Euphytica **114:** 25–36, 2000.

Quantitative trait loci analysis in *Theobroma cacao* using molecular markers. Inheritance of polygenic resistance to *Phytophthora palmivora* in two related cacao populations

Polygenic resistance to Phytophthora palmivora *in cacao*

Dominique Crouzillat[1], Wilbert Phillips[2], Paul J. Fritz[3] & Vincent Pétiard[1]
[1]*Centre de Recherche Nestlé Tours, 101 Avenue Gustave Eiffel, Notre Dame-d'Oé, B.P.9716, 37097 Tours Cedex 2, France;* [2]*Centro Agronomico Tropical de Investigacion y Enseñanza, CATIE, Unit of Biotechnology, P.O. Box 7170, Turrialba, Costa Rica;* [3]*Penn State University, Department of Food Science and the ACRI cocoa molecular biology laboratory. University Park, PA 16802, U.S.A.*

Received 8 April 1999; accepted 21 January 2000

Key words: Cacao, disease resistance, genetic mapping, *Phytophthora palmivora*, QTL

Summary

Two related segregating populations of *Theobroma cacao* L. were analysed for their resistance to *Phytophthora palmivora*. The first F_1 population was obtained by crossing two susceptible cacao clones of Catongo (a highly homozygous genotype) and Pound 12 (a highly heterozygous genotype) and the second population was obtained by backcrossing a single F_1 tree with Catongo. The genetic maps obtained for each population were compared. The F_1 map includes 162 loci and the backcross has 140 loci. The two maps, F_1 and BC_1, exhibit high co-linear loci organisation covering respectively, 772 and 944 cM. *Phytophthora* resistance was assessed by measuring the size increase of a lesion at five (DL5) and ten days (DL10) after pod inoculation. Six different QTL were detected in the F_1 and BC_1 populations. One QTL was found in both populations, and appeared to be a major component of disease resistance, and explaining nearly 48% of the phenotypic variance in the F_1 population. The absence of some QTL detection in the BC_1 in comparison with the F_1 population is due to the lack of transmission of the favouring alleles for these QTL from the single F_1 tree used for the backcross. The phenotypic variance explained by the action of the quantitative trait alleles indicated that genetic factors of both major and minor effects were involved in the control of the character studied. QTL conferring increased resistance to *Phytophthora* were identified in both susceptible parents, suggesting the presence of transgressive traits and the possibility of selection in cacao. Pleiotropic and epistatic effects for the QTL were also detected. Finally, the use of marker assisted selection (MAS) in cacao breeding programs is discussed.

Introduction

Cacao, *Theobroma cacao* L. (Sterculiaceae) originated in the tropical rainforest of equatorial America, probably at the foot of the Andes in the upper reaches of the Amazon river (Mossu, 1992), but is now cultivated in all tropical lowlands of the world. The world has some five million hectares of cacao and they are mainly managed by small holders. Cacao production is seriously affected by five major diseases (Van der Vossen, 1997 for review) that annually destroy up to 40% of the total crop. Witches'broom (*Crinipellis perniciosa*) and moniliasis fungus (*Moniliophthora roreri*) are present only in some countries of tropical America; cacao swollen shoot virus (CSSV) is a disease reported for the first time in 1936 in Africa; and vascular-streak dieback (*Oncobasidium theobromae*) is a cacao disease only found in Indonesia, Malaysia and Papua New Guinea. The most important disease is the black pod disease, or *Phytophthora* pod rot, which has been the primary fungal disease affecting cacao production since the 1920's. The most important pathogens are *Phytophthora palmivora*, which is present world-wide, and *Phytophthora megakarya*, re-

stricted to some African countries. Chemical control of such diseases is expensive and unattractive from commercial and environmental points of view. Chemical control can be used as a temporary measure, but resistance breeding is a better long-term solution. Progress has been made recently on the nature and variation of host resistance, based on artificial inoculation tests and field scores carried out on *P. palmivora* (Iwaro, 1997a; Phillips-Mora, 1996; Luz et al., 1996; N'Goran, 1996). Similar work has also been published on *P. megakarya* (Nyassé et al., 1995, 1996; Cilas et al., 1996).

Based on these different studies it appears that natural disease incidence records are strongly influenced by environmental effects. That made it necessary to develop new screening tests for reducing the environmental variance. Artificial inoculation of fully developed attached pods has become the most widely accepted test, with the rate of lesion development being the best indication of intrinsic resistance to black pod. The resistance appears to be of a quantitative nature and up to now no genotypes have been found with complete resistance to black pod disease.

There is evidence for various resistance genes acting against *Phytophthora* on cacao. Warren (1994) postulated the existence of a minimum of five unlinked loci involved in the inheritance of the resistance to *P. palmivora* in cacao. It seems that resistance to black pod is due to various genes more additive than dominant (Enriquez & Salazar, 1987; Tan & Tan, 1990).

In this report, QTL analysis of resistance to *Phytophthora* was investigated in two related populations. The objectives of the study were (1) to identify QTL affecting this trait and (2) to investigate the possibilities of marker assisted selection (MAS) on disease resistance in *Theobroma cacao*.

Materials and methods

Mapping populations

A genetic map was generated from each of two related cacao populations. The first map was originated from an intraspecific cross between two vegetatively propagated cacao clones: Catongo and Pound 12 (Alpizar et al., 1986; Morera et al., 1994). This mapping population consisted of 55 F_1 trees, planted in 1977. Catongo is a highly homozygous, self-compatible, white-seeded and white-flowered, lower Amazon Forastero. Pound 12 is a self-incompatible, purple-seeded and purple-flowered, highly heterozygous upper Amazon Forastero.

The second map was obtained from a single F_1 plant, produced from a cross of Catongo and Pound 12, used as the male parent in a backcross to Catongo (Morera et al., 1991; Fritz et al., 1995). This population of 131 trees was previously used for QTL analysis of agronomic characteristics (Crouzillat et al., 1996).

Both populations were established in the same area at CATIE (Centro Agronomico Tropical de Investigacion y Enseñanza) in Turrialba, Costa Rica.

DNA extraction and molecular markers

Total genomic DNA was extracted as previously described (Crouzillat et al., 1996). Three main types of molecular markers were used for the mapping of the two progenies: RFLP, RAPD and AFLP. RFLP and RAPD markers were used on both maps, whereas AFLP markers were only tested on the F_1 map. RFLP and RAPD protocols were the same as described previously (Crouzillat et al., 1996) except that only markers with less than 15% of missing data were retained. A RFLP probe for the cacao chitinase gene (CCCHIT) was obtained from cacao DNA amplification using a genomic clone sequence from accession code U30324 of the GCG database (Genetics Computer Group, USA). The two primer sequences used were 5′-GCTGAGCAGTGTGGACGGC-3′ and 5′-CCTCTGGTTGTAGCAGTCGA-3′. These primers allowed the amplification, via PCR, of a single DNA fragment of 1050 bp. Twenty one cacao RFLP probes (gTcCIR or cTcCIR) previously mapped on another progeny (Lanaud et al., 1995) were kindly provided by CIRAD, France.

