$$Y_{gpb} = \mu + X_g + Z_p + W_b + H_{pxg} + \varepsilon$$

where Y = data vector, g = vector of random effect from genotypes, b = vector of random effect from blocks, p = vector of fixed effect from pathogens, and pxg = vector of random effect from pathogen and genotypes interaction. X, Z, W and H were genotypes, pathogen, block and interaction matrix, respectively, and ε = experimental error.

The variance component model (Cruz et al. 2004) was considered to be:

$$\sigma_f^2 = \sigma_g^2 + \varphi_p^2 + \sigma_{gxp}^2 + \sigma_b^2 + \sigma_e^2$$

where σ_f^2 = phenotipic or total variance; σ_g^2 = genetic variance; φ_p^2 = pathogen variance; σ_{gxp}^2 = plant-pathogen interaction variance; σ_b^2 = block variance and σ_e^2 = environmental variance. The variance is expressed by σ^2 in random components and by φ^2 in fixed component. The correction factor (fc) was calculated as $f_c = p/(p-1)$ where p = pathogen's species number. The broad-sense heritability (H^2) was estimated with these components (Table 1).

3 Results and Discussion

The analysis of variance of the data obtained for progeny, parent and control plants demonstrates that there were statistically significant differences based on the F test at 1 % probability level for blocks, plants, species and species×plant interaction (Table 1). Therefore, there were different responses from parents and control plants regarding reaction to the two species tested.

The estimated variance components were phenotipic or total variance σ_f^2=4.496; genetic variance σ_g^2=3.626; pathogen variance φ_p^2=0.000; plant-pathogen interaction variance σ_{gxp}^2=0.575; block variance σ_b^2=0.005 and environmental variance σ_e^2=0.290. We estimated the herdability (H^2) in 0.8066 ou 80.66 % by leaf disk assay, while based on fruit evaluation (detached pods test) it is cited to be 0.51 (Thevenin et al. 2005). In fact, small variations in results may occur between the different inoculation methods. For example, Pinto et al. (2007) attributed the observed differences for pod and stem inoculations to several factors, such as the seasonal effects of temperature and different rating scales. Additionally, we must also consider that heritability may vary among different kinds of populations or genotypes and assay environments.

When comparing the reaction of parents with Sca-6 (resistant) and Catongo (susceptible) controls against the two species of the pathogen (*P. citrophthora* and *P. palmivora*), the highest level of resistance was verified for Sca-6 and the lower level for Catongo, and they were statistically different from each other when inoculated with *P. citrophthora* (Table 2). Parent TSH-1188 was statistically more resistant to *P.citrophthora* than Catongo, however, it was more susceptible than Sca-6. CCN-51 did not differ statistically from Sca-6

Table 1 Analysis of variance of the levels of infection of two *Phytophthora* species in parental clones TSH-1188 and CCN-51, resistant (Sca-6) and susceptible (Catongo) controls, and the 282 plants from crossing TSH-1188× CCN-51

Source of variation	DF	Mean square	EM
Plants	285	16.76**	$\sigma_e^2+pr\sigma_g^2$
Pathogen	1	2.73**	$\sigma_e^2+rf_c\sigma_{gxp}^2+g\,\sigma_b^2+gr\,\varphi_p^2$
Plant*Pathogen	285	1.59**	$\sigma_e^2+rf_c\sigma_{gxp}^2$
Block/pathogen	7	1.61**	$\sigma_e^2+g\,\sigma_b^2$
ERROR	1741	0.29	σ_e^2
CV (%)	27.43		

p number of pathogens, *r* number of repetitions, *fc* correction factor, *g* number of genotypes, *b* number of blocks, σ^2 random variance, φ^2 fix variance

**Significant at 1 % probability

Table 2 Means of the levels of infection obtained from inoculating leaf disks of parental (TSH-1188 and CCN-51) and resistant (Sca-6) and susceptible (Catongo) controls with *P. citrophthora* and *P. palmivora*

Clones	*P.citrophthora*	*P.palmivora*	Mean
Catongo	3.20 a	1.00 a	2.10
TSH-1188	1.83 b	0.70 a	1.26
CCN-51	1.53 bc	0.83 a	1.18
SCA 6	0.85 c	0.33 a	0.59
Average	1.85	0.71	

Means followed by the same letter do not differ between themselves at 5 % probability based on *T* test (LSD =0.75)

Table 3 Number of plants of the progeny TSH-1188× CCN-51 classified per level of infection after inoculation with *P. citrophthora* and *P. palmivora*, compared with the infection levels of resistant (Sca-6) and susceptible (Catongo) controls

