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Breeding for resistance to *Meloidogyne exigua* in *Coffea arabica* by introgression of resistance genes of *Coffea canephora*

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Breeding for resistance to root-knot nematode *Meloidogyne exigua* in coffee may help in controlling this nematode, which causes substantial harvest losses throughout Latin America. Accessions of *Coffea arabica*, *C. canephora* and lines derived from the Timor Hybrid (wild *C. arabica* × *C. canephora* interspecific hybrid) were tested in a greenhouse for resistance to gall formation by counting individual galls on the roots of plants inoculated with second-stage juveniles of a *M. exigua* population from Costa Rica. The level of introgression from *C. canephora* was also assessed on 28 resistant and susceptible genotypes in a molecular study with amplified fragment length polymorphism (AFLP) markers. The frequency of resistant plants was very low or even nonexistent in *C. arabica* and very high in *C. canephora* with $> 78\%$ immune plants and 100% resistant plants. Several lines derived from the interspecific hybrid revealed a high level of resistance similar to that observed in the parent *C. canephora* species. A molecular study of Timor Hybrid-derived lines revealed high variability for the number of markers present in the study genotypes. A larger number of introgression markers was found in the group of resistant genotypes than in the susceptible genotypes. However, there were also lines with little introgression that had retained resistance. Based on genetic distances calculated from these markers, two groups of lines were revealed: those derived from Timor Hybrid CIFC1343, and those derived from Timor Hybrids CIFC832/1 and CIFC832/2, which may indicate the existence of different resistance genes in the two groups. The segregations observed in F_1 and F_2 progeny may be explained by at least a dominant gene. The lines derived from the Timor Hybrid are a worthwhile source of resistance to *M. exigua* that can be exploited to improve *C. arabica* with the help of molecular-assisted selection.

Keywords: AFLPs, breeding, *Coffea*, marker-assisted selection, resistance, root-knot nematode

Introduction

Root-knot nematodes of the genus *Meloidogyne* are frequently and abundantly found on Arabica coffee trees (*Coffea arabica*) in Central America (Campos *et al*., 1990; Anzueto *et al*., 1993). Recent studies confirmed the great diversity of potentially pathogenic species in the region (Hernández *et al*., 1996), of which *M. exigua* is the most common. This species is widespread in South America (Sosa-Moss, 1985; Campos *et al*., 1990; Leguizamon, 1990) and is dominant in Costa Rica (Flores & López, 1989). In Costa Rica, the attacks cause general weakening of the tree with an estimated drop in yields ranging from 10 to 20% (Bertrand *et al*. 1997). In Central America, all the cultivated varieties (namely, Typica, Bourbon, Caturra, Catuai, Costa Rica 95 and IHCAFE90) are susceptible.

C. arabica is characterized by a relatively low genetic diversity that is attributable to its allotetraploid origin, reproductive biology and evolution (Lashermes *et al*., 1999). In relation to resistance to *M. exigua*, Curi *et al*. (1970) has shown, with a Brazilian population, that most genotypes are susceptible. In contrast, resistance was discovered in the *C. canephora* species (Curi *et al*., 1970; Morera & López, 1987; Chaverri, 1987). Since the second half of the 20th century, most breeding programmes implemented worldwide (Brazil, Colombia, Kenya, Costa Rica, Honduras) have attempted to transfer characters of resistance to rust (*Hemileia vastatrix*), root-knot nematodes (*Meloidogyne* spp.), and coffee berry disease (*Colletotrichum kahawae*) into

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cultivars of *C. arabica* from the Timor Hybrid. This tree, which originated from the island of Timor (Bettencourt, 1973), is derived from a wild interspecific cross between *C. arabica* ($2n = 2x = 44$) and *C. canephora* ($2n = 2x = 22$). Several progeny have been used in breeding programmes: origins CIFC832/1, CIFC832/2 and CIFC1343. Those origins have been crossed with commercial varieties such as Caturra or Villa-Sarchi to produce Catimors or Sarchimors. The F_1 hybrid is selfed and a pedigree programme is implemented over more than five generations. Based on this strategy, several cultivars have been released in Brazil (IAPAR 59), Colombia (Colombia cultivar) and Central America (IHCAFE 90 and Costa Rica 95) (Bertrand *et al.*, 1999). Certain lines selected for their resistance to leaf rust are also resistant to *M. exigua (*Fazuoli *et al.*, 1977; Fazuoli & Lordello, 1978; Bertrand *et al.* (1997); Gonçalvez & Pereira, 1998; Silvarolla *et al.*, 1998). With the recent development of molecular markers of gene introgression from *C. canephora* (Lashermes *et al.* 2000), it is now possible to envisage genetic diversity studies, and the marking of resistance genes. The benefits obtained from marker-selection depend on several factors, such as the presence of independent single resistance genes and the degree of linkage between the markers and the target gene.