AFLP assays were performed as described by Gibco BRL, using the AFLP small genome primer kit. The *Eco*RI primers were end labelled using [γ^{32}P] ATP and T4 polynucleotide kinase. AFLP reactions were performed for 36 cycles with the following cycle profile: a 30 s DNA denaturation step at 94 °C, a 30 s annealing step and a one minute extension step at 72 °C. The annealing temperature during the first cycle was 65 °C, and subsequently reduced each cycle by 0.7 °C for the next 12 cycles and was then kept at 56 °C for the remaining 23 cycles. DNA amplifications were performed in a Braun 60/2 thermocycler. An equal volume of formamide dye (20 μl) was added to the final reaction products. Aliquots (3 to 6 μl) of this mixture were loaded on a 6% acrylamide gel with

1 × TBE and 7.5 M urea. Gels were run at 270 volts for approximately 14 h, dried on filter paper under vacuum at 65 °C for 1 h and finally exposed to X-ray films using standard techniques.

Phenotypic markers

Two segregating phenotypic traits (anthocyanidin synthesis and self-compatibility) were recorded for the BC_1 population and were mapped. Only the self-compatibility trait segregates in the F_1 progeny (Crouzillat et al., 1996). It was determined by controlling self-pollination of at least ten-isolated cacao flowers per tree and recording the percentage of successful self-pollination after 10 days.

Fungus inoculum preparation and artificial inoculation

The highly virulent *Phytophthora palmivora* isolate named CATIE 194 in conjunction with an artificial inoculation method developed at CATIE (Phillips-Mora & Galindo, 1989; Phillips-Mora, 1996) were used for detecting the reaction of the trees against *P. palmivora*.

The inoculum was obtained using a 125 ml Erlenmeyer containing 25 ml Agar-V8 juice-$CaCO_3$ (31.8, 20 and 0.3%) culture medium. The inoculum suspension was prepared using 9 to 12 days old isolate to which 15 ml distilled water (10 °C) was added. The flask was then placed in a growth chamber for 30 min at 5 °C and then, transferred to another growth chamber at 25 °C. The suspension was calibrated using a hematocytometer for which a solution's aliquot was coloured with methylene blue to immobilize the zoospores (Phillips-Mora & Galindo, 1989).

The suspension should be used within 45 min after preparation as zoospores encyst easily, reducing the infection ability. The inoculation was performed on two opposite points located in the 'equator' of five month-old fruits. A Whatman paper disc of 1 cm diameter already submerged in the previously shaken suspension was placed on each point (0.05 ml/disc). Pods were then covered with a polyethylene transparent bag containing a paper towel and 50 ml distilled water, to act as a humid chamber. The water and the towel were removed after two days.

Different artificial inoculation events were carried out in the two cacao populations in order to get a confident data set for quantifying the reaction to *Phytophthora* for all the trees under study. There were twelve and fifteen events, respectively, in the F_1 and the BC_1 populations from October 1994 to November 1995. All the available pods were inoculated in each event. Fruits of the parents of the two crosses were also inoculated during the same periods.

Disease severity evaluation

The disease severity was obtained by measuring the diameter size (cm) of the larger lesion in each fruit at five (DL5) and ten days (DL10) after inoculation. A tree by tree average was obtained using all the data collected throughout the different inoculation events. The ten days (DL10) evaluation is used to qualify the reaction to *Phytophthora*; five days (DL5) evaluation allows the study of the lesion development. A resistant clone is defined as one with a DL10 from 0 to 3 cm, moderately resistant with DL10 from 3 to 6, moderately susceptible with DL10 from 6 to 9, and susceptible with DL10 greater than 9.

Mapping and quantitative trait analysis

Crouzillat et al. (1996) and Lanaud et al. (1995) had previously mapped some of the markers used in this study. The F_1 and BC_1 maps include 162 and 140 loci, respectively, mainly from RFLP markers. Due to the high level of homozygosity of Catongo, which was used as the female parent in the two related crosses, the two genetic maps of F_1 and BC_1 were obtained respectively from male parents: Pound 12 and the single F_1 tree selected for the backcross.

Each marker was tested against the expected segregation ratio using a χ^2 goodness of fit. Linkage analysis was performed using the program MAPMAKER 2.0 (Lander et al., 1987). Kosambi's mapping function (Kosambi, 1944) was used to convert the recombination frequency into map distance (cM). The pairwise analysis obtained from MAPMAKER was used to assign markers to linkage groups with a recombination frequency < 0.3 and a (LOD) > 4.

Individuals from the F_1 and BC_1 populations were monitored for DL5 and DL10 data. Each trait was assayed for normal distribution with d'Agostino Omnibus test that combines the tests for skewness and kurtosis (D'Agostino et al., 1990) using NCSS 97 statistical software.

Two methods of QTL detection were used: analysis of variance (ANOVA) and interval mapping. For one-way ANOVA, single markers were used as treatments to test for an association between mapped markers and quantitative traits in the two progenies using Q-gene software (Nelson, 1997). A $p < 0.005$ value was used as the threshold for considering the

