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QTLs DETECTION UNDER NATURAL INFECTION OF *Moniliophtora perniciosa* IN A CACAO F2 PROGENY WITH SCAVINA-6 DESCENDANTS

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Studies to determine the genetic bases to witches' broom disease (WBD) resistance in *Theobroma cacao* L. were carried out in order to identify different genetic sources of resistance and to improve the efficiency of selection using molecular markers. A major QTL for WBD resistance and linked to Scavina 6 - the mains source of resistance, was detected in chromosome 9 from a F_2 Scavina 6 x ICS 1 progeny. In this paper, phonotypical quantification of vegetative and flower cushions brooms was done in the same progeny growing in two fields: F_2-1M and F_2-1C; respectively with 62 individuals and 142 individuals, observed over a period from 2003 to 2008. QTLs analyses were carried out with MapQTL 5.0 software to confirm the presence in chromosome 9 of the previously detected QTL. A significant instability of this QTL was observed with a LOD and the percentage of variation explained decreasing and moving in the confidence interval of mTcCIR157. Others QTLs of resistance were also detected such as one in Ch2, which was found in an opposite direction. These new QTLs analyzes, under natural infections of *M. perniciosa*, suggest a change in the host and pathogen relationships.

Key words: QTL mapping, cacao witches' broom disease*, Theobroma cacao* L.

Detecção de QTLs sob infecção natural de *Moniliophtora perniciosa* na progênie F2 com descendencia Sca 6. Estudos para determinar as bases genéticas da resistência à vassoura-de-bruxa do cacaueiro (VBC) a fim de identificar diferentes fontes genéticas de resistência e melhorar a eficiência da seleção por meio de marcadores moleculares. Um QTL de efeito maior para resistência a VBC foi detectado no cromossomo 9 em uma descendência F_2 do cruzamento Scavina 6 x ICS 1. Neste trabalho foram realizadas quantificações fenotipicas do número de vassouras vegetativas e almofadas florais, nesta mesma progênie, em dois campos experimentais F_2-1M e F_2-1C, com 62 e 142 indivíduos, respectivamente, no período de 2003 e 2008. As análises de QTLs foram realizadas com o software MapQTL 5.0 para confirmar a presença de um QTL localizado no Ch9, entretanto os resultados indicam uma instabilidade significativa deste QTL com o valor do LOD e a porcentagem de variação explicada reduzida, movendo-se no intervalo de confiança do marcador mTcCIR157. Outros QTLs de resistência também foram detectados, destacando-se um QTL no Ch2, que foi encontrado em sentido oposto. A detecção de novos QTLs sob condições de infecção natural de *M. perniciosa*, sugere mudança nas relações hospedeiro e patógeno.

Palavras-chave: mapeamento de QTLs, Vassoura-de-bruxa do cacaueiro, *Theobroma cacao* L.

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Introduction

Witches' broom disease (WBD) of cacao, caused by the basidiomycota *Moniliophthora pernicosa* (Stahel) Aime-Mora (Aime and Phillips-Mora, 2005) is the main phytopathological problem for cacao production in Brazil (Figure 1). This disease was detected in 1989 in the state of Bahia, the main Brazilian cacao-growing region (Pereira et al., 1989). Different sources of resistance to *M. perniciosa* have been identified (Pires et al., 1999), among them the Scavina (Sca 6) selections which are one of the most important sources of resistance of several cacao producing countries including Brazil, Bahia. Among Sca 6 descendants, the TSH´s genotypes such as TSH516, have shown a significant increase in the number of flower cushions broms (FCB) and vegetative brooms (TB) (Gramacho et al., 2003; Pires, 2003). Whereas, genotypes originated from other sources have showed stability over time. The effects of Sca 6 resistance genes are maintained when associated with resistance genes from other sources (Pires et al., 2012).

The aid of classical and DNA technology using co-dominant markers such as single sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs), using genetic mapping and phenotypic data of a progeny, have allowed the identification of genomic regions linked to the expression of various traits, named quantitative trait loci (QTLs). The main interest of QTL detection in perennial crops is due to the potential of marker-assisted selection (MAS). However, in most cases, a good deal more information on QTL will be required before they can be successfully utilized for MAS, for example QTL stability over time.