The aims of the present study were: (i) to verify that resistance sources to *M. exigua* are very rare or nonexistent in *C. arabica* and that *C. canephora* is resistant; (ii) to confirm that the resistance genes present in the lines derived from the interspecific Timor Hybrid are widespread and came from *C. canephora*; (iii) to examine the possibility of developing a marker-assisted selection programme based on molecular markers of gene introgression from *C. canephora.*

Materials and methods

Nematode populations and inoculation

The nematode population came from a severely infested plot in which the coffee trees had average infestation levels of 1700 second-stage juveniles (J2) per gram of roots. The plot was located at the CICAFE experimental station (Barva de Heredia, Costa Rica) at 1100 m above sea level in a humid tropical climate with a 3–4 month dry season and a mean temperature of 22–23°C. The population used in this study was characterized from three samples by enzymatic electrophoresis for four enzyme systems (esterase, malate dehydrogenase, superoxide dismutase, and glutamate-oxaloacetate transaminase) (Hernández *et al.*, 1996). The samples revealed the esterase phenotype VF1 of *M. exigua* (Esbenshade & Triantaphyllou, 1985) and the enzyme phenotypes N1, N1a and H1, respectively, for the malate dehydrogenase, superoxide dismutase, and glutamate-oxaloacetate transaminase systems.

Nematodes were extracted from roots by the mist technique (Hooper, 1970). Once the coffee seedlings reached the two cotyledon leaf stage, they were transferred to 250-mL pots containing a 2 : 1 mixture of disinfected soil and sand (pH 5·2–5·6). The inoculum was applied to the collar of each coffee seedling using a micropipette when the plants were between 2 and 3 months old (two pairs of leaves). Nematode inoculum concentration was determined from a 1-mL aliquot and 3000 ± 200 J2 were applied per pot. Observations were carried out 5 or 6 months after inoculation.

Experiments and plant material

The trials were carried out in a greenhouse in Costa Rica at a relative humidity of 70–90% and a mean temperature of 25°C. The objective of trial 1 was to confirm that the species *C. arabica* does not provide genes of resistance to *M. exigua*. The seeds came from Ethiopian accessions collected in the centre of origin of the species by Guillaumet & Halle, 1978) in 1966 or by the FAO in 1965 (FAO, 1968) and kept in the CATIE field genebank in Costa Rica. Twenty-nine accessions were assessed by taking at random the open-pollinated progeny of a single tree. In trial 2, the objective was to confirm that the species *C. canephora* is resistant to the nematode. The plant material came from *C. canephora* accessions ($2n = 2x = 22$). Ten accessions kept in the CATIE genebank and six accessions from a genebank in French Guiana were tested using seeds taken at random for each origin from a single tree in the collection. The objective of trial 3 was to confirm that resistance is widespread in lines derived from different hybridizations between accessions of Timor Hybrid populations (CIFC832/1, CIFC832/2, CIFC1343) and commercial cultivars (Caturra, Villa-Sarchi). A study on progeny segregation was carried out in trial 4, by studying one pure line (IAPAR59 in F_8), one F_1 progeny obtained by crossing one resistant plant of cv. IAPAR59 by the susceptible cv. CR95, and one F_2 progeny obtained by self-crossing a resistant F_1 hybrid, T5296 × T4759. The number of plants per family was in the range 13–25 for trials 1, 2 and 3 and 273–365 for trial 4. Cv. CR95, a Catimor line well-known for its susceptibility, was used as the susceptible control.