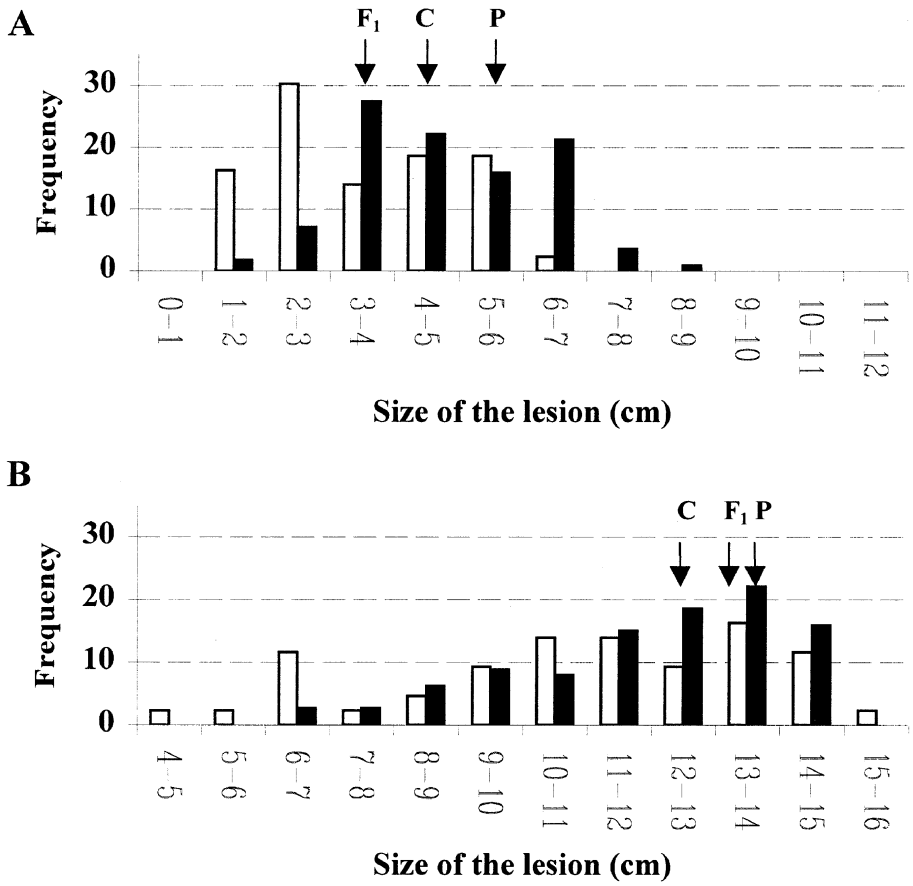

Figure 1A-B. Frequency distribution for *Phytophthora* resistance in F_1 and BC_1 progenies. **A**, Diameter size (cm) of the lesion at five days (DL5), **B**, Diameter size of the lesion at ten days (DL10). The data values for Catongo (C), Pound12 (P) and the tree (F_1) used for the backcross are indicated by arrows. White and black histograms represent, the F_1 and BC_1 distribution traits, respectively. The average DL5 and DL10 values are respectively: 3.6 and 11 for the F_1 progeny and 4.8 and 12 for the BC_1 individuals.

likely presence of a QTL near a marker. In addition, QTL were detected according to the method of interval mapping (Lander & Botstein, 1989) using the computer package MAPMAKER/QTL version 1.1 (Lincoln et al., 1992). A putative QTL map location was assigned to the linkage group using a LOD threshold of 2.

The proportion of the total phenotypic variance attributable to each QTL was estimated via linear regression. Total percentage of phenotypic variance explained for each *Phytophthora* component by all significant QTL was calculated with a multiple regression analysis, using the trait as dependent variable and the previously identified markers, linked to a QTL, as independent variables.

Epistasis analysis

Two-way interactions were evaluated for each significant QTL for the traits DL5 and DL10 and all segregating loci via two-way ANOVA using SAS statistical software (SAS institute, 1988) with a significance threshold of $p < 0.01$.

Results

Analysis of Phytophthora resistance in two related populations

The two parental clones Catongo and Pound 12 and the F_1 tree used as the male parent for the BC_1 cross, were classified as susceptible since their DL10 values were 12.8, 14 and 13.6 cm, respectively. The

frequency distributions for the lesion size for the F_1 and BC_1 populations are shown in Figure 1. Statistic tests based on skewness and kurtosis rejected normality distribution ($p<0.05$) for DL5 and DL10 data. A bimodal distribution tendency observed in the DL5 data suggested the effect of a major genetic factor. The DL5 and DL10 values on the two progenies show a continuous range from resistant to susceptible trees suggesting the action of transgressive QTL. Comparison of average DL5 and DL10 values for the F_1 and BC_1 crosses indicated that the F_1 individuals had a higher level of resistance than the BC_1 progeny (Figure 1).

The linkage maps and QTL analysis

Despite it higher number of loci, the F_1 map was characterised by 12 linkage groups instead of 10 compared to the BC_1 map. The Ic extra linkage group (Figure 2) was produced using only four RFLP markers, which were heterozygous for Catongo, and represented the small part (16.9 cM) of linkage group 1 from the female parent (Catongo). The two other extra F_1 linkage groups are due, at least partly, to the low number of F_1 trees (55 versus 131 for the BC_1 cross) which reduces the significance threshold for the linkage between markers. For example, a recombination frequency (Θ) of 0.2 gave the LOD score values of 4.4 and 11 for the F_1 and BC_1 population, respectively. Consequently, the linkage threshold of significance (LOD $>$ 4) gave a Θ value of 0.22 for F_1 and 0.33 for the BC_1 map, respectively.

Using the 59 shared loci between the two crosses it was possible to assign these two other extra F_1 linkage groups as part of linkage groups 3 and 8 of the cacao genetic map. The F_1 and BC_1 maps (Figure 2) covered 772 and 944 cM, respectively. This size difference can be explained by at least three factors. First, the markers used in this study do not cover exactly the same areas in the two maps. Secondly, the two linkage groups from the F_1 map each contained a gap of at least 35 cM. Finally, the overall comparison of the two point distance data of the 59 shared loci between the two populations indicate that the estimated distance ratio (F_1 / BC_1 = 0.87) could reflect some differences in the frequency of crossovers on the two progenies.

One and twelve markers on the F_1 and BC_1 maps respectively do not fit an expected 1: 1 segregation ratio ($p<0.05$). It appears that all the markers with a distortion of segregation from the BC_1 map show a preferential transmission of Pound 12 alleles. These markers are not randomly distributed, for example seven of these markers on the BC_1 map are located on chromosome 1 and three of them are on chromosome 8 (Figure 2).

Comparative analysis of the location of shared loci (Figure 2) also indicated the co-linearity of the two genetic maps studied. The exceptions were the loci CCG2222 and T20, respectively, (linkage groups 5 and 8). This observation demonstrates that the lower number of individuals in the F_1 population relative to the BC_1 population do not influence the order of the loci.

The F_1 and BC_1 genetic maps were obtained mainly by using RFLP (77.2 and 56.5% respectively) and RAPDs or AFLPs (22.2 and 42.1%, respectively). Only minor contribution was due to the phenotypic markers (0.6 and 1.4%). The non-random mapping location of PCR markers, as shown for example on the end of linkage group 1 or on the median part of linkage group 9, could underline the chromosomal DNA particularity near the telomeres or centromeres.

Due to the high correlation between the DL5 and DL10 traits for the F_1 and BC_1 populations (R^2 = 0.74 and 0.67, respectively) some of the same QTL could be involved in these two traits. Considering both populations, the study revealed six different QTL associated with the *P. palmivora* resistance (Table 1). They are numbered from 1 to 6 (Figure 2) and located on five different linkage groups. Only one is mapped in the same linkage group area for the two progenies. This QTL (②) was detected on linkage groups 2 and II near the shared loci CCG1135 for the DL5 and DL10 traits.

Individual QTL explained between 7.4 to 47.9% of the total variance with a significance level varying from 0.0035 to less than 0.0001. These results suggest the action of several QTL with both minor and major effects on *Phytophthora* resistance. For an individual ANOVA test, a genome-wide significance level of 5% respectively corresponds, for the F_1 and BC_1 populations, to the thresholds of p =0.00032 and p =0.00037. The p-value threshold (<0.005) chosen in this study minimises the risk of type I error (declaring a non significant marker significant) and most of the six QTL detected are significant with lower p-values.

