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Novel sources of witches' broom resistance (causal agent *Moniliophthora perniciosa*) from natural populations of *Theobroma cacao* from the Brazilian Amazon

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Abstract Witches' broom is a severe disease of *Theobroma cacao* L. (cacao), caused by the basidiomycete *Moniliophthora perniciosa*. The use of resistant cultivars is the ultimate method of control, but there are limited sources of resistance. Further, resistance from the most widely used source ('Scavina 6') has been overcome after a few years of deployment. New sources of resistance have been intensively searched for in the Amazon basin. Here, we evaluated for witches' broom resistance, cacao accessions from various natural cacao populations originally collected in the Brazilian Amazon. Resistance of 43 families was evaluated under nursery and/or field conditions by artificial or natural infection, respectively, based on disease incidence. Screening for resistance by artificial inoculation under nursery conditions appeared to be efficient in identifying these novel resistance sources, confirmed by natural field evaluation over a nine-year period. The increase in natural field infection of 'Scavina 6' was clearly demonstrated. Among the evaluated families with the least witches' broom incidence, there were accessions originally collected from distinct river basins, including the Jamari river ('CAB 0371'; 'CAB 0388'; 'CAB 0392'; and 'CAB 0410'); Acre ('CAB 0169'); Javari ('CAB 0352'); Solimões ('CAB 0270'); and from the Purus river basin, the two most outstanding resistant accessions, 'CAB 0208' and 'CAB 0214'. The large genetic diversity found in cacao populations occurring at river basins from Acre and Amazonas states, Brazil, increased the chance that the selected resistant accessions would be genetically more dissimilar, and represent distinct sources of resistance to *M. perniciosa* from 'Scavina 6'.

Keywords Basidiomycete · Cocoa · *Crinipellis perniciosa* · Natural population

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Introduction

The basidiomycete *Moniliophthora perniciosa* (Marasmiaceae sensu stricto) (Aime and Phillips-Mora 2005) [Syn. *Crinipellis perniciosa* (Stahel) Singer; Tricholomataceae] is the causal agent of the witches' broom disease of *Theobroma cacao* L. (cacao). This severe cacao pathogen is endemic to the Amazon basin, and upon introduction into producing regions, the disease devastated local industries in Surinam, Trinidad, Ecuador, and more recently in Bahia, Brazil (Andebrhan et al. 1999). The fungus is presently found in all South American and Caribbean cacao producing countries, with estimated losses reaching over 250,000 ton year^{-1} (Bowers et al. 2001). There are current serious concerns about the potential risk of *M. perniciosa* introduction into West Africa, a region responsible for over 70% of the world cacao production.

The long-term method of witches' broom control will be through genetic resistance, as chemical and biological controls are currently inefficient and costly, and phytosanitary pruning of infected tissues is a stop-gap measure (Purdy and Schimdt 1996; Rudgard et al. 1993). Evaluation of resistance has traditionally been based on incidence and/or severity of symptoms (Frias et al. 1995), and mechanisms of resistance remain largely unknown (Leal et al. 2007). Only recently some biochemical and physiological aspects of the pathogenesis are being investigated and elucidated (Mondego et al. 2008; Rincones et al. 2008).

The accessions 'Scavina 6' and 'Scavina 12', originally collected in Peru (Bartley 2005), were characterized as resistant in Trinidad in the 1950s, with resistance appearing as mostly monogenic, with 'Scavina 6' supposedly as homozygous dominant, and 'Scavina 12' as an heterozygote (Bartley 1986; Rudgard et al. 1993). By genetic mapping, a major resistance locus was identified at linkage group nine of the cacao consensual map (Brown et al. 2005; Faleiro et al. 2006). 'Scavina 6' has been the only recognized source of resistance widely used in cacao breeding programs; however, this resistance was overcome in Ecuador, Peru and the Brazilian Amazon after few years of deployment (Bartley 1986; Wheeler and Suarez 1993). New sources of resistance have been intensively searched for in the Amazon region from Ecuador, Peru, and Brazil for the last 70 years (Bartley 2005; Rudgard et al. 1993). A systematic collection to obtain a representation of the genetic diversity of spontaneous and semi-spontaneous cacao from the whole Brazilian Amazon region was conducted by the Brazilian government from 1976 to 1991 (Almeida et al. 1995; Sereno et al. 2006). As a result, a germplasm collection was established by the "Comissão Executiva do Plano da Lavoura Cacaueira" (CEPLAC) at the "Estação de Recursos Genéticos José Haroldo" (ERJOH), in Marituba, Para state (PA), currently holding 1,817 accessions (denominated as Cacao Amazon Brazil or CAB), of which 940 were of clonal origin and 877 derived from open-pollinated seedlings, representing 36 river basins of the 186 Brazilian Amazon basins (Almeida et al. 1995; Bartley 2005).

The development of a large scale inoculation spray method using controlled concentration of viable spores (Frias et al. 1995) stimulated the establishment of witches' broom resistance screening programs in Brazil, which were complemented with field evaluation. In Marituba (PA), screening for resistance started with the evaluation of 521 clonal CAB accessions under high inoculum pressure upon field conditions (Fonseca and Albuquerque 2000), followed by artificial inoculation of seedlings derived from either open-pollinated pods or from controlled cross under nursery conditions. Similar resistance evaluation of CAB accessions was initiated in Southern Bahia, at first by artificial inoculation under nursery condition using the automated spray method (Frias et al. 1995), followed by screening under field conditions using families obtained from open-pollinated pods (Paim et al. 2006). After six years of field evaluation, progenies from seven CAB accessions ('CAB 0064', 'CAB 0066', 'CAB 0156', 'CAB 0194', 'CAB 0195', 'CAB 0269' and 'CAB 0274') appeared as significantly more resistant than 'Scavina 6' or 'Scavina 12' (Paim et al. 2006). Here, we hypothesized that cacao accessions originally collected from river basins at the Upper Amazonian region would contain resistant individuals, particularly from Upper and Middle Solimões, Purus and Acre river basins, where *M. perniciosa* and *T. cacao* may share a common center of diversity (Purdy and Schimdt 1996). Our evaluation of CAB accessions from those regions led to the identification of important novel sources of resistance to witches' broom, namely 'CAB 0214'; 'CAB 0208'; and 'CAB 0270'.

