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ORIGINAL PAPER

Selection and parasite evolution: a reproductive fitness cost associated with virulence in the parthenogenetic nematode *Meloidogyne incognita*

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Abstract Selection in plant parasites for virulence on resistant hosts and the resulting effects on parasite fitness may be considered as a driving force in host-parasite coevolution. In the present study, we tested the hypothesis that a fitness cost may be associated with nematode virulence, using the interaction between the parthenogenetic species *Meloidogyne incognita* and tomato as a model system. The reproductive parameters of near-isogenic lines of the nematode, selected for avirulence or virulence against the tomato *Mi* resistance gene, were analysed and combined into a reproductive index that was taken as a measure of fitness. The lower fitness of the virulent lines on the susceptible tomato cultivar showed for the first time that a measurable fitness cost is associated with unnecessary virulence in the nematode. Although parthenogenesis should theoretically lead to little genetic variability, such cost may impose a direct constraint on the coevolution between the plant and the nematode populations, and suggests an adaptive significance of trade-offs between selected characters and fitness-related traits. These results indicate that, although plant resistance can be broken, it might prove durable in some conditions if the virulent nematodes are counterselected in susceptible plants, which could have important consequences for the management of resistant cultivars in the field.

Keywords Evolution of virulence · Fitness · Host–Pathogen interaction · Plant resistance · Root-knot nematode · Trade-off

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Introduction

Host plant resistance has been used extensively as a crop protection strategy against many pests and pathogens because it is cost effective and considered to be environmentally sound (Fritz and Simms 1992). Ideally, plant resistance should be durable, i.e., should provide an efficient protection against the target organism during prolonged and widespread use in an environment conducive to disease development (Johnson 1981). However, such a control strategy is frequently made inefficient due to the rapid emergence in pathogen populations of new variants that are able to overcome the plant resistance (Enjalbert et al. 2005; Isakeit and Jaster 2005). In the context of phytopathology, such pathogen genotypes that develop and reproduce on host plants carrying resistance genes are considered as 'virulent' (Shaner et al. 1992). Throughout this paper, we use 'virulence' in the standard plant pathology sense, meaning the nominal ability of the nematode to cause a susceptible response on a host plant carrying a given resistance gene. This is quite different from the animal host-parasite literature, where virulence refers to the severity of effects on an infected host. In plant–pathogen interactions, virulence has been identified in fungi, bacteria, virus and nematodes (Castagnone-Sereno 2002; Parlevliet 2002; Lecoq et al. 2004).

Since the pioneering works of Van der Plank (1968, 1975)) on the genetic basis of plant disease and resistance, pathogen fitness has extensively been considered as the driving force responsible for evolution in a pathosystem. Basically, Van der Plank hypothesized that unnecessary virulence in a pathogen will result in a fitness cost when it infects a host plant without the corresponding resistance, a concept known as 'stabilizing selection' (Crill 1977). In this context, fitness can be defined as the ability of an organism to survive and reproduce, i.e., to pass its genes to the next generation (Holliday 2001). In further models developed to understand the coevolutionary aspects of plant–pathogen interactions, the cost of virulence has regularly been introduced as an important component of fitness, and calculations showed that pathogen fitness should be reduced on both plant genotypes with and without the corresponding resistance (Leonard and Czochor 1980; Thrall and Burdon 2003). More recently, experimental evidence supported the prediction that a plant resistance gene in rice would be a durable gene because of a fitness cost in a bacterial pathogen strain showing adaptation (i.e. virulence) to that gene (Vera Cruz et al. 2000). Thus, the durability of resistance genes could be predicted by evaluating the pathogen's fitness cost of virulence (Leach et al. 2001).

More generally, pathogen populations with a high evolutionary potential (i.e. able to produce offspring with new recombinant genotypes can adapt to new environmental conditions) have been suggested to be more likely to overcome genetic resistance than pathogen populations with a low evolutionary potential (offspring with few recombinant genotypes). Accordingly, pathogens having the greatest risk of breaking down resistance genes should have (1) a mixed reproduction system (i.e. both sexual and asexual reproduction), (2) a high potential for gene flow, (3) large effective population sizes, and (4) high mutation rates (McDonald and Linde 2002). Finally, it is now known that analysis of pathogen variation, including fitness costs, will allow the selection of the most effective combinations of resistance genes, and that monitoring of pathogen populations will allow the impact of different resistance genes and strategies to be observed, facilitating rapid responses when resistance breaks down (Michelmore 2003).