Comparisons of the QTL of DL5 and DL10 indicated that the action of some QTL is not constant during time, for example, BC_1 QTL ① was only detected after ten days, while QTL ② exhibited a remarkable

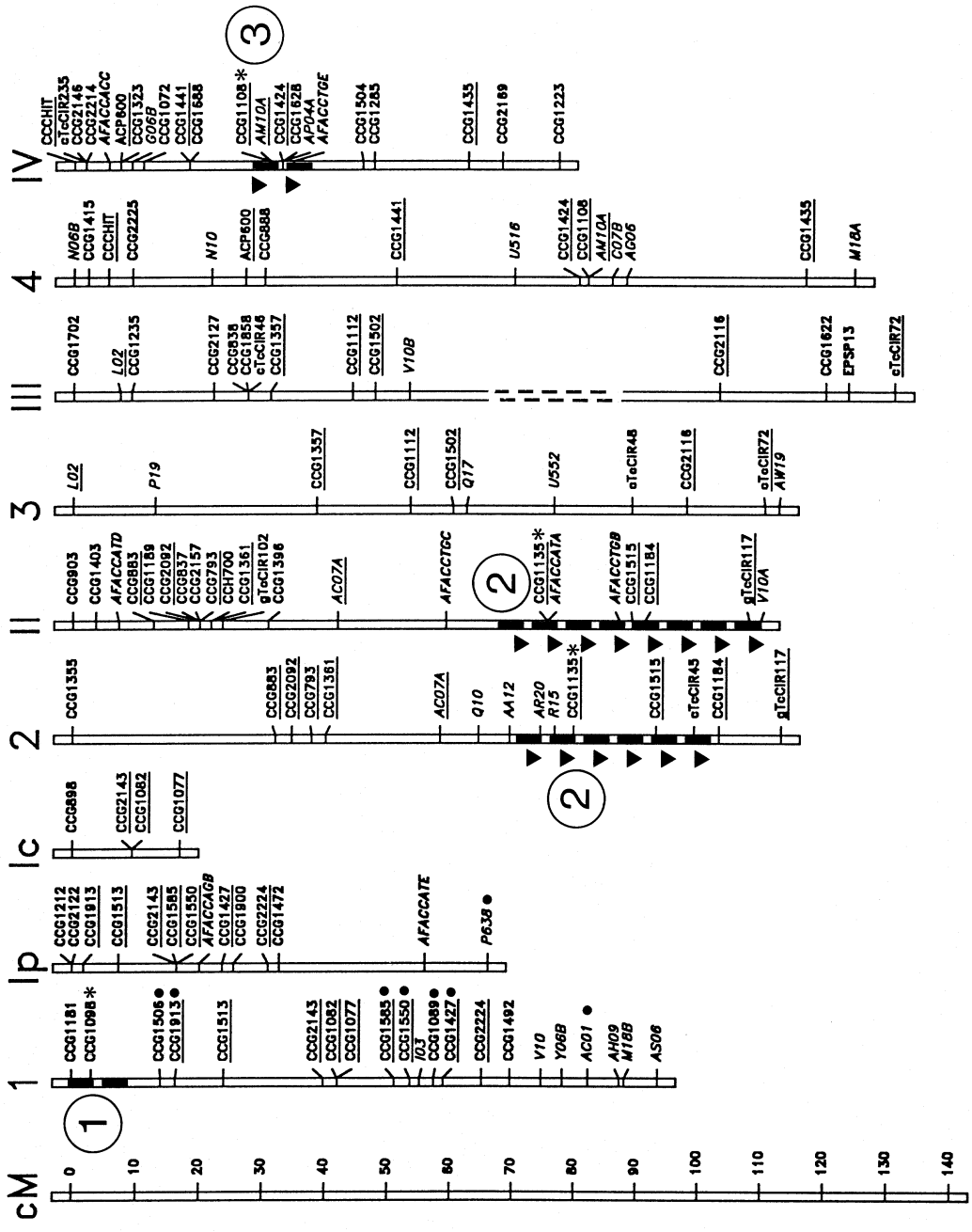

Figure 2. Comparative F_1 and BC_1 linkage maps and *Phytophthora* resistance putative QTL location. BC_1 and F_1 linkage groups are noted with Arabic (1 to 10) or roman (I to X) numbers, respectively. Ip and Ic are the linkage group I of the parents Pound 12 and Catongo respectively. BC_1 map included 140 loci (79 RFLP, 59 RAPD and 2 phenotypic markers) while the F_1 map displayed 162 loci (125 RFLP, 23 RAPD, 13 AFLP and 1 phenotypic marker). RFLP loci are indicated by ccg, ccc, gTc or cTc labels and with five specific codes as CCH700, EPSP13, ACP600, CHS600 and INV1000. AFLP loci on F_1 linkage groups are labelled as AFACC. The two phenotypic markers (*Anth* and *Autoc*) are mapped on linkage group 5, and the remaining loci come from RAPD markers. The 59 shared loci (51 RFLP, 7 RAPD and 1 phenotypic marker) are underlined,

