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Isofemale Line Analysis of *Meloidogyne incognita* Virulence to Cowpea Resistance Gene *Rk*

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Abstract: Isofemale lines (IFL) from single egg masses were studied for genetic variation in *Meloidogyne incognita* isolates avirulent and virulent to the resistance gene *Rk* in cowpea (*Vigna unguiculata*). In parental isolates cultured on susceptible and resistant cowpea, the virulent isolate contained 100% and the avirulent isolate 7% virulent lineages. Virulence was selected from the avirulent isolate within eight generations on resistant cowpea (lineage selection). In addition, virulence was selected from avirulent females (individual selection). Virulence differed ($P \leq 0.05$) both within and between cohorts of IFL cultured for up to 27 generations on susceptible or resistant cowpea. Distinct virulence profiles were observed among IFL. Some remained avirulent on susceptible plants and became extinct on resistant plants; some remained virulent on resistant and susceptible plants; some changed from avirulent to virulent on resistant plants; and others changed from virulent to avirulent on susceptible plants. Also, some IFL increased in virulence on susceptible plants. Single descent lines from IFL showed similar patterns of virulence for up to six generations. These results revealed considerable genetic variation in virulence in a mitotic parthenogenetic nematode population. The frequencies of lineages with stable or changeable virulence and avirulence phenotypes determined the overall virulence potential of the population.

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

The root-knot nematode, *Meloidogyne incognita* (Kofoid and White) Chitwood, reproduces by obligate mitotic parthenogenesis (apomixis) and is polyploid (Triantaphyllou, 1981). Genetic information is passed from mother to progeny in the absence of meiosis and sexual reproduction, and the lack of recombination events suggests that the progeny of a single female should be genetically identical. Therefore, populations are thought to consist of lineages of genetically homogenous individuals although lineages may differ from each other, as indicated for some other clonal organisms. For example, the flagellate parasite *Trypanosoma cruzi,* vectored by triatomine bugs and the causal agent of Chagas' disease, is thought to consist of multiclonal populations comprised of clonally reproducing lineages that together define the population structure of a given region or within a vector or host (Oliviera et al., 1998). Genetic variability among clonal lineages of *T. cruzi* is believed to be a factor in differences in its host-parasite interaction (Macedo and Pena, 1998). Welch and Meselson (2000) examined every copy of a given gene present in the genome of individuals of asexual Bdelloid rotifers. They found extensive divergence at allelic sequences beyond that normally due to recombination and genetic drift, resulting in strikingly different genomes among individuals, contrary to the idea that individuals would be nearly clonal.

In a mitotic parthenogen, i.e., organisms such as *M. incognita,* field populations are a mixture of distinct isofemale lineages derived from single females cohabiting a given location. The general view that clonal propagation by mitotic parthenogenesis in *M. incognita* limits genetic variability and is therefore a disadvantage, by preventing the organisms from adapting and evolving rapidly (Blok et al., 1997a), is not supported by its documented behavior as a successful parasite (Trudgill, 1997; Trudgill and Blok, 2001). The possibility exists that populations of *M. incognita* are not comprised of lineages of genetically homogeneous individuals but are a composite of several diverging lineages arising from a common ancestor. Observations in related work revealed variability in virulence of *M. incognita* to the resistance gene *Rk* in cowpea. Populations of *M. incognita* in some fields with a cropping history of resistant cowpea were virulent, whereas in other fields *M. incognita* remained avirulent (Roberts et al., 1995). In addition, progressive loss of virulence to gene *Rk* occurred in an *M. incognita* isolate during about 25 generations of culture on susceptible tomato (Roberts and Matthews, 1995). Genetic diversity in these same and other *M. incognita* isolates was revealed by hierarchical analysis of mtDNA variable number tandem repeats, with only 7% diversity among the isolates but 60% and 30% of the total genetic diversity occurring within individuals and within isolates, respectively (Whipple et al., 1998).

Virulent isolates have been selected from avirulent populations of *M. incognita* that overcome *Mi*-mediated resistance in tomato (Castagnone-Sereno et al., 1996; Jarquin-Barberena et al., 1991; Riggs and Winstead, 1959). Castagnone-Sereno et al. (1994b) observed substantial variation among 63 isofemale lines of *M. incognita* for heritable virulence to *Mi* in tomato; two lineages increased in virulence and the remainder expressed little or no response to selection, 62% of which became extinct within three generations. Virulence to gene *Mi* selected from a generally avirulent population was stable in the absence of *Mi* (Jarquin-Barberena et al., 1991), and this stable virulence was confirmed over 18 generations (Castagnone-Sereno et al., 1993). Overall, several lines of evidence from different studies of (a)virulence to gene *Mi* suggest that virulence may arise multiple times and is not based on a single mutational

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event, but rather is based on different genetic determinants depending on the population or lineage of origin (Abad et al., 2003).