A field assessment was carried out on 28 trees that were more than 10 years old, representing 28 Catimor and Sarchimor lines, in generations F_4 to F_7. These trees were assessed on two occasions, in November 1996 and October 1999, in trials at the CICAFE experimental station, under conditions of severe infestation (800–4000 nematodes per gram of roots), using the protocols described by Bertrand *et al.* (1997). For each tree, the first 5–10 cm of soil were removed in a radius of ≈ 80 cm from the trunk. The unit observed was one secondary root around 30 cm in length with a diameter of 3–5 mm, with its root hairs. Each tree had four to six root units that could be observed in the first few centimetres of soil. Following these observations, the genotypes for which no galls were observed were scored as resistant (R), and those for which at least one gall was found were scored susceptible (S).

AFLP protocol

Leaf samples were taken from the 28 trees characterized for their resistance in the field and representing 28 introgressed lines. The amplified fragment length polymorphism (AFLP) procedure was performed as described in Lashermes *et al.* (2000). Aliquots of 500 ng genomic DNA were digested using two restriction enzymes, *Eco*RI and *Mse*I. The linker-adapter and primer sequences were as described by Vos *et al.* (1995), with minor adaptation for coffee DNA. Preamplification was carried out with 1-bp extension primers. Selective amplification of restriction fragments was conducted using primers with three selective nucleotides. One of these primers (*Eco*RI primer) was end-labelled with γ-[33]-ATP using T4 polynucleotide kinase. A total of 42 AFLP primer combinations was used (sequences indicated in Lashermes *et al.* 2000). Amplification products were separated on 6% denaturing polyacrylamide gels. The gels were dried and exposed to Kodak Bio Max X-ray film. Amplification products were separated on 6% denaturing polyacrilamide gels. The gels were dried and exposed to Kodak Bio Max X-ray film.

Histological techniques

Histological sections were cut to observe plant reactions to the parasite. The samples were fixed in FAA (formaldehyde alcohol, acetic acid and distilled water) for 48 h. They were then dehydrated in a series of ethanol (70–100%), 1 h in each bath, embedded in HistoresinÔ at 4°C overnight, and then moulded. Sections 3 mm thick were cut and stained with toluidine blue or naphthol blue black.

Analysis of data

Numbers of root galls were assessed using a six point gall index (GI) scale; the classes were GI = 0, 0 galls; GI = 1, 1–2 very small galls; GI = 2, 3–10 very small galls; GI = 3, 11–30 small and large galls; GI = 4, 31–100 small and large galls; GI = 5, over 100 small and large galls. It was considered that plants scoring 0, 1, 2 in this index were resistant (R) and those classed in 3, 4 and 5 were susceptible (S). Chi-squared tests were carried out when necessary, based on plant frequencies on the six-point scale, to compare groups of plants.

The AFLP amplification products were designated according to the restriction enzymes, the primer combination used and in order of decreasing fragment size. Only AFLP bands showing a clear polymorphism were scored as present (1) or absent (0). As previously described (Lashermes *et al.* 2000), the additional marker band (i.e. not detected in any of the accessions of *C. arabica* and observed in accessions of *C. canephora*) detected in the Timor Hybrid-derived genotypes analysed were considered as introgressed markers from *C. canephora*. Genetic distances (*GD*) between genotypes were estimated as follows:

$$GDxy = (Nx + Ny)/(Nx + Ny + Nxy)$$

where Nx is the number of introgressed bands in line x and not in line y, Ny is the number of introgressed bands in line y and not in line x, and Nxy is the number of introgressed bands in lines x and y. Analysis by the neighbour-joining tree construction method (Saitou & Nei, 1987) was performed with the TREECOM software package (Van de Peer & Wachter, 1994).

The number of markers for each Timor Hybrid group was statistically analysed by a one-way analysis of variance (ANOVA) and compared using the least significant difference test (LSD) at $P = 0{\cdot}05$.

Results

C. arabica (trial 1)

None of the accessions of *C. arabica* tested were resistant. All had a majority of plants in classes 4 and 5 of the gall index. Of the 523 individuals tested, only nine (2%) had a gall index of < 3. That represented either resistant plants or plants for which inoculation was a failure. Some accessions appeared as susceptible as the control. It should be noted that 22 accessions had more than two plants in class 3, as opposed to none for the control. The histological sections cut according to plant class did not reveal any differences between the different classes of susceptible plants (GI = 3–5). The histological patterns were identical. The sectioned galls revealed several females deeply buried in the vascular bundles. These females were associated with large to very large egg masses. The giant syncytial cells were well formed and abundant.