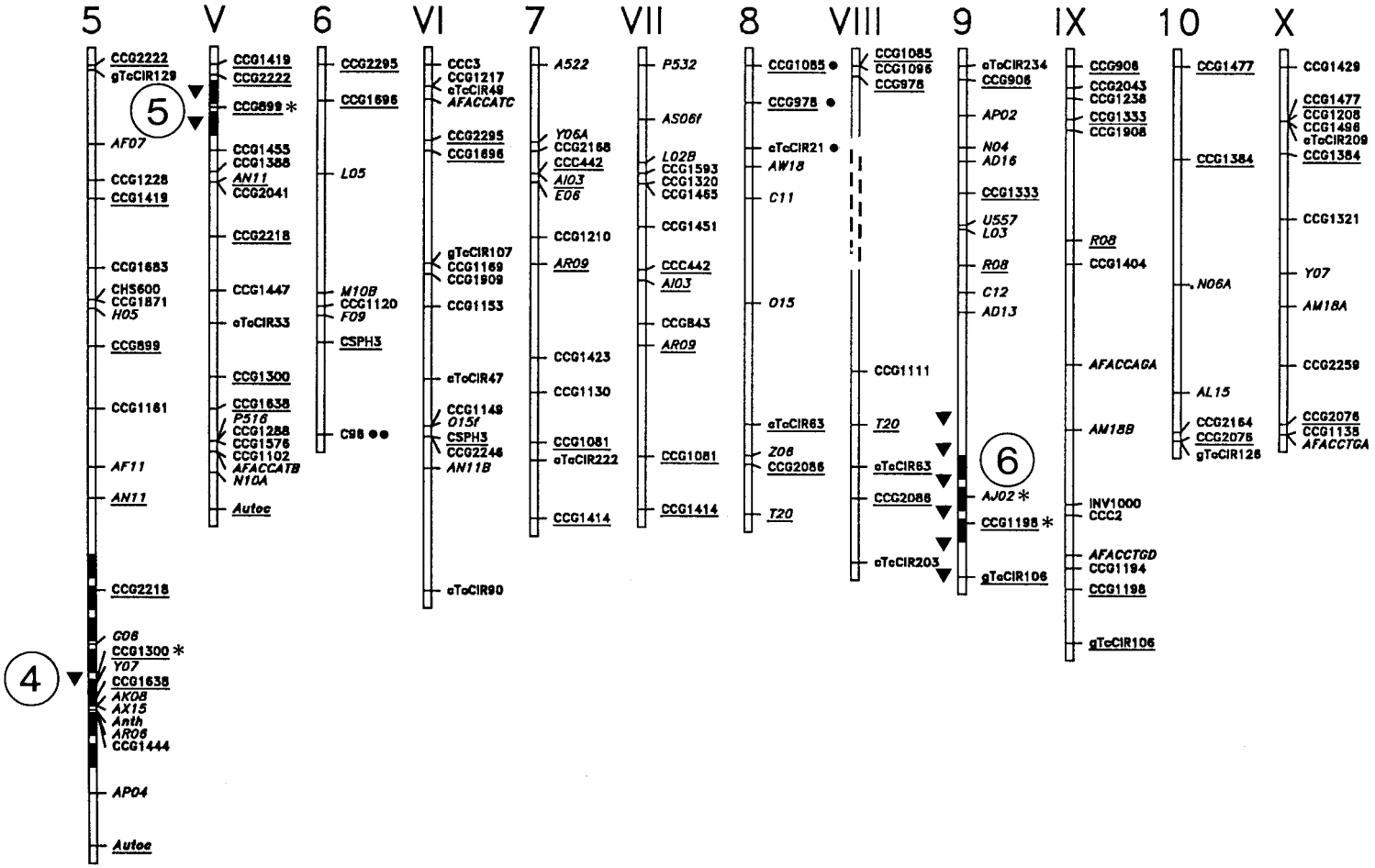

Figure 2. Continued. and were used to assign the linkage group of the two genetic maps. Dotted lines on F_1 linkage groups III and VIII indicated the presence of a gap on each of these two chromosomes but the presence of shared markers with BC_1 map allow the identification, the orientation and the relative position of the two parts of these chromosomes. Loci marked • and •• deviated significantly from a 1: 1 segregation ratio at $p < 0.05$ and $p < 0.01$ respectively. The numbers on the left of the figure represent the cumulative map distance in cM (Kosambi function). QTL map positions of DL5 (▶▶▶) and DL10 (■■) are indicated as previously described in materials and methods. QTL codes (① to ⑥) near the linkage groups are showed as referenced in Table 3. The closest marker linked to a QTL is shown (★).

Table 1. QTL data for *Phytophthora* resistance components from BC_1 and F_1 progeny

Progeny	Trait	QTL code	Locus	Chr.	R^2 (%)	p-value	LOD	A (cm)	N1	B (cm)	N2
BC_1	DL5	2	CCG1135	2	11.1	0.0003	3.2	5.3	59	4.3	54
		4	CCG1300	5	7.4	0.0035	1.9	4.5	58	5.2	55
		6	AJ02	9	11.4	0.0003	3.7	5.3	58	4.3	55
	DL10	1	CCG1098	1	10	0.0006	2.6	11.2	50	12.6	63
		2	CCG1135	2	12.2	0.0002	3.4	12.7	59	11.2	54
		4	CCG1300	5	13.2	0.0001	3.4	11.2	58	12.7	55
		6	CCG1198	9	8.8	0.0015	2.5	12.6	56	11.3	57
F_1	DL5	2	CCG1135	II	42.4	<0.0001	5.4	4.5	21	2.7	21
		3	CCG1108	IV	23.5	0.0010	2.6	2.9	19	4.2	24
		5	CCG899	V	22.2	0.0014	2.4	3	23	4.3	20
	DL10	2	CCG1135	II	47.9	<0.0001	5.3	12.9	21	9	21
		3	CCG1108	IV	19.8	0.0028	2.3	9.6	19	12.1	24
		5	CCG899	V	23.1	0.0011	2.5	9.7	23	12.5	20

A-Homozygote, B-Heterozygote, N1 and N2-Number of plants in each class, R^2-Percent phenotypic variance attributable to a marker. Usually only one marker for a chromosome, associated with the higher percentage of phenotypic variance explained, is shown. LOD value attributable to a QTL is also given.

Table 2. Summary of QTL data for *Phytophthora* resistance

Progeny/trait	QTL's number	Chromosome	Variance (%)	N	p value
BC_1/DL5	3	2, 5 & 9	27.5	113	<0.0001
F_1/DL5	3	II, IV & V	47.7	42	<0.0001
BC_1/DL10	4	1, 2, 5 & 9	39.8	113	<0.0001
F_1/DL10	3	II, IV & V	51.6	42	<0.0001

Percent phenotypic variance explained for each resistance trait component and each segregating population was performed with multiple regression analysis.

stability within the two progenies tested for DL5 and DL10 traits.

The percentage of total variance (Table 2) demonstrated for each trait is higher in the F_1 population compared to the BC_1 population, this difference may be explained, at least in part, by the low number of individuals in the F_1 cross.

Determination of the parental genotype for Phytophthora *QTL resistance*

In order to gain further information on the six different putative QTL detected in this study a detailed genetic analysis was done to characterise the QTL genotype of Catongo, Pound 12 and the F_1 single tree used for the backcross. The transmission of each QTL was followed using the flanking markers within the two populations and the genotype of the F_1 tree was established. This approach was facilitated by the fact that Catongo is highly homozygous, and that the small part of its genome detected as heterozygous was not close to a QTL. The results of this analysis (Table 3) indicate that alleles conferring resistance to *Phytophthora* from Catongo are detected for QTL ① and ④, and from Pound 12 for QTL ②, ③, ⑤, and ⑥. This indicates the mixed origin of the sources of resistance observed in these populations.

QTL ①, ④ and ⑥ have the same allelic forms for the three parents studied. The results suggested that Catongo and Pound12 are homozygous but with different alleles since there is no QTL segregation at the F_1 level. All the F_1 individuals are heterozygous (a/b) for these three QTL, explaining why they were only detected in the BC_1 segregation.

On the contrary, the two QTL ③ and ⑤, were detected as heterozygous (a/b) in Pound 12 as segregation was observed at the F_1 level. Molecular analysis indicated that the chromosome which carries the favouring allele was not transmitted by the F_1 tree used to produce the BC_1 population and explains the absence of this QTL detection in this population.

