BREAKDOWN OF SCAVINA RESISTANCE IN BAHIA CAUSED BY THE EVOLUTION *MONILIOPTHORA PERNICIOSA*

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ABSTRACT

Resistant clones to witches' broom disease of cacao (WBD), caused by the basidiomycete fungus *Moniliophthora perniciosa* (Mp) is the most practical and cost efficient strategy for WBD management, but their use is currently restricted to very limited clones, mostly, Scavina's descendants. Scavina resistance has proved inadequate or unstable, as, for example, in Rondônia in the Amazon basin, in Ecuador during the 1980s, and in Bahia in 2002. In this paper, we present evidences, through several independent studies, that the Scavinas' resistance have been overcome because of changes in the pathogen population. Using molecular markers in studies of 40 isolates of the fungus, collected from brooms from five resistant and two susceptible cocoa genotypes, a clear genetic differentiation was observed between fungal isolates from primarily resistant clones and from susceptible ones. Further, a study carried out to characterize temporal genetic variability of *Mp* populations in Bahia, Brazil, over four consecutive years (2001 to 2004), in several locations, have shown that there was a shift in the genetic composition of the Mp populations. Pathogenic variability through cross inoculation experiments using -isolates derived from Scavinas (Scavina 6 and descendants), non scavina, and from SIC, a susceptible clone, showed that Scavina isolates caused more disease on the Scavinas genotypes, whereas the isolate derived from SIC was less pathogenic on the Scavinas and more pathogenic on its respective host. These results allowed us to conclude that the increase in susceptibility in Scavina descendants was the result of the buildup of resistance-breaking pathogen's strains capable of overcoming the resistance of Scavina. Also, temporal observations of natural infections between 2003 and 2010 in a F_2 Scavina 6 x ICS1 showed that the major QTL identified in the LG9 had its effect decreased year by year; moving from a LOD of 9 in 2003 to a non significant LOD in 2010. The same results were found under artificial inoculations with specific strains of *Mp* and using a new F_2 population created in 2007. The present study provides the first evidences that Scavinas WB resistance breakdown is due to the adaptation of the *Mp* field populations. These studies, besides proving the change in the Mp population in Bahia, they are also a warning call that the efficiency of resistant cultivars in WBD management is limited by Mp variability and an incentive to design breeding programs for durable resistance. Different sources of resistance have been identified and are being used in the cacao breeding program to associate distinct genes of resistance.

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INTRODUCTION

The current challenge of the cacao breeding program for Witches´ broom disease (WBD), caused by the basidiomycete *Moniliophthora perniciosa*, is to achieve resistance durability. The extent of the difficulty of achieving durable resistance is highlighted by the fact that most varieties deployed are mainly Scavina descendants. Scavina-6 has been the main available source of resistance in Bahia since the WBD arrival in Bahia, in 1989 (Pereira et al., 1990). In Trinidad, despite the increase in witches´ broom severity in some populations, the progenies of Scavina-6 showed resistance to the local strains (Laker et al., 1987).

On the other hand, Scavina resistance has proved inadequate or unstable, as for example in Ecuador (Bartley, 1981) and Peru (Rios-Ruiz, 1989) in the 1980s, in Brazil in Rondônia State (Anderbrhan et al., 1998) and Bahia in 2002 (Pires 2003, Gramacho et al, 2008). Studies of epidemics on crops have shown that plant pathogens can quickly adapt to their host plant and overcome resistance genes mainly when one source of resistance is consistently used. In this paper, we present evidences, through several independent studies, that the Scavinas' resistance have been overcome because of changes in the pathogen population.

MATERIAL AND METHODS

Fungal isolation and DNA extraction Isolations were derived either from vegetative brooms or axillary brooms collected at random in the field. Trees were marked and GPS points registered. Small tissue pieces taken from the edges of diseased tissues were incubated in Petri dishes on potato dextrose agar (PDA) medium amended with 0·5 g L^1 of streptomycin at 20–25°C. Growing mycelium was transferred to PDA and to Malt 50% for 12 days for DNA extraction. Mycelia were filtered through Whatman No. 1 filter paper, washed twice with TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0), and then lyophilized. Total DNA was extracted as described by Zolan and Pukilla (1986). Quality and quantity of the DNA was determined by spectrophotometer and eletrophoresis (Varian Cary 50 Spectrophotometer), respectively. The DNA samples were diluted to 10 ng l^{-1} and stored at -20^{o}C for further use. RNA was eliminated by adding RNAse in a final concentration of 10 mg mL^{-1} (Ribonuclease A).