C. canephora (trial 2)

All the *C. canephora* progenies tested proved to be 100% resistant. Of the set of 387 plants tested, 303 (78%) did not show any symptoms, 67 (17·3%) were in class 1 and 17 (4%) were in class 2. Sections cut from small galls in classes 1 and 2 showed that different situations were apparent, namely poor nematode development with a single female per gall and poorly developed egg masses or abnormalities due to incomplete development of the giant cells when compared with that observed in controls.

Lines derived from the Timor Hybrid (trial 3)

Evaluation for resistance to *M. exigua* of Catimor and Sarchimor lines, derived from the Timor Hybrid, revealed a highly contrasting response. Six lines were classed as resistant and two as susceptible. Among the resistant plants (193 plants), a majority were in class 0

(71%), and a smaller proportion of plants in class 1 or 2 (29%). In the two susceptible lines, there were a few plants in class 3, unlike the control, which had none. The histological sections revealed similar behaviour to those previously observed in *C. canephora* for the group of resistant plants or in *C. arabica* for the group of susceptible plants.

Relations between introgressed markers and resistance to *M. exigua*

A total of 113 introgressed marker bands (i.e. additional bands inherited from *C. canephora*) were observed among the Timor Hybrid-derived genotypes studied. The mean number of markers introgressed per genotype was 20·9, with extremes of one marker only for genotype T17929–37 introgression markers for genotype T17936 (Table 1). The genotypes divided into 12 susceptible and 16 resistant. A larger number of markers was found in the group of resistant genotypes than in the susceptible genotypes. Of the 16 resistant genotypes, 11 (i.e. 69%) had 20 or more markers, whereas among the susceptible plants, the proportion was 33·3%. The origins derived from Timor Hybrid CIFC832/1 had statistically fewer markers, on average, than the other two origins (Table 2). The number of markers was 13·0 for CIFC832/1, as opposed to 22·1 and 23·4 for origins CIFC832/2 and CIFC1343. Resistant genotypes were found among the three Timor Hybrid origins (Table 2). It should be noted that some genotypes had few introgression markers, but they were nonetheless resistant (T17925, T17930). The proportion of resistant genotypes was higher in the group of lines derived from Timor Hybrid CIFC1343, as shown in Table 2 (chi-squared = 5·61; $P < 0·06$).

Table 1 Evaluation of 28 introgressed genotypes of *C. arabica* for resistance to a *Meloidogyne exigua* population of Costa Rica, by number of AFLP markers

No.	Code	Markers (*n*)	Parent HdT[a]	R/S[b]
1	T18122	2	1	S
2	T18123	32	3	S
3	T18126	23	3	S
4	T18127	8	1	S
5	T18137	25	2	S
6	T18139	14	2	S
7	T17924	32	3	R
8	T17925	14	3	R
9	T17926	28	3	R
10	T17927	30	3	R
11	T17928	26	3	S
12	T17929	1	3	S
13	T17935	16	3	R
14	T17936	37	3	R
15	T17937	33	3	R
16	T17940	19	3	R
17	IAPAR cv	21	2	R
18	CR 95 cv	12	1	S
19	T18121	8	1	S
20	T18130	31	1	R
21	T5175	17	1	S
22	T17930	14	3	R
23	T17931	30	3	R
24	T17933	16	3	R
25	T18140	20	2	S
26	T18141	20	2	R
27	T5296	27	2	R
28	T16784	28	2	R

[a] Parent HdT, derived from the Timor Hybrid; 1, CIFC832/1; 2, CIFC832/2; 3, CIFC1343.

[b] R, resistant; S, susceptible.

Table 2 Mean number of introgression markers and distribution of genotypes susceptible and resistant to the nematode *M. exigua*, depending on the origin of the Timor Hybrid. CIFC832/1, CIFC832/2 and CIFC1343.

Timor Hybrid	Mean number of markers[a]	Number of susceptible genotypes	Number of resistant genotypes
CIFC832/1	13·00 b	5	1
CIFC832/2	22·14 ab	3	4
CIFC1343	23·40 a	4	11

[a] Means followed by the same letter do not differ significantly ($P = 0·05$) according to the LSD test.

