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Genetic variation in *Meloidogyne incognita* virulence against the tomato *Mi* resistance gene: evidence from isofemale line selection studies

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Abstract Resistance to the parthenogenetic root-knot nematode *Meloidogyne incognita* is controlled in tomato by the single dominant gene *Mi*, against which virulent pathotypes are able to develop. Isofemale lines (i.e., families) were established from a natural avirulent isolate of *M. incognita* in order to study the genetic variability and inheritance of the nematode virulence. From the progeny of individual females, the production of egg masses on the root system of the *Mi*-resistant tomato 'Piersol' was analyzed in artificial selection experiments. A family analysis revealed, after two successive generations, a strongly significant variation between the 63 isofemale lines tested, and the results obtained for the mothers and their daughters were also significantly correlated. These results together clearly demonstrate the existence of a genetic variability and inheritance for this character. In a second experiment, a four-generation selection was performed on 31 other isofemale lines. The results revealed a significant response to selection apparently limited only to the two families able to produce, in first generation, a significant minimal egg-mass number on the resistant cultivar.

Key words Tomato · *M. incognita* · Virulence
Genetic variation · Inheritance

Introduction

Meloidogyne incognita (Kofoid and White) Chitwood is one of the most damaging root-knot nematodes, affecting a large number of crop species from tropical to temperate regions (Sasser 1979). Its biological cycle is characterized by the absence of meiosis, the females reproducing by obligate mitotic parthenogenesis (Triantaphyllou 1971). Eggs are deposited by the female outside of the root tissue in a gelatinous matrix. Second-stage juveniles (J2) hatch from these eggs and invade new rootlets. They penetrate the cortex and establish themselves in the vascular cylinder where they induce the formation of giant cells upon which they feed. After three further moults, the nematodes develop into pyriform females usually able to produce 500 to 1000 eggs. The use of resistant cultivars that inhibit nematode reproduction is the most efficient way of controlling *Meloidogyne* spp., especially on vegetables, for which many resistance genes are available (Fassuliotis 1979). However, the occurrence of naturally virulent nematode populations able to overcome plant resistance has already been reported. This is particularly true for tomato, where resistance to root-knot nematodes is thought to be controlled by a single dominant gene designated *Mi* (Gilbert and McGuire 1956) and for which many virulent pathotypes have been identified in the field (Netscher 1977; Prot 1984; Sikora et al. 1973).

Due to the absence of recombination events, all of the J2 constituting the progeny of a single female are thought to be genetically identical. It has nevertheless been demonstrated that some of them, issuing from an avirulent female, were able, under artificial selection conditions, to become virulent and overcome the *Mi* resistance gene of tomato (Bost and Triantaphyllou 1982; Jarquin-Barberena et al. 1991; Riggs and Winstead 1959; Roberts and Thomason 1986; Triantaphyllou and Sasser 1960). In these studies, the plant-nematode interaction was analyzed using populations (i.e., J2 produced from many females) as inoculum sources, but no information about the genetic basis of virulence was supplied. In particular, the behavior against *Mi* of sister J2 hatched from single egg masses was not investigated.

In the work presented here, we used isofemale lineages (i.e., founded from individual females) from a naturally non-virulent population of *M. incognita* to study whether there is genetic variability in nematode virulence against the tomato *Mi* resistance gene. For this, we set up experimental selection experiments to determine whether the occurrence of nematode development on resistant plants was

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subject to, or independent of, clonal sources. In a second study, the influence of the selection pressure of resistant plants, maintained over four successive generations, was tested in relation to the inheritance and variability of the virulence response among J2 issued from randomly selected mothers.

Materials and methods

Biological material

A *M. incognita* population from Adiopodoumé, Ivory Coast, increased from a single female and characterized according to its isoesterase electrophoregram (Dalmasso and Bergé 1978), was chosen for the experiments on the basis of its natural avirulence against the *Mi* resistance gene of tomato. Before the experiments were initiated, this nematode population was reared on the susceptible tomato cultivar 'Saint Pierre', which served as the control in all further analyses. Selection for virulence was performed on the near-isogenic resistant cultivar 'Piersol', which carries the *Mi* gene.