In tomato, resistance to the major root-knot nematode species *Meloidogyne arenaria*, *M. incognita* and *M. javanica* is controlled by a single dominant gene named *Mi* (Braham and Winstead 1957), and all the modern fresh-market and processing resistant tomato cultivars carry this gene (Williamson 1998). Although it is still highly efficient in most agronomical situations, the intensive use of the *Mi* gene for more than 60 years, along with the pathogenic variability of root-knot nematodes, raises concern about the durability of the resistance (Roberts 1995; Castagnone-Sereno 2002). Nematode biotypes virulent against the *Mi* gene have recently been described from most of the tomato growing areas in the world (Kaloshian et al. 1996; Eddaoudi et al. 1997; Ornat et al. 2001). Although *M. incognita* reproduces asexually, the selection of *Mi*-virulent lineages from progenies of avirulent nematodes was demonstrated under laboratory conditions (Jarquin-Barberena et al. 1991), suggesting an unexpected capacity of adaptive evolution in this clonal organism. The aim of this work was to determine whether there are fitness costs associated with the ability of the nematode to overcome the tomato *Mi* resistance gene, by comparing the reproductive parameters of isogenic lines that differ only for their virulence on a set of resistant and susceptible host genotypes. Such costs could impose a direct constraint on the coevolution between the plant and the pathogen populations, and also could have important consequences for the management of plant resistance in the field.

Materials and methods

Biological material

Cultivated tomato, *Lycopersicon esculentum* L., was used for testing reproductive fitness of the nematodes. Two cultivars were used, the susceptible cultivar Saint Pierre and the near-isogenic resistant cultivar Piersol carrying the *Mi* gene (Laterrot 1975).

In order to minimize the influence of the pathogen genetic background on its life history traits, we selected two *M. incognita* populations originating from very different geographic regions (Kursk, Russia and Morelos, Mexico, respectively), both avirulent against the tomato *Mi* resistance gene. To eliminate any potential within-population heterogeneity, a line was raised from each field population, starting from the progeny of a single female as follows. Single females were carefully dissected from the root tissues with their egg-mass, which was then used to reinoculate a tomato plant. Because of the mitotic parthenogenetic mode of reproduction of *M. incognita* (Triantaphyllou 1985), the second-stage juveniles (J2) that hatched from each egg-mass were considered as a clonal line. From each avirulent line, a near-isogenic line (NIL) was selected for virulence by successive re-inoculation on the *Mi*-resistant tomato cultivar Piersol, according to the procedure originally described by Jarquin-Barberena et al. (1991). In this study, we used two pairs of avirulent and virulent NIL that had been selected on Saint Pierre and Piersol, respectively, for 40 successive generations (Fig. 1). Prior to multiplication, each line was specifically identified according to its isoesterase electrophoretic pattern (Dalmasso and Bergé 1978).