Species	Infection level	Individual number	Significant difference of progeny to	
			Sca-6	Catongo
P. citrophthora	0.00–2.00	159	ns	*
	2.10–4.35	92	*	ns
	4.40–5.00	31	*	*
P. palmivora	0.00–1,50	130	ns	ns
	1.60–2,20	35	*	ns
	2.25–5,00	117	*	*

*Significantly different from Sca-6 (Catongo) based on Dunnett's test (5 % significance)

Ns Not significantly different from Sca-6 (Catongo) based on Dunnett's test

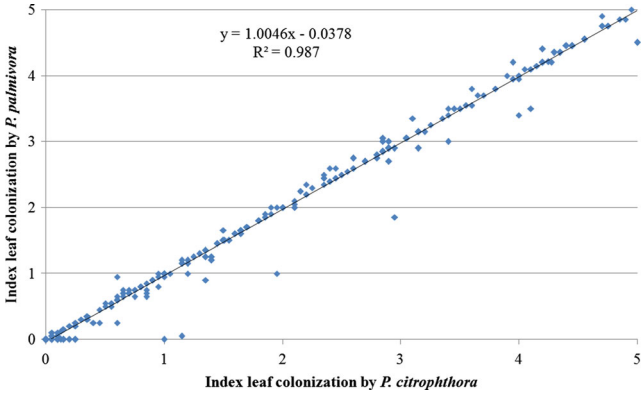

Fig. 1 Relationship between colonization by *Phytophthora citrophthora* and *P. palmivora* on leaf disks of cocoa progenies from crossing TSH 1188 and CCN51

regarding resistance level. For both species, the level of infection in CCN-51 was approximately double of that for Sca-6 (Table 2).

When comparing the averages obtained by the four clones regarding both *Phytophthora* species tested, one can observe that although lacking statistical comparison, the mean levels of infection obtained for *P. citrophthora* (1.81) is higher than double that obtained for *P. palmivora* (0.71), demonstrating that *P. citrophthora* is a more aggressive species to these clones than *P. palmivora.* Luz and Yamada (1985) observed this same proportion when establishing a range of scores to assess resistance to *Phytophthora* in fruits. Other authors (Campelo and Luz 1981; Lawrence 1983; Luz 1989) also reported higher virulence of *P. citrophthora* in relation to other species affecting cocoa trees in Bahia, *P. palmivora* and *P. capsici*, and this is the main reason for choosing *P. palmivora* and *P. citrophthora* for the present work. *Phytophthora palmivora* is a cosmopolitan species, and *P. citrophthora* is more aggressive, according to tests performed by Luz (1989). Furthermore, Luz et al. (2003) demonstrate the growth of both species populations in Bahia in recent years, and the decline of *P. capsici.*

The reactions of all 282 plants assessed for both *Phytophthora* species tested was stratified in three categories according to the level of infection (Table 3). The categories created for both species were different due to their different level of aggressiveness. The means of each category of plants were then compared with those of controls Catongo and Sca-6.

The 159 plants most resistant to *P. citrophthora* (Scores =0.00 to 2.00, Table 3) did not differ statistically from the resistance control (Sca-6) and were more resistant than the susceptibility control, based on Dunnett's test (p=0.005). The other 123 progeny plants, on the other hand, revealed to be less resistant than Sca-6. However, among these 123 plants, 92 were statistically as susceptible as the susceptibility control (Catongo), and 31 were statistically more susceptible. Therefore, although parents do not differ between themselves as to *P. citrophthora* infection level, there was segregation of this characteristic in the progeny. The possible reason is heterozygosis in one or both of the parents for resistance