The objective of this work was to determine the variation in virulence to resistance gene *Rk* in cowpea of two populations of *M. incognita*. We studied virulence levels over multiple generations of culture on resistant and susceptible plants in (i) the parental isolates and their subcultures on resistant and susceptible plants, (ii) cohorts of isofemale lines derived from these parental isolates, and (iii) single descent lines derived from some of the characterized isofemale lines.

Materials and Methods

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

Nematode 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."

Parental isolates: The Avirulent and Virulent isolates were cultured continuously in the greenhouse on susceptible 8685 for five 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 was inoculated onto resistant CB46 (Av-Res). 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.

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. Isofemale lines 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).

Virulence assays: All assays were performed 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 and 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 an upright vertical position. Plants were transferred to a growth chamber maintained at 16 hours daylight and 8 hours darkness, 26.7 ± 0.6 °C constant temperature, and watered as needed until inoculation.

Eggs were recovered from roots by maceration of the total root system in 0.5% NaOCl solution (Hussey and Barker, 1973). When second-stage juveniles (J2) were used for inoculum, eggs were hatched for 4 to 5 days prior to inoculation by a modified Baermann funnel technique, using filter paper-lined mesh screens nested in petri dishes incubated at 26 ± 1.0 °C. Root systems were inoculated 11 to 13 days after planting. Specific inoculum quantities are described for each experiment.

Inoculated plants were watered as needed, and Hoagland's solution (Hoagland and Arnon, 1950) was added to plants 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. Egg production per root system was assessed by counting in three replicate aliquots eggs recovered after maceration of the total root system in 0.5% NaOCl solution (Hussey and Barker, 1973). Susceptible 8685 was used as a control to determine the total number of egg masses produced in a compatible interaction and to calculate percentage virulence.

Experiment one: To test for variability in virulence between the parental isolates in the presence or absence of resistance gene *Rk* over successive generations, virulence assays were performed at generations 2, 5, 13, 18, and 25. Plants in full-size pouches were inoculated with 1,000 motile J2/root system. Experiments were performed using a complete randomized design with five to 10 replicates. Fewer replications in some treatments

after generation 5 were due to insufficient inoculum for 10 replicates.

Experiment two: To test for variability in virulence among isofemale lines in the presence or absence of resistance gene *Rk* over successive generations, virulence assays were performed at generations 4, 12, 16, 21, and 27. The assay at generation 4 was performed in half-size pouches inoculated with 900 motile J2/root system. After generation 12, a subset of IFL was used representing different levels of virulence expression and egg-mass production on susceptible cowpea based on generation 4 results. Assays at generations 16, 21, and 27 were performed on the subset of IFL using a full-size pouch inoculated with 1,000 motile J2/root system, in a complete randomized design with five to ten replicates. Experiments performed in half-size pouches generally resulted in less egg-mass production per root system compared with full-size pouches, even though root size and weight were not different at time of inoculation and termination. Missing data at generations 16, 21, and 27 were due the extinction of the IFL or insufficient inoculum for 10 replicates.

Experiment three: To determine variability in progeny of an isofemale line, single descent lines (SDL) were established from three IFL (Av-03S, Av-03R, Av-06S) at generation 22. Each SDL was established by inoculating one egg mass obtained at generation 22 onto a 16- or 17-day-old cowpea root system. The egg mass was placed in contact with the primary root about 2.5 cm below the top of a half-size growth pouch. From each IFL, 32 SDL were established on resistant CB46 plants and 32 on susceptible 8685 plants. The SDL were cultured repeatedly on susceptible or resistant cowpea over six successive generations. Egg masses produced on resistant CB46 were each transferred onto a separate CB46 plant at each generation. Each egg-mass transfer on resistant CB46 was matched with an egg mass produced on susceptible 8685 transferred separately onto an 8685 plant. Cultures were maintained under the same conditions of the virulence assays. Numbers of egg masses per root system were counted 35 or 36 days after inoculation.

Statistical analysis: An analysis of variance (ANOVA) was performed using Fisher's Protected LSD test to isolate significant paired differences in mean values. Statistical analysis of virulence data was performed on $\log_{10}$ [(egg masses produced on resistant cowpea) + 1] – $\log_{10}$ (mean egg masses produced on susceptible cowpea). All statistical analysis was made using Minitab Release 13 statistical software (MiniTab Co., State College, PA).