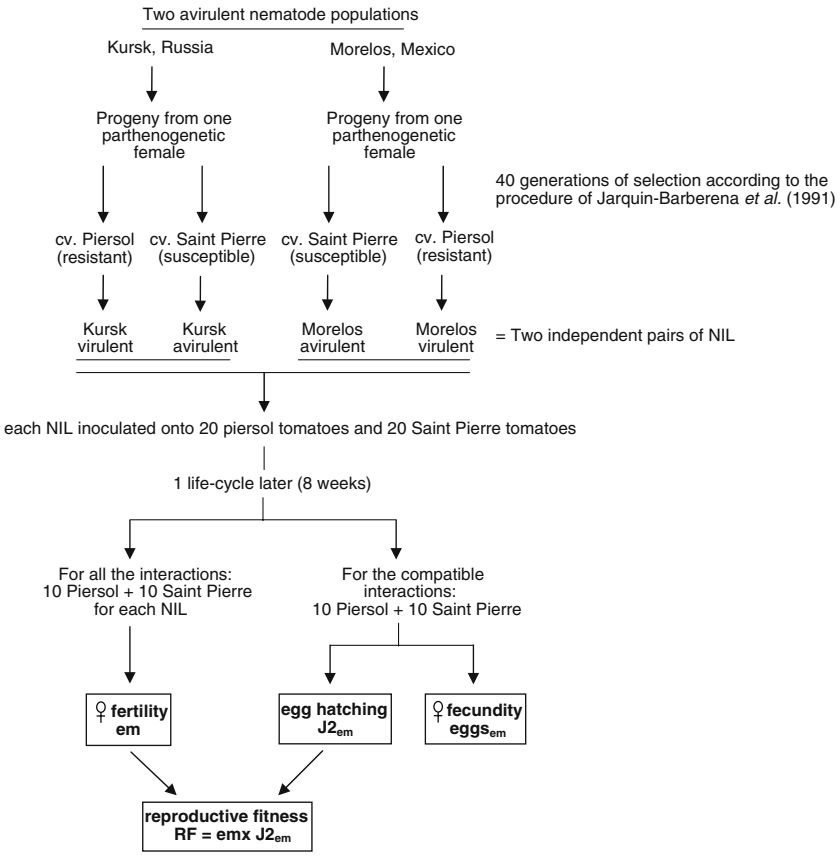

Fig. 1 Experimental design for the estimation of reproductive fitness in *Meloidogyne incognita* near-isogenic lines avirulent and virulent against the tomato *Mi* resistance gene. For details, see text

Experimental design and measurements

The experimental design is schematically shown in Fig. 1. Experimentation was conducted in a growth chamber maintained at 20 ± 1°C, with 16-h days of light. Tomato seeds were germinated in steam-sterilized sandy soil in flats, and two-weeks-old seedlings were transplanted singly into 50-ml plastic tubes containing the same substrate and allowed to establish for two to three weeks before inoculation. In order to avoid any influence of density-dependent effects on nematode reproduction parameters, a fixed number of infective J2 was used to inoculate the tomato plantlets. For that purpose, in each nematode × plant combination tested, 20 replicate plants received each 25 J2 using strictly-controlled miniaturized tube test culture and inoculation conditions (Castagnone-Sereno et al. 1994a). Female fertility was evaluated as the number of egg masses produced (a fertile female produces one egg mass, while an unfertile female produces no egg mass). Eight weeks after inoculation, the root systems of 10 plants for each nematode × plant combination were gently washed free from soil, placed in cold eosin yellow (0.1 g/L water) and stirred for 30 min to stain egg masses. Numbers of egg masses per root system were counted by eye.

For each compatible nematode × plant combination (i.e., those in which eggs were produced), females fecundity was further evaluated as the number of eggs per egg mass (i.e., the number of eggs produced by each female). From each tomato plant, one egg mass was randomly picked up from the roots (i.e., 10 egg masses selected at random for each nematode × plant combination), and the eggs were separated from the gelatinous matrix in 0.9% NaOCl, mounted in water between a glass side and a cover slip and counted under a stereomicroscope.

Nematode egg hatching was studied using the 10 remaining tomato plants for each compatible nematode × plant combination (i.e., those in which eggs were produced). From each tomato plant, one egg mass was randomly picked up from the roots (i.e., 10 egg masses selected at random for each nematode × plant combination), and individually deposited on a 150-μm nylon sieve in a plastic Petri dish containing tap water. Freshly hatched J2 were collected by removing the water every three days and counted under a stereomicroscope. Using this procedure, tap water was changed in the Petri dishes every three days to keep optimal oxygeneization of the egg masses. Preliminary experiments indicated that hatching success in water is similar to hatching success in the soil conditions (data not shown).

Statistical analysis

Since each repetition for each nematode line cannot be considered to be truly independent replicates (Hurlbert 1984; Heffner et al. 1996), we computed the average value of the three parameters measured for each nematode line and used these average values for the statistical analysis of the different effects tested only. On graph presentations, however, the original data were used to compute and plot standard errors of these average values.