Materials and methods

Plant material

Families derived from open-pollinated pods from accessions originally collected at the Brazilian Amazon (CAB series) were evaluated for witches' broom resistance. Based on the initial resistance screening under field conditions at Marituba, PA (Fonseca and Albuquerque 2000), a total of 43 CAB accession families (Table 1) was evaluated for resistance, including 23 originally collected in the Amazonas state; 12 from Rondônia state; five from Acre state; two from Pará state; and one from an unknown origin (Almeida et al. 1995; Table 1). The screening assays included a susceptible (the albino mutant cultivar 'Catongo') and resistant ('Scavina 6' and 'Scavina 12') standard controls (Frias et al. 1995). Evaluation of resistance was conducted under nursery conditions at ERJOH from CEPLAC, Marituba, PA, Brazil (1°21′S; 48°18′W), while nursery and field evaluations were conducted at the "Centro de Pesquisa do Cacau" (CEPEC) from CEPLAC, Ilhéus, Bahia state (BA), Brazil (14°47′S; 39°13′W).

Families of seedlings derived from open-pollinated pods were evaluated for resistance under nursery conditions in CEPEC, Ilhéus (BA) in two assays. The first experiment (established on September 1998) tested 'CAB 0153'; 'CAB 0160'; 'CAB 0208'; 'CAB 0219'; 'CAB 0221'; 'CAB 0233'; 'CAB 0327'; 'CAB 0328'; 'CAB 0329'; 'CAB 0334'; 'CAB 0356'; 'CAB 0364'; 'CAB 0383'; 'CAB 0388'; 'CAB 0486'; and 'CAB 0992', using 'Catongo' (susceptible), 'Scavina 6' and 'Scavina 12' (resistant) as standard controls. The experimental design was a complete randomized block, with four blocks, every one with 19 families in plots of 28 seedlings each. The second experiment (established on October 1998) evaluated families obtained from 'CAB 0012'; 'CAB 0148'; 'CAB 0153'; 'CAB 0160'; 'CAB 0180'; 'CAB 0181'; 'CAB 0186'; 'CAB 0193'; 'CAB 0198'; 'CAB 0214'; and 'CAB 0501', using 'Catongo', 'Scavina 6', 'Scavina 12' and 'Theobahia' (a resistant hybrid progeny derived from the cross 'Scavina 6' × 'ICS 1') as controls. This assay was also conducted in complete randomized block design, with four blocks, with 15 families in plots of 28 seedlings each. The next trial (February 1999) was conducted in ERJOH, Marituba, PA, to evaluate families derived from controlled crosses between resistant accessions ('CAB 208 × CAB 214'); between resistant and susceptible accessions ('CAB 0214 × ICS 39'; 'CAB 0208 × PA 195'; 'PA 195 × CAB 0208';'CAB 0270 × ICS 39'; 'ICS 39 × CAB 0270'), between two susceptible accessions ('PA 195 × ICS 39'); and from open-pollinated crosses ('CAB 0049'; 'CAB 0191'; 'CAB 0208'; 'CAB 0214'; and 'CAB 0313') using 'Catongo' as the susceptible control. This experiment was conducted with four blocks, every one with 13 families in plots of 25 seedlings. Finally, 24 CAB families derived from open-pollinated pods ('CAB 0031'; 'CAB 0169'; 'CAB 0186'; 'CAB 0208'; 'CAB 0213'; 'CAB 0214'; 'CAB 0219'; 'CAB 0232'; 'CAB 0233'; 'CAB 0260'; 'CAB 0270'; 'CAB 0275'; 'CAB 0307'; 'CAB 0309'; 'CAB 0327'; 'CAB 0332'; 'CAB 0334'; 'CAB 0352'; 'CAB 0371'; 'CAB 0388'; 'CAB 0392'; 'CAB 0410'; 'CAB 0531'; and 'CAB 0992) were evaluated for resistance under natural field conditions at CEPEC, Ilhéus, BA, comparing with the resistant control families derived from 'Scavina 6', 'Scavina 12' and ' Theobahia' ('Scavina 6' × 'ICS 1') for 9 years (2000–2008). The field experimental design was a complete randomized block, with four blocks, every one with 28 families in plots of 12 plants each.

M. perniciosa inoculum production for artificial inoculations

Dry infected cacao shoots (dried brooms) were collected either at the area of CEPEC in Ilhéus, BA, or at ERJOH, Marituba, PA. Brooms were exposed to alternating wet-dry cycles (12 h) to induce basidiocarp production. Basidiospores were collected into a 16% glycerol solution, filtered through a 0.45 μm membrane, and the retained spores were resuspended in 2 ml 16% glycerol solution, and stored in liquid nitrogen (Frias et al. 1995).

Evaluation of resistance by artificial inoculation under nursery conditions

For the first and second experiments, 21-day-old seedlings from open-pollinated pods were inoculated by spraying 1 ml of a basidiospore suspension (7.5×10^4 spores ml^{-1}), followed by incubation at a humid chamber for 24 h, as described by Frias et al. (1995).

Table 1 List of 43 ‘CAB’ accessions used as parents of open-pollinated families evaluated for resistance to *M. perniciosa*, with description of respective original location of collection, river basin and Brazilian state of origin