The dendrogram (Fig. 1) calculated from the genetic distance estimated from the introgression markers illustrated the distances calculated between the genotypes derived from the three Timor Hybrid origins. The lines derived from origin CIFC1343 are grouped together except for T17929 which has only one introgression marker. The Sarchimors T18141 and T18140, which are very close genetically (same generation until F_4) differed in their resistance reaction.

Resistance trait segregation

A study of the reaction of one pure line, one F_1 progeny and one F_2 progeny is shown in Table 3. Plants derived from the fixed line were all resistant. Of the plants derived from the F_1 progeny of a resistant plant crossed with a susceptible plant, there was only one susceptible plant out of the 274 plants tested. Among the F_2 progenies, segregation was seen that was similar to a 3 : 1 or 12 : 4 type Mendelian model (chi-squared = 0·07, $P > 0·93$) indicating the existence of at least one dominant gene for resistance against *M. exigua.*

Discussion

The results revealed very different levels of resistance to *M. exigua* in the two species studied. In *C. arabica*, 98% of the plants tested proved to be susceptible and 100% of the *C. canephora* progenies were resistant. This result confirmed the observations by Curi *et al.* (1970) in Brazil. A low percentage of plants was found in class 3

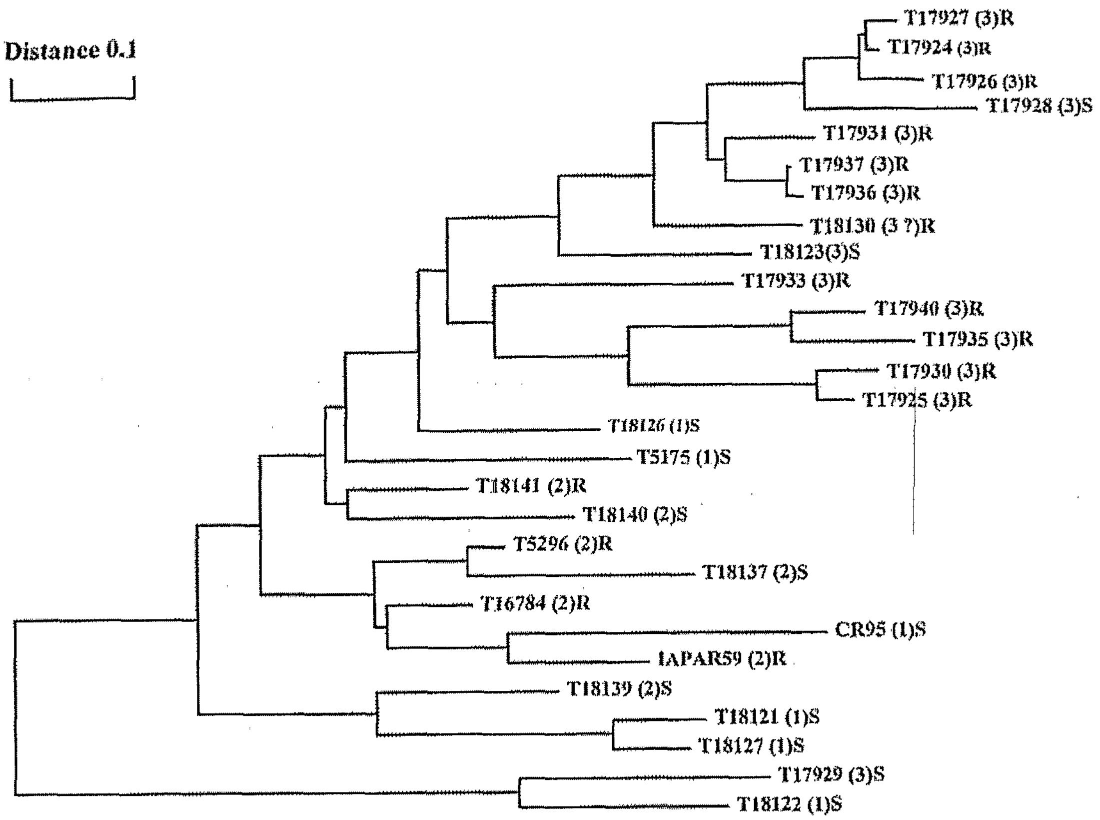

Figure 1 Dendrogram of the introgressed Arabica genotypes generated by the neighbour-joining method using genetic distance based on introgression of AFLP marker bands. Genotypes were observed to be (R) resistant or (S) susceptible to *M. exigua*. The Timor Hybrid progenitor used in the introgression programme is indicated in parenthesis as 1, 2 and 3 for CIFC832/1, CIFC832/2 and CIFC1343, respectively.