QTL ② is the most interesting QTL as it is detected as a major component of *Phytophthora* resistance in both crosses. Molecular studies show that the favourable resistance allele (b) found on the Pound 12 homologue chromosome II was transmitted to the F_1 individual which was used for the backcross, and so explains the presence of this QTL in both populations.

Table 3. Parental genotype QTL determination for *Phytophthora* resistance

QTL code	Chr.	Catongo	Pound 12	F_1 individual	QTL genotype favouring the trait	Comments
①	1	a/a	b/b	a/b	a/a	Only BC_1 QTL detection
②	2 & II	a/a	a/b	a/b	a/b	BC_1 & F_1 QTL detection
③	IV	a/a	a/b	a/a	a/b	Only F_1 QTL detection
④	5	a/a	b/b	a/b	a/a	Only BC_1 QTL detection
⑤	V	a/a	a/b	a/a	a/b	Only F_1 QTL detection
⑥	9	a/a	b/b	a/b	a/b	Only BC_1 QTL detection

a and b are the QTL alleles deduced from the genetic analysis of the two populations studied.

Table 4. Summary of two-way ANOVA to detect interactive effects between different loci in the BC_1 progeny

Trait	Markers in interaction	Linkage groups	P	R^2	HH[a]	N	AH[a]	N	HA[a]	N	AA[a]	N
DL5	CCG1135 (QTL ②)-ACP600[b]	2–4	0.0055	6.2%	*4.05*	28	5.79	25	4.63	23	4.93	34
	AJ02 (QTL ⑦)-cTcCIR72	9–3	0.0013	8%	*3.87*	30	5.51	34	5	24	5.02	24

[a] Combination of two loci with heterozygous status (HH), two loci with homozygous status (AA) or two loci with the first marker as homozygous and the second marker as heterozygous (AH) and conversely the heterozygous status for the first marker and the homozygous status for the second marker (HA). Data are expressed in centimetres. In many cases, other interactions of markers closely linked to the first one were significantly associated to the trait, probably due to the presence of only one QTL localised within the cluster. Accordingly, we considered that QTLs were identified by the marker loci that displayed the highest R^2 score. Underlined type indicates the more resistant classes. N is the number of plants for each class.
[b] The interaction CCG1135 and the marker cluster N10, CCG2225, CCCHIT.

Detection of interactions between QTL and markers

Two-way ANOVA detected two genomic regions with interactive effects within the BC_1 population (Table 4). These epistatic effects may explain up to 8% of the phenotypic variance. The interactions were characterised between one QTL, identified by the marker loci that displayed the highest R^2 value, and one locus on another linkage group. The more resistant trees always corresponded to symmetrical combinations of heterozygous loci status (HH) for DL5 trait (Table 4).

An interesting interaction effect was found for the trait DL5, between the marker CCG1135 linked to QTL ② and the cluster of markers represented by ACP600, because one of the mapped markers in this region of the linkage group 4 is a cacao chitinase gene (CCCHIT). Although this locus gene does not exhibit the highest interaction probability score ($p = 0.0076$) and location about 21.7 cm from the ACP600 locus, it could be considered as a candidate gene involved in a global plant resistance process despite its intermediate probability score.

No significant interaction between DL10 QTL and marker loci was detected, suggesting that epistatic effects occur mainly at earlier stages of infection.

Discussion

Phytophthora *resistance assessment*

Previous cacao evaluation using pod inoculation on 350 cultivars classified only 7% as resistant and one of the best genotypes (CATIE-1000) came from the cross between Pound 12 and Catongo (Phillips-Mora, 1996). These two parental genotypes, used for the creation of the cacao populations, were defined as susceptible. This suggests the presence of transgressive *Phytophthora* resistance genes in both parents. Since field tests use adult fruiting trees, the evaluation of *Phytophthora* resistance is time and space consuming some predictive assays were developed. Recently, comparative studies using cacao leaves and pods in order to define the relationship between *Phytophthora* resistance components were reported (Nyassé et al., 1995; Iwaro et al., 1997b). It was hypothesised that the poor relationship found between pod and leaf response at the penetration stage of infection (passive resistance) was due to the differential stomata density on these two organs (Iwaro, 1995). In contrast, a high positive correlation was obtained between pod and leaf resistance at the post-penetration stage of infection. This suggested that a systemic mechanism conferred

an intrinsic resistance to both leaves and pods of cacao. Early screening using leaf assays could be useful to screen large cacao populations and reduce the number of plants to be assessed at the pod level in a secondary selection stage.

Genetic mapping and QTL determination

Both genetic maps exhibited a high co-linear organisation of shared markers. A two-point comparison using these shared loci indicates a difference of 13% in the frequency distribution of crossovers in male meioses of Pound 12 for the F_1 progeny and the F_1 individual used for the BC_1 population. Such a significant higher frequency of recombination was also found in Brassica species (Kearsey et al., 1996) in female meiosis compared with male meiosis. The different frequency of recombination observed can not be due to sex differences because Catongo was the female parent for the two segregating populations.

Six different QTL were detected for the F_1 and BC_1 populations. These QTL are located on five linkage groups of the cacao genetic map. This result confirms the polygenic nature of the resistance as previously suggested (Spence & Bartley, 1966; Rocha, 1974; Partiot 1975; Enriquez & Salazar, 1987; Tan & Tan, 1990; Warren, 1994).

One QTL located on linkage group 2 was detected in both populations and had a major effect on resistance, especially for the F_1 progeny. It was probably responsible for the bimodal shape of the distributions for DL5 and DL10 traits studied.

The absence of transmission of QTL ③ and ⑤, from the F_1 to the BC_1 population is due to the lack of the favouring alleles in the F_1 individual used for the backcross. This situation occurs often in allogamous species such as cacao and restricts the application of QTL for breeding.

Differential effects of QTL

The QTL detected were different in their individual quantitative effects (R^2 values). QTL ② explained 42 to 48% of the phenotypic variance within the F_1 population, while intermediate QTL ranked between 10 and 24%. The minor QTL explained only 7 to 9% of the variance, and were probably only detected in the BC_1 population due to the larger size of the population studied. Involvement of a major QTL in quantitative disease resistance has been detected in pea (Dirlewanger et al., 1994) and rice (Wang et al., 1994) suggesting that complete resistance could be found in annual crops and that a resistance gene could be monogenic and race-specific. Most crops show intermediate or partial resistance against pathogens.

This study underlines the contribution of the two susceptible parents Catongo and Pound 12 for the transgressive expression of the resistance of cacao to *P. palmivora*. A similar situation was previously reported for pepper (Lefebvre & Palloix, 1996), maize (Bubeck et al., 1993; Freymark et al., 1994), mungbean (Young et al., 1993), and tomato (Danesh et al., 1994), indicating that susceptible plants can also be a genetic source of variability for disease resistance. Such genetic factors were not used in breeding because their phenotypic contributions were considered too weak. However, the use of molecular markers could facilitate an exhaustive detection and the management of such trait.