Results

Experiment 1—virulence profiles in the parental isolates: Mean egg masses per susceptible plant produced by the parental isolates and their subcultures at generations 2, 5, 13, 18, and 25 ranged from 122 to 130, 125 to 132, 136 to 163, 37 to 179, and 114 to 185, respectively. Virulence differed ($P \leq 0.05$) among the four subcultures of the parental isolates on resistant plants at generations 2, 5, and 18 (Fig. 1). Comparisons at generations 2 and 5 were between isolates Av-Susc and Vir-Susc only because their subcultures maintained on resistant plants (Av-Res and Vir-Res) were not initiated until after generation 5. Virulence of isolate Av-Susc was lower ($P \leq 0.05$) than virulence of the other isolates at all assay dates, remaining low (<7%) and relatively unchanged (stable avirulence) during 18 generations of culture on susceptible cowpea.

Virulence of isolates Vir-Susc and Vir-Res remained high (61% to 121%) during 13 and 25 generations, respectively (Fig. 1). This stable virulence was found during repeated culture on either susceptible (isolate Vir-Susc) or resistant (isolate Vir-Res) cowpea.

Virulence in avirulent isolate Av-Res increased from 1.2% to 113% within eight generations of culture on resistant CB46 (Fig. 1). Virulence of Av-Res was similar to that observed for Vir-Susc at generation 13. Thereafter, the virulence of isolate Av-Res declined from generations 18 to 25, but the overall level of virulence remained high and did not differ from that expressed in the Vir-Res isolate at generations 18 and 25. No differences in virulence were observed between isolates Av-Res and Vir-Res at generations 18 and 25.

Experiment 2—virulence profiles of isofemale lines at generation 4: Mean egg masses per susceptible plant at generation 4 produced by the 27 IFL started from the Avirulent isolate and maintained on susceptible 8685 plants ranged from 8 to 91. Variation in virulence was observed at generation 4 ($P \leq 0.05$) among these 27 IFL (Table 1). Two IFL expressed higher ($P \leq 0.05$) virulence (89.6% to 121.0%) than the other IFL, 16 IFL expressed low virulence that varied ($P \leq 0.05$) from

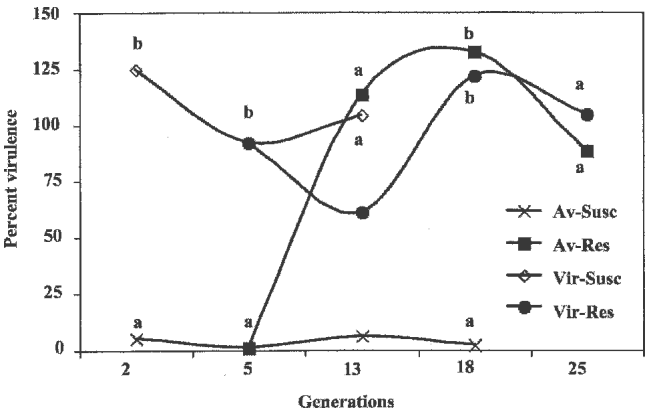

Fig. 1. The percent virulence to gene *Rk* of *Meloidogyne incognita* parental isolates and subcultures maintained continuously on susceptible or resistant (*Rk*) plants for up to 25 generations. Isolates Av-Res and Vir-Res did not exist at generations 2 and 5. Isolates Av-Susc and Vir-Res were omitted from statistical analysis at generation 13 due to insufficient replication. Values at the same generation with the same letter are not different at $P \leq 0.05$ using Fisher's Protected LSD.

TABLE 1. Percentage virulence at generation 4 of *Meloidogyne incognita* isofemale lines (IFL) started from the 'Avirulent' and 'Virulent' parental isolates.

IFL (Avirulent)	% Virulence	IFL (Virulent)	% Virulence
Av-10	121.0 ± 20.7	Vir-50	140.6 ± 21.6
Av-06	89.6 ± 17.9	Vir-55	128.2 ± 21.6
Av-23	5.6 ± 1.7	Vir-53	127.6 ± 26.4
Av-08	5.3 ± 1.4	Vir-56	126.4 ± 24.4
Av-26	3.6 ± 1.6	Vir-60	124.0 ± 26.4
Av-28	3.2 ± 1.6	Vir-57	121.2 ± 28.9
Av-17	2.9 ± 1.6	Vir-46	117.3 ± 21.6
Av-11	2.3 ± 1.5	Vir-41	104.8 ± 26.4
Av-13	2.2 ± 1.8	Vir-51	82.6 ± 22.9
Av-19	1.4 ± 1.6	Vir-45	77.2 ± 24.4
Av-24	1.4 ± 1.6	Vir-49	70.1 ± 22.9
Av-21	1.3 ± 1.7	Vir-34	68.3 ± 28.9
Av-16	0.8 ± 1.4	Vir-58	67.8 ± 22.9
Av-05	0.6 ± 1.5	Vir-59	46.9 ± 21.6
Av-02	0.4 ± 1.4	Vir-31	D
Av-27	0.3 ± 1.5	Vir-32	D
Av-03	0.3 ± 1.5	Vir-33	D
Av-14	0.3 ± 1.5	Vir-35	D
Av-01	0.0	Vir-36	D
Av-04	0.0	Vir-37	D
Av-07	0.0	Vir-39	D
Av-12	0.0	Vir-40	D
Av-15	0.0	Vir-42	D
Av-18	0.0	Vir-43	D
Av-22	0.0	Vir-44	D
Av-25	0.0	Vir-47	D
Av-29	0.0		