Experimental procedures and evaluation

To test the hypothesis that a genetic determinism may be involved in *M. incognita* virulence against the *Mi* gene, we used experimental procedures derived from the 'isofemale line methods' of Parsons (1980). Each isofemale line (i.e., family) is founded by a single female. Then, the character to be studied is measured on several individuals in each line. Finally, the mean values are compared with a standard ANOVA procedure. If the null hypothesis is rejected, the character can thus be considered as a family feature, which strongly suggests the occurrence of a genetic basis of the observed variability.

All experiments were conducted in a climate chamber at a mean temperature of 23°C. Tomato seeds were germinated in steam-sterilized sandy soil in flats, and 2-week-old seedlings were transplanted singly into 50-ml plastic tubes containing the same substrate; these allowed to establish for 2 weeks before inoculation.

Egg masses that developed on susceptible tomato roots were randomly selected and allowed to hatch individually in distilled water. Each progeny (i.e., isofemale line or family), which consisted of about 800 infective J2, was used to inoculate 20 'Piersol' and 5 'Saint Pierre' tomatoes at a rate of 25 J2 per plant. These miniaturized tube test conditions have been tested previously and shown to give reproducible results (Castagnone-Sereno et al. 1993). The inoculum was pipetted in the water suspension onto the soil surface around the stem base; this was followed by light watering. Eight weeks after inoculation, the washed root systems were placed in cold eosin yellow (0.1 g/l H_2O) and stirred for half an hour to stain the egg masses. The number of egg masses per root system was counted.

A first experiment was performed to test whether the variability in the virulence character is under genetic control. For that purpose, the response of non-virulent *M. incognita* J2 on resistant tomatoes was assessed over two successive nematode generations (mothers and daughters). Sixty-three avirulent females (i.e., families), preliminary kept on susceptible tomatoes, were allowed to hatch in water. Each individual avirulent progeny obtained (i.e., mothers) was tested on the resistant cultivar 'Piersol', but only egg masses that developed on the susceptible control 'Saint Pierre' were selected to found the next generation. Juveniles hatched from those egg masses (i.e., daughters) were tested on resistant tomatoes again. The variability in nematode virulence among the 63 families was evaluated at both the mother and daughter generations.

A second experiment was carried out to determine whether there is variability among newly-obtained virulent *M. incognita* progenies (i.e., families) when the selective pressure of the *Mi* gene is maintained over successive generations. Thirty-one new families, each derived from a single avirulent female, were individually inoculated onto the resistant cultivar 'Piersol'. From each family and at each generation developed on 'Piersol', 1 egg mass was picked from the root system of the most attacked plant and the hatched J2 re-inoculated on the same resistant cultivar. This selective procedure was repeated over four successive generations.

Statistical analysis

For the first experiment, inter-family variability for virulence was analyzed at both the mother and daughter generations with a simple ANOVA. A mother-daughter correlation analysis was performed on the same set of data using a Spearman rank correlation. For the second experiment, variance in virulence was studied with a two-way ANOVA with isofemale lines (i.e., families) and generations as factors. All statistical analysis were performed using SAS software (1985).

Results

Data collected in both experiments consisted of the number of egg masses present on the root system of each 'Piersol' tomato plant analyzed, with 20 replicates for each family. Egg-mass numbers were corrected according to those found on the susceptible cultivar 'Saint Pierre'. The purpose of this correction was to compensate for any J2 viability differences that may have arisen during preparation of the inoculum, since any preinoculation condition which suppressed J2 infectivity on the standard susceptible cultivar should have suppressed infectivity on the resistant one proportionately. For any given isofemale line, the correction factor may act upon the intensity of parasitism (i.e., nematode aggressivity) but will not modify the presence or absence of parasitism (i.e., nematode virulence). Therefore, as the proportion of J2 showing abnormally low reproduction on the control tomatoes was very small (less than 10% over the two experiments) in comparison to those that normally developed, the influence of the correction factor with respect to nematode virulence was not significant in most cases and had no consequence on the statistical analyses (data not shown).