In the cacao breeding programs, QTLs analyses have been carried out for various traits (disease resistance, agronomics and quality traits) from various progenies. The main results of these QTL studies were synthesized in a meta-analysis made by Lanaud et al. (2009). In Bahia, Brazil, at the Cacao Research Center (CEPEC/CEPLAC, Comissão Executiva do Plano da Lavoura Cacauiera), QTL studies were carried out to detect resistance genes for Witches' broom disease of cacao (WBD) using an F_2 progeny from Sca 6 x ICS 1. A main QTL, located on chromosome 9, has been identified with based on field observation on the average number of vegetative brooms per tree between 1996 and 2002 (Brown et al., 2005; Faleiro et al., 2006). LOD score and the percentage of variation explained by the QTL were respectively: 10.5 and 51%.

Figure 1. Symptoms of witches' broom disease of cacao. (A) Terminal broom and (B) Flower cushion broom.

We report herein new results obtained from QTL analysis on this same progeny in two different fields at CEPEC/CEPLAC. An investigation of temporal QTL stability (from 2003 to 2008) has also been carried out considering the number of vegetative and flower cushion brooms under natural infection.

Materials and Methods

Plant Material

One hundred and forty-two F2 Sca 6 x ICS 1 individuals (8 years old), randomly distributed in two experimental fields, were used in this study. This segregating F_2 population was produced at the Cacao Research Center (CEPEC) of CEPLAC in Bahia, Brazil through self-pollinations of the clone TSH516, a selection from the cross between Sca 6 (resistant to WBD) and ICS 1 (susceptible to WBD). The first F_2 field, herein named F_2-1M, included the 82 individuals in which the first QTL analysis were carried out by Brown et al. (2005) and Faleiro et al. (2006). The second experimental field (8 years old), named F_2-1C, included the 82 individuals of the first population (F_2-1M) plus 60 new individuals (not observed in the first QTL study) to finally constitute a population of 142 individuals. In the F_2-1C, each genotype was replicated three times by grafting on adult cacao tree, randomly distributed in three blocks.

Phenotypic measurements under natural infection

The individuals of the two field populations F_2-1M and F_2-1C, were observed during 6 years from 2003 to 2008 (two times a year) for the number of terminal vegetative (TB) and flower cushion brooms (FCB) aiming to compare the performance of the populations growing in different conditions, i.e., trees age and inoculum pressure, during the period.

Besides the number of brooms, the trunk diameter at 50 cm height was also evaluated aiming to correct TB by the number of potential infection points, which is associated with the vigor of the tree. The tree vigor was assessed in 2008, from the measurement of the cross-section of the trunk in F_2-1M field and the grafted part of the tree in F_2-1C field. This trait was noted as C-Sec-Troncor/Yr = 2008. The phenotypic data was adjusted by regression considering the number of brooms proportional to the trunk diameter of each individual.

Genetic map

The genetic matrix used in this study was made considering the 142 individuals and 188 co-dominants markers mainly represented by SSRs and some homologous genes of resistance used in the map by Brown et al. (2005). Markers as SSR-ESTs described in two previous studies obtained from the interactions between Cacao-*Moniliophthora perniciosa* (Lima et al., 2010) and Cacao-*Ceratocystis cacaofunesta* (Santos et al., 2013) were also mapped.

Statistical analyses and detection QTL

Descriptive statistical parameters, variances, normality of phenotypic traits and others specific programs as the regression taking into account the number of brooms proportional to the difference of the cross-section of each individual, were obtained using SAS (Statistical Analysis System, version 9.1.3, SAS Institute Inc., Cary, NorthCarolina, USA).