Values are means of 5 to 10 replicates, plus or minus one standard error ($SE = \sqrt{(MSE/[(1/n_i)+(1/n_j)])}$ calculated from the analysis of variance. For Avirulent and Virulent IFL, degrees of freedom for error (df_e) were 131 and 88, respectively.

D = a starting egg mass in which egg hatch did not occur over 7 days; therefore an IFL was not established.

TABLE 2. Mean egg masses per resistant cowpea plant of *Meloidogyne incognita* isofemale lines over 27 generations grouped by virulence profiles.

	Generation			
Isofemale line[a]	4	16	21	27
Type A				
Av-28S	0.3b	6.8a	2.8a	1.7ab
Av-04S	0 a	5.3a	nd	3.7ab
Av-16S	0.5b	10.7a	nd	2.1ab
Av-03S	0.2b	nd[b]	nd	3.6ab
Type B				
Vir-41S	47.3d	nd	156.5c	162.8d
Av-10S	53.8d	nd	nd	78.3c
Type C				
Av-03R	0.2b	259.0c	222.6d	176.2d
Type D				
Vir-49S	18.5c	66.7b	7.6ab	0.7a
Av-06S	45.3d	nd	44.3b	D[c]
Type E				
Av-19S	0.1b	70.7b	29.4ab	4.4b
Vir-59S	11.7bc	nd	nd	156.3d
Vir-58S	41.4d	nd	nd	98.2c

[a] "Avir" or "Vir" are isofemale lines derived from the Avirulent or Virulent parental isolate, respectively. "S" or "R" following an IFL represents continuous culture on a susceptible or resistant cowpea, respectively. Isofemale lines grouped according to virulence profiles (see Fig. 2). Types A and B are stable for avirulence and virulence, respectively. Type C changed from low to high virulence. Type D changed from high to low virulence. Type E increased in virulence in the absence of selection pressure. Values within a column followed by the same letter are not different at $P \leq 0.05$ based on Fisher's Protected LSD test.

[b] Not determined at this generation.

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

0.3% to 5.6% virulence, and 9 IFL did not reproduce (0% virulence) on resistant cowpea. The two highly virulent IFL had virulence levels within the range of the virulence expressed by IFL started from the Virulent isolate (Table 1).

Variation in virulence was observed at generation 4 among the 26 IFL that were started from the Virulent isolate and maintained on susceptible 8685 plants ($P \leq 0.05$) (Table 1). Eggs within egg masses from 12 of these IFL were unable to hatch during 7 days of incubation and were considered extinct at generation 1. The remaining 14 IFL from the Virulent isolate varied ($P \leq 0.05$) from 47% to 141% virulence at generation 4 (Table 1). These 14 IFL produced from 13 to 61 mean egg masses per susceptible plant at generation 4.

Experiment 2—virulence profiles over 27 generations: Mean egg masses per susceptible plant produced by 12 IFL in virulence tests at generations 4, 16, 21, and 27 ranged from 8 to 67, 106 to 175, 108 to 176, and 67 to 160, respectively. Only IFL Av-06S showed a significant change in egg mass production on susceptible plants that influenced the virulence rating. The mean egg masses per resistant plant of the 12 IFL in these tests are given in Table 2. Six virulence profiles were observed among IFL over 27 generations. Three of these profiles were described previously for the parental isolates (Fig. 1), being stable avirulence, stable virulence, and a change from low to high virulence expression. These profiles are shown for the IFL in Figures 2A, B, and C, respectively. The stable avirulence profile was observed in four IFL (Av-03S, Av-04S, Av-16S, and Av-28S) when cultured on susceptible cowpea for up to 27 generations (Fig. 2A). Virulence did not differ ($P > 0.05$) within each line between sampling times. Two IFL (Av-10S and Vir-41S) maintained a stable profile of 105% to 120% virulence that did not differ ($P > 0.05$) among sampling times during 27 generations on susceptible 8685 plants (Fig. 2B). Isofemale line Av-03R changed ($P < 0.05$) from low to high (0.3% to 148%) virulence within 12 generations on resistant CB46 (Fig. 2C). Thereafter, Av-03R virulence remained unchanged ($P > 0.05$) from generations 16 to 27.

