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Fitness of Virulent *Meloidogyne incognita* Isolates on Susceptible and Resistant Cowpea

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Abstract: A study of life-history traits was made to determine factors associated with the fitness of *Meloidogyne incognita* isolates virulent to resistance gene *Rk* in cowpea. Egg hatch, root penetration, egg mass production, and fecundity (eggs per egg mass) of avirulent and virulent phenotypes were compared among *M. incognita* isolates, isofemale lines, and single descent lines over multiple generations on resistant and susceptible cowpea. Variation ($P \leq 0.05$) in both hatch and root penetration rates was found among isolates at a given generation. However, this variation was not consistent within nematode lines among generations, and there was no correlation with level of virulence, except for penetration and virulence on resistant cowpea at generation 20. Resistant and susceptible cowpea roots were penetrated at similar levels. Differences in reproductive factors on resistant plants were correlated with levels of virulence expression. In some isofemale lines, single descent lines, and isolates, lower ($P \leq 0.05$) rates of egg mass production and fecundity on susceptible cowpea were associated with virulence to *Rk,* indicating a trade-off between reproductive fitness and virulence. Other virulent nematode lines from the same isolates did not have reduced reproductive ability on susceptible cowpea over 27 generations. Thus, virulent lineages varied in reproductive ability on susceptible cowpea, contributing to adaptation and maintenance of virulence within *M. incognita* populations under stabilizing selection.

Key words: cowpea, fitness, genetic variation, *Meloidogyne incognita,* resistance, root-knot nematode, selection, *Vigna unguiculata,* virulence.

Host plant resistance is a preferred method of controlling the root-knot nematode *Meloidogyne incognita.* One form of resistance to *M. incognita* in cowpea (*Vigna unguiculata*) is conferred by the dominant major gene *Rk* (Fery and Dukes, 1980). Virulence (the ability to overcome a resistance gene) to gene *Rk* has been reported in some *M. incognita* populations (Roberts et al., 1995), leading to a search for additional resistance genes in cowpea (Ehlers et al., 2000; Roberts et al., 1996).

Differences have been reported in the behavior of virulent nematodes in the absence of the specific resistance gene. Virulence of *M. incognita* to gene *Mi* in tomato was stable for the virulent phenotype when cultured for up to 9 months (about 6 generations) (Riggs and Winstead, 1959) or 18 generations (Castagnone-Sereno et al., 1993) on susceptible tomato. In contrast, an *Rk*-virulent *M. incognita* population declined in virulence during continuous greenhouse culture on susceptible cowpea for at least 25 generations even though the viability of the culture remained high (Petrillo et al, 2006; Roberts and Matthews, 1995). This observation suggested that virulence was associated with reduced relative fitness compared to avirulent forms, resulting in negative selection in the absence of the resistance gene (Petrillo et al, 2006; Roberts and Matthews, 1995).

Measurements of life-history traits at key points in the life cycle of *M. incognita,* including hatch, root penetration, and reproduction factors, may provide insight into the factors that affect nematode fitness. Relatively little has been reported on host effects on egg hatch of *Meloidogyne* spp., except that a diapause was observed in *M. javanica* eggs produced on poor hosts compared to favorable hosts (Huang and Pereira, 1994). Penetration of *M. incognita* into roots of susceptible and resistant plants varies depending upon the host. In some cases, penetration by avirulent *M. incognita* into resistant roots was lower than into susceptible roots (Bleve-Zacheo et al., 1998; Khan and Khan, 1991; Lawrence and Clark, 1986). Migration of juveniles from resistant roots after initial penetration accounted for some of the observed differences (Moura et al., 1993; Schneider, 1991). In contrast, similar levels of penetration into susceptible and resistant roots have been reported for avirulent and virulent *M. incognita* populations (Creech et al., 1995; Jarquin-Barberena et al., 1991; Riggs and Winstead, 1959; Windham and Williams, 1994). In most reported studies, a post-infection resistance response is triggered by initiation of host cell modification and nematode feeding.

Comparisons of egg mass production and fecundity (number of eggs per egg mass) on susceptible and resistant hosts have given mixed results. Reproduction rate was lower on resistant than susceptible plants of selected virulent isolates of *M. incognita* (Castagnone-Sereno et al., 1994, 1996). In contrast, selected virulent isolates had diminished egg mass production and fecundity on susceptible tomato compared to their avirulent parental isolate (Jarquin-Barberena et al., 1991). Fecundity and egg mass production on susceptible tomato progressively increased to near parental ability after 21 generations of culture on resistant tomato. Diminished fecundity on resistant compared to susceptible tomato was reported in some natural and selected virulent *M. incognita* isolates (Castagnone-Sereno et al., 1994), whereas fecundity on resistant and susceptible tomato did not differ in other *M. incognita* isolates (Bost and Triantaphyllou, 1982; Castagnone-Sereno et al., 1994).

Reduced fitness with increased virulence has been reported in several host-pathogen systems. Resistance

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gene *Bs2* in pepper matches strains of *Xanthomonas campestris* pathovar *vesicatoria* expressing avirulence gene *avrBs2* (Minsavage et al., 1990). Growth on susceptible pepper of an *avrBs2*-mutant of *X. c. vesicatoria* was similar to wild-type growth on the resistant host (Kearney and Staskawicz, 1990). Complementation of the *avrBs2*-mutant with a plasmid-borne copy of *avrBs2* restored growth on susceptible pepper to near wild-type, thus gene *avrBs2* was needed for full virulence of *X. c. vesicatoria* on susceptible pepper. In *X. oryzae* pv. *oryzae (Xoo),* loss of function of avirulence gene *avrXa7* resulted in virulence of *Xoo* to rice carrying resistance gene *Xa7* (Vera Cruz et al., 2000). Loss of function of *avrXa7* led to reduced aggressiveness to rice in general and an inability to increase or persist in field populations. Loss of *avrXa10* function resulting in virulence against rice resistance gene *Xa10* was not associated with a fitness penalty. These two systems are similar to the contradictory observations in *M. incognita*. Recently, spore production and transmission potential were found to be negatively correlated with virulence in the rust fungus *Melampsora lini* to resistance genes in the wild flax plant host *Linum marginale,* demonstrating a trade-off among aggressiveness (fitness) and virulence (Thrall and Burdon, 2003).