To examine the effects of selection for virulence on nematode fitness, a nested analysis of variance (ANOVA) was performed using nematode lines (i.e., originating from different geographic location), nematode (a)virulence (nested within lines) and tomato cultivars as factors, and female fertility, female fecundity and egg hatching as the dependent variables. Pairwise tests were performed by choosing appropriate contrasts, and an overall significance level of 5% was maintained using a modified Bonferroni procedure (Hochberg 1988). All the corresponding statistical computations were done using the SAS/STAT package (SAS Institute Inc. 1990).

To estimate the reproductive fitness (RF) of the nematode avirulent and virulent NILs, we used the following formula: $RF = em \times J2_{em}$, where em = number of egg masses per root system and $J2_{em}$ = number of hatched J2 per egg mass, as previously evaluated.

We used bootstrap resampling to evaluate and compare average RF values and their standard deviation. The combination of em and $J2_{em}$ values from different root systems was legitimate because previous analysis had shown that differences between repeated experiments were small and mostly non-significant (P. Castagnone-Sereno, unpublished data). For each nematode × plant interaction, bootstrap was performed as follows: 10 em values and 10 $J2_{em}$ values were randomly sampled with replacement (Efron and Tibshirani 1993; Goodnight and Schwartz 1997), and used to calculate 10 corresponding RF values as described above. The newly obtained dataset, for all the nematode × plant combinations, was then subjected to an ANOVA. Following this procedure, we produced 500 resampled datasets, and

generated a bootstrap distribution of the 500 F values. Average RF values were further compared with Fisher's protected least significant difference test (PLSD, at $P = 0.05$).

If we assume that the fitness of the avirulent nematode lines on the susceptible host is maximal, then the fitness cost of the selected virulent lines on the susceptible host (C_h) may be estimated as follows: C_h (%) = $1–RF_V/RF_A$, where RF_A and RF_V are the RF of the avirulent and the virulent line, respectively, both estimated on the susceptible host.

Results

As expected, both avirulent lines from Kursk and Morelos were unable to reproduce on the resistant cultivar Piersol. Conversely, the selected lines exhibited high reproductive rates on Piersol (average (±SE) egg mass numbers per plant = 21.23 (±0.98) and 23.75 (±0.68) for the selected lines from Kursk and Morelos, respectively; Fig. 2). This result indicates that selection for virulence was successful, virulence being defined as the ability of the parasite to reproduce (i.e., for a *M. incognita* female to produce one egg-mass) on a resistant cultivar. However, in the successful plant–nematode interactions analyzed, the effects of the nematode lines, their (a)virulence nested within lines, and the tomato cultivars on which they were inoculated, were not significant for *M. incognita* fertility (Table 1).

Further statistical analyses were conducted to compare the fecundity of the reproducing females. Only the six compatible interactions were taken into account, since no reproduction occurred when the two avirulent NIL were inoculated onto the resistant tomatoes. The average numbers of eggs per egg mass ranged from 207.80 to 630.50 (Fig. 3). Again, the nematode lines, their (a)virulence nested within lines, and the tomato cultivars on which they were inoculated had no significant effects on *M. incognita* fecundity (Table 1).

Egg hatching of the avirulent and virulent NIL was followed on the susceptible cultivar Saint Pierre for over 30 days and exhibited little to no difference, the number of recovered J2 being always slightly higher or equivalent for the avirulent lines compared to the virulent ones (data not shown). However, in contrast to previous results, nematode lines, their (a)virulence nested within lines, and the tomato cultivars on which they were inoculated had significant effects on the hatching of *M. incognita* eggs (Table 1). In particular, the number of hatched J2 on the

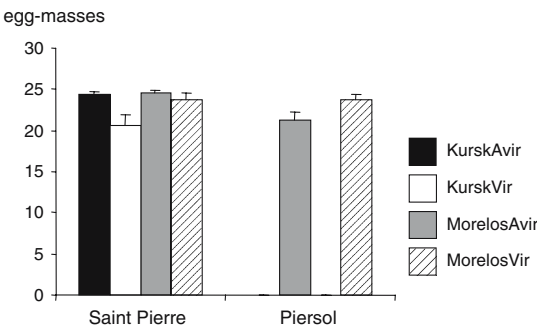

Fig. 2 Average (+SE) Fertility of *Meloidogyne incognita* females on tomato genotypes. KurskAvir, MorelosAvir: avirulent lines; KurskVir, MorelosVir: virulent near-isogenic lines. Saint Pierre: susceptible cultivar; Piersol: *Mi*-resistant cultivar