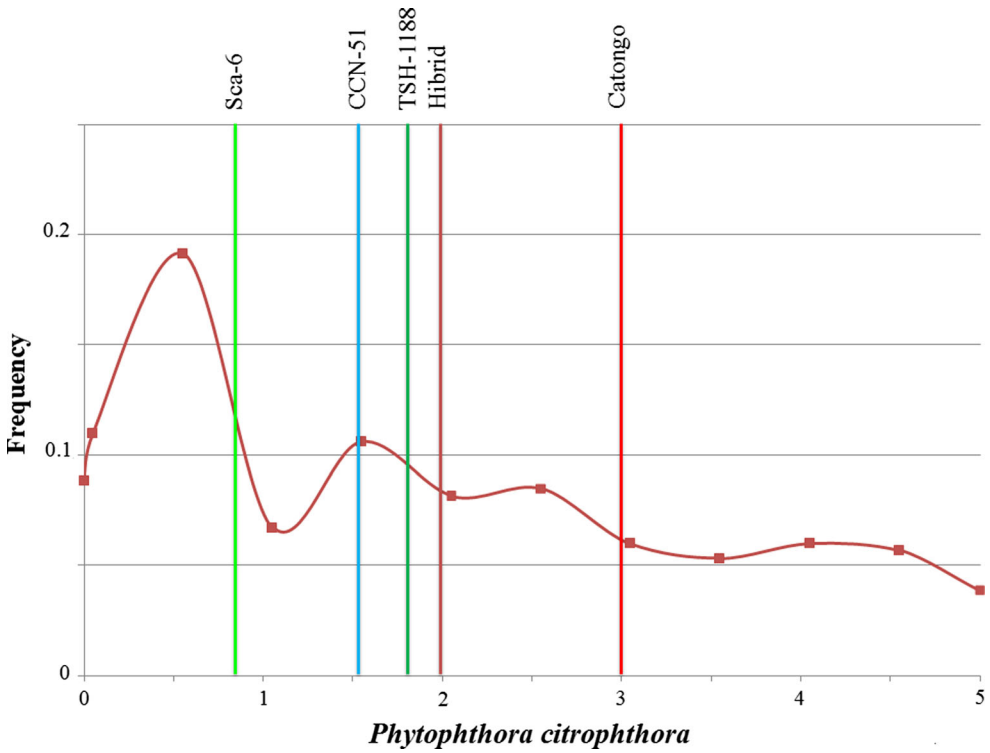

Fig. 2 Frequency distribution of genotypes in average level of infection on leaf disks inoculated with *Phytophthora citrophthora*, with TSH 1188 and CCN51 as control parents, and Catongo and Sca-6 as susceptible and resistant controls, respectively

genes to *P. citrophthora*. However, this affirmation must be confirmed by further studies.

With regard to *P. palmivora*, the controls did not differ statistically between themselves based on *t* test (Table 2). In the progeny, 130 plants did not differ from either control; however, 117 plants were statistically more susceptible than both controls based on Dunnett's test (Table 3).

There was high frequency of plants with the lowest level of infection (0.8) in relation to other levels for both species. The distribution of plants in the other levels was more or less similar for both species. The curves observed were skewed towards higher resistance for both species, suggesting that few genes are involved in controlling resistance to both species. Many authors (Blaha and Lotodé 1976; Dantas Neto et al. 2005; Enriquez and Salazar 1980; Rodriguez et al. 1985) suggest the existence of a polygenic control for resistance to *P. palmivora*. In some crosses, the phenotypic distribution of segregating individuals is closer to normal (Dantas Neto et al. 2005) than others (this study). In addition to methodological differences, it is possible that each population reveals different sets of polymorphisms located in different genes with effects on resistance to black pod.

There is high correlation between resistance to *P. citrophthora* and *P. palmivora* (Fig. 1). This correlation is suggested because the lesion establishment is dependent of stomatal frequency and pore length with a correlation of 0.93 (Iwaro et al. 1997). The integrated assessment with other agronomical characteristics may lead to the reduction of problems caused both by *P. palmivora* and *P. citrophthora*. In the preliminary selection, *P. citrophthora* can be used for allowing a higher range of selection of resistant genotypes.

Distribution of this progeny was on average more susceptible than both parents (Fig. 2) being transgressive, because there were other genotypes that were much more resistant than Scavina 6 (resistance standard) and much more susceptible than Catongo (susceptible standard). Since character has no addictive predominance or of quantitative nature, the formation of new populations with more resistant individuals should produce new segregating populations with means lower than parents. However, there is possibility of selecting individuals that are more resistant, through vegetative propagation of the more resistant individuals.

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References

ABC, CNPC & Coopercacau (2001) Pacto do Cacau - Programa de Recuperação da Lavoura Cacaueira. Resumo da Proposta de Readequação. CEPLAC, Ilhéus

Bahia RC, Corrêa RX, Santos RC, Machado RCR, Luz EDN, Araújo IS, Ahnert D (2013) Inheritance of the number of ovules per ovary and selection of cacao genotypes. Am J Plant Sci 4:1387–1392

Blaha G, Lotodé R (1976) Un critêre primordial de sélection du cacaoyer au Cameroun: la resistance à la pourriture brune des cabosses. Variations des réactions à la maladie en liaison avec les dones écologiques et l'état physiologique des fruits. Café Cacao Thé 20: 97–116

Campelo AMFL, Luz EDMN (1981) Virulência de *Phytophthora spp*. em frutos destacados de cacau da cultivar comum. Fitopatol Bras 6: 587