The objective of this study was to determine life history traits that led to the decline of *Rk*-virulence in *M. incognita* isolates. Hatch, penetration, and reproduction of avirulent and virulent phenotypes were compared among *M. incognita* greenhouse isolates, field populations, isofemale lines, and single descent lines to determine levels of relative fitness. Host influence on life history traits was examined by challenging *M. incognita* isolates and lines with continuous culture on susceptible or resistant cowpea genotypes.

Materials and Methods

Plant materials: Cowpea genotypes 'CB46', 'H8-9', and '8685' were used throughout the study. CB46 carries the dominant gene *Rk* (Fery and Dukes, 1980) for resistance to *M. incognita,* whereas H8-9 and 8685 are susceptible to *M. incognita*.

Nematode parental isolates: Two *M. incognita* race 1 isolates were collected 6 years apart from the same field site near Denair, Stanislaus County, California, where cowpea cultivars with gene *Rk* had been grown frequently in preceding years. Virulence to gene *Rk* of these isolates was indexed as the numbers of egg masses produced on CB46 divided by the numbers on 8685, expressed as a percentage. The *Rk*-avirulent isolate (Muller 89) was about 75% virulent when collected from the field in 1989. However, prior to the start of this study, this isolate declined to about 5% virulence during 6 years of continuous culture on susceptible tomato, representing ≥25 generations (Roberts and Matthews, 1995). The *Rk*-virulent isolate (Muller 95) was about 120% virulent when collected from the field in 1995. These isolates are referred to hereafter as Avirulent and Virulent.

Nematode isolates under greenhouse culture: The Avirulent and Virulent isolates from tomato were continuously cultured in the greenhouse on susceptible 8685 for 5 generations, being transferred to new plants at each generation. At inoculation of the sixth generation, eggs from the Avirulent isolate were divided into two portions. One portion was inoculated onto susceptible 8685 (Av-Susc) and the other portion onto resistant CB46 (Av-Res). The Avirulent isolate on susceptible cowpea and Avirulent isolate on resistant cowpea were started from the Avirulent isolate. The Virulent isolate subcultures on susceptible (Vir-Susc) and resistant (Vir-Res) cowpea were started in the same manner at the sixth generation. The Av-Susc and Vir-Susc isolates and the Av-Res and Vir-Res isolates were continuously cultured on susceptible 8685 and resistant CB46 plants, respectively, in 15-cm-diam. pots in the greenhouse for the duration of the study. This group of isolates is referred to as the greenhouse isolates.

Nematode isofemale lines: Twenty-seven and 26 isofemale lines (IFL) were started from the Avirulent and Virulent isolates, respectively. Each IFL was started from a single egg mass inoculated onto an individual plant. The IFL were continuously cultured in the greenhouse for 27 generations, being recultured onto new plants at each generation. All IFL were cultured on susceptible 8685 for the first four generations. At the fifth generation, eggs from each IFL were divided into two portions and inoculated onto 8685 and CB46. The portion of each IFL inoculated onto 8685 was designated as "Av-#S" or "Vir-#S" and onto CB46 as "Av-#R" or "Vir-#R" (Av denotes an IFL from the Avirulent isolate and Vir from the Virulent isolate, # denotes the original IFL number, and S and R denote culturing on susceptible 8685 or resistant CB46). Eggs within egg masses from 12 IFL from the Virulent isolate were unable to hatch during 7 days of incubation. Thus, 27 and 14 IFL from the Avirulent and Virulent isolates, respectively, were available for experiments. Using this procedure, two near-identical lineages were formed from each IFL. Sub-sets of these IFL were used in the life history trait experiments.

Nematode single descent lines: Egg masses produced at generation 21 of IFL Av-03S, Av-03R, and Av-06S were used to start 23, 30, and 18 single descent lines (SDL, equivalent to multiple IFL from an existing IFL), respectively. Each SDL from each IFL was started by inoculating one egg mass produced at generation 21 onto a separate 16 or 17-day-old 8685 root system. The egg mass was placed about 2 cm below the top of the pouch and touching the taproot. Lines Av-03S (low virulence) and Av-03R (high virulence) originated from the same isofemale lineage but differed due to repeated culturing for 17 generations on susceptible 8685 and resistant CB46, respectively. Line Av-06S changed from high to

low virulence during 17 generations of culturing on 8685.

Nematode isolates under field conditions: Cowpea genotypes H8-9 or CB46 were planted in microplots at the University of California Agricultural Experiment Station, Riverside, California, during the 1996 to 2000 seasons. A microplot was planted with the same cowpea genotype over the five seasons. Eggs from the Avirulent and Virulent parental isolates at generation 5 were used to inoculate the microplots. In 1996, each microplot was inoculated 3 weeks after planting with 160,000 eggs of the Avirulent isolate, the Virulent isolate, or a 1:1 (80,000:80,000 eggs) mixture of the Avirulent and Virulent isolates (Mixed isolate). Each of the six nematode × cowpea combinations was represented by a single microplot containing 12 plants. Nematode populations in microplots planted with susceptible H8-9 and inoculated with the Avirulent, Virulent, and Mixed isolates are referred to as the AS, VS, and MS populations, respectively. Nematode populations in microplots planted with resistant CB46 and inoculated with the Avirulent, Virulent, and Mixed isolates are referred to as the AR, VR, and MR populations, respectively. The six populations are referred to as the field populations.

Assays of life-history traits: For all experiments, eggs were recovered from nematode-infected roots by maceration of the total root system in 0.5% NaOCl solution (Hussey and Barker, 1973). To obtain juveniles for inoculation, eggs were hatched using a modified Baermann funnel technique consisting of a galvanized wire mesh screen lined with filter paper and nested in a petri dish incubated at 26 ± 1.0 °C.

Numbers of juveniles per root system, egg masses per root system, and eggs per root system were assayed using a modified growth-pouch technique (Ehlers et al., 2000) in full-size (12.5 by 15.0 cm) or half-size plastic growth-pouches containing paper inserts. Half-size pouches were made by cutting the paper insert into halves vertically, then sealing the plastic pouch vertically into halves with a plastic bag sealer.