for a large number of Ethiopian accessions of *C. arabica*, whereas there were none for the control. The resistance evaluation scale greatly influences the interpretation of the results. Presence of minor genes in the Ethiopian accessions could account for class differences. However, microscopic symptoms did not differ between class 3 and classes 4 or 5. It should be also noted that the control was a particularly early selected line, whose development in the nursery was vigorous. In contrast, the accessions of Ethiopian origin mostly gave rather weak trees, with relatively late development (unpublished data). The difference in vigour could explain the differences in reaction to the nematode. The more roots the plant had, the more the nematode developed.

All the *C. canephora* introductions were classed as resistant, with a very large proportion (78·3%) of plants in class 0 (immune). However, 17·3% and 3·6% of plants were found in classes 1 and 2, respectively. Microscopy revealed that classes 1 and 2 had varying symptoms reflected by the existence of a single female and giant cells developed to varying degrees. Detailed studies of host/nematode reactions should enable a more precise description to be made of the physiological bases of resistance. This would be particularly useful for finding out whether these various reactions are controlled by different genes. Plants from the variety Nemaya derived from the cross between T3561 (2–1) and T3751 (1–2) were 91% of symptom-free plants,

Table 3 Evaluation of one pure line, and one F_1 and F_2 progeny of *Coffea* for resistance to an *M. exigua* population from Costa Rica. Resistance was based on a six-point scale assessed on the roots of each plant, evaluated 5–6 months after inoculation. Plants with gall indices (GI) of 0 to 2 were considered as resistant and those with gall indices (GI) of 3 to 5 were considered susceptible.

Name	Type of population	Type of cross	Number of plants tested	Number of resistant plants	Number of susceptible plants
IAPAR 59	Pure line (F_8)	R × R	273	273	–
IAPAR59 × CR95	F_1 progeny	R × S	274	273	1
T5296 × T4759	F_2 progeny	Self-crossing (R × S)	365	276	89
CR95 (control)	Pure line (F_8)	S × S	25	–	25

suggesting low nematode invasion. These progenies have also been selected for their resistance to *Meloidogyne* spp. in El Salvador and to *M. incognita* in Guatemala and are distributed as rootstocks for cultivated *C. arabica* varieties (Bertrand *et al.*, 1999).

Catimor and Sarchimor lines are frequently resistant to *M. exigua*, which confirms earlier results (Bertrand *et al.*, 1997; Gonçalvez & Pereira, 1998; Silvarolla *et al.*, 1998). The results obtained here showed that the level of resistance involved could be quite similar to that found in *C. canephora*. The Arabica parents of these lines do not reveal resistance to *M. exigua*, and supports the suggestion by Gonçalvez & Pereira (1998) and Silvarolla *et al.* (1998) that the resistance genes were introgressed from *C. canephora* and gave resistance to a large proportion of lines derived from the interspecific hybrid. However, among the 28 study genotypes, which came from the three Timor Hybrid origins, substantial variability in terms of introgression marker content was revealed. Some genotypes, such as T17929, had virtually no introgressed content. Moreover, this genotype is susceptible to leaf rust, Coffee Berry Disease and *M. exigua* (Bertrand *et al.* 1997). Despite a few noteworthy exceptions, such as genotype T18123, it would seem that the genotypes that had the largest number of introgression markers were also those that had the greatest chance of having retained the resistance genes. Conversely, for the genotypes with limited introgression, resistance was less frequent. However, it should be noted that resistant genotypes exist that revealed little introgression, which suggests that genes of resistance to *M. exigua* do not have strong linkages with the introgressed chromosome fragments from which they came. If it is considered that the study genotypes effectively represented the lines from which they were derived, it can be taken that all or part of the resistance genes would have been conserved in more than half of the lines. To the authors' knowledge, there has not been any rational selection of the lines for resistance to the nematode *M. exigua*. The loss or conservation of introgressed resistance genes is doubtless due more to the randomness of choices made by breeders and the number of generation during the pedigree selection process than to a rational choice by breeders. For example, genotypes T5175 and T8667, which represent cultivars IHCAFE90 and CR95, respectively, on which considerable selection pressures have been exerted (selection for productivity and resistance to leaf rust up to F_7), revealed few introgression markers and were susceptible to *M. exigua*. On the other hand, resistance to *M. exigua* has been fixed in cultivar IAPAR59. In this case, the frequent existence of the nematode in the trials in Brazil would have favoured plants possessing such resistance. For lines derived from CIFC1343, most of which make up the multiline Colombia variety, conservation of resistance in most of the lines is largely due to the few generations of selection at the time of their distribution (Castillo & Moreno, 1986).