Table 1 Analysis of variance of *Meloidogyne incognita* fertility (egg mass production), fecundity (eggs/egg mass) and hatching

	Fertility			Fecundity			Hatching success		
	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Line	1	0.01	0.9214	1	21.04	0.1366	1	2155.01	0.0137
(A)virulence[a]	2	1.10	0.4376	2	23.24	0.1451	2	981.09	0.0226
Tomato cultivars	1	2.47	0.2144	1	22.11	0.1334	1	1015.37	0.0200
Error	3			1			1		
Corrected total	7			5			5		

[a]The (a)virulence effect was nested within nematode lines

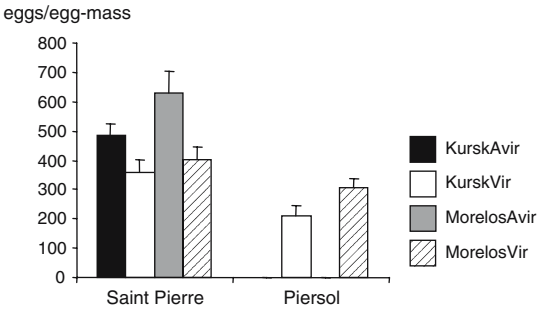

Fig. 3 Average (+SE) fecundity of *Meloidogyne incognita* females on tomato genotypes. KurskAvir, MorelosAvir: avirulent lines; KurskVir, MorelosVir: virulent near-isogenic lines. Saint Pierre: susceptible cultivar; Piersol: *Mi*-resistant cultivar

susceptible tomato cultivar was higher in avirulent lines compared to their virulent counterparts ($P = 0.0542$ and $P = 0.0280$ for the NIL from Kursk and Morelos, respectively; Fig. 4).

Estimation of nematode RF for both avirulent and virulent NIL was done by combining the numbers of egg masses per root system and the numbers of hatched J2 per egg mass. When these traits were analyzed separately, no clear trend about avirulent versus virulent nematodes inoculated onto susceptible versus resistant tomatoes could be inferred (Figs. 2 and 4). However, the combination of these two independent measures into a single value, using the bootstrap resampling procedure, did allow detection of significant differences between the RF levels of the avirulent

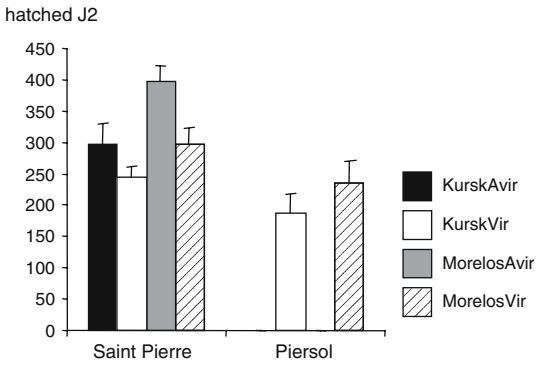

Fig. 4 Average (+SE) egg hatching of *Meloidogyne incognita* on tomato genotypes. KurskAvir, MorelosAvir: avirulent lines; KurskVir, MorelosVir: virulent near-isogenic lines. Saint Pierre: susceptible cultivar; Piersol: *Mi*-resistant cultivar

and virulent NIL inoculated on the susceptible tomatoes (on 500 bootstrap replicates, 57 and 132 were below the threshold, for the NIL from Kursk and Morelos origins, respectively, instead of the 5 replicates expected in a 1% threshold test; Fig. 5). For the virulent NIL of both origin, RF was significantly reduced compared to the corresponding avirulent NIL. The virulence cost on the susceptible tomatoes was estimated at 30.0% and 27.1% for the virulent lines from Kursk and Morelos, respectively.