Cowpea seeds were germinated directly in the pouches. At planting, approximately 5 ml or 13 ml of tap water was added to each half-size or full-size pouch, respectively. Pouches were placed in a plastic folder (two pouches per folder) held in a vertical position. Plants were maintained in a growth chamber at 16 hours daylight and 8 hours darkness, 27 ± 0.6 °C constant temperature, and watered once or twice per day until inoculation. A complete randomized design was used.

Hatch: Percentage hatch was determined for the original egg mass of an IFL and at generations 12 and 17 of the greenhouse isolates and 6 Virulent and 10 Avirulent IFL. Roots were wrapped in a moist paper towel, sealed in a plastic bag, and stored at 15 °C for 8 to 9 days before egg extraction. Hatching was performed in triplicate by placing 10,000 eggs on a galvanized wire-mesh screen lined with blue filter paper and incubating in a mist chamber. Based on preliminary tests indicating only trace amounts of hatch occurred after 7 to 10 days using this system, hatched J2 at generations 12 and 17 were collected for 10 days and counted.

Penetration: Penetration of susceptible 8685 and resistant CB46 roots was determined at generations 13 and 20 for the greenhouse isolates and IFL in a half-size pouch. In a preliminary study, 300 J2/root system was found to be an optimum inoculum level. Eggs were hatched for 3 days to obtain juveniles. Six replicates of each cowpea genotype were inoculated with 300 motile J2/root system 12 days after planting. Following inoculation, plants were watered once or twice per day. After 5 days, roots were stained with acid fuchsin (Byrd et al., 1983) and observed under the microscope to count the juveniles that penetrated the root system.

Reproduction of greenhouse isolates: Numbers of egg masses and eggs per root system, and eggs per egg mass on susceptible 8685 and resistant CB46 were determined at generations 2, 5, 13, 18, and 25. Experiments had a complete randomized design with 4 to 10 replicates. Missing data after generation 5 resulted from insufficient inoculum.

Eggs were hatched for 4 to 5 days prior to inoculation. Each root system was inoculated with 1,000 motile J2 11 to 13 days after planting, watered once or twice per day, and Hoagland's solution (Hoagland and Arnon, 1950) was added every 4 days. After 28 days, each pouch was inundated for 1 hour with 75 mg erioglaucine/liter (Sigma Chemical Co., St. Louis, MO) to stain egg masses for counting. Eggs per root system were assessed by maceration of the total root system in NaOCl solution (Hussey and Barker, 1973) and then counting eggs in three replicates of 0.3-ml aliquots taken from a total volume of 200 ml. Eggs per egg mass were calculated as the quotient of eggs per root system divided by egg masses per root system.

Reproduction of IFL: Numbers of egg masses and eggs per root system, and eggs per egg mass on susceptible 8685 and resistant CB46 were determined at generations 4, 12, 16, 21, and 27. Half-size pouches were used at generation 4. The procedure described to assess the reproduction of the greenhouse isolates was used except that a root system in a half-size pouch was inoculated with 900 J2. A complete randomized design with five to 10 replicates was used. At generation 12, IFL were categorized according to virulence to gene *Rk* and egg mass production on susceptible cowpea with a representative subset from each category advanced for further experimentation. Gaps in data after generation 12 are due to the extinction of the IFL or insufficient inoculum.

Reproduction of SDL: Numbers of egg masses and eggs per root system, and eggs per egg mass on susceptible 8685 and resistant CB46 were determined for progeny

of an IFL (single descent lines, SDL) at generation 22. Assays were made 35 or 36 days post-inoculation, with the same procedure used to assess reproduction of the greenhouse isolates.

Reproduction of field populations: Samples of the field populations collected from roots at harvest in 1998, 1999, and 2000 were evaluated for numbers of egg masses and eggs per root system and eggs per egg mass on susceptible H8-9 and resistant CB46. The procedure described to assess the reproduction of the greenhouse isolates was used except inoculation was with 900 J2/root system. Each nematode × cowpea genotype treatment was replicated 7 times.

Statistical analysis: Analysis of variance (ANOVA) was performed using Fisher's Protected LSD to isolate significant paired differences in mean values. Egg count and fecundity data were transformed to $\log_{10}$ (eggs + 1) and $\log_{10}$ [(eggs + 1)/egg mass], respectively, before analysis. The relationships among virulence and fitness parameters were evaluated using regression analysis. All statistical analysis was made using Minitab Release 13 statistical software (MiniTab Co., State College, PA).

TABLE 1. Percentage cumulative hatch of *Meloidogyne incognita* isofemale lines (IFL) and greenhouse isolates when founded and at generations 12 and 17.

Isolate or IFL	Generation		
	Founding egg mass	12	17
Av-Res.	nd[a] (1)	2.0 ± 2.9 b (113)	4.7 ± 6.1 bcde (132)
Vir-Susc.	nd (95)	2.5 ± 2.9 b (105)	nd (nd)
Vir-Res.	nd (95)	2.8 ± 2.9 bcd (61)	3.6 ± 6.1 ab (123)
Av-Susc.	nd (1)	4.4 ± 2.9 fg (5)	4.6 ± 6.1 bcde (2)
Vir-41S	2.0 (105)	3.4 ± 2.9 de	7.3 ± 6.1 f (118)
Av-06R	6.5 (90)	0.4 ± 2.9 a	D[b] (nd)
Av-06S	6.5 (90)	3.1 ± 2.9 cd	4.8 ± 6.1 bcde (56)
Av-04S	11.0 (0)	3.2 ± 2.9 cde	6.3 ± 6.1 ef (5)
Av-10S	18.1 (121)	2.8 ± 2.9 bcd	nd (nd)
Av-10R	18.1 (121)	5.3 ± 2.9 h	D (nd)
Av-03R	19.3 (0.3)	3.2 ± 2.9 cd	4.3 ± 6.1 bc (150)
Av-03S	19.3 (0.3)	4.5 ± 2.9 fgh	5.5 ± 6.1 cde (1)
Vir-55S	20.6 (128)	2.0 ± 2.9 b	3.2 ± 6.1 ab (nd)
Vir-59R	21.9 (47)	0.4 ± 2.9 a	6.3 ± 6.1 def (nd)
Vir-59S	21.9 (47)	3.0 ± 2.9 cd	nd (85)
Av-16S	24.1 (0.8)	4.0 ± 2.9 ef	3.3 ± 6.1 ab (7)
Vir-58S	28.8 (68)	0.8 ± 2.9 a	nd (85)
Av-28S	30.8 (3)	5.2 ± 2.9 gh	2.1 ± 6.1 a (6)
Vir-49S	59.3 (70)	6.3 ± 2.9 i	2.1 ± 6.1 a (57)
Av-19S	67.0 (1)	3.0 ± 2.9 cd	2.4 ± 6.1 a (58)