Epistasis controls a small but significant part ($p < 0.01$) of the resistance of cacao to *P. palmivora*. Rice (Wang et al., 1994) and pepper (Lefebvre & Palloix, 1996) are some of the few examples where epistasis was detected in plant resistance to pathogens. Epistasis in cacao was effective only at the first stage of the infection (DL5) and became non-significant after 10 days (DL10) suggesting that this type of interaction is limited in time. Epistasis requires looking for interaction effects between all genome loci and highlights the influence of a genetic background in the expression of a QTL. QTL ②, which is subject to some epistasic influence, showed a major effect in the F_1 population (42 to 47.9%) but only an intermediate effect (11 to 12%) in the BC_1 population. This data reflects the effect of the genetic background, which could regulate the expression of a given QTL according to gene combinations. Such genetic interaction is a good example of the difficulties of QTL management.

Another interesting characteristic of this study is that QTL ②, ③ and ⑤ located on linkage groups II, IV and V, respectively, are overlapping with three QTL detected for pod index in the F_1 population (Crouzillat et al., in press) which influences the size of the pod and the beans. This relationship was underlined by a highly significant correlation index between these two traits ($R = -0.63$, $p < 0.001$). At present, it is not possible to conclude whether these QTL correspond to pleiotropic genes or to clusters of tightly linked genes. Iwaro et al., (1997c) have studied the influence of pod morphology on *P. palmivora* resistance in cacao and have shown that some pod characteristics were highly correlated with the frequency, but not with the size, of the lesions on the pods.

Future use of molecular markers in cacao for disease resistance

Further studies are needed to determine the main genetic components for black pod resistance. The evidence produced so far is based on segregating families derived from medium resistant or susceptible parental genotypes. Future genetic analyses using F_2 or BC_1 populations from crosses between highly resistant and susceptible parents may detect fewer genes with larger effects (Van der Vossen, 1997). The establishment of related molecular studies would enhance the efficiency of marker assisted selection especially regarding the accumulation of resistance genes in one genotype in order to increase resistance level and the durability of such new cacao cultivars. Of course, such breeding schemes should be part of the current breeding programmes based on recurrent selection. The identification of resistance genes from different genetic populations by early screening in cacao germplasm collection using leaf-screening test would be of great value.

The identification of genes involved in black pod resistance can be used to understand the molecular and genetic bases for cacao disease resistance. Such an approach could be greatly facilitated since the cloning and the characterisation of several candidate genes was realised in tomato and flax (Ori et al., 1997; Anderson et al., 1997). The comparison of the sources of resistance between annual and perennial crops could accelerate the rate at which novel and durable resistance can be added to a crop species such as cacao.

Acknowledgements

The authors are grateful to Michel Rigoreau, Laurence Bellanger, Bertrand Ménard and Estelle Lerceteau for their helpful contribution with the molecular analyses. We thank Jim Mac Carthy, Peter Bucheli and Mary Evans for commenting on the manuscript. The field work of this research would not have been possible without the professional skills and dedication of José Castillo Fernàndez, Antonio Alfaro, Abel Chacón Garcia and Manrique Gonzàlez.

References

Alpizar, L., H.W. Fassbender, J. Heuveldop, H. Fölster & G.A. Enriquez, 1986. Modelling agroforestry systems of cacao (*Theobroma cacao*) with laurel (*Cordia alliodora*) or poró (*Erythrina poeppigiana*) in Costa Rica. Agroforestry systems 4: 175–189.

Anderson, P.A., G.J. Lawrence, B.C. Morrish, M.A. Ayliffe, E.J. Finnegan & J.G. Ellis, 1997. Inactivation of the flax rust resistance gene *M* associated with loss of a repeated unit within the leucine-rich repeat coding region. Plant Cell 9: 641–651.

Bubeck, D.M., M.M. Goodman, W.D. Beavis & D. Grant, 1993. Quantitative trait loci controlling resistance to gray leaf spot in maize. Crop Sci 33: 838–847.

Cilas C., D. Berry, D. Paulin, J. N'Goran & E. Djiekpor, 1996. La résistance à la pourriture brune des cabosses au Cameroun, en Côte d'Ivoire et au Togo. Bilan d'évaluation au champs. In: Proc. INGENIC workshop on the contribution of disease resistance to cocoa variety improvement, Salvador-Bahia, Brazil, 25–26/11/1996.

Crouzillat, D., E. Lerceteau, V. Pétiard, J. Morera, H. Rodriguez, D. Walker, W. Phillips, C. Ronning, R. Schnell, J. Osei & P. Fritz, 1996. *Theobroma cacao* L.: a genetic linkage map and quantitative trait loci analysis. Theor Appl Genet 93: 205–214.

Crouzillat, D., B. Ménard, A. Mora, W. Phillips, V. Pétiard, Quantitative trait analysis in *Theobroma cacao* using molecular markers. Yield QTL detection and stability over 15 years. Euphytica, in press.

D'Agostino, R.B., A Belanger, R.B. Jr. D'Agostino, 1990. A suggestion for using powerful and informative tests of normality. The American Statistician 44(4): 316–321.

Danesh, D., S. Aarons, G.E. McGill & N.D. Young, 1994. Genetic dissection of oligogenic resistance to bacterial wilt in tomato. Mol Plant-Microbe Interact 7: 464–471.

Dirlewanger, E., P.G. Isaac, S. Ranade, M. Belajouza, R. Cousin & D. de Vienne, 1994. Restriction fragment length polymorphism analysis of loci associated with disease resistance genes and developmental traits in *Pisum sativum* L. Theor Appl Genet 88: 17–27.

Enriquez, G.A. & L.G. Salazar, 1987. Cacao varietal resistance to *Phytophthora palmivora* and its inheritance at Turrialba, Costa Rica. In: Proceedings of the meetings of the American regional group on *Phytophthora palmivora* on cacao. Enriquez G.A. and Zentmyer G.A. eds, Technical series. Technical report CATIE 126.

Freymark, P.J., M. Lee, C.A. Martinson & W.L. Woodman, 1994. Molecular-marker-facilitated investigation of host-plant response to *Exserohilum turcicum* in maize (*Zea mays* L): components of resistance. Theor Appl Genet 88: 305–313.

Fritz, P.J., J. Osei, J. Lainez, H. Rodriguez, D.R. Walker, J. Morera & R. Lastra, 1995. A backcross population for genetic analysis of *Theobroma cacao*. Turrialba, Costa Rica 45(1–2): 49–56.