Discussion

Our results indicate that selection for virulence against the tomato *Mi* resistance gene occurs in the asexual nematode *M. incognita*, and that costs are associated with virulence on susceptible hosts (i.e., without the resistance gene). In previous studies, no differences could be found between fecundity (i.e., the number of egg masses produced) of virulent root-knot nematodes on susceptible vs. resistant plants (Castagnone-Sereno et al. 1994a; Tzortzakakis et al. 1998). These data tend to indicate that the number of egg masses in itself is a poor indicator of nematode reproductive potential when fitness is considered. Clearly, the RF index used in the present work, which combined elementary life history traits of the nematodes, gave a more accurate evaluation of their overall reproductive potential. Although the occurrence of a fitness cost associated to virulence in relation to the durability of plant resistance has been documented in fungal, bacterial and viral pathogens (Leach et al. 2001; McDonald and Linde 2002; Parlevliet 2002), few data were available so far for plant-parasitic nematodes. It concerned mainly cyst nematodes, *Heterodera* spp. and *Globodera* spp., and provided contradictory information; the virulent parasites exhibiting (Lange et al. 1993; Lasserre et al. 1996) or not exhibiting (Turner 1990; Beniers et al. 1995) a decrease in their reproductive potential. Unlike *M. incognita*, these nematode species reproduce sexually, and the results of the present study therefore constitute the first evidence of a reproductive fitness cost associated to virulence in a mitotic parthenogenetic nematode.

Because it generates diversity through recombination, sexual reproduction is considered to play a major role in adaptive evolution of organisms. In particular,

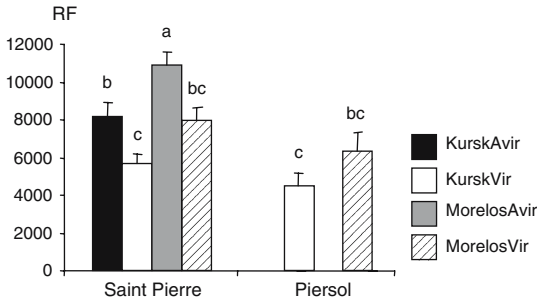

Fig. 5 Average (+SE) reproductive fitness of *Meloidogyne incognita* on tomato genotypes. KurskAvir, MorelosAvir: avirulent lines; KurskVir, MorelosVir: virulent near-isogenic lines. Saint Pierre: susceptible cultivar; Piersol: *Mi*-resistant cultivar. Values with different letters are significantly different ($P < 0.01$)

pathogen populations that undergo sexual reproduction are believed to be more likely to overcome plant genetic resistance than those reproducing by obligate parthenogenesis (McDonald and Linde 2002). However, the increasing amount of experimental data has provided evidence of the dynamic nature and adaptive potential of eukaryotic clonal genomes, including root-knot nematodes, through non-sexual mechanisms involving, among others, transposable elements, chromosomal rearrangements or karyotype variation (Lushai et al. 2003; Castagnone-Sereno 2006). The *M. incognita*-tomato pathosystem used in this study showed that parthenogenetic root-knot nematodes have developed mechanisms for generating the variability necessary to ensure parasitic success in varying environments (i.e. a new host genotype harbouring a resistance gene), and thus can experience trade-offs just like sexual species. According to Muller's Ratchet, accumulation of mutations, in the long term, should gradually lead to an inexorable decline in mean fitness of clonal animals, up to their extinction (Haigh 1978). Although the genetic basis of virulence is unknown in parthenogenetic root-knot nematodes, isofemale line selection experiments suggested that a polygenic system is involved (Castagnone-Sereno et al. 1994b), which implies that transition from avirulence to virulence cannot result from one single mutation. If one assumes that sexual reproduction would probably be a superior way than parthenogenesis to enable the fixation of virulence, then the polyploid, apomictic root-knot nematode *M. incognita* appears as an exception. Although virulence-associated fitness costs may reduce its adaptive ability, *M. incognita* is a far more superior plant parasite than the closely related diploid, sexual *Meloidogyne* spp., with an ubiquitous distribution and wide host range encompassing most flowering plants (Trudgill and Blok 2001). We suggest that the advantages of clonality, probably in close relationship with polyploidy, may have been underestimated in this nematode species.