Values in parentheses are virulence percentages of lines at generation 4 (for founding egg masses), generation 12 (in hatch at generation 12 column), and generation 16 (in hatch at generation 17 column). Isolates or IFL preceded with "Av" or "Vir" were started from the Avirulent or Virulent isolates, respectively; those followed with "S" and "Susc" or "R" and "Res" were continuously cultured on susceptible or resistant cowpea, respectively. Each founding egg mass was hatched for 7 days. Generations 12 and 17 values are the mean of three replicates of 10,000 eggs hatched over 10 days. Means ranked in ascending order according to hatch of founding egg mass. Values shown plus or minus one standard error mean ($SE=\sqrt{(MSE/n_i)}$ calculated from the analysis of variance. Means within a column followed by the same letter are not different ($P \leq 0.05$) using Fisher's Protected LSD.

[a] nd = not determined.

[b] D = an IFL that became extinct among generations 12 and 17.

TABLE 2. Correlative relationships for hatch, penetration, and reproduction of *Meloidogyne incognita* isofemale lines (IFL) based on virulence status, host status, and generation.

Compared variables	Correlation[a]	R^2	*P*
Hatch			
Generation 12 vs. generation 17	—	0.23	0.136
Hatch generation 17 vs. virulence generation 16	—	0.00	0.976
Penetration			
Susceptible vs. resistant (generation 13)	0.912	0.83	0.000*
Susceptible vs. resistant (generation 20)	0.530	0.28	0.094*
Generation 13 vs. generation 20 (susceptible)	—	0.07	0.469
Generation 13 vs. generation 20 (resistant)	—	0.19	0.213
Penetration susceptible (generation 20) vs. virulence (generation 21)	—	0.13	0.435
Penetration resistant (generation 20) vs. virulence (generation 21)	0.701	0.49	0.079*
Reproduction[b]			
No. egg masses vs. virulence (generation 16)	0.459	0.21	0.007*
Fecundity vs. virulence (generation 16)	–0.340	0.12	0.053*
No. egg masses vs. virulence (generation 27)	—	0.01	0.387
Fecundity vs. virulence (generation 27)	–0.363	0.13	0.001*

* Significant ($P \leq 0.10$) relationship among the variables using simple regression analysis.

[a] Correlation not determined if not significant.

[b] Reproduction parameters on susceptible cowpea.

RESULTS

Hatch: Hatching rates ranged from 2.0% to 67.0% in the egg masses used to start the isofemale lines (IFL), 0.4% to 6.3% at generation 12, and 2.1% to 7.3% at generation 17 (Table 1). Percentage hatch varied ($P \leq 0.05$) among all IFL and among IFL started from the Avirulent or Virulent isolates at generations 12 and 17. However, hatch was not correlated among generations 12 and 17 for either the IFL (Table 2) or the greenhouse isolates (data not shown). Hatch of the Av-Susc isolate was higher ($P \leq 0.05$) than the other greenhouse isolates at generation 12 but did not differ at generation 17 (Table 1). Hatch of an IFL at generation 17 was not correlated with virulence at generation 16 (Table 2). Hatch was not affected by continuous culture on resistant compared to susceptible cowpea plants.

Penetration: Penetration into susceptible and resistant roots was similar at generation 13, ranging from 3.1% to 30.0% on susceptible and 1.3% to 37.1% on resistant plants (Table 3). Penetration into susceptible and resistant roots at generation 20 ranged from 14.3% to 44.4% and 5.0% to 48.4%, respectively (Table 3). Penetration into susceptible and resistant roots by an IFL showed a positive correlation at both generations 13 and 20 (Table 2). However, differences ($P \leq 0.05$)

TABLE 3. Percentage of *Meloidogyne incognita* juveniles of isofemale lines (IFL) and greenhouse isolates that penetrated roots of susceptible and resistant cowpea at generations 13 and 20.

	Susceptible		Resistant	
Isolate or IFL	13	20	13	20
Vir-55S	nd[a]	21.3 ± 12.1 bc (nd)	nd	16.9 ± 9.4 abc
Av-Susc.	nd (5)	33.7 ± 12.1 de (1)	nd	26.6 ± 9.4 bcde
Av-04S	3.1 ± 11.6 a	18.7 ± 12.1 abc (5)	1.3 ± 8.1 a	21.7 ± 9.4 abcd
Av-06S	6.2 ± 11.6 ab	19.8 ± 12.1 bc (25)	9.0 ± 9.9 abc	13.7 ± 9.4 ab
Av-28S	7.0 ± 10.4 ab	14.3 ± 12.1 ab (3)	4.1 ± 8.1 ab	5.0 ± 9.4 a
Av-03S	12.3 ± 9.5 abc	44.4 ± 12.1 e (1)	9.8 ± 8.9 bc	24.2 ± 9.4 bcde
Av-Res.	13.3 ± 9.5 bcd (113)	nd (132)	25.7 ± 8.1 f	nd
Vir-49S	15.1 ± 9.5 bcd	20.6 ± 12.1 bc (56)	15.5 ± 8.1 cde	32.5 ± 9.4 cdef
Av-10S	18.3 ± 9.5 cd	35.0 ± 12.1 de (118)	22.9 ± 8.1 e	48.4 ± 9.4 g
Av-16S	19.7 ± 9.5 cd	23.7 ± 12.1 bcd (7)	13.5 ± 8.1 cd	23.4 ± 9.4 bcd
Av-06R	21.6 ± 9.5 de	D[b] (nd)	16.1 ± 8.1 cde	D
Av-03R	22.2 ± 9.5 de	19.4 ± 12.1 bc (125)	19.2 ± 8.1 def	24.2 ± 9.4 bcde
Vir-41S	22.3 ± 9.5 de	26.6 ± 12.1 cd (105)	21.6 ± 8.1 ef	38.0 ± 9.4 ef
Av-19S	30.0 ± 9.5 e	26.6 ± 12.1 cd (20)	37.1 ± 8.1 g	19.8 ± 9.4 abcd