RAPD analysis. Five primers OPH20, OPH13, OPI19, OPI14, and OPL7obtained from Operon Technologies (Alameda, California) that generated reproducible polymorphism and clear banding patterns were used to amplify DNA of all isolates. RAPD analysis with each of the primers was repeated twice and only consistent and reproducible banding patterns were scored. PCR reactions were performed in 25 uL containing 10 mM of Tris-HCl (pH 8.3), 2.4 mmoL. L^{-1} of $MgCl_2$, 0.25 mM. L^{-1} of each dNTP, 0.4 μM of each primer, 0.5 uL of Taq DNA polymerase and approximately 30 ng of genomic DNA. Amplifications were conducted in a GeneAmp PCR System 9600 (Perking-Elmer) under the following conditions: 30 cycles of 1 min at 92°C, 1 min at 35°C, for 2 min at 72°C. Amplification products were separated by electrophoresis at 110 V for 2 h on BET-stained 1.2% agarose gel in 1X Tris-borate EDTA buffer, and visualized under UV light.

Temporal Studies. *Moniliophthora perniciosa* isolates, around twenty-five per year, were obtained from susceptible genotypes in Southeast Bahia, Brazil during four consecutive years (2001 to 2004). At total, 89 isolates were obtained. Isolates from the same year were considered as being of the same population. Also natural and artificial disease incidence were observed on a hundred and fifty individuals of the F2 (SCA6 x ICS 1) population from 2003 to 2010 and mapping was performed with MapQTL v. 4.0.

Evolution of the pathogen pathogen population. Isolates were derived from seven *Theobroma cacao* resistant genotypes (CCN-10, MOQ-216, Playa Alta-4, TSH-565, TSH-1188) and two susceptible ones (SIC-2, EEG-8). A total of 40 isolates were obtained. These isolates are referred here as clone-isolate. The genotypes were selected according to their witches´ broom response, genetic origin and relatedness with the Scavinas. Fungal isolations were performed as above. Cluster analysis was re carried by SAS using PROC MDS and SAS graph (SAS Institute, 1998) using Jaccard similarity coefficient.

Genetic Analysis. Amplified DNA fragments were scored as either present (1) or absent (0) and the computation of similarity coefficients were processed as a diploid model with two alleles per locus (Lynch, 1994). In addition, factorial analyses were used to assigned isolates to RAPD groups (clusters); further each defined group of isolates was considered as a separate population allowing examining the distribution of isolates based on the year of collection using the Nei's genetic distance (1978). This matrix was subjected to an un-weighted paired group method with arithmetic average (UPGMA) to generate a dendrogram using software NTSYS-pc Version 2.02 (Rohlf, 1998). the distribution of genetic diversity, the Nei's genetic identity (h) (1973) within and across populations, were computed with the PopGene32 software (Yeh et al., 2000). The analysis of molecular variance (AMOVA) was used to estimate the total variance and variance within populations, among populations within regions, and among regions (Excoffier *et al.* 1992). The variance components were statistically tested by nonparametric randomization tests using 1,000 permutations.

Pathogenicity testes. For the artificial inoculation a drop of 20 µL of $2x10^5$ basidiósporos/mL of clone-isolate inocula was deposited onto cacao rooted cuttings of the resistant (TSH1188, CCN51and CCN10) clones and on seedlings of Catongo (susceptible) according to Surujdeo-Maharaj et al. (2003). Clone-isolates inocula were obtained from cacao resistant genotypes-Scavina descendent (isolates Ceplac/CEPEC i_346, i_346, i_487, i_1734), non-descedent (i_339 and i_593) and susceptible ones (i_343) according to Niellla, et al., 2000. After inoculation, the plants of all treatments remained for 24 h in humid chamber, with temperatures around 25 °C and 100% RH, afterword they were transferred to the green house until the end of the experiment. Disease incidences were evaluated 60 days after the inoculation.

RESULTS AND DISCUSION

The temporal studies over four consecutive years (2001 to 2004), in several locations, have shown that there was a shift in the genetic composition of the *M. perniciosa* populations. The distribution of the 89 isolates in the factorial planes (1,2,3) is presented in Figure 1A. Each of the first three axes explains a limited part of the total variability: 26.85%, 17.62%, and 8.4%, respectively. Some isolates had identical multicharacter patterns, therefore shared the same coordinates, i.e. year 1(2001) and 2 (2003). However, isolates from year 4 (2004) always merged around the axis 3. Although, this population seems genetically more uniform, it was the most distant (Figure 1B) and genetically different from the populations of the other years (Theta (θ) for year4 x year1= 0.33 and year 4 x year 2= 0.51, $P<0.05$).