QTL analyses were carried out with MapQTL software, version 5.0 (Van Ooijen, 2004) using the three procedures proposed by this software: (i) "Kruskal-Wallis (KW)" for a nonparametric mapping analyses comparable to an analysis of variance, (ii) "Interval mapping" method developed by Lander and Bostein (1989) and (iii) "MQM or ResMQM mapping" a method based on multiple-QTL models (Jansen and Stam, 1994) using maker cofactors determined by the procedure "Automatic Cofactor Selection" of MapQTL 5.0. For the analysis procedure in simple and composite interval mapping, the significance threshold of the LOD score was determined from the permutation test procedure proposed and using 10.000 interactions. Using a nonparametric procedure (KW), we considered a significant QTL from the K* value for a threshold corresponding to a significance P-value of 0.005, also represented by four stars in Tables and Figures.

Results

The first objective of this study was to evaluate, under natural conditions, the evolution of WBD for a

period of six years, from 2003 to 2008. In both fields, the average number of TB and FCB per tree was relatively low from 2003 to 2005 and markedly increased from 2006 to 2008, therefore, characterizing two periods (Figure 2 a, b). For the second period (2006-2008) the curves of the two types of broom were different between themselves. In F_2-1M field, where the disease was observed since 1996, the TB curve is below the FCB curve. In the F_2-1C field, where the observation of the disease initiated in 2003, an inverse situation occurred.

Analyses with cumulative data from 2003 to 2008, revealed variation in the average number of brooms per tree between fields, which were, respectively, for TB and FCB: 107.7 $\pm$ 78.3 and 165.9 $\pm$ 185.8 in F_2-1M (62 genotypes) and 60.7 $\pm$ 42.5 and 43.8 $\pm$ 54.7 in the F_2-1C (142 genotypes replicated three times). The areas of trunk section in these two fields, evaluated in 2008, were: 108.2 $\pm$ 61.97 in F_2-1M and 56.4 $\pm$ 28.6 cm^3 in F_2-1C.

QTL analyses were performed using both individual year data and cumulative data for the periods 2003 to 2005, 2006 to 2008 and 2003 to 2008. Table 1 shows the QTLs associated with WBD in both fields. In the F_2-1M fields, only one QTL was observed from 2003 to 2005 on chromosome 9. This QTL refer to the main QTL located on chromosome 9 and centered on mTcCIR35, mentioned by Brown et al. (2005) and Faleiro et al. (2006), which in this paper has been named *qWBD-9-1.* QTLs identified in the F_2-1C field were mainly located on chromosomes 2 and 9 for the different years and also for cumulative data (Table 1, Figure 3). In the F_2-1C, three QTLs, significant only with KW analyses, were observed; one on chromosome 2 and two in chromosome 9. They were located on the top of chromosome 9, with a peak near mTcCIR266 and LOD = 2.9 (Table 1).

Analyzing the average TB (mTB) data on the first period 2003-2005, the main *qWBD-9-1* was significantly detected. The LOD values and the