Values in parentheses are virulence of lines at generation 13 (in penetration at generation 13 column) and generation 21 (in penetration at generation 20 column). Percentage of juveniles in the root system 5 days after inoculation with 300 J2 per root system. Means ranked in ascending order of penetration on susceptible cowpea at generation 13. Means within a column followed by the same letter are not different ($P \leq 0.05$) using Fisher's Protected LSD. Values are plus or minus one standard error ($SE = \sqrt{(MSE)/[(1/n_i)+(1/n_j)]}$) calculated from the analysis of variance. At generation 13 on susceptible plants, values are means of four to six replicates and $df_e = 55$, where df_e denotes degrees of freedom for error. At generation 13 on resistant plants, values are means of four to six replicates and $df_e = 57$. At generation 20, values are means of six replicates.

[a] nd = not determined.

[b] D = an IFL that became extinct among generations 12 and 17.

among penetration into resistant and susceptible roots at generation 20 were observed for Av-03S, Av-10S, Vir-41S, and Vir-55S, despite the positive correlation. Penetration into susceptible or resistant roots at generation 13 was not correlated with penetration into susceptible or resistant roots at generation 20 (Table 2). Within these ranges, penetration of susceptible or resistant roots at generations 13 and 20 varied ($P \leq 0.05$) among IFL started from the Avirulent or Virulent isolates and among all IFL (Table 3).

IFL Av-03S and Av-03R, started from IFL 03 from the Avirulent isolate, differed ($P \leq 0.05$) in ability to penetrate susceptible roots at both generations and to penetrate resistant roots at generation 13 (Table 3). IFL Av-06S and Av-06R, started from IFL 06 from the Avirulent isolate, differed ($P \leq 0.05$) in ability to penetrate susceptible roots but not to penetrate resistant roots at generation 13. Penetration into susceptible and resistant roots was not different for Av-06S at generations 13 and 20 and for Av-06R at generation 13. Av-06R became extinct among generations 13 and 20.

Penetration of resistant roots by IFL at generation 20 was positively correlated with their virulence to gene *Rk* at generation 21, but penetration of susceptible roots at generation 20 was not correlated with virulence at generation 21 (Table 2). Host status did not affect the ability of an IFL to penetrate susceptible or resistant roots.

Reproduction of greenhouse isolates: Numbers of egg masses of the Vir-Susc and Av-Susc isolates on susceptible plants at generations 2 and 5 did not differ,

TABLE 4. Egg masses and eggs per egg mass on susceptible cowpea by *Meloidogyne incognita* greenhouse isolates over 25 generations.

	Generation				
Isolate	2	5	13	18	25
			Egg masses/root system		
Av-Susc	122 a (4)	125 a (1)	153[c] (5)	37 a (2)	nd (nd)
Vir-Susc	130 a (125)	132 a (90)	163 a (105)	nd (nd)	nd (nd)
Av-Res[a]	nd[b] (4)	nd (1)	136 a (113)	111 b (132)	186 b (90)
Vir-Res[a]	nd (125)	nd (90)	155[c] (61)	179 c (123)	114 a (105)
			Eggs/egg mass		
Av-Susc	311 b	369 a	567[c]	360 a	nd
Vir-Susc	140 a	291 a	384 a	nd	nd
Av-Res	nd	nd	397 a	450 a	379 b
Vir-Res	nd	nd	482[c]	444 a	304 a

Av-Susc and Vir-Susc are the Avirulent and Virulent isolates, respectively, continuously cultured on susceptible cowpea; Av-Res and Vir-Res are the Avirulent and Virulent isolates, respectively, continuously cultured on resistant cowpea. Values are means of four to ten replicates. Means under the same subheading within a column followed by the same letter are not different ($P \leq 0.05$) using Fisher's Protected LSD. Values in parentheses are virulence percentages at generation 2, 5, 12, 16, and 25 in egg masses per root system generation columns 2, 5, 13, 18, and 25, respectively.

[a] Av-Res and Vir-Res did not exist at generations 2 and 5.

[b] nd = not determined.

[c] Value given for reference but omitted from statistical analysis due to lack of replication.

whereas fecundity (eggs per egg mass) of Vir-Susc was lower ($P \leq 0.05$) than that of Av-Susc at generation 2 but not at later generations (Table 4). Differences in reproduction on susceptible plants among isolates were not found at generation 13. At generation 18, Av-Susc produced fewer egg masses than Av-Res and Vir-Res (Table 4). At generation 25 on susceptible plants, numbers of egg masses and eggs per egg mass were both lower ($P \leq 0.05$) for isolate Vir-Res than isolate Av-Res (Table 4).

Reproduction of field populations: In all seasons, no differences in numbers of egg masses produced on susceptible cowpea were observed among the field populations (AR, VR, MR) from microplots planted with resistant cowpea (Table 5). For populations grown on susceptible cowpea (AS, VS, MS), egg masses produced on susceptible cowpea did not differ from inoculation in 1996 to harvest in 1998, but in both the 1999 and 2000 seasons, the VS population produced fewer egg masses than the AS population (Table 5). Numbers of eggs per egg mass produced on susceptible plants did not show any consistent differences among populations over the five seasons. The AS, VS, and MS populations did not differ in 1996 and 1999. In 1998 VS produced more eggs per egg mass than AS and MS, and in 2000 AS and VS produced more eggs per egg mass than MS. The populations AR, VR, and MR did not differ in 1996, 1998, and 2000; only in 1999 did AR produce more eggs per egg mass on susceptible plants than VR and MR (Table 5).

TABLE 5. Egg masses and eggs per egg mass on susceptible cowpea in growth pouches of *Meloidogyne incognita* isolates over five seasons after inoculation into field microplots.