Genetic variability of nematode virulence

The production of egg masses on 'Piersol' resistant tomatoes was assessed for two successive generations of J2 that had previously never been submitted to the selection pressure of the *Mi* gene (natural avirulent nematodes for the mothers, nematodes reared on the susceptible cultivar used as control for the daughters). Substantial variations among the 63 isofemale lines analyzed in this first experiment were observed for both generations, with mean values ranging from 0.0 to 18.9 and 0.0 to 19.0 egg masses per plant for mother and daughter families, respectively (Fig. 1). Results of the ANOVA performed on these data indicated that for both generations the variations observed in nematode virulence appeared to be a family feature (Table 1). This strongly suggests that this variability is under a genetic control.

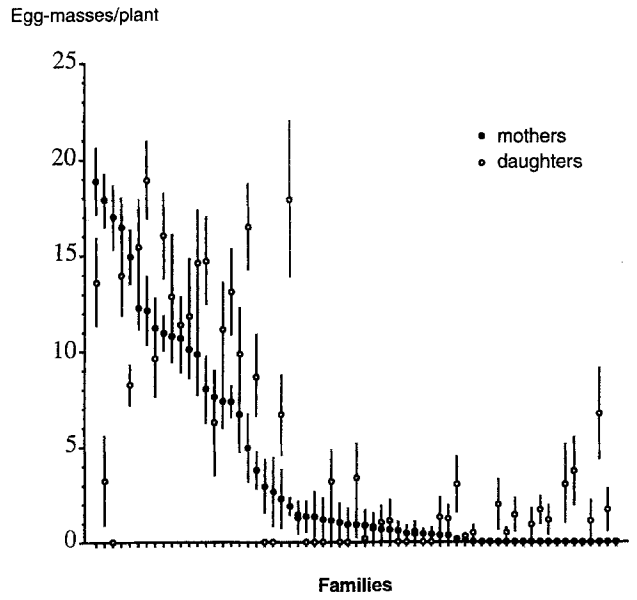

Fig. 1 Variation in the average virulence on 'Piersol' resistant tomatoes of 63 isofemale lines tested over two successive generations. Families are ordered according to decreasing average values in the mother generation. *Bars* are standard errors

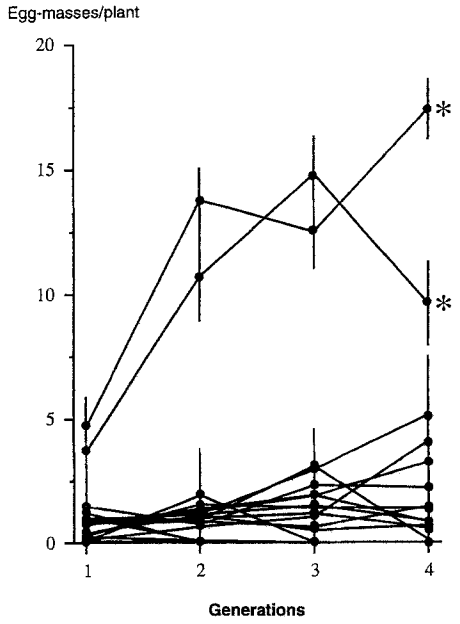

Fig. 3 Response of 31 isofemale lines for egg-mass production over four successive generations of selection on 'Piersol' resistant tomatoes. * indicate families that respond significantly to selection (for details see text). *Bars* are standard errors

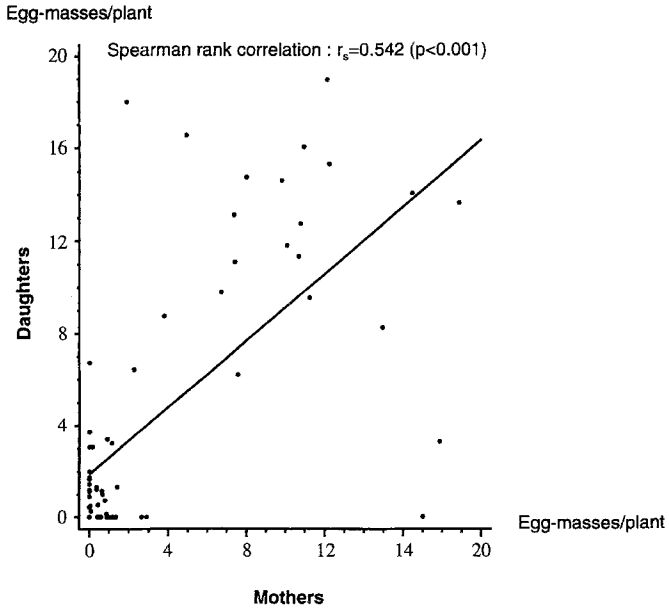

Fig. 2 Mother-daughter regression analysis for the mean numbers of egg masses produced by 63 isofemale lines on 'Piersol' resistant tomatoes