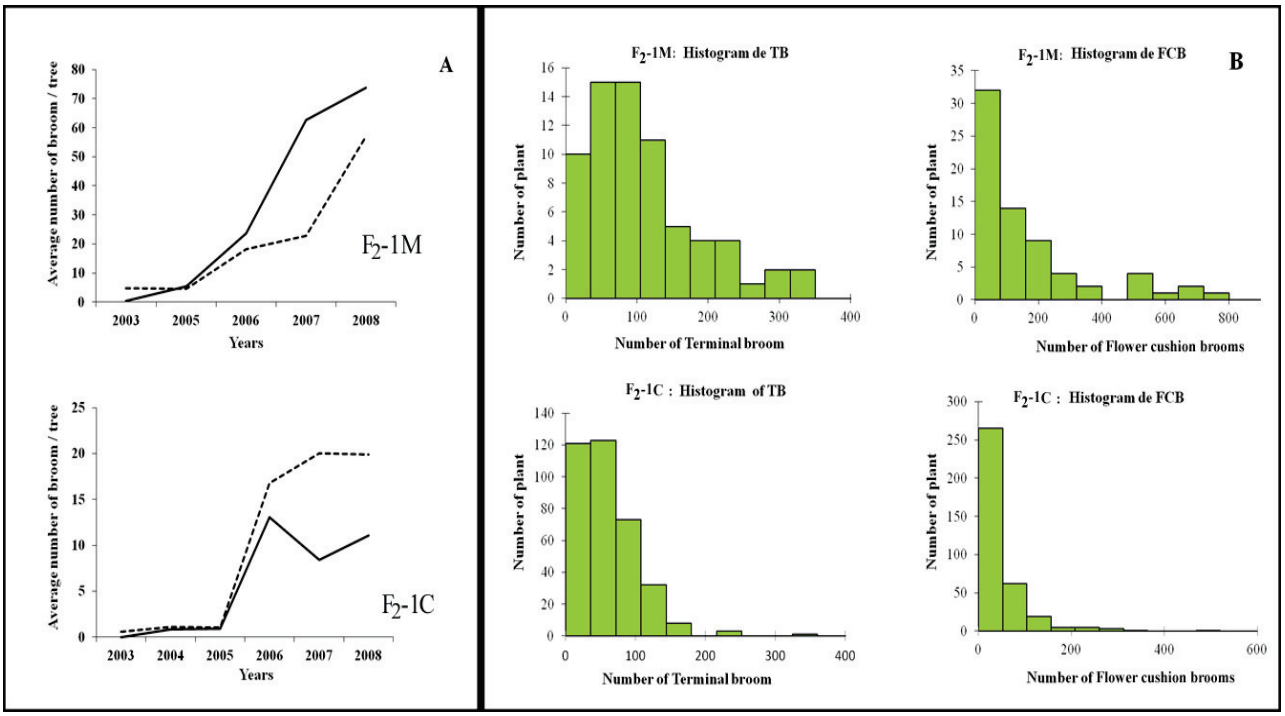

Figure 2. Evolution for vegetative and flower cushion brooms under natural infection in two fields (F_2-1M and F_2-1C) at the Cacao Research Center: A- Disease progress from 2003 to 2008 and B- Frequency distribution for total number of brooms over 6 years. F_2-1M = field population with 82 individuals in which the main QTL analysis were carried out by Brown et al. (2005) and Faleiro et al. (2006); F_2-1C = field population with 142 individuals, formed by the 82 individuals of the F_2-1M population plus 60 new individuals (not observed in the first QTL study).

percentage of variance explained by the QTL were respectively 5.52 and 16.9% (Table 1). QTL analyzes from mTBcorr/Yr = 2006-2008 data, showed that the QTL *qWBD-9-1* was detected in the same confidence interval, but the peak of the LOD moved to mTcCIR157, which is located at 4.8 cM of the mTcCIR35 marker. As shown in Figure 2, the numbers of both types of brooms (TB and FCB) have strongly increased during this period and the LOD value and the percentage explained by the QTL *qWBD-9-1* were lower: 3.06 and 8.7%, respectively (Table 1).

Otherwise, as shown in Figure 4, from 2003 to 2008, the evolution of the LOD values of each locus (SSR-markers) located in the region of the QTL *qWBD-9-1* fluctuated and declined. The average number of TB for individuals from the heterozygous class at the mTcCIR157 allele marker was 42.2 brooms (data not shown) and represented the most resistant plants while the average number TB of the individuals from homozygous Sca 6 class and homozygous ICS 1 class, were respectively, 55.1 and 66.2 brooms (data not shown).

Results obtained, from cumulative data mTBcorr/Yr = 2003-2005 with the QTL *qWBD-9-1* (centered on mTcCIR35) have shown that the averages number of TB of the individuals from the homozygous Sca 6, homozygous ICS 1 and heterozygous classes were respectively, 1.9, 5.3 and 2.4 (data not shown).