Cruz CD, Regazzi AJ, Carneiro PCS (2004) Modelos biométricos aplicados ao melhoramento genético, 3rd edn. Editora UFV, Viçosa

Dantas Neto A, Corrêa RX, Monteiro WR, Luz EDMN, Gramacho KP, Lopes UV (2005) Caracterização de uma população de cacaueiro para mapeamento de genes de resistência à vassoura-de-bruxa e podridão parda. Fitopatol Bras 30:380–386

Dias LAS (2001) Melhoramento genético do cacaueiro. FUNAPE, Viçosa

Enriquez GA, Salazar LG (1980) Cocoa varietal resistance to *Phytophthora palmivora* and its inheritance at Turrialba, Costa Rica. Proceedings. CATI, Turrialba

Guest D (2007) Black pod: diverse pathogens with a global impact on cocoa yield. Phytopathology 97:1650–1653

ICCO, International Cocoa Organization (2013) Annual Report. p 64

Iwaro AD, Sreenivassan TN, Umaharan P (1997) *Phytophthora* resistance in cacao (*Theobroma cacao*): influence of pod morphological characteristics. Plant Pathol 46: 557–565

Iwaro AD, Butler DR, Eskes AB (2006) Sources of resistance to *Phytophthora* pod rot at the International CocoaGenebank, Trinidad. Genet Resour Crop Evol 53:99–109

Lawrence JS (1983) Virulência relativa das espécies de *Phytophthora* que causam podridão-parda na Bahia. Fitopatol Bras 8:595

Luz EDMN (1989) The roles of five species of *Phytophthora* in infection and disease of roots, stems, and pods of *Theobroma cacao* L. D.Sc. Thesis. University of Florida, Gainesville

Luz EDMN, Silva SDVM (2001) Podridão-parda dos frutos, cancro e outras doenças causadas por *Phytophthora* no cacaueiro. In: Luz EDMN, Santos AF, Matsuoka K, Bezerra JL (eds) Doenças causadas por *Phytophthora* no Brasil. Livraria Editora Rural, Campinas, pp 175–265

Luz EDMN, Yamada MM (1985) Índice para avaliar a reação de cultivares de cacau a *Phytophthora* spp. Theobroma 14:181–188

Luz EDMN, Silva SDVM, Yamada MM, Pires JL, Braga MCT, Lopes UV, Bezerra JL, Brugnerotto MIB (1996) Research on cacao resistance to black pod disease in Bahia, Brazil - 1980 to 1995. In: International workshop on the contribution of disease resistance to cocoa variety improvement, Proceedings… Reading, UK. INGENIC.

Luz EDMN, Ram A, Rocha CSS, Freitas DB (2003) Aumento da frequência de ocorrência de *Phytophthora citrophthora* em cacaueiros no sul da Bahia. Fitopatol Bras 28:214–215

Luz EDMN, Paim M, Cerqueira AO, Souza JT (2010) Genetic diversity, distribution and pathogenicity of *Phytophthora* species on cacao in Brazil. In: Pereira JL, Lopes UV (eds) The use of molecular biology techniques in search for varieties resistant to witches' broom disease of cacao. Common Fund for Commodities, Amsterdam, pp 99–110

Nyassé S, Cilas C, Herail C, Blaha G (1995) Leaf inoculation as an early screening test to assess cacao (*Theobroma cacao* L) resistance to *Phytophthora* black pod disease. Crop Prot 14: 657–663

Pinto LRM, Silva SDVM, Yamada MM (2007) Evaluation of phenotypic stability of resistance to *Phytophthora* spp. in cacao clones. Fitopatol Bras 32:453–457

Rodriguez R, Enriquez GA, Soria VJ (1985) Herencia de la reacción del cacao a la pudrición de la mazorca causada por *Phytophthora palmivora* (Butl.) Butl. In: Workshop, Grupo Internacional de *Phytophthora*, Resumos… CEPLAC, Ilhéus

Santos ESL, Cerqueira-Silva CBM, Clement DPL, Luz EDMN (2009) Identificação de resistência genética do cacaueiro à podridão-parda. Pesq Agrop Brasileira 44:413–416

Santos ESL, Cerqueira-Silva CBM, Clement DPL, Luz EDMN (2011) Resistance gradient of Black pod disease in cocoa and selection by leaf disk assay. Crop Breed Appl Biotechnol 11:297–303

SAS Institute (1989) SAS/STAT user's guide, version 6.4. SAS institute Inc, Cary

Thevenin J-M, Umaharan R, Surujdeo-Maharaj S, Latchman B, Cilas C, Butler DR (2005) Relationships between black pod and witches' broom diseases in *Theobroma cacao*. Phytopathology 95:1301–1307