Moreover, Fig. 2 shows that there is a highly significant correlation between the egg-mass numbers produced on 'Piersol' by nematodes over two successive generations for the 63 isofemale lines tested (Spearman rank correlation, r_s=0.542, P< 0.001). This result is in agreement with the variation in *M. incognita* virulence revealed above by the family analysis.

Table 1 ANOVA for the mean number of egg masses produced by 63 isofemale lines on resistant tomatoes 'Piersol' inoculated with 25 juveniles over two successive generations

Source of variation	Mothers			Daughters		
	df	Variances	*F*	df	Variances	*F*
Isofemale line	62	596.34	29.3***	62	568.43	14.8***
Error	1187	20.34		1094	38.53	
Total	1249	48.93		1156	66.95	

*** Significance at P=0.0001

Response of *M. incognita* to artificial selection over successive generations

New isofemale lines were established as described above and independently selected for highest egg-mass production on 'Piersol' over four generations. The kinetics of change for the 31 families tested are shown in Fig. 3. Two distinct responses to selection can be observed. Two families significantly showed a rapid increase in their ability to reproduce on resistant tomatoes, with final (after the fourth generation) and average (for the four generations together) egg-mass numbers of 17.5 and 12.1 for one and 10.5 and 9.9 for the other respectively. It is also noteworthy that both of these lineages showed a significantly higher mean egg-mass number after the first generation on the resistant tomato (3.7 and 4.7, respectively) compared to the other lines. On the other hand, the remaining families tested (94% of the total) expressed little or no response

Table 2 ANOVA for the mean number of egg masses produced by 31 isofemale lines on resistant tomatoes 'Piersol' inoculated with 25 juveniles over four successive generations

Source of variation	df	Variances	*F*
Isofemale line (1)	30	389.10	25.8***
Generation (2)	3	459.64	30.5***
Interaction (1)×(2)	45	25.76	1.7**
Error	1278	15.08	
Total	1356	24.69	

Significance at ** P=0.0027 and *** P=0.0001

to selection, with 41%, 52% and 62% of them even being lost after one, two or three generations on the resistant tomato, respectively.

An ANOVA was used on these data to determine the extent to which selection pressure versus genetic influences affect *M. incognita* virulence. For this, the dependant variable was the egg mass numbers produced on 'Piersol', with isofemale lines and successive generations as factors (Table 2). The results of this analysis indicate the existence of a strong influence by both factors, the significant differences between families confirming those reported in the first experiment. Moreover, a significant interaction occurred between the isofemale line and generation terms, which indicates that response to the selective pressure of the *Mi* gene over successive generations is not the same for each genotype. This interaction was no longer significant when the ANOVA was run without the two families that strongly responded to selection (data not shown), which is in agreement with the occurrence of a set of two families, one responding and one another not responding to selection.

Discussion

By the successive multiplication of progenies from individual *M. incognita* females on the resistant tomato cultivars 'Small Fry' and 'Nematex', a step-by-step increase in nematode multiplication rate was observed by Bost and Triantaphyllou (1982). A subsequent analysis over 12 and 21 generations of two other *M. incognita* populations on the resistant variety 'Piersol' confirmed those results and indicated that J2 penetration, rate of reproduction, and female fecundity also gradually increased during selection (Jarquin-Barberena et al. 1991). These two experiments described both the acquisition and evolution of nematode virulence but did not provide information about the genetics of this character. Triantaphyllou (1987) suggested the occurrence of several genes submitted to an unusually high frequency of mutations with small favorable effects on the same or different loci in one or more homologous chromosomes, but he did not have any strong argument to demonstrate this hypothesis.