From cumulative data of average FCB between 2006 and 2008 (Table 1), a QTL was detected just under LOD = 3.28. This QTL was also detected on chromosome 9, centered on the RGH2 marker. The additive effect for this QTL was 8.92, with favorable alleles from Sca 6 (Table 1). QTL detected on chromosome 2 from cumulative data between 2006 and 2008 (Table 1) was near thems EST12. The LOD values for mTBcorr/Yr = 2006-2008 and mTBcorr/Yr = 2003-2008 were respectively 3.49 and 4.91 and the variance explained were 11.7 and 15.6 (Table 1). The additive effect of this QTL was negative (Table 1), therefore, in opposite direction to the others QTLs mainly detected on chromosome 9. The mean numbers of TB for the individuals from the homozygous classes Sca 6 and ICS

Table 1. Genomic locations of quantitative trait loci (QTL). Results obtained from natural infections from 2003 and 2008 from F_2-1 population. Two mapping methods were used; the method considered most appropriate for each QTL is given. The MQM method mapped it to the site identical to SIM, with effects and percent variance explained similar to Res. MQM

F2-1 Fields	Traits field observations	Ch*	QTL Position			Peak or Nearest Marker	LOD peak value	Mapping Method	% variance Explained	additive effect
			Marker-1	Peak	Marker-2					
F_2-1M	mTB-0305	9	0	7.73	13.52	mTcCIR266	2.89 (ns)	IM	19.30	4.854
F_2-1M	mTB-0305	9	30.44	38.64	46.22	mTcCIR35	2.54 (ns)	IM	17.10	5.951
F_2-1M	mTB-0305	9	0	7.73	13.52	mTcCIR266	2.97 (ns)	ResMQM	15.90	4.617
F_2-1M	Sec-2008	9	44.22	53.16	55.98	mTcCIR178	3.39 (ns)	MQM	16.30	3.603
F_2-1C	mTB-2004	2	29.99	38.66	45.32	msEST12	2.98 (ns)	MQM	11.50	-0.749
F_2-1C	mTB-2006	2	29.99	37.66	45.32	msEST12	3.11 (ns)	MQM	11.10	-6.593
F_2-1C	mTB-2008	2	29.99	34.66	45.32	msEst12	3.45	MQM	10.60	-7.361
F_2-1C	mTB-0608	2	29.99	38.66	45.32	msEST12	3.49	MQM	11.70	-16.489
F_2-1C	mTB-0308	2	29.99	38.66	45.32	msEST12	4.91	MQM	15.60	-18.431
F_2-1C	mTB-2003	9	30.44	35.44	41.44	mTcCIR35	9.03	MQM	32.60	0.765
F_2-1C	mTB-2004	9	30.44	38.64	41.44	mTcCIR35	4.64	MQM	14.40	0.750
F_2-1C	mTB-2007	9	35.44	41.44	44.22	mTcCIR157	3.70	MQM	9.70	1.405
F_2-1C	mTB-0305	9	29.99	38.64	41.47	mTcCIR35	5.52	MQM	16.90	1.631
F_2-1C	mTB-0608	9	38.64	41.47	44.22	mTcCIR157	3.06 (ns)	MQM	8.70	5.546
F_2-1C	mTB-0308	9	38.64	41.47	44.22	mTcCIR157	4.15	MQM	11.30	8.921
F_2-1C	mFCB-2005	9	55.98	68.542	80.19	RGH2	5.49	IM	17.20	10.209
F_2-1C	mFCB-0608	9	55.98	68.542	80.19	RGH2	3.28 (ns)	IM	10.60	19.619
F_2-1C	Sec-2008	9	53.16	55.166	67.54	mTcCIR8	5.44	MQM	17.40	4.725