Based on genetic distances calculated from molecular markers, two groups of lines depending on the origins of the Timor Hybrid were revealed: firstly the group of lines derived from CIFC1343, and secondly the groups of lines derived from CIFC832/1 and CIFC832/2. Differences between the two groups may have been due to introgressed chromosome fragments of a different nature. In the latter case, notably when seeking resistance markers, the possibility will have to be considered that the resistance genes in CIFC1343 are different from those in CIFC832/1 and CIFC832/2. Genetic studies of host–parasite relations in cultivated plants with respect to resistance to *Meloidogyne* very often revealed that a limited number of genes are operating (Sidhu & Webster, 1981) and more recently the same has been shown by Roberts *et al.* (1996) in *Vigna unguiculata*, Wang & Goldman (1996) in carrot and Cap *et al.* (1993) in tomato. In coffee, Anzueto *et al.* (2000) also suggested the existence of only two complementary genes for resistance to *M. incognita* in Guatemala. The results presented here indicate the existence of at least one dominant gene for resistance to *M. exigua*. The high frequency of resistant lines suggest the possibility of either using lines derived from the Timor Hybrid directly as a variety, or intraspecific crossing programmes, notably with the Ethiopian accessions, which often reveal resistance to *M. incognita* (Anzueto *et al.* 2000) or to *M. arabicida* (unpublished data), so as to create vigorous and resistant F_1 hybrids (Bertrand *et al.*, 1999). It should be possible to detect DNA markers linked with qualitative resistance to *M. exigua* and one approach, namely bulk segregant analysis (Halden *et al.*, 1997), is currently being pursued.

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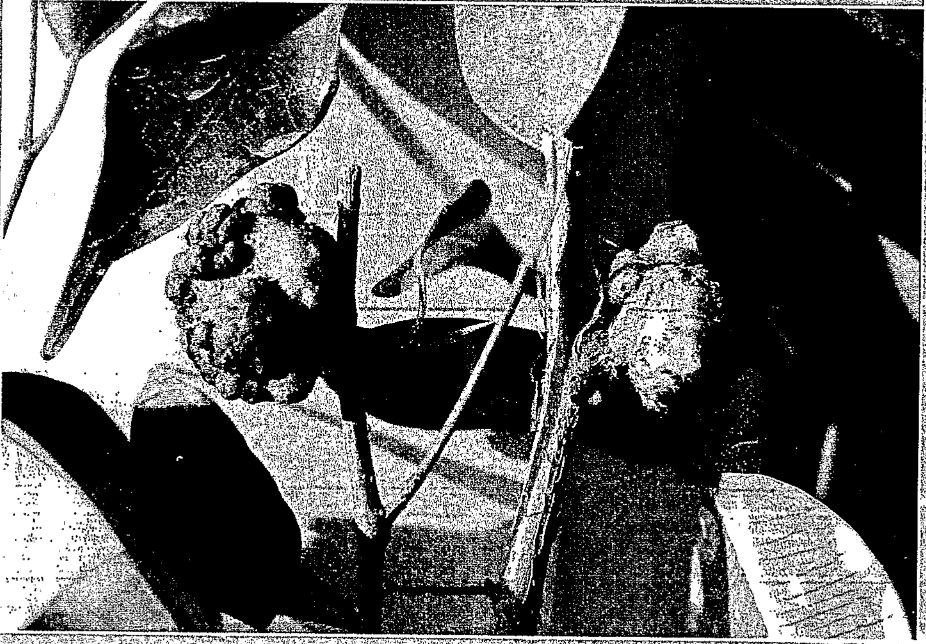

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