In our investigation, an isofemale line method was used in selection experiments designed to investigate possible genetic determinism in the variability of *M. incognita* virulence against the tomato *Mi* resistance gene. The results revealed (1) a high level of variation in the ability of isofemale lines to reproduce on tomatoes carrying the *Mi* gene, (2) a strong correlation between previously unselected mothers and daughters for this character, and (3) a significant response to selection apparently limited only to the families able to produce in the first generation a significant minimal egg-mass number on the resistant cultivar. All of these arguments brought together clearly demonstrate both the occurrence of genetic variability in virulence against the tomato *Mi* resistance gene between isofemale lines and the genetic inheritance of this character from one generation to the other, thus confirming its genetic determinism in the nematode population studied.

Virulence here was estimated as the mean number of egg masses produced on resistant tomato plants, i.e., the ability of the nematode to reproduce. Many successive biological steps are involved from the inoculation with J2 to the occurrence of egg masses on the root system, among which root surface recognition, root tissue penetration, feeding site establishment, and all the possible interactions between these events. As the phenotype investigated should be the result of many of these steps, it is thus not possible to state more precisely which elementary biological feature (or their interaction) is under genetical control.

The gene-for-gene concept of coevolution, in the case of a plant-pathogen interaction, is based on the occurrence of complementary loci in both of the organisms involved: each resistance gene of the host is matched by a corresponding avirulence gene in the pathogen (Ellingboe 1981). Virulence in the pathogen is generally under the genetical control of the recessive allele of the dominant avirulence gene (Keen 1990). In the case of plant-parasitic nematodes, the gene-for-gene theory has been demonstrated for the interaction between *Globodera rostochiensis* and the *H1* resistance gene found in *Solanum tuberosum* ssp. *andigena*. The F_2 segregation in crossing experiments of virulent and avirulent lines showed that virulence to the *H1* gene is controlled by a single major recessive gene (Janssen et al. 1991). It is clear that Mendelian genetic approaches cannot be used in the case of parthenogenetic organisms, which does not allow this kind of information to be provided for *Meloidogyne*. Nevertheless, results shown here revealed the occurrence of a continuous variation (Fig. 1), which may suggest that a polygenic system is involved.

Two mechanisms may be proposed to explain the genetic variation in virulence reported here. The first hypothesis involves a change in virulence allele(s) frequencies during selection and suggests the occurrence of virulence genes at a reasonable frequency in the natural avirulent nematodes that responded to selection. Such a preadaptative mechanism is to be correlated with the results of the second experiment: two isofemale lines were rapidly selected over the 31 tested, and both of them showed a significant higher mean egg-mass number after the first generation on the resistant tomato. Moreover, the absence of significant interaction in the ANOVA conducted without

these two families strongly suggested that the variability of the influence of the selection pressure over generations revealed in the global ANOVA was only due to these two families. However, the fact that a laboratory-selected virulent strain of the same *M. incognita* population was not able to revert back to an avirulent phenotype in the absence of the *Mi* -gene selection pressure over 18 successive generations (Castagnone-Sereno et al. 1993) makes this hypothesis difficult to definitely accept, as the relative high frequency of fixed virulence genes in avirulent nematodes seems unconvincing.

The second hypothesis is related to gene amplification. Variation in chromosome numbers and ploidy levels is not uncommon and is well documented in the genus *Meloidogyne* (see Triantaphyllou 1985 for a review), and could also be associated to some kind of selective amplifications of virulence alleles in some lineages. The process of gene amplification has already been proposed in the case of an apomictic clone of the aphid *Myzus persicae* selected for increased esterase activity (Bunting and Van Emden 1980) and could be related in our case to the stepwise acquisition of nematode virulence over successive generations (Bost and Triantaphyllou 1982; Jarquin-Barberena et al. 1991), but such an hypothesis can not be used easily to explain the high variability at a given generation. Anyway, no molecular evidence of amplification associated with *Meloidogyne* virulence is actually available.

Although the exact solution to this problem remains unknown, it is possible that such a phenomenon is not restricted to *M. incognita* and has not yet been detected in other models because most of the work on selection is carried out on sexually reproducing organisms.

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