	Season			
Isolate[a]	1996 (Inoculum)	1998	1999	2000
		Egg masses/root system		
AS	125 a (1)[b]	72 a (4)	104 b (2)	160 b (2)
VS	132 a (92)	88 a (26)	38 a (40)	117 a (58)
MS	nd[c]	72 a (68)	91 b (39)	121 a (57)
AR	125 a (1)	91 a (175)	89 b (122)	165 b (129)
VR	132 a (92)	78 a (198)	79 b (127)	151 b (153)
MR	nd	123 a (116)	78 b (190)	146 b (172)
		Eggs/egg mass		
AS	369 a	304 b	294 bc	563 b
VS	291 a	432 c	301 bc	641 b
MS	nd	207 a	293 bc	412 a
AR	369 a	343 bc	339 c	463 a
VR	291 a	303 b	208 a	431 a
MR	nd	368 bc	245 ab	380 a

[a] AS, VS, and MS are the Avirulent, Virulent, and Mixed isolates, respectively, from microplots continuously planted to susceptible (S) cowpea. AR, VR, and MR are the Avirulent, Virulent, and Mixed isolates, respectively, from microplots continuously planted to resistant (R) cowpea. In 1996 (inoculum), values are the same because AS and AR were inoculated from the Avirulent isolate and VS and VR were inoculated from the Virulent isolate. Populations were not tested at harvest of the 1996 and 1997 seasons. Values are the means of seven replicates. Means under the same subheading within a column followed by the same letter are not different ($P \leq 0.05$) using Fisher's Protected LSD.

[b] Values in parentheses are percent virulence of the isolate at the given season.

[c] nd = not determined.

Reproduction of IFL at generations 4 and 27: Numbers of egg masses on susceptible plants at generation 4 of IFL from the Avirulent (27 IFL) and Virulent (14 IFL) isolates ranged from 8 to 91 and 13 to 61, respectively (data not shown); these ranges varied ($P \leq 0.05$) over a continuum within each of the two groups of IFL. The variation in egg mass production was not correlated with virulence to gene *Rk* at generation 4. The egg mass numbers at generation 4 of 12 IFL advanced to generation 27 are given in Table 6, together with the eggs per each egg mass used to start the IFL at generation 4.

Numbers of egg masses and eggs per egg mass on susceptible cowpea both differed ($P \leq 0.05$) among IFL at generation 27 (Table 6). Grouping the IFL according to their *Rk*-virulence profiles over the 27 generations (Petrillo and Roberts, 2005) did not indicate a relationship among egg mass production or fecundity with virulence to *Rk* (Table 6). However, regression analysis showed a significant negative correlation among fecundity and virulence on susceptible cowpea at both generations 16 ($P = 0.053$) and 27 ($P = 0.001$) (Table 2). The relationship among egg mass production and virulence was inconsistent, being positively correlated at generation 16 ($P = 0.007$) and not signifi-

TABLE 6. Egg masses and eggs per egg mass on susceptible cowpea in growth pouches of *Meloidogyne incognita* isofemale lines (IFL) over 27 generations grouped by virulence profiles.

	Generation 4		Generation 27	
Virulence group IFL	Egg masses/ root system	Eggs/ egg mass	Egg masses/ root system	Eggs/ egg mass
Type A				
Av-28S	8 a (3)	2434[a]	160 d (1)	467 de
Av-04S	47 bcd (0)	442	79 a (5)	nd[b]
Av-16S	65 cd (1)	278	135 d (1)	426 cde
Av-03S	67 d (0.3)	231	135 d (3)	471 e
Type B				
Av-10S	45 bcd (121)	669	67 a (117)	239 ab
Vir-41S	45 bcd (105)	267	137 d (118)	392 cde
Type C				
Av-03R	67 d (0.3)	231	127 cd (140)	357 cd
Type D				
Vir-49S	26 abc (70)	330	92 ab (0)	421 cde
Av-06S	51 cd (90)	370	D[c] (nd)	D
Type E				
Av-19S	9 a (1)	751[a]	80 a (2)	333 bc
Vir-59S	25 ab (47)	119	127 bcd (125)	323 bc
Vir-58S	61 d (68)	141	96 abc (104)	228 a

IFL preceded with "Av" or "Vir" were started from the Avirulent or Virulent isolates, respectively; those followed with "S" or "R" were continuously cultured on susceptible or resistant cowpea, respectively. IFL are grouped according to virulence profiles (Petrillo and Roberts, 2005): Types A and B are stable avirulent and virulent phenotypes, respectively. Type C changed from a low to high virulent phenotype on resistant cowpea. Type D changed from a high to low virulent phenotype on susceptible cowpea. Type E increased in virulence on susceptible cowpea. Values within a column followed by the same letter are not different at ($P \leq 0.05$) using Fisher's Protected LSD. Fecundity data transformed to $\log_{10}$ [(Eggs + 1)/egg mass] before statistical analysis. Values not followed by a letter were omitted from statistical analysis due to inadequate replication. Values in parentheses are percent virulence of the isolate at the given generation.

[a] Value skewed due to low egg mass count (see text for further explanation).

[b] nd = not determined.

[c] D = isofemale line that became extinct between generations 21 and 27.