Accessions	Locality of collection	River basin	State
CAB 0012	Seringal Muqui, Presidente Médici	Ji-Paraná	Rondônia
CAB 0031	Barcarena	Acará	Pará
CAB 0049	Benevides	Guamá	Pará
CAB 0148	Rio Acre	Acre	Acre
CAB 0153	Rio Acre	Acre	Acre
CAB 0160	Rio Acre	Acre	Acre
CAB 0169	Rio Acre	Acre	Amazonas
CAB 0180	Ji-Paraná and Ouro Preto	Ji-Paraná	Rondônia
CAB 0181	Rio Tarauacá	Tarauacá	Acre
CAB 0186	Rio Tarauacá	Embira	Acre
CAB 0191	Rio Purus	Purus	Amazonas
CAB 0193	Rio Purus	Purus	Amazonas
CAB 0198	Rio Purus	Purus	Amazonas
CAB 0208	Rio Purus	Purus	Amazonas
CAB 0213	Rio Purus	Purus	Amazonas
CAB 0214	Rio Purus	Purus	Amazonas
CAB 0219	Ariquemes	Jamari	Rondônia
CAB 0221	Ariquemes	Jamari	Rondônia
CAB 0232	Ariquemes	Jamari	Rondônia
CAB 0233	Ariquemes	Jamari	Rondônia
CAB 0260	Ariquemes	Jamari	Rondônia
CAB 0270	Tefé	Solimões	Amazonas
CAB 0275	Middle Solimões	Solimões	Amazonas
CAB 0307	Parnás, Copeá and Carapanatuba	Solimões	Amazonas
CAB 0309	Parnás, Copeá and Carapanatuba	Solimões	Amazonas
CAB 0313	Parnás, Copeá and Carapanatuba	Solimões	Amazonas
CAB 0327	Middle Solimões near Tefé	Solimões/Amazonas	Amazonas
CAB 0328	Middle Solimões near Tefé	Solimões/Amazonas	Amazonas
CAB 0329	Middle Solimões near Tefé	Solimões/Amazonas	Amazonas
CAB 0332	Middle Solimões near Tefé	Solimões/Amazonas	Amazonas
CAB 0334	Upper Solimões near Tabatinga	Solimões/Amazonas	Amazonas
CAB 0352	Rio Javari and affluents	Javari	Amazonas
CAB 0356	Rio Javari and affluents	Itaquí	Amazonas
CAB 0364	Rio Içá	Içá	Amazonas
CAB 0371	Ariquemes	Jamari	Rondônia
CAB 0383	Ariquemes	Jamari	Rondônia
CAB 0388	Ariquemes	Jamari	Rondônia
CAB 0392	Ariquemes	Jamari	Rondônia
CAB 0410	Ariquemes	Jamari	Rondônia
CAB 0486	Upper médio Japurá	Japurá	Amazonas
CAB 0501	Upper médio Japurá	Japurá	Amazonas
CAB 0531	Upper médio Japurá	Japurá	Amazonas
CAB 0992	Unknown	Unknown	–

The experiment using seedlings derived from controlled pollination assay was conducted at ERJOH, Marituba, PA, and evaluated the most promising CAB accessions crossed among themselves or with susceptible genotypes (‘ICS 39’ and ‘PA 195’), using 45-day old seedlings for inoculation. In this case, flushing apical meristems were inoculated with 30 μl of a basidiospore suspension (10^5 spores ml^{-1}), followed by incubation at a humid chamber for 24 h. Evaluation of disease incidence was conducted 60 days after inoculation in comparison with susceptible controls. Level of resistance of each family was estimated by the average proportion of seedlings (disease incidence), exhibiting one or more of the evaluated symptoms, which included presence of terminal and/or axillary brooms; and/or seedlings exhibiting swollen shoot and/or pulvinus (Frias et al. 1995).

Statistical analyses of witches’ broom resistance by artificial inoculation under nursery conditions

The variable estimated for the first three experiments referred to the number of seedlings Y_i, $i = 1, 2,\ldots,n$, that displayed symptoms among m_i plants to compare the proportion of seedlings with symptoms (disease incidence) among the various families. Proportion data can be analyzed using a binomial regression model (McCullagh and Nelder 1989), assuming that the number of seedlings with symptoms Y_i, from a total of m_i is a random variable. The standard binomial model assumes that $Y_i \sim \mathrm{Bin}(m_i, \pi_i)$ where π_i is the probability of a seedling expressing the symptom, with mean $\mu_i = E(Y_i) = m_i\pi_i$ and variance $\mathrm{Var}(Y_i) = m_i\pi_i(1 - \pi_i)$. The generalized linear model allows to model expected proportions π_i in terms of explanatory variables x_i (family effects and covariates), modelled in terms of $g(\mu_i/m_i) = \eta_i = x_i'\boldsymbol{\beta}$, where g is suitable link function, and $\boldsymbol{\beta}$ is the vector of parameters. The most common link function is the ‘logit’ link $g(\mu_i/m_i) = \ln[\mu_i/(m_i - \mu_i)] = \ln[\pi_i/(1 - \pi_i)]$. For a well-adjusted model, it is expected that the residual deviance would be approximately equal to the number of degrees of freedom. To test the significance of the family effect, progenies deviance were compared to $\chi^2_{v,\mathrm{p}}$ value with υ degrees of freedom at $\alpha = 0.05$ significance level. After estimating the parameters by maximum likelihood, the expected probability to exhibit disease symptoms is $\hat{\pi}_i = \frac{\exp(\hat{\eta}_i)}{1+\exp(\hat{\eta}_i)}$ and the expected number of seedlings with symptoms is equal to $\hat{\mu}_i = m_i\hat{\pi}_i$. Each family contrast was compared to reference families (‘Catongo’; ‘CAB 0208’; ‘CAB 0214’; ‘CAB 0214 × ICS 39’; or ‘CAB 0208 × PA 195’) using the test of Wald (Dobson 2002).

Evaluation of resistance under natural field conditions

Seedlings from the families with superior performance in the nursery evaluation were transferred to the field at the CEPEC station in Ilhéus, BA, planted at 3 × 1.5 m spacing under highly infected old cacao trees. The 24 CAB families plus three resistant accessions (‘Scavina 6’; ‘Scavina 12’; and ‘Theobahia’) and one susceptible (‘Catongo’) were evaluated every two weeks for the number of points of infection, which were tagged to avoid re-counting. Evaluations occurred for nine years, from January 2000 to December 2008. The variable considered was the number of infected shoots (vegetative broom) per family per year. Because the number of surviving trees differed among families after field planting, the average number of infected shoots was corrected for this factor. The number of infected shoots per year was analyzed considering each year, and interpreted as nine distinct variables using Proc GLM—MANOVA (SAS Institute 1988). The interpretation of year and family × year interaction effects was conducted using repeated measurement analyses (Proc GLM—REPEATED). To better recognize the family × year interaction effect, each one of the nine years was successively removed from the analyses to compare individual contributions to the interaction by their effects on the F test. Every family was compared by contrast with the resistant controls ‘Scavina 6’; ‘Scavina 12’; and ‘Theobahia’.