*Ch=chromossome

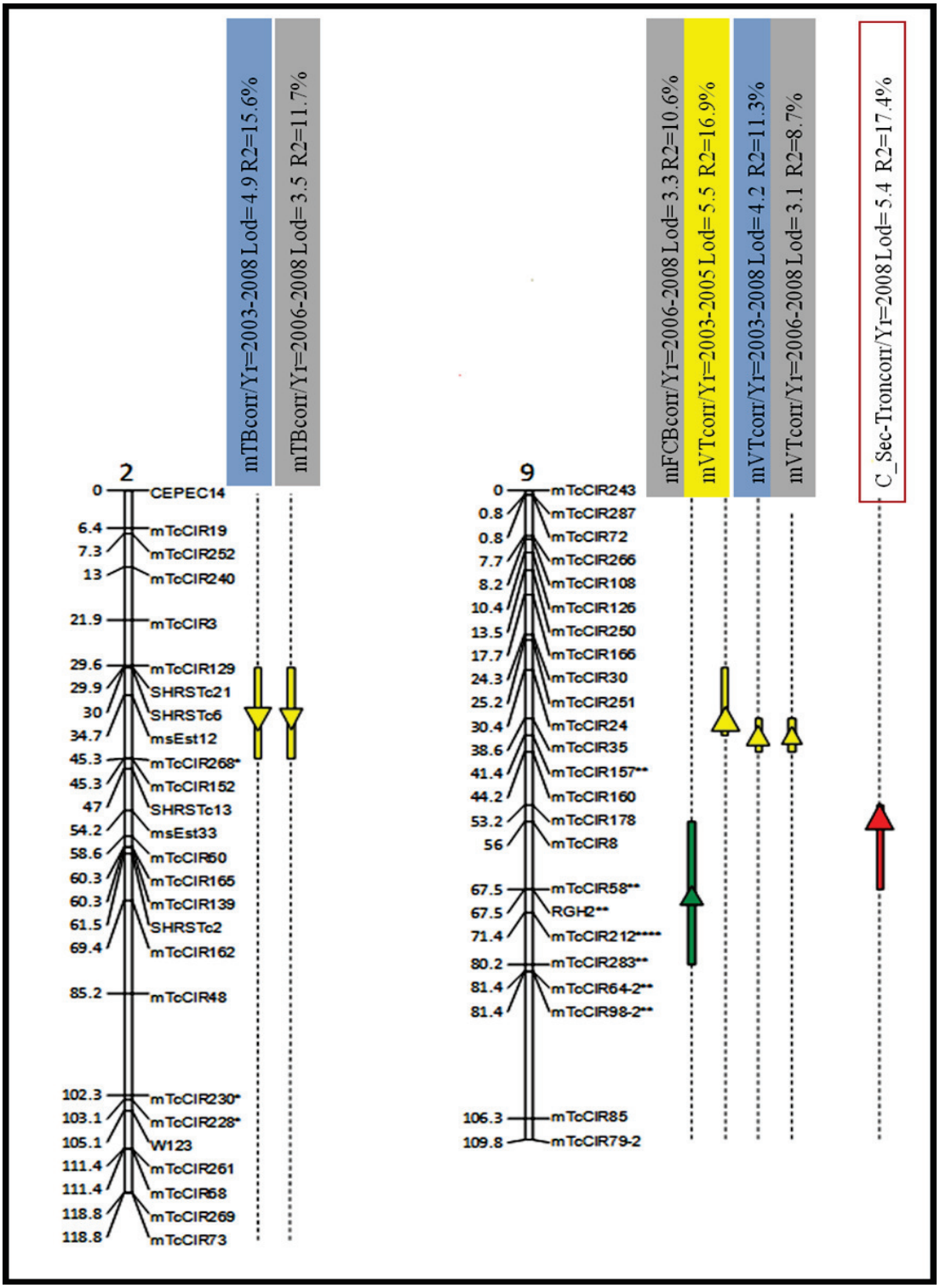

Figure 3. Quantitative trait loci (QTLs) map for chromosomes 2 and 9 based on natural infection data collected 2003 to 2008. mTBcorr and mFCBcorr = average number of vegetative brooms and flower cushion brooms corrected by the measurement of the trunk section. Yr = year. R2 = amount of phenotypic variation in resistance to Witches' broom disease explained by the QTL. C-SEC-Troncorr = section of the trunk corrected.