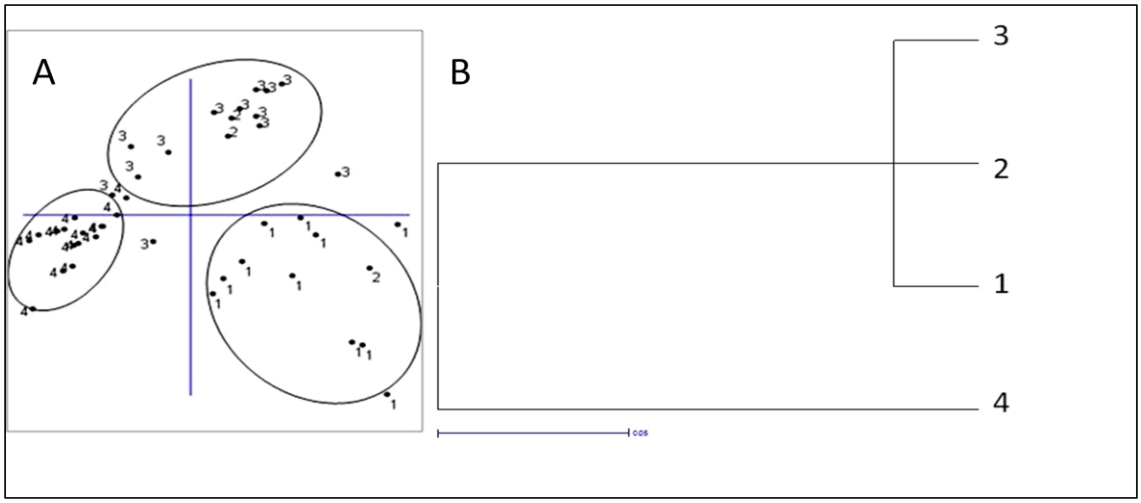

Figure 1 - Three-dimensional principal coordinates (1/2 and 1/3) plot of 89 *Moniliophthora perniciosa* isolates (A) and dendrogram Based Nei's (1978) genetic distance method (UPGMA) (B) generated from random amplified polymorphic DNA data for four population (year of collection) of *Moniliopthora perniciosa*. Number 1,2,3, and 4 refers to the year which isolates were collected:2001,2002,2003 and 2004; respectively.

Thereafter, WBD arrival in Bahia, about 150,000 hectares of susceptible varieties have since been replaced, as a emergency measure, with a single resistant source (Scavina descendants) as clones. Thus, we propose that selection pressure imposed by the cacao host has caused a population shift in the 2004 population for specific adaptation to the local host genotypes. This is supported by the fact that the population of the pathogen in resistant genotypes are genetically different from those of susceptible

genotypes, by studying the 40 isolates (strains) of the fungus, collected from brooms from five resistant (CCN-10, MOQ-216, Playa Alta-4, TSH-565, TSH-1188) and two susceptible genotypes (SIC-2, EEG-8).In this study it was observed a clear genetic differentiation between fungal isolates from resistant clones and those from susceptible ones. Both cluster analysis, carried by SAS, and analysis of molecular variance (AMOVA) revealed that most of the genetic variation was found between individuals within populations and about 7% of variation was located between populations. Moreover, populations differed genetically ($p<0.05$), and the average gene diversity (h) over loci was higher in clone-isolates derived from resistant clones ($h=0.17\pm0.08$) and lower in clone-isolates derived from susceptible clones ($h=0.07 \pm0.04$).