Results

Evaluation of witches’ broom resistance by artificial inoculation under nursery conditions

The first experiment evaluated sixteen CAB families for witches’ broom resistance by automated spray inoculation (Frias et al. 1995) in comparison to the resistant control standards ‘Scavina 6’ and ‘Scavina

12', and the susceptible 'Catongo'. The average proportion of seedlings exhibiting symptoms (disease incidence) ranged from 13% ('Scavina 12') to 64% ('CAB 0356'), with the susceptible standard control presenting an average of 51% of disease incidence (Table 2). Based on the Wald test for contrasting the average disease incidence of each family against the susceptible reference, eight CAB families ('CAB 0356'; 'CAB 0327'; 'CAB 0364'; 'CAB 0328'; 'CAB 0383'; 'CAB 0334'; 'CAB 0992'; and 'CAB 0160') did not significantly differ from 'Catongo' for the proportion of seedlings exhibiting symptoms (Table 2). On the other hand, 'CAB 0329', 'CAB 0221', 'CAB 0233', 'CAB 0153', 'CAB 0388', 'CAB 0219', and 'CAB 0486' families did not statistically differ for disease incidence from 'CAB 0208', the CAB family with the lowest disease incidence (Table 2). The mean proportion of seedlings with symptoms for these eight CAB families ('CAB 0329'; 'CAB 0221'; 'CAB 0233'; 'CAB 0153'; 'CAB 0388'; 'CAB 0219'; 'CAB 0486'; and CAB 0208') were similar to the disease incidence observed for the resistant controls 'Scavina 12' and 'Scavina 6' (Table 2).

In the second experiment, eleven CAB families were evaluated in comparison to 'Catongo' and 'Scavina' standard control genotypes (Table 3) using the automated spray system. The 'Catongo' family displayed the highest proportion of seedlings with witches' broom symptoms (71%; Table 3), while 'CAB 0214' had the lowest disease incidence (13%; Table 3). The proportion of seedlings with symptoms for 'Catongo' was higher in this experiment in comparison to the previous one (71 vs. 51%). When the proportion of seedlings with symptoms of the CAB families was compared with 'Catongo', only four CAB families ('CAB 0181'; 'CAB 0012'; 'CAB 0186'; and 'CAB 0214') displayed significantly differences for disease incidence (Table 3). 'CAB 0160' did not differ from the susceptible control 'Catongo' for disease incidence, as observed for the first experiment (Table 2). On the other hand, 'CAB 0153' did not differ from the susceptible control for disease incidence in this assay (Table 3), but was significantly distinct in the previous experiment (Table 2). 'CAB 0214' did not significantly differ for disease incidence from the resistant control accessions 'Scavina 12', 'Theobahia', 'Scavina 6'

Table 2 Comparison of disease incidence among families in relation to the proportion of seedlings exhibiting symptoms ($n = 28$), and estimated probability of significant differences for pairwise contrasts in relation to the susceptible control 'Catongo' or the resistant 'CAB 0208' for the first experiment conducted at CEPEC, Ilhéus, BA, Brazil

Accession	Mean proportion of seedlings with symptoms	*P* value for contrast with Catongo	*P* value for contrast with CAB 0208
CAB 0356	64.0	0.133	6.8×10^{-7}***
CAB 0327	58.0	0.426	1.1×10^{-5}***
Catongo	51.0	–	1.9×10^{-4}***
CAB 0364	48.0	0.766	5.1×10^{-4}***
CAB 0328	43.0	0.371	3.2×10^{-3}**
CAB 0383	41.0	0.313	4.7×10^{-3}**
CAB 0334	40.0	0.233	7.5×10^{-3}**
CAB 0992	39.0	0.196	9.8×10^{-3}**
CAB 0160	38.0	0.163	0.012*
CAB 0329	32.0	0.040*	0.071
CAB 0221	31.0	0.020*	0.087
CAB 0233	29.0	0.016*	0.132
SCA 6	28.0	0.012*	0.162
CAB 0153	27.0	0.009**	0.196
CAB 0388	26.0	0.005**	0.271
CAB 0219	21.0	6.0×10^{-4}***	0.706
CAB 0486	19.0	4.2×10^{-4}***	0.799
CAB 0208	18.0	1.9×10^{-4}***	–
SCA 12	13.0	2.6×10^{-5}***	0.496

Level of significance: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$

Table 3 Comparison of disease incidence among families in relation to the proportion of seedlings exhibiting symptoms ($n = 28$), and estimated probability of significant differences for pairwise contrast in relation to the susceptible control 'Catongo' and the resistance reference 'CAB 0214' for the second experiment conducted at CEPEC, Ilhéus, BA, Brazil

Accession	Mean proportion of seedlings with symptoms	P value for contrast with Catongo	P value for contrast with CAB 0214
Catongo	71.0	–	3.2×10^{-5}***
CAB 0501	69.0	0.797	2.8×10^{-5}***
CAB 0148	54.0	0.193	2.0×10^{-5}***
CAB 0180	51.0	0.122	5.0×10^{-4}***
CAB 0153	49.0	0.090	9.0×10^{-4}***
CAB 0198	49.0	0.090	9.0×10^{-4}***
CAB 0193	48.0	0.091	1.0×10^{-3}**
CAB 0160	47.0	0.073	1.0×10^{-3}**
CAB 0181	40.0	0.023*	8.0×10^{-3}**
CAB 0012	40.0	0.024*	7.0×10^{-3}**
CAB 0186	35.0	9.0×10^{-3}**	0.025*
Theobahia	37.0	6.0×10^{-3}**	0.194
SCA 12	23.0	3.0×10^{-3}**	0.321
SCA 6	18.0	1.0×10^{-4}***	0.194
CAB 0214	13.0	3.2×10^{-5}***	–

Level of significance: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$

(Table 3), with the 'CAB 0214' family appearing as the most resistant.

The two CAB accessions ('CAB 0214' and 'CAB 0208') with the lowest incidence of disease at the previous two assays were then further evaluated by testing both accessions in controlled crosses with susceptible or resistant genotypes to validate their behaviour against *M. perniciosa* infection by artificial inoculation using the agar drop method. Families from controlled crosses between the two supposedly resistant accessions ('CAB 0208 × CAB 0214'); or by crossing both accessions with susceptible accessions ('ICS 39'or 'PA 195') were obtained and inoculated (Table 4). The accession 'CAB 0270' also demonstrated a good level of resistance in other assays (data not shown), and it was also included in crosses with susceptible genotypes ('CAB 0270 × ICS 39'; 'ICS 39 × CAB 0270'). Families derived from crosses between the two susceptible accessions ('ICS 39' and 'PA 195') were also included.