Three virulence profiles found in the IFL were not observed in the parental isolates. In IFL Vir-49S and Av-06S a change ($P < 0.05$) from high to low virulence occurred during 21 and 27 generations, respectively, on susceptible 8685 (Fig. 2D). The decline in virulence differed between IFL Vir-49S and Av-06S. Isofemale line Vir-49S decreased in ability to reproduce on resistant plants from generations 16 to 27 while retaining ability to reproduce on susceptible plants. In contrast, Av-06S

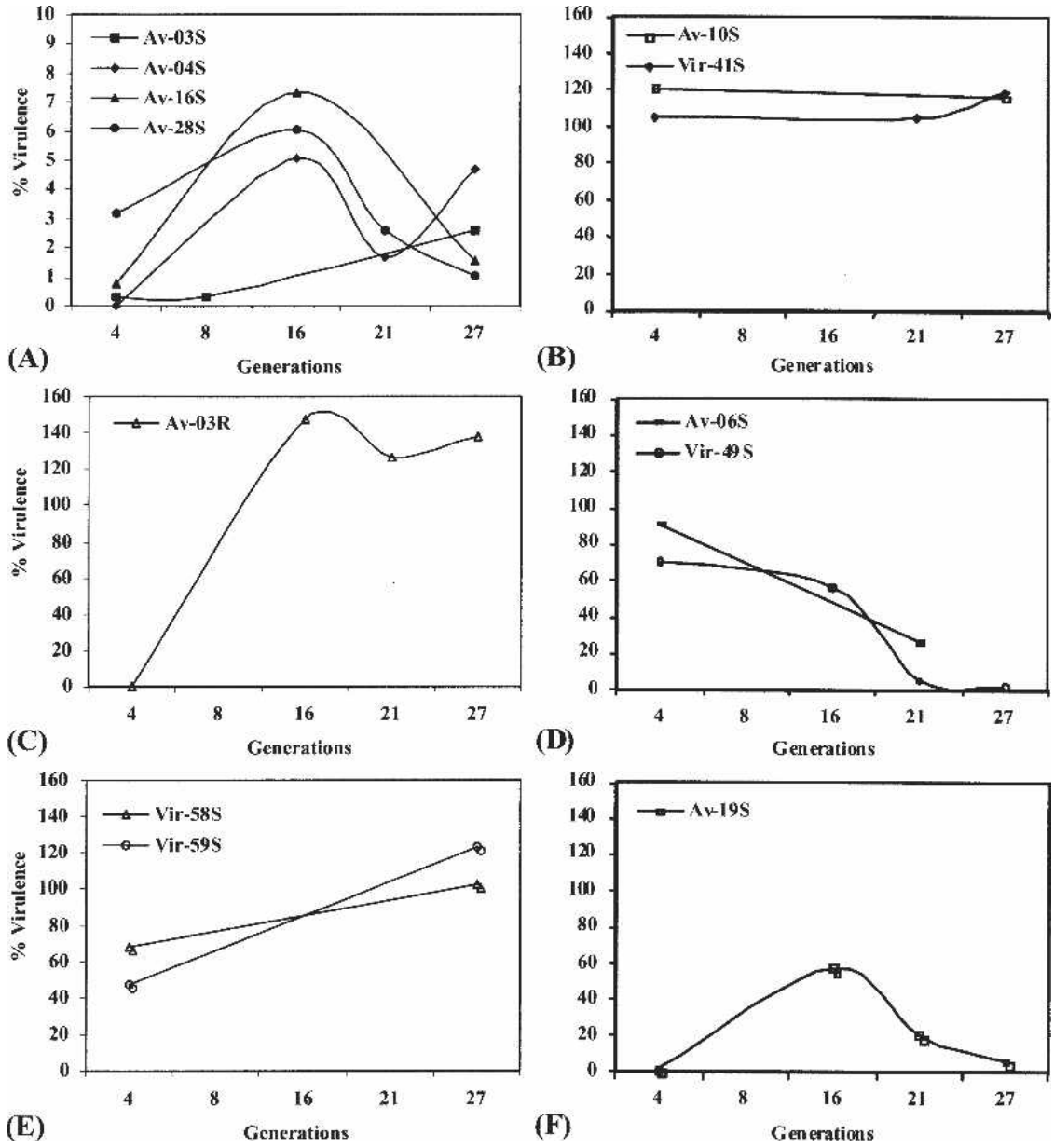

FIG. 2. Virulence profiles of *Meloidogyne incognita* IFL over 27 generations. A) Stable avirulence. B) Stable virulence. C) Change from low to high virulence. D) Change from high to low virulence. E) Increase in virulence in the absence of selection. F) Increase then decline in virulence in the absence of selection. Avir and Vir lines derived from the Avirulent and Virulent isolates, respectively. S and R indicate continuous culture on susceptible and resistant cowpea, respectively.

produced egg masses on resistant cowpea while its egg-mass production on susceptible cowpea increased nearly 4-fold. In another profile, virulence in IFL Vir-58S and Vir-59S increased ($P < 0.05$) from 67% to 102% and from 45% to 122%, respectively, during 27 generations on susceptible 8685 (Fig. 2E). In yet another virulence profile, IFL Av-19S increased (generation 4 to 16; $P < 0.05$) then declined (generation 16 to 21 and 21 to 27; $P < 0.05$) in virulence during 27 generations on susceptible 8685 (Fig. 2F). The changes in virulence of Av-19S were due to ability to produce egg masses on resistant cowpea.