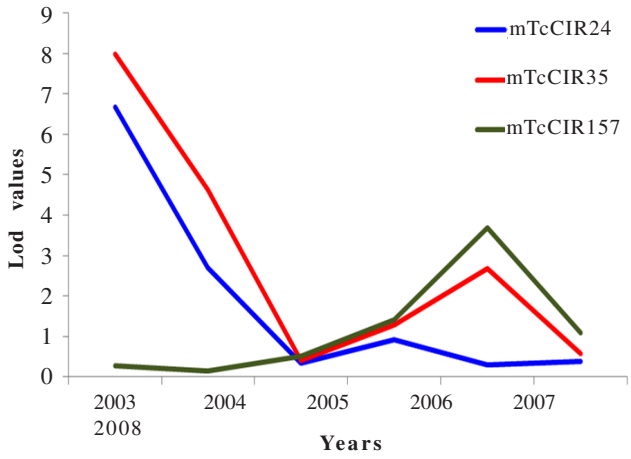

Figure 4. Evolution of the LOD values of each locus Single Sequence Repeats (SSR-markers) located in the main region of the main quantitative trait locus *qWBD-9-1,* throughout the years.

1 and heterozygous class TSH516, were respectively: 86.4, 49.6, and 64.0 (data not shown); therefore, the most resistant individuals came from the homozygous ICS 1 class.

Discussion

We have showed a decrease in the effect of the main QTL *qWBD-9-1* over time. In the F_2-1M context, the main QTL *qWBD-9-1*, which was detected by Brown et al. (2005) and Faleiro et al., (2006) with a LOD = 10.5 and 51.1% of variance explained using the cumulative data of TB between 1996 and 2002 showed a drastic fall of the significance level when the remaining 62 individuals of this field were evaluated for WBD during the period of 2003 and 2008. In the context of the F_2-1C field, on this same period, QTL results showed that at the beginning (2003 to 2005), *qWBD-9-1* was clearly identified with a good significance (LOD = 5.5 and 17% of variance explained) and decreased when WBD increased during the period of 2006 to 2008 (Table 1). During this second period (2006-2008) the evolution curves of both types of broom showed in F_2-1C that the TB curve was above the FCB curve. A reverse situation was found in the F_2-1M field. The situation found for the older F_2 tree (62 tree of F_2-1M) may be compared to the results obtained by Pires (2003) showing that the TSHs clones, such as TSH 516 or TSH 565, which have Sca 6 as an ancestor, showed an increase in the number of FCB compared to the number of TB. In F_2-1C, the trees were younger (installed in 2001 from grafting) and the curves of both kind of brooms showed an increased increment. Probably in the initial years the disease appears mainly on the canopy branches and later infects the flower cushions (FCB). Analyzing the period 2006-2008 in F_2-1C, we observed also a change in the host pathogen relationship. At first concerning a slight displacement of the LOD peak of the QTL *qWBD-9-1* from mTcCIR35 towards mTcCIR157 locus, where a major effect for resistance came respectively from the homozygous Sca 6 and the heterozygous class.

From clones involving Sca 6 and ICS 1, as THS 516 and TSH 565, Pires et al. (2012) analyzing total brooms collected showed differences of responses to resistance to WBD, *which* was probably due to the origin of both possible alleles of Sca 6 in association with the ICS 1 alleles. Otherwise, the QTL detected in chromosome 2 (near msEST12) showed in opposite direction of the QTL *qWBD-9-1.* This result is also interesting for the Trinitario origin of ICS 1 that combines alleles from Forastero and Criollo origin (Motamayor et al., 2003). Probably the resistance is provided by the Criollo allele of ICS 1. It is also interesting to mention that results from clone trials obtained by Pires et al., (2012) showed that the clone Chuao 120, from the Chuao region in Venezuela and probably resulting from a Criollo hybridization, like a Trinitario clone, imparted resistance to WBD. However these hypothesis require to be strengthened with a fine mapping of the region analyzed with genome data made from a pure Criollo clone B97/61/B2 from Belize (Argout et al., 2011).

The results presented in this study based on natural infection of WBD, showed an evolution of QTL *qWBD-9-1* and a changing in the relationship between the host and the pathogen, perhaps a response to selection pressure. It would be possible to verify if there is a good correlation between observations from natural infections and artificial inoculations. Also, using appropriate *M. perniciosa* strains carry out a fine mapping of the more interesting QTL regions detected.

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