In this experiment, the mean proportion of seedlings exhibiting symptoms ranged from 8% ('CAB 0208 × CAB 0214') to 91% ('PA 195 × ICS 39'). When disease incidence for all families was contrasted with the susceptible control 'Catongo', two groups were formed (Table 4). The first group exhibited a larger proportion of infected seedlings, ranging from 79 to 91% (Table 4), and it was formed by families from the controlled crosses between the susceptible accessions 'PA 195 × ICS 39', together with those derived from open-pollinated families of 'CAB 0313' and 'CAB 0049', and the susceptible reference 'Catongo'. The other group, with smaller proportion of infected seedlings, ranging from 8 to 62% (Table 4), included mostly families from open-pollinated pods or controlled crosses involving either 'CAB 0208', 'CAB 0214', or 'CAB 0270', plus the family derived from 'CAB 0191'. When disease incidence expressed as the proportion of seedlings with symptoms from all families were contrasted with those from the 'PA 195 × CAB 0208', there were no significant differences for the reciprocal 'CAB 0208 × PA 195'; and families from open-pollinated crosses of 'CAB 0191'; 'CAB 0214'; and 'CAB 0208' (Table 4). The families 'CAB 0214 × ICS 39' and 'CAB 0208 × CAB 0214' presented the lowest disease incidence, and significantly differed for disease incidence when contrasted with all the other families (Table 4).

In general, the experiments conducted using artificial inoculation under nursery conditions pointed that families derived from open pollinated or controlled crosses involving 'CAB 0208', 'CAB 0214', or 'CAB 0270' displayed a smaller proportion of infected seedlings, either for experiments conducted

Table 4 Comparison of disease incidence among families in relation to the proportion of seedlings exhibiting symptoms ($n = 25$), and estimated probability of significant differences for pairwise contrasts in relation to the susceptible control 'Catongo' and the reference families 'PA 195 × CAB 0208', 'CAB 0214 × ICS 39', 'CAB 0208 × CAB 0214'. This assay was conducted at ERJOH, Marituba, PA, Brazil

Families	Overall proportion of seedlings with symptoms	*P* value for contrast with Catongo	*P* value for contrast with PA 195 × CAB 208	*P* value for contrast with CAB 0214 × ICS 39	*P* value for contrast with CAB 0208 × CAB 214
PA 195 × ICS 39	91.0	0.406	4.63×10^{-15}***	$<2.00 \times 10^{-16}$***	$<2.00 \times 10^{-16}$***
Catongo	88.0	–	4.60×10^{-15}***	$<2.00 \times 10^{-16}$***	$<2.00 \times 10^{-16}$***
CAB 0313	84.0	0.563	7.14×10^{-14}***	$<2.00 \times 10^{-16}$***	$<2.00 \times 10^{-16}$***
CAB 0049	79.0	0.108	4.89×10^{-10}***	1.99×10^{-15}***	1.65×10^{-15}***
CAB 0270 × ICS 39	62.0	5.26×10^{-8}***	1.14×10^{-16}***	4.68×10^{-12}***	3.71×10^{-12}***
ICS 39 × CAB 0270	48.0	5.26×10^{-8}***	7.00×10^{-3}**	2.03×10^{-7}***	1.15×10^{-7}***
CAB 0208 × PA 195	33.0	6.45×10^{-12}***	0.443	2.00×10^{-4}***	1.20×10^{-4}***
CAB 0191	29.0	4.52×10^{-13}***	0.833	1.30×10^{-3}**	7.20×10^{-4}***
PA 195 × CAB 0208	27.0	4.60×10^{-15}***	–	1.50×10^{-3}**	8.00×10^{-4}***
CAB 0214	26.0	2.05×10^{-15}***	0.872	2.00×10^{-3}**	1.20×10^{-3}**
CAB 0208	25.0	5.75×10^{-15}***	0.819	3.00×10^{-3}**	1.80×10^{-3}**
CAB 0214 × ICS 39	9.0	$<2.0 \times 10^{-16}$***	1.50×10^{-3}**	–	0.7998
CAB 0208 × CAB 0214	8.0	$<2.0 \times 10^{-16}$***	8.00×10^{-4}***	0.7998	–

Level of significance: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$

in Bahia (trial 1 and 2) or Para state (trial 3), using local *M. perniciosa* isolates.

Evaluation of resistance under natural field conditions

Analyses of witches' broom resistance of CAB families derived from open-pollinated crosses was then carried out under natural infection of field conditions at CEPEC, Ilhéus, BA, by counting the number of infected shoots (brooms) every two weeks for nine years. The experiment contained seedlings from 24 CAB families, which exhibited superior performance during nursery evaluation using artificial inoculation, and included three resistant controls ('Scavina 6'; 'Scavina 12'; and 'Theobahia') plus a susceptible one ('Catongo'). Nonetheless, as most of the 'Catongo' plants did not survive after a few years of cultivation under high inoculum conditions, this control was not further considered in this experiment.

When the level of resistance was estimated based on the average number of infected vegetative shoots per plant, highly significant differences among families were detected by the Wilks' λ test ($P < 0.0001$) using multivariate analyses (Tables 5, 6). The effect of year was also highly significant (Wilks' λ test; $P < 0.0001$, repeated measurement analyses), as there was a trend to increase the number of infected shoots with year, as it can be seen in Fig. 1 for a few families. The interaction between families and year was also significant for number of infected sites per plant based on Wilks' λ test, repeated measurement analyses ($P = 0.0001$), because the rate of increase in infected sites per plant per year was distinct among the various families.