Experiment 2—extinction of isofemale lines: Extinctions occurred at different rates in the cohorts of IFL of the Avirulent (AS, AR) and Virulent (VS, VR) isolates during 25 generations following subdivision and culturing on resistant and susceptible cowpea (Fig. 3). In 24 of the 25 IFL started from the Avirulent isolate that expressed low virulence initially (Table 1), extinction occurred within eight generations during attempted culture on resistant cowpea (AR IFL). Only one IFL was able to overcome gene *Rk* (Fig. 3). The two virulent AS IFL cultured on resistant cowpea (Av-06R, Av-10R) both became extinct by generation 17. Among the 27 AS IFL cultured on susceptible 8685 plants, only 3.7% extinction occurred at generation 4 and 11.1% by generation

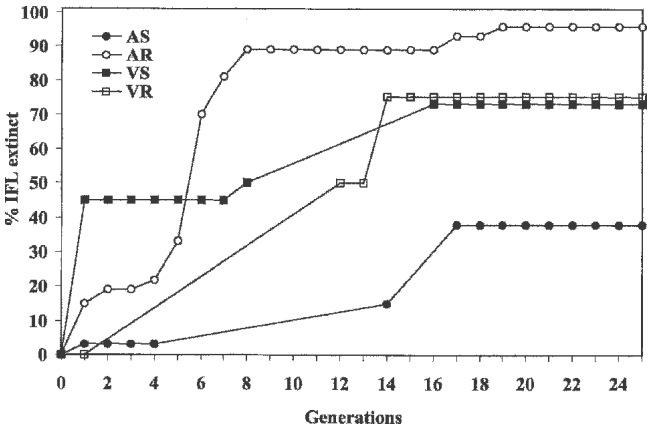

FIG. 3. The percent of *Meloidogyne incognita* IFL of the Avirulent and Virulent isolates that became extinct during 25 generations after being subdivided and cultured on susceptible and resistant cowpea. AS and AR IFL started from the Avirulent isolate cultured on susceptible and resistant cowpea, respectively; VS and VR IFL started from the Virulent isolate cultured on susceptible and resistant cowpea, respectively.

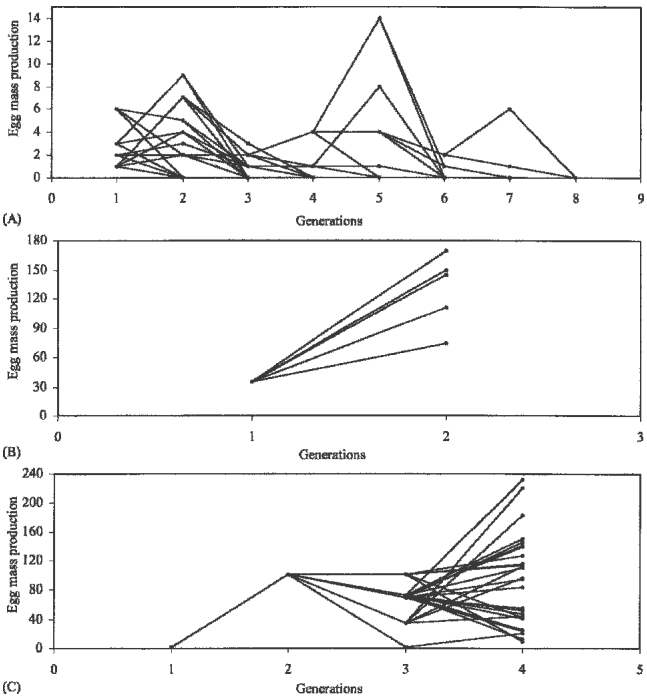

FIG. 4. *Meloidogyne incognita* egg masses per root system on resistant cowpea of 32 single descent lines (SDL) started from IFL Av-06S. A) Lineages that became extinct (stable avirulence) after 2 to 8 generations. B) An SDL with high egg mass production indicated in five of its randomly selected progeny at generation 2 (stable virulence). C) An SDL with initially low egg mass production (generation 1) whose progeny ranged from low to high egg mass production in generations 2 to 4 (change from low to high virulence). When available, up to six egg masses were transferred to the next generation from each lineage.