It is widely known that parasite-mediated selection can significantly affect genetic variation of the host. In the animal literature, this type of selection has been shown to drive frequency-dependent cycling of host and parasite genotypes, and thus helps explaining the maintenance of genetic polymorphism in host and parasite populations (Lively 1999). In plant-pathogen systems, a similar cycling is not necessarily expected, because the underlying genetics is assumed to follow the gene-for-gene model. However, if there is a cost associated with parasite virulence, frequency-dependent selection can still occur (Agrawal and Lively, 2003). Recently, a clear negative relationship between virulence and spore production has been demonstrated in the fungus *Melampsora lini*, which may be a central factor in maintaining genetic variation in pathogen populations (Thrall and Burdon 2003). Although the avirulence determinants have not yet been functionally characterized, the *M. incognita*-tomato interaction is considered to be highly specific. Hence, although a fitness cost is associated with virulence, a stabilizing feedback between host and nematode genotypes should allow both avirulent and virulent strains to persist in the population, in agreement with theoretical modelling predictions (Kirchner and Roy 2002). Differences in life-history traits of individual host–parasite interactions are likely to have significant impacts on the ecological and evolutionary dynamics of such interactions (Thrall et al. 2005). In parallel to the results reported here, we evaluated the survivorship and the mobility of the same avirulent and virulent NIL and did not find any difference for these two biological traits (unpublished results). Although further studies are needed to ascertain the magnitude of the influence of both the host and pathogen genetic backgrounds on

nematode reproductive fitness, it is clear that such an issue is of crucial importance to provide a more comprehensive view of the evolutionary dynamics of the *M. incognita*–tomato interaction.

Extensively used since the 1950s in many fresh-market and processing tomato cultivars, and although virulent populations have been reported from several tomato-growing areas in the world, the *Mi* gene has remained largely durable (Trudgill and Blok 2001), even under agronomical conditions a priori favourable to the selection of nematode virulent populations (i.e., tomato monoculture or short rotations in warm climate areas; Roberts, 1995). In addition, the routine testing in our laboratory of hundreds of *M. incognita* populations collected from the field over the years has confirmed the extreme scarcity of virulent nematodes in nature (unpublished data). This observed situation, in which virulent isolates are indeed present but at a very low frequency, is an argument in favour of a selective disadvantage of virulence. In that respect, the estimation of the magnitude of the cost associated with virulence is central to the study of the spread of the gene(s) involved in virulence because of its impact on the relative fitness of carriers of such gene(s). Moreover, although no reliable information is available on the relative prevalence of resistant *versus* susceptible tomato cultivars in use worldwide, it is well admitted that resistance to nematodes is currently under utilized, particularly in developing countries where tomato constitutes a major crop (Starr et al. 2002). This implies that the (virulent) nematodes may commonly encounter susceptible hosts, which should contribute to maintain avirulence in the populations.

Data reported here suggest that the lines that have been selected to overcome the *Mi* resistance gene could have a compromised ability to compete with other lines on susceptible cultivars, as they would reproduce less efficiently. However, co-infections of tomatoes with mixtures of avirulent and virulent nematodes were not performed, and competition between avirulent and virulent nematodes is probably of outstanding importance to evaluate the risk of propagation of virulence in the field. From an experimental point of view, the lack of molecular markers to differentiate between avirulent and virulent individual nematodes constitutes a serious drawback to follow the outcome of such competition experiments. Moreover, our results were obtained under laboratory conditions and are still to be validated in field situations. Clearly, further work is needed to evaluate all the theoretical and practical aspects of the fitness cost associated to virulence in these nematodes, which involves critical events of its life cycle, in order to prolong the durability of plant resistance genes. Host mixtures have been proposed as an alternative method to slow down the evolution of virulence in pathogens (Burdon 1987), although some experimental work did not support the expectation that a cultivar mixture would slow down the rate of evolution in the pathogen population (Zhan et al. 2002). Similarly, computer simulations and field testing have shown that spatial refuges of susceptible corn fields of a patchwork planting regime can delay the evolution of corn borer resistance to maize varieties that express insecticidal toxins from *Bacillus thuringiensis*, although this prediction has occasionally been controversial (Andow and Zwahlen 2006, for review). In conclusion, from the results of the present work, we can reasonably hypothesize that management strategies based on alternation of *Mi*-resistant and susceptible tomatoes would delay the selection of nematode virulence, which would in turn enhance the durability of the plant resistance.

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