Overall, the families from 'CAB 0208', 'CAB 0214' and 'CAB 0270' displayed the lowest level of witches' broom incidence (Fig. 1; Table 5). During the nine years of evaluation, families derived from 'CAB 0208', 'CAB 0214' and ' CAB 0270' presented lower incidence of shoot infection than most of the other CAB families (Table 5). Among the three resistant controls, 'Scavina 12' presented successively more infection in shoots than the other resistant controls, with an increasing trend over the years. Similar tendency was observed for 'Scavina 6', which showed increasing number of infected shoots especially after 2004, while 'Theobahia', a hybrid derived from 'Scavina 6' had increasing number of shoots with symptoms in average, but at lower rate of

Table 5 Adjusted mean number of infected shoots (vegetative brooms) per plant per year in families of the CAB series in Ilhéus, Bahia, Brazil

Family	2000	2001	2002	2003	2004	2005	2006	2007	2008
CAB 0031	6.196	1.876	5.500	3.013	3.098	3.167	18.901	26.167	23.008
CAB 0169	1.717	1.976	1.148	0.873	0.732	0.452	4.678	13.584	11.547
CAB 0186	4.189	2.451	3.463	2.615	4.798	6.858	15.969	28.664	22.621
CAB 0208	1.201	0.187	1.190	0.291	0.040	0.055	1.166	7.680	5.636
CAB 0213	3.422	1.851	6.263	3.115	3.631	4.958	10.102	30.564	23.221
CAB 0214	0.382	0.398	0.117	0.167	0.160	0.205	1.210	2.306	2.406
CAB 0219	2.813	1.297	3.281	2.464	4.651	3.630	20.742	26.449	19.829
CAB 0232	6.310	2.973	5.069	4.432	5.284	7.253	17.189	36.040	26.915
CAB 0233	5.586	1.108	3.599	2.618	3.375	4.227	11.988	27.252	23.462
CAB 0260	5.204	2.824	4.331	2.726	3.237	8.224	14.695	34.423	28.308
CAB 0270	2.065	0.226	1.026	0.642	0.571	1.110	3.252	5.490	5.754
CAB 0275	6.003	2.217	3.514	2.748	5.398	5.376	16.566	34.026	25.288
CAB 0307	5.014	2.275	2.100	0.993	3.004	3.557	9.267	18.703	13.339
CAB 0309	5.312	2.134	1.940	1.717	2.339	3.803	11.405	24.523	18.069
CAB 0327	6.654	4.612	4.704	2.510	3.541	3.082	13.134	27.157	16.729
CAB 0332	4.842	1.417	2.180	1.878	3.405	3.451	7.379	23.804	15.790
CAB 0334	7.022	3.126	7.917	4.578	5.595	7.810	22.194	51.221	34.110
CAB 0352	3.802	1.908	3.763	1.824	2.689	3.090	10.181	11.963	9.874
CAB 0371	1.955	0.779	1.958	1.490	2.111	3.897	15.709	26.384	26.438
CAB 0388	3.022	1.003	2.659	1.498	2.486	3.672	8.828	33.719	21.032
CAB 0392	3.234	0.975	1.300	0.989	1.085	2.518	5.772	11.312	7.319
CAB 0410	3.244	0.741	3.527	1.716	1.524	2.848	6.366	20.061	19.652
CAB 0531	7.385	3.935	6.741	5.253	6.826	9.649	20.141	36.605	34.269
CAB 0992	5.286	5.161	4.308	2.443	3.433	4.972	15.684	20.040	14.459
SCA 12	0.350	0.481	0.864	1.322	5.210	3.323	10.127	21.515	19.675
SCA 6	0.296	0.538	1.471	1.889	3.178	5.961	17.589	26.316	28.473
Theobahia	1.466	0.574	1.387	1.037	2.429	2.968	4.629	6.053	7.914

increase compared to both Scavinas (Fig. 1). The level of infection for 'CAB 0208'; 'CAB 0214'; and 'CAB 0270' was consistently inferior to all the resistant controls (Tables 5, 6; Fig. 1).

In terms of probability of differences for the average number of shoot infection plant^{-1} year^{-1} for 9 years between the CAB families with lowest incidence ('CAB 0208'; 'CAB 0214'; and 'CAB 0270') and the resistant controls (Table 6), the three CAB families were significantly more resistant than 'Scavina 12', while not significantly differing from 'Scavina 6' and 'Theobahia', and these CAB families did not differ among themselves (Table 6). But 'CAB 0214' was consistently the most resistant family and significantly differed from all the other CAB families, except for 'CAB 0169', 'CAB 0208', 'CAB 0270', 'CAB 0352', 'CAB 00371', 'CAB 0388', 'CAB 0392', 'CAB 0410' (Table 6). 'CAB 0208' and 'CAB 0270' presented the same pattern as 'CAB 0214', except that both did not significantly differ from 'CAB 0307', 'CAB 0309', 'CAB 0332' (Table 6).

Seven of the CAB families were evaluated in one of the three previous assays, and in general, all maintained the same evaluation. The 'CAB 0327', 'CAB 334' and 'CAB 0992' families did not differ for the average number of infected seedlings from the susceptible control 'Catongo' in the first assay (Table 2), whereas during the field evaluation, the average number of infected points per plant were highly significantly different from the resistant controls (Tables 5, 6). Similarly, 'CAB 0388' did not differ from 'CAB 0208' for disease incidence in the

Table 6 Probability of significance estimated for the contrast in number of infected shoots (vegetative brooms) per plant per year (for 9 years) between the families derived from ‘CAB 0208’, ‘CAB 0214’ and ‘CAB 0270’ with all CAB families, ‘Scavina 6’, ‘Scavina 12’ and ‘Theobahia’

Family	CAB 208 Pr > F[a]	CAB 214 Pr > F[a]	CAB 270 Pr > F[a]
CAB 0031	<0.0001	<0.0001	0.0002
CAB 0169	0.7879	0.8012	0.6527
CAB 0186	0.0005	0.0001	0.0049
CAB 0208	–	0.9717	0.9874
CAB 0213	0.0074	<0.0001	0.0026
CAB 0214	0.9717	–	0.9209
CAB 0219	0.0003	0.0001	0.0011
CAB 0232	<0.0001	<0.0001	<0.0001
CAB 0233	0.0046	<0.0001	0.0285
CAB 0260	<0.0001	<0.0001	0.0002
CAB 0270	0.9874	0.9209	–
CAB 0275	<0.0001	<0.0001	0.0002
CAB 0307	0.0290	0.0050	0.1309
CAB 0309	0.0221	0.0044	0.1383
CAB 327	<0.0001	<0.0001	<0.0001
CAB 332	0.0382	0.0045	0.1321
CAB 334	<0.0001	<0.0001	<0.0001
CAB 352	0.0925	0.0211	0.3336
CAB 371	0.0491	0.0146	0.0807
CAB 388	0.3306	0.0357	0.2174
CAB 392	0.5687	0.4002	0.9737
CAB 410	0.5847	0.0694	0.6175
CAB 531	<0.0001	<0.0001	<0.0001
CAB 992	<0.0001	<0.0001	<0.0001
Scavina 6	0.1020	0.1796	0.0836
Scavina 12	0.0001	0.0003	0.0001
Theobahia	0.6416	0.7625	0.8825