14, rising to 37% at generation 17 and remaining at that level to generation 25 (Fig. 3). Of the two virulent AS IFL cultured on susceptible cowpea, one (Av-06S) became extinct by generation 17 and the other (Av-10S) was viable at generation 25. Of the 26 IFL from the Virulent isolate on susceptible 8685 plants (VS IFL), 46% were extinct at generation 1 because viable progeny were not produced. The rate of extinction rose to 73% at generation 16 and remained at that level to generation 25 (Fig. 3). Of the 26 IFL from the Virulent isolate on resistant CB46 plants (VR IFL), 50% were extinct at generations 12 and 13, rising to 75% at generation 14 and remaining at that level to generation 25 (Fig. 3).

Experiment 3—virulence in single descent lines: Single descent lines (SDL) were produced from IFL and assayed for virulence to confirm results of the IFL experiments. Variation ($P \leq 0.05$) in egg-mass production on resistant cowpea was observed among SDL of an IFL. Percent extinction of SDL started from Av-06S, Av-03S, and Av-03R was 47%, 72%, and 6%, respectively, after one generation on resistant cowpea.

Patterns of egg-mass production on resistant cowpea of the 32 SDL started from Av-06S resembled previously described virulence profiles of IFL, including stable avirulence, stable virulence, and change from low to high virulence during culture on resistant CB46 (Fig. 4). Eighty-four percent (27) of Av-06S SDL became extinct within eight generations on resistant CB46 (Fig. 4A). Four SDL (12.5%) from Av-06S were virulent at generation 1, producing >30 egg masses on resistant plants (not shown). Five egg masses from one of the four virulent SDL were advanced to generation 2. Virulent progeny were produced from all five egg masses, confirming virulence in this SDL (Fig. 4B).

One (3%) of the Av-06S SDL at generation 1 was avirulent and produced one egg mass on resistant CB46; this egg mass produced 101 egg masses on resistant cowpea at generation 2 (Fig. 4C). Six of the 101 egg masses were advanced to generation 3, and their progeny produced from 2 to 101 egg masses (Fig. 4C). Two to five egg masses from each of the six lineages (total of 27 egg masses) were advanced to generation 4, and their progeny produced 9 to 221 egg masses on resistant plants (Fig. 4C). This SDL was now virulent and produced egg masses on resistant CB46 within a similar range to the numbers of egg masses produced on susceptible cowpea. The SDL from Av-03S produced a similar range of egg masses on resistant cowpea as shown for SDL of Av-06S in Figure 4A. All SDL started from Av-03S became extinct within six generations on resistant cowpea. In contrast, 91% of the SDL from Av-03R were able to produce egg masses on resistant CB46 at levels >10% of the mean egg mass production on susceptible 8685 (data not shown). The two cohorts of SDL from Av-03S and Av-03R (IFL derived from the same egg mass 22 generations earlier) differed ($P \leq 0.05$) in frequency of virulent individuals.

Discussion

In related work we had observed variability in virulence of *M. incognita* to the resistance gene *Rk*. Populations of *M. incognita* in some fields with a cropping history of resistant cowpea were virulent whereas in other fields *M. incognita* remained avirulent (Roberts et al., 1995). In addition, progressive loss of virulence to gene *Rk* occurred in an *M. incognita* isolate during about 25 generations of culture on susceptible tomato (Roberts and Matthews, 1995). These observations do not support the general dogma that reproduction by obligate mitotic parthenogenesis in *M. incognita* is a disadvantage, by preventing the organism from adapting and evolving rapidly and thereby being an evolutionary dead-end (Blok et al., 1997a). In the present study, considerable variation was found in isolates of *M. incognita,* the dynamic profiles of which over many generations indicated a complex process of positive and negative selection, maintenance, and balance of avirulent and virulent genotypes. These results showed that selection of reproductively viable virulent individuals from avirulent *M. incognita* isolates of field populations can occur when these populations are exposed repeatedly to host plants carrying the *Rk* gene. Further, our experiments with isofemale and single descent lines demonstrated that virulent lines can be selected from the progeny (egg mass) of an avirulent female.