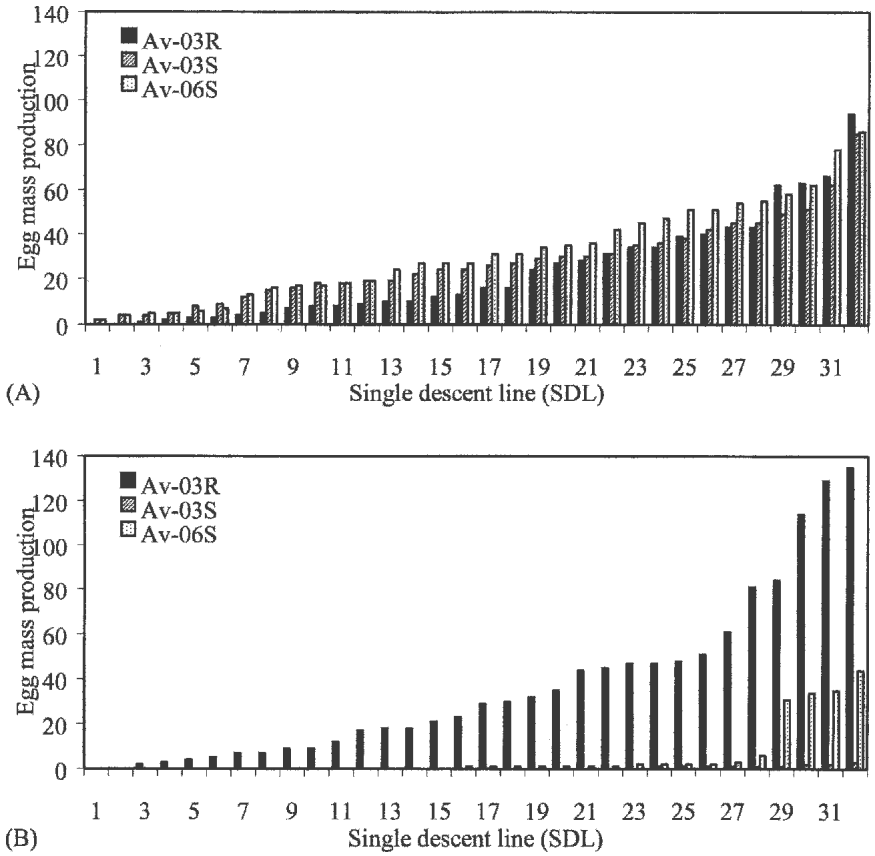

FIG. 1. Numbers of egg masses per root system on susceptible (A) and resistant (B) cowpea of *Meloidogyne incognita* single descent lines (SDL) started from isofemale lines (IFL) Av-03R, Av-03S, and Av-06S at generation 21. Isofemale lines Av-03S and Av-03R were started from the same isofemale lineage at generation 4 and cultured for 17 generations on susceptible and resistant cowpea, respectively.

cant at generation 27 ($P = 0.387$) (Table 2). Trends in reproduction factors were not apparent among generations 4 and 27 (data for generations 12, 16, and 21 not shown) for a given IFL.

Reproduction of SDL: Numbers of egg masses produced on susceptible and resistant cowpea plants varied ($P \leq 0.05$) among SDL started from an IFL (Fig. 1). The mean numbers of egg masses on susceptible plants of SDL cohorts from the three IFL did not differ ($P > 0.05$) due to the continuous, overlapping ranges of scores (Table 7). However, alignment of the values for each cohort of SDL in ranked order (Fig. 1) indicated that the SDL derived from the virulent Av-03R IFL cultured continuously on resistant cowpea had a lower overall reproductive potential than the SDL derived from avirulent IFL Av-03S and Av-06S, both of which were cultured repeatedly on susceptible cowpea plants. The SDL started from the virulent Av-03R had lower ($P \leq 0.05$) fecundity (eggs per egg mass) on susceptible cowpea than the SDL started from the avirulent Av-03S or Av-06S IFL (Table 7). The SDL from virulent Av-03R produced more ($P \leq 0.05$) egg masses on resistant cowpea than the SDL started from avirulent Av-03S or Av-06S. The fecundity of SDL from Av-03R was higher ($P \leq 0.05$) on resistant plants than on susceptible plants.

DISCUSSION

The current experiments were designed to determine the extent of variation in life-history traits among

TABLE 7. Egg masses and eggs per egg mass on susceptible and resistant cowpea of *Meloidogyne incognita* single descent lines (SDL) derived from isofemale lines (IFL) Av-03S, Av-03R, and Av-06S.

IFL parent of SDL	Egg masses/root system		Eggs/egg mass	
	Susceptible	Resistant	Susceptible	Resistant
Av-03S[a] (2)[b]	27.5 ± 3.8 a	0.4 ± 4.0 a	794 ± 60 b	nd[c]
Av-03R[a] (125)	23.6 ± 3.8 a	36.5 ± 4.0 b	490 ± 53 a	611[d]
Av-06S (27)	32.2 ± 3.8 a	2.1 ± 4.0 a	985 ± 68 b	nd

Values for egg masses on susceptible and resistant cowpea are means of 32 SDL. Values for eggs per egg mass on susceptible cowpea are means of 23, 30, and 18 SDL for Av-03S, Av-03R, and Av-06S, respectively. Values shown plus or minus one standard error mean (SE = $\sqrt{(MSE/[(1/n_i) + (1/n_j)])}$ calculated from the analysis of variance. Means down a column with the same letter are not different ($P \leq 0.05$) using Tukey's Test. Statistical analysis performed on eggs per egg mass data transformed to $\log_{10}$ [(Eggs + 1)/egg mass].

[a] IFL Av-03S and Av-03R were started from the same isofemale lineage at generation 4 but differ due to repeated culturing for 17 generations on susceptible and resistant cowpea, respectively.

[b] Values in parentheses are percent virulence at generation 21 of the IFL used as SDL parents.

[c] nd = not determined.

[d] Fecundity of Av-03R SDL was higher ($P \leq 0.05$) on resistant than susceptible cowpea using Tukey's Test. Mean of 30 replicates and standard error mean (SE = $\sqrt{(MSE/n_i)}$ equals plus or minus 40 calculated from the analysis of variance.

nematode isolates known to be avirulent or virulent to gene *Rk* in cowpea and, further, to determine the extent to which such variation is associated with the (a)virulence phenotype. We had shown previously that the original parental isolate ('Avirulent' in this study) had declined in virulence to gene *Rk* from a level of about 75% virulence when first collected from the field, to about 5% virulence following continuous culturing on susceptible tomato plants for at least 25 generations (Petrillo et al., 2006; Roberts and Matthews, 1995). This sequential loss of virulence suggested that virulence was associated with a negative cost to fitness of the virulence phenotype relative to avirulent forms. Our current analysis of isolates and lines grown under greenhouse and field conditions over multiple generations revealed extensive variation among isolates in hatch, root penetration, and reproduction traits, and that host status affected some of the observed variation.

Hatch varied among isolates or IFL at each generation, but within a lineage the variation was not constant from generation to generation. These results indicated that the variation in hatching, whereas important in determining the relative contribution of the lineage to the next generation, was not a heritable condition and was not correlated with the (a)virulence status of the isolate or lineage or to repeated culture on resistant or susceptible plants. Variation in hatch is expected as this trait is influenced by several abiotic and biotic factors. The level of hatch, which was generally quite low in the later generations, was not associated with virulence to *Rk* or to repeated culture on susceptible or resistant cowpea. Differences in hatching could reflect the percent of eggs in diapause (de Guiran, 1979) or numbers of nonviable eggs.