[a] Probability of incurring in error by accepting the hypothesis of difference between families with nine annual mean of shoot infection, considering Wilks’ λ test at 5%

first experiment (Table 3) and did not differ from ‘CAB 0208’ and ‘CAB 270’ during field evaluation (Tables 5, 6). ‘CAB 0186’ significantly differed from ‘CAB 0214’ during the second assay (Table 3), and again during the field experiment (Tables 5, 6). On the other hand, ‘CAB 0219’ and ‘CAB 0233’ were shown to not significantly differ for disease incidence from ‘CAB 0208’ in the first assay (Table 2), while during field assessment of resistance, both families appeared to significantly differ for disease incidence from ‘CAB 0208’ (Tables 5, 6).

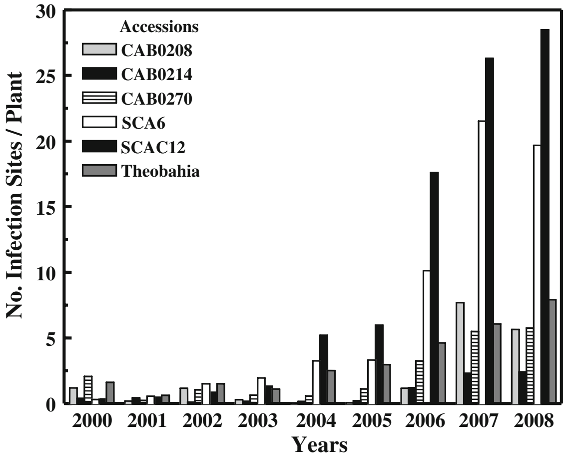

Fig. 1 Adjusted mean number of infected shoots (‘vegetative brooms’) per plant for each year (2000–2008) for the most resistant CAB families (‘CAB 0214’; ‘CAB 0208’; and ‘CAB 0270’) in comparison to the resistant control families (‘Scavina 6’; ‘Scavina 12’; ‘Theobahia’)

Discussion

The identification of novel dependable sources of resistance to witches’ broom disease is urgently required to secure a sustainable cacao industry, not only in South America and the Caribbean, but also to prevent a potential collapse of the industry from an accidental introduction of *M. perniciosa* into disease-free regions of West Africa and Southeast Asia, responsible for over 85% of the current world cacao production. The search for witches’ broom resistance started in Ecuador in the 1920s, after the establishment of the disease in the coastal zone, through mass selection of seedlings derived from symptomless trees (*Refractarios*) (Zhang et al. 2008), followed by expeditions to the Amazon valley to collect disease-free semi-cultivated and spontaneous cacao in 1937 and 1942 (Pound 1943). All the resulting planting material was eventually sent to Trinidad for evaluation, and among the various accessions, only ‘Scavina 6’ and ‘Scavina 12’ were described as remarkably resistant (Bartley 1986). Because of the poor agronomic characteristics, ‘Scavina 6’ and ‘Scavina 12’ were not directly used as clones, but were crossed to develop hybrid varieties or clones, such as the ‘Trinidad Selected Hybrids’ (TSH) and ‘Trinidad Selected Amazons’ (TSA) clonal series, successfully cultivated worldwide until today. TSH clones have been commercially used in Trinidad, where they appeared to have had a major role in the reduction of witches' broom incidence (Laker et al. 1988), while more recently, in Bahia, Brazil, CEPLAC recommended 'TSH 565' and 'TSH 516' as major resistant clones and distributed seeds of 'Theobahia', a hybrid variety derived from the 'Scavina 6 × ICS 1' cross.

However, when 'Scavina 6' was re-introduced in Ecuador, resistance to *M. perniciosa* was overcome after only a few years in the field (Bartley 1986), a trend also observed in the Brazilian Amazon (Fonseca and Albuquerque 2000) and Peru (Rios-Ruiz 2001), likely because of the occurrence of more aggressive isolates. It has been proposed that *M. perniciosa* might have co-evolved with *Theobroma* hosts in the Upper Amazon region, where both may share a common center of diversity (Purdy and Schimdt 1996), similarly to other cacao pathogens, such as *M. rorei* and *Ceratocystis cacaofunesta* (Engelbrecht et al. 2007). Here, we demonstrated the increasing level of infection of 'Scavina 12' and 'Scavina 6' families under field conditions in Bahia over a nine-year period (Fig. 1), a anecdotal development noticed by local farmers and researchers by the increasing infection of some of the cultivated 'Scavina 6'-derived resistant clones, such as 'TSH 565' and 'TSH 516'. Noteworthy, Marita et al. (2001) described 'Scavina 6' and 'Scavina 12' as resistant based on data obtained from field evaluation at the same location in Bahia, Brazil, but collected previous to 1998. The increased level of infection might derive from a possible widespread of more virulent isolates, or from an increase in inoculum pressure by the intensification of the witches' broom epidemic. The original introduction of *M. perniciosa* in Bahia was detected in 1989, and it was demonstrated to be derived from at least two distinct isolates (Andebrhan et al. 1999). But, later introduction of other isolates cannot be discarded as phytosanitary restrictions of plant material movement from the Amazon into Bahia were relaxed after the first disease outbreak. It is possible that during the last 20 years, with the increasing cultivation of planting material derived from 'Scavina 6', isolates were continuously selected for aggressiveness or new isolates were brought in, or further, inoculum pressure increased steadily by the widespread of the disease. In fact, Shaw and Vandenbon (2007) demonstrated that 'Scavina 6' families were highly susceptible to an isolate (APC3) originally collected in Bahia, when inoculated at a very high inoculum concentration (16,000 spores per shoot), confirming that virulent isolates occur in the region. The resistance breakdown from 'Scavina 6' and 'Scavina 12' clearly reinforces about the risk of depending on single sources for long-term resistance deployment in a perennial crop.