Iwaro, A., 1995. Mechanisms of resistance to *Phytophthora palmivora* (Butler) Butler in cacao (*Theobroma cacao* L.) and their genetic basis. Ph.D. thesis. The University of the West Indies, Trinidad and Tobago.

Iwaro, A., P. Umaharan & T. Sreenivasan, 1997a. Inheritance of foliar resistance to *Phytophthora palmivora* (Butler) Butler in cacao (*Theobroma cacao* L.). Euphytica 96: 377–383.

Iwaro, A., T. Sreenivasan & P. Umaharan, 1997b. Foliar resistance to *Phytophthora palmivora* as an indicator of pod resistance in *Theobroma cacao*. Plant disease 81(6): 619–624.

Iwaro, A., T. Sreenivasan & P. Umaharan, 1997c. *Phytophthora* resistance in cacao (*Theobroma cacao*): Influence of pod morphological characteristics. Plant Pathology 46: 557–565.

Kearsey, M.J., L.D. Ramsay, D. Jennings, D.J. Lydiate, E. Bohuon & D.F. Marshall, 1996. Higher recombination frequencies in female compared to male meioses in *B. oleracea*. Theor Appl Genet 92: 363–367.

Kosambi, D., 1944. The estimation of map distances from recombination values. Ann Eugen 12: 172–175.

Lanaud, C., A. Risterucci, J. N'Goran, D. Clement, M. Flament, V. Laurent & M. Falque, 1995. A genetic linkage map of *Theobroma cacao* L. Theor Appl Genet 91: 987–993.

Lander, E.S., P. Green, J. Abrahamson, A. Barlow, M. Daly, S. Lincoln & L. Newburg, 1987. MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1: 174–181.

Lander, E.S. & D. Botsein, 1989. Mapping mendelian factors underlying quantitative traits using RFLP linkage maps. Genetics 121: 185–199.

Lawrence, J., 1978. Evaluation of methods for assessing resistance of cacao (*Theobroma cacao* L.) cultivars and hybrids to *Phytophthora palmivora* (Butler) Butler. In: Boletin Tecnico 62. 46 p. Itabuna, Bahia, Brasil.

Lefebvre, V. & A. Palloix, 1996. Both epistatic and additive effects of QTLs are involved in polygenic induced resistance to disease: a case study, the interaction pepper- Phytophthora capsici leonian. Theor Appl Genet 93: 503–511.

Lincoln E., M.J. Daly, E.S. Lander, 1992. Mapping genes controlling quantitative traits using Mapmaker/QTL version 1.1: a tutorial and reference manual. Whitehead Institute Technical Report, Cambrige, 2nd edition.

Luz, E., M. Yamada, S. Silva, J. Pires, U. Lopes, L. Pinto, W. Monteiro, M. Braga, G. Melo & M. Brugnerotto, 1996 Research on cocoa resistance to black pod in Bahia, Brazil. In: Proc. INGENIC workshop on the contribution of disease resistance to cocoa variety improvement, Salvador-Bahia, Brazil, 25–26/11/1996.

Morera, J., A. Paredes & A. Mora, 1991. Germoplasma de cacao en el CATIE entre 1947 y 1991. In: CATIE/IICA. a II: generación y transferencia de tecnología. San Jose, Costa Rica. 49 pp.

Mossu, G., 1992. Cocoa. London. Macmillan Education. 103 pp.

N'Goran, J., I. Kebe, M. Tahi, D. Paulin, D. Clement & A. Eskes, 1996. Pathology and breeding research on resistance to black pod in Côte d'Ivoire. Proc. INGENIC workshop on the contribution of disease resistance to cocoa variety improvement, Salvador-Bahia, Brazil, 25–26/11/1996.

Nelson, J.C., 1997. QGENE: software for marker-based genomic analysis and breeding. Mol Breed 3: 239–245.

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

Nyassé, S., N. Bidzanga, G. Blaha, D. Berry, C. Cilas, D. Despréaux & A. Eskes, 1996. Update on the work on resistance of cocoa to *Phytophthora megakarya* in Cameroun. In: Proc. INGENIC workshop on the contribution of disease resistance to cocoa variety improvement, Salvador-Bahia, Brazil, 25–26/11/1996.

Ori, N., Y. Eshed, I. Paran, G. Presting, D. Aviv, S. Tanksley, D. Zamir & R. Fluhr, 1997. The *I2C* family from the wilt disease resistance locus *I2* belongs to the nucleotide binding, leucine-rich repeat superfamily of plant resistance genes. Plant Cell 9: 521–532.

Partiot, M., 1975. La résistance horizontale du cacaoyer au *Phytophthora* sp. Méthodes d'évaluation précoce. Café, Cacao, Thé 19(2): 123–136.

Phillips-Mora, W. & J. Galindo, 1989. Método de inoculación y evaluación de la resistencia a *Phytophthora palmivora* en frutos de cacao (*Theobroma cacao*). Turrialba (Costa Rica) 39(4): 488–496.

Phillips-Mora, W., 1996. Studies on resistance to black pod disease (*Phytophthora palmivora*) at CATIE. In: Proc. INGENIC workshop on the contribution of disease resistance to cocoa variety improvement, Salvador-Bahia, Brazil, 25–26/11/1996.

Rocha, H.M., 1974. Breeding cacao resistance to *Phytophthora palmivora*. In: P.H. Gregory (Ed.), *Phytophthora* Disease of Cocoa, pp. 211–218, Longman, London.

SAS Institute, Inc., 1988. SAS Users guide: Statistics. SAS Institute, Cary, N.C.

Spence, J.A. & B.G. Bartley, 1966. Testing of breeding material of *Theobroma cacao* for resistance to black pod disease (*Phytophthora palmivora*). Second session of FAO Technical working party on cocoa production and protection. FAO.

Tan, G.Y. & W.K. Tan, 1990. Additive inheritance of resistance to pod rot caused by *Phytophthora palmivora* in cocoa. Theor Appl Genet 80: 258–264.

Van der Vossen, H., 1997. Strategies of variety improvement in cocoa with emphasis on durable disease resistance. International group for genetic improvement of cocoa. University of Reading, UK. 32 p.

Wang, G.L., D.J. Mackill, J.M. Bonman, S.R. McCouch, M.C. Champoux & R.J. Nelson, 1994. RFLP mapping of genes conferring complete and partial resistance to blast in a durably resistant rice cultivar. Genetics 136: 1421–1434.

Warren, J.M., 1994. Estimation of the number of loci involved in the inheritance of resistance to *Phytophthora palmivora* (Butl.) Butl. In the leaves of *Theobroma cacao*. Plant Pathology 43: 73–79.

Young, N.D., D. Danesh, D. Menancio-Hautea & L. Kumar 1993. Mapping oligogenic resistance to powery mildew in mungbean with RFLPs. Theor Appl Genet 87: 243–249.