Rates of penetration into both susceptible and resistant cowpea roots varied among isolates and IFL at a given generation. Similar findings were reported among selected virulent *M. incognita* isolates and their avirulent parental isolate for penetration into susceptible and resistant tomato (Jarquin-Barberena et al., 1991). However, a given isolate or IFL penetrated into susceptible and resistant roots at a similar level. Equivalent penetration rates into roots of susceptible and resistant plants by *M. incognita* populations have been reported for several hosts (Creech et al., 1995; Lawrence and Clark, 1986; Windham and Williams, 1994). Thus, the resistance response of cowpea to avirulent or virulent *M. incognita* phenotypes did not involve ability to penetrate into resistant roots, confirming that expression of resistance by gene *Rk* is a post-infection process. Rates of penetration into susceptible and resistant roots by an isolate or IFL were not constant among generations, and as such the variation did not appear to be heritable. Root penetration was not correlated with the (a)virulence status of the isolates and lines, indicating that, like hatching, ability to penetrate roots did not contribute to the dynamic nature of virulence gain or loss in these *M. incognita* populations.

Variation in reproductive factors (i.e. egg mass production, egg production, and fecundity) on susceptible cowpea was often associated with virulence to gene *Rk*. Reproductive factors on susceptible and resistant cowpea varied among *M. incognita* IFL and isolates. Differences in reproductive factors on resistant plants were often correlated with levels of virulence expression. This is expected because virulence enables reproduction on resistant plants. We also observed that changes over multiple generations in reproductive factors on susceptible and resistant cowpea were often influenced by repeated culture on susceptible or resistant cowpea.

Of particular importance, lower rates of egg mass production and fecundity (eggs per egg mass) on susceptible cowpea were associated with higher virulence levels in some IFL, SDL, and isolates. This negative correlation among reproductive fitness and virulence in some nematode lines provides an explanation for the decrease of virulence to *Rk* that occurred over 25 generations in the original *M. incognita* field isolate when cultured continuously on susceptible tomato plants (Petrillo et al., 2006; Roberts and Matthews, 1995). Previously we had suggested that a fitness cost was associated with *Rk*-virulence in these *M. incognita* isolates. Our tests of the virulence dynamics of the IFL, SDL, and isolates over multiple generations provided evidence that the original field isolates were a mixture of both avirulent and virulent nematode lineages (Petrillo and Roberts, 2005). The current results indicated that a lower relative reproductive fitness of at least some virulent lineages, particularly in fecundity rates, was the primary reason for the frequency of virulent individuals in the population to decrease over multiple generations in the absence of the resistance gene. These findings are similar to previous reports of lowered reproductive ability on susceptible tomato in a selected *Mi*-virulent *M. incognita* isolate (Jarquin-Barberena et al., 1991) and in a *Bs2*-virulent strain of the bacterium *X. c. vesicatoria* on susceptible pepper (Kearney and Staskawicz, 1990). Diminished host range in *M. incognita* also has been associated with virulence—loss of ability to reproduce on susceptible pepper occurred in an *M. incognita* isolate selected for virulence to resistance gene *Mi* (Castagnone-Sereno et al., 1996).

Importantly, not all IFL, SDL, and isolates had reduced reproductive ability on susceptible cowpea associated with virulence. This supports earlier reports in which virulence did not decline when virulent *M. incognita* isolates were cultured on susceptible tomato for up to 9 months (about 6 generations) (Riggs and Winstead, 1959) or 18 generations (Castagnone-Sereno et al., 1993). No differences in fecundity and egg mass production on susceptible tomato were reported in a

selected virulent *M. incognita* isolate and the unselected avirulent parental isolate (Castagnone-Sereno et al., 1994, 1996). Clearly, virulence is not consistently associated with a specific reproductive level on susceptible cowpea. Rather, virulent phenotypes vary in reproductive ability on susceptible cowpea and this contributes to adaptation and maintenance of virulent genotypes within *M. incognita* populations.

Previous analysis of virulence of IFL derived from the Avirulent isolate that underwent reduced virulence on susceptible tomato (Petrillo and Roberts, 2005) revealed that 7% of the IFL were virulent, matching the virulence level of the isolate as a whole after 25 generations on tomato. One of these virulent IFL, Av-06S, was included in the present study and was used to develop a set of SDL at generation 22. Analysis of both the IFL and its derived cohort of SDL showed that its virulence was stable, with no association of reduced reproductive fitness. This lineage had remained virulent for at least 47 generations since the original field collection (25 generations on susceptible tomato plus 22 on susceptible cowpea). Presumably, the virulent lineages in the original isolate with lower reproductive fitness diminished or became extinct over 25 generations on tomato, whereas the few surviving virulent lineages such as Av-06S were adapted to survive and compete in the population, without the disadvantage of lowered reproductive fitness. The gradual or progressive loss of virulence over many generations can be explained by this pattern of fitness variation among the virulent lineages of the obligate mitotic parthenogenetic *M. incognita*. Thus, evidence is found for both stabilizing selection (see Caswell and Roberts, 1987) and long-term adaptation and maintenance of virulence in *M. incognita*.

The phenotypic variation observed in *M. incognita* in this study is comparable to the high level of variability in reproductive traits reported in some other obligate parthenogens, including *Artemia parthenogenetica* (Browne and Hoopes, 1990) and *Pycnoscelus surinamensis* (Niklasson and Parker, 1994). In molecular studies, genetic diversity in *M. incognita* isolates has been reported among isolates and within isolates (Semblat et al., 1998) and individuals (Whipple et al., 1998). In other obligate parthenogens, populations of *A. parthenogenetica* (Browne, 1992; Perez et al., 1994), *P. surinamensis* (Parker et al., 1977), and *Trypanosoma cruzi* (Tibayrenc and Ayala, 1999) were determined to be highly divergent within and among populations using various molecular techniques. The phenotypic variation observed in IFL and SDL of the present study can be compared to other parthenogenetic animals that display genetic variation. Mutation and multiple (i.e. hybrid) origin (Fu et al., 1998, 2000) have been reported as the underlying causes of such genetic diversity. Similar processes are likely to be involved in *M. incognita* because phenotypic changes often occurred within a short period.

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