Families derived from open-pollinated or controlled crosses from 'CAB 0214' and 'CAB 0208' exhibited the highest level of resistance among the tested CAB accessions in all experiments conducted using artificial inoculation under nursery conditions, independently from isolate origin (Bahia or Para). The superior performances of 'CAB 0214' and 'CAB 0208' families in terms of reduced infection and symptom development were confirmed during the nine-year evaluation under field conditions in Bahia, while the resistance observed for families derived from 'CAB 0270' under nursery conditions conducted in Para was also confirmed under field conditions in Bahia (Fig. 1). The accessions 'CAB 0208', 'CAB 0270' and 'CAB 0214' were able to transmit high levels of resistance to *M. perniciosa* to offspring, even when crossed with highly susceptible genotypes, such as 'ICS 39' or 'PA 195'(Table 4), suggesting dominance. The resistance from 'Scavina 6' was initially considered to be dominant and predominantly monogenic (Bartley 1986). Indeed, a major dominant quantitative resistance locus (QRL) was identified in 'Scavina 6' by molecular mapping, at linkage group 9 of the consensual map, responsible for up to 51% of the phenotypic variance for resistance (Brown et al. 2005; Faleiro et al. 2006). However, another minor QRL was detected on linkage group 1, accounting for 6.7% of the variance, with dominance for the susceptible parent 'ICS1' (Brown et al. 2005). Our results indicated a clear distinction for witches' broom resistance in the field between 'Scavina 6' and the hybrid family 'Theobahia', derived from a cross with 'ICS 1', which maintained a limited disease incidence, at levels similar to the most resistant CAB families during the nine year evaluation (Fig. 1). Therefore, it appears that the performance of 'Theobahia' may originate from the presence of additional resistance gene(s) derived from the susceptible 'ICS 1', including the one identified by Brown et al. (2005), in addition to the major gene inherited from 'Scavina 6', resulting in a complete distinct infection behavior from the resistant parent under field condition. Our own results from genome mapping of two families derived from crosses between 'CAB 0214' or 'CAB 0208' with the highly susceptible 'ICS 39' indicated that, besides a major QRL, additional QRLs were identified at distinct linkage groups and loci (Paulo Albuquerque and Antonio Figueira, unpublished). Current models for plant-pathogen interaction indicate that qualitative and quantitative resistances are governed by similar genetic mechanisms, with the so-called vertical or major gene resistance may be overlapped by quantitative disease resistance loci (Poland et al. 2009).

Overall, the screening based on artificial inoculation showed a good correlation with the final families' performance for resistance in the field. From the nine families evaluated under both conditions (artificial inoculation in the nursery and natural field conditions), seven ('CAB 0186'; 'CAB 0208'; 'CAB 0214'; 'CAB 0327'; 'CAB 0334'; 'CAB 0388'; and 'CAB 0992') maintained the same resistance classification between evaluations. The two discrepant results were those from 'CAB 0219' and 'CAB 0233', which did not differ from 'CAB 0208' for mean proportion of seedlings with symptoms after artificial inoculation, but significantly differed for adjusted mean number of infected shoots per plant from 'CAB 0208' during field evaluation. In this case, it appeared that the first experiment were less effective in discriminating susceptible from resistant families, possibly because the level of incidence was lower than normal, as 'Catongo' exhibited only a 51% proportion of seedlings with symptoms, while rates of 71 and 88% were observed for the second and third experiment, respectively. Therefore, artificial inoculation under nursery conditions appeared as a good predictor for field performance if an efficient inoculation system is adopted, especially in avoiding escapes.

From the nine CAB families here identified with better field resistance against *M. perniciosa* infection, the top five derived from accessions originally collected at Upper Amazonian river basins, with one from the Acre river ('CAB 0169'); two from Purus ('CAB 0208'; 'CAB 0214'); one from the Javari river ('CAB 0352'); and one from the Solimões river basin ('CAB 0270') (Table 1; Almeida et al. 1995). The other four derived from accessions collected at the Jamari river basin in Rondônia state ('CAB 0371'; 'CAB 0388'; 'CAB 0392'; CAB 0410). From the 40 CAB families evaluated for resistance by Paim et al. (2006), the seven best were originally from the Caeté/Iaco and Acre river basin (three accessions) and Purus (two accessions), Solimões or Japurá river basins. Other cacao accessions from the Brazilian Amazon exhibiting resistance to witches' broom had been previously reported, mainly from the 'Cruzeiro do Sul' series, collected originally at the Jurua river basin, Acre state (Marita et al. 2001). Our results corroborated the original hypothesis that resistance to witches' broom is more frequent for accessions originating from the Upper Amazon region, particularly from Upper and Middle Solimões, Purus and Acre river basins, but additional potential sources of resistance were identified in accessions from the Jamari river basin in Rondônia state.

The distinct resistance behaviour of 'CAB 0214', 'CAB 0208', and 'CAB 0270' in relation to 'Scavina 6' and 'Scavina 12' under field conditions in Bahia strongly suggested that alternative genes of resistance might be operating in these novel resistance sources. The large genetic diversity detected for cacao populations occurring at river basins from Acre and Amazonas states (Motamayor et al. 2008; Sereno et al. 2006) increased the chance that resistant accessions from these areas would be genetically more dissimilar, and represent distinct sources of resistance to *M. perniciosa* from Scavina. The occurrence of resistant families from accession originally sampled at the Jamari river basin at the Rondonia state, a distant area from the Upper Amazon region demonstrated that potential sources of resistance might be more widespread than previously assumed. The resistant CAB families here identified mostly belong to the Purus genetic cluster in the classification proposed by Motamayor et al. (2008). The novel CAB resistance sources can be used to pyramid resistance genes to increase durability and stability of the resistance (Paim et al. 2006).

In conclusion, this work was able to identify nine promising novel sources of witches' broom resistance, originally collected at the Brazilian Amazon region. Ongoing work using genetic mapping has identified QRL for the two most promising sources 'CAB 0214' and 'CAB 0208', whereas defense-related genes have been identified and characterized from both sources (Leal et al. 2007).

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