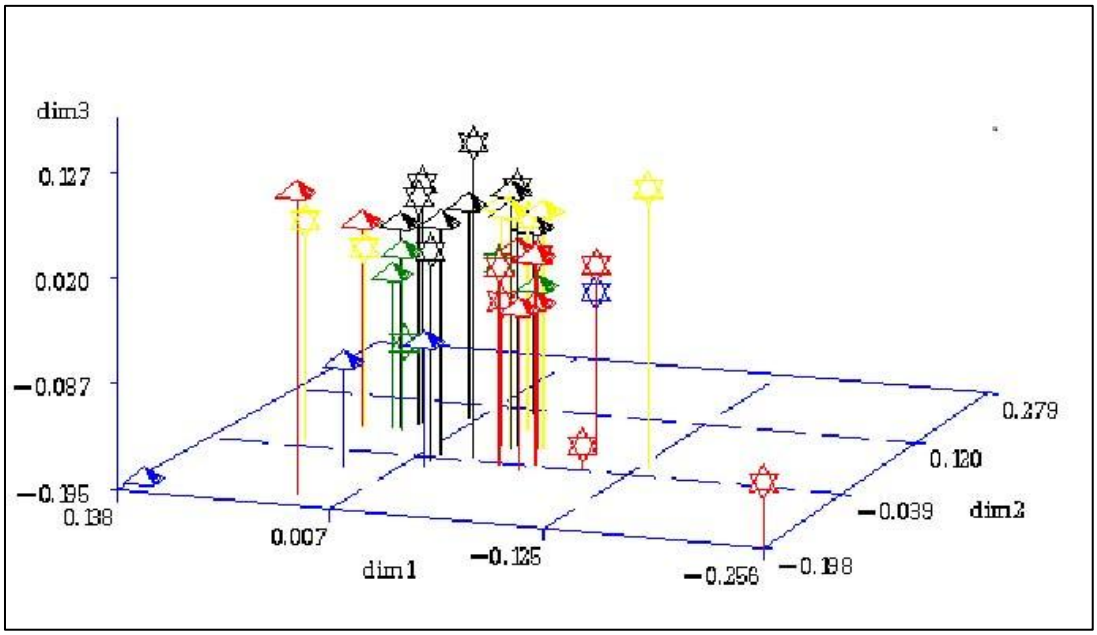

Figure 2. Graphical dispersion of the 40 clone-isolates obtained by MDS-SAS function along first three dimensions axes based on RAPD data using Jaccard's similarity coefficient (Jaccard, 1902). Black= clone-isolates derived from the susceptible genotype - SIC2; colors clone-isolates derived from the resistant genotypes CCN 10, MOQ 216, Playa Alta 4, TSH 565 e TSH1188.

Also temporal observations of natural infections between 2003 and 2010 in a F_2 Scavina 6 x ICS1 population showed that the major QTL identified in the LG9 had its effect decreased year by year; moving from a LOD of 9 in 2003 to a LOD of 3.9 in 2010 (Clement et al 2012). Pathogenic variability through cross inoculation experiments using isolates derived from resistant (Scavina and non-scavina descendants) and susceptible sources, showed that the Scavina-descendent isolates (346, 1734 and 487) were the most pathogenic on SIC2 ($P<0,05$), whereas the clone-isolate derived from the susceptible source (343) was less pathogenic (Figure 3).

These studies, besides proving the shift in *M. perniciosa* population in Bahia, they are also a warning call that the efficiency of resistant cultivars in WBD management is limited by *M. perniciosa* variability. Under this scenario WB resistance genes from different sources needs to be associated to increase the durability of field resistance.

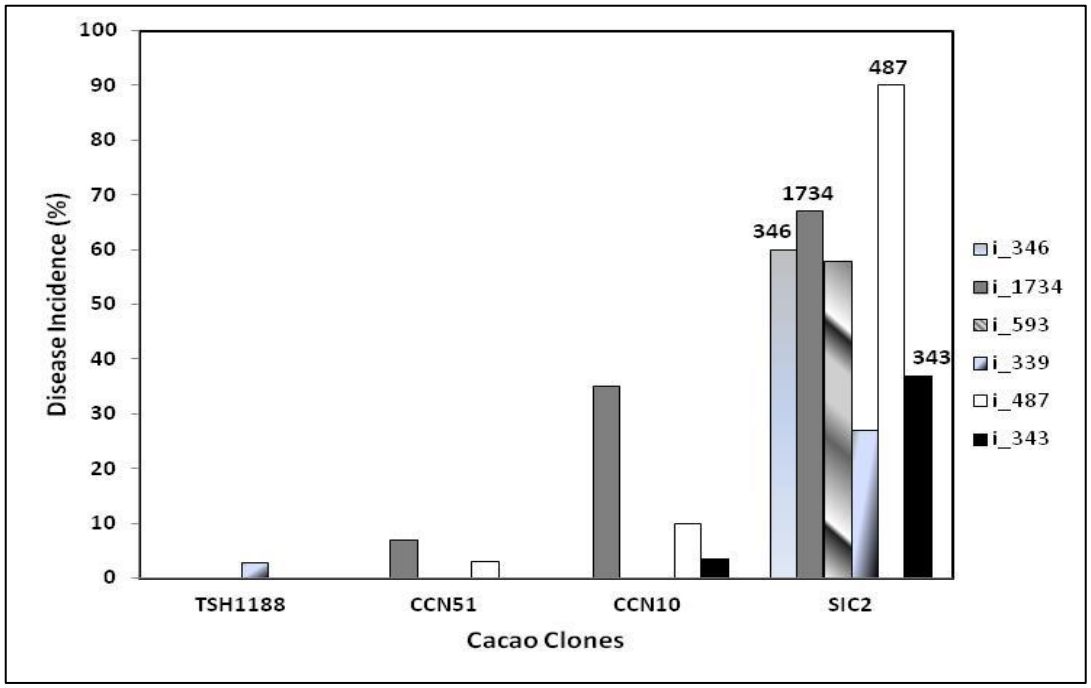

Figure 3. Pathogenicity of *Moniliophthora perniciosa* isolates on cacao clones; i= derived isolates from suceptible genotypes (343) and from resistant genotypes scavina descendent (346,1734 and 487) and non-scavina escendent (593, and 339).

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