The selection of virulent individuals from avirulent populations of *M. incognita* exposed to resistant plants also has been reported for resistant tomato (Jarquin-Barberena et al., 1991; Netscher, 1976; Riggs and Winstead, 1959) and pepper (Castagnone-Sereno et al., 1996). Genetic variation for interaction with the *Mi* gene in tomato was found among individuals of *M. incognita* isolates (Castagnone-Sereno et al., 1994b), whereas molecular evidence of genetic variation in the Avirulent isolate used in this study was presented by Whipple et al. (1998) (isolate D in their study) and for other *M. incognita* isolates (Blok et al., 1997b; Castagnone-Sereno et al., 1994a; Dalmasso et al., 1991). Studies on sexually reproducing nematodes showed that selection of virulent populations from avirulent populations occurred during repeated exposure of *Globodera pallida* to resistant *Solanum* hybrids (Schouten and Beniers, 1997; Turner et al., 1983; Turner and Fleming, 2002) and of *Heterodera avenae* to a resistant oat cultivar (Lasserre et al., 1996). Therefore, selection for virulence to resistance genes deployed in crop cultivars can occur in both sexual and asexual nematode forms, although its occurrence will depend on the specific nematode-plant interaction in each case.

Several dynamic forces appear to be at work in guiding the genetic constitution of *M. incognita* populations. The analysis of the Avirulent isolate revealed that it is comprised of a mixture of virulent and avirulent lineages, as indicated by isofemale line analysis. The frequency of virulent lineages will determine the overall virulence potential of the population. Thus, the low frequency of virulent lines in the Avirulent isolate corresponds closely with the index of virulence for the parent isolate, as does the high frequency of virulent lines in the Virulent isolate. It follows that at any given time of cropping season or sampling, the level of virulence of the field population to the resistance gene will be determined largely by the frequency and magnitude of the virulent and avirulent component lineages in the population. Phenotypes (lineages) that occur within a population at low frequencies may be masked at the population level and go undetected. The variation observed among isofemale lines on resistant cowpea confirms similar findings of Castagnone-Sereno et al. (1994b), who reported significant variation among IFL of *M. incognita* in their ability to reproduce on tomato with gene *Mi*.

Our finding from analysis of IFL that progeny of a single egg mass are not identical for their virulence phenotype and that these differences are heritable was confirmed by tests with the single descent lines from IFL Av-06S. One viable egg mass on a resistant plant from the progeny of a single avirulent female led to a virulent lineage, within which a range of virulence phenotypes was apparent after four generations. Presumably the virulence of such an individual arose by mutation, transposition, or some other gene rearrangement of the avirulent form. These results indicate that progeny of a single female are a mixture of genotypes representing the component clonal lineages that can adapt and be selected, thus changing the frequency of genes in the population and leading to changes in the overall constitution of the gene pool. All the other SDL started from the same avirulent IFL failed to develop on resistant cowpea and were extinct within 8 generations. These SDL profiles matched the results of Castagnone-Sereno et al. (1994b), who tested 31 IFL independently selected over 4 generations for virulence to resistance gene *Mi* in tomato. They found two distinct virulence profiles, in which two IFL showed a rapid increase in virulence and the remaining IFL expressed little or no response to selection.

The variation across IFL was maintained over 27 generations in our study, even though the frequency of virulent individuals within a given IFL changed due to host selection. This suggests that genetic variation is maintained within a population, and individual lineages within the population are adapting to selective forces, thus leading to sustained existence of the population as a whole. The virulence profiles of the IFL and SDL indicated that populations are made up of a genetically diverse group of individuals having unique genomes that lead to the perpetuation of a range of virulence phenotypes within a population. Our finding of

both stable and unstable virulent lineages supports the suggestion from other studies that virulence may arise multiple times and is based on different genetic determinants (Abad et al., 2003).

We identified at least five virulence profiles by observing the IFL over 27 generations, and they revealed a dynamic process of maintaining genetic variability for virulence. Some lineages had stable avirulence, apparently lacking the virulence condition initially and not responding to selection from the resistance gene. They became extinct when challenged with the resistance gene, unable to reproduce on resistant plants. Populations of *M. incognita* in fields in which virulence is not selected could consist of lineages with only this profile.

In a second profile, stable virulence, virulent IFL retained virulence when cultured for 27 generations in the absence of the selection pressure. It is not known whether the relative fitness of these virulent lineages in mixed populations with avirulent lineages would allow their perpetuation over multiple generations. Castagnone-Sereno et al. (1993) found virulence to gene *Mi* in tomato was stable in laboratory-selected and wild virulent *M. incognita* isolates after 18 generations of culturing on susceptible tomato. Similar results have been reported for other *M. incognita* populations (Castagnone-Sereno et al., 1994b, 1996; Jarquin-Barberena et al., 1991; Riggs and Winstead, 1959) and for *G. pallida* (Turner, 1990). Jarquin-Barberena et al. (1991) selected two *Mi*-virulent individuals from a phenotypically avirulent population through selection on resistant tomato for 12 and 21 generations. This virulence was stable during nine generations on susceptible plants in the absence of the selection pressure from gene *Mi*.

The third profile was a change from low to high virulence on resistant plants, as described for the Av-Res parental isolate due to lineage selection, and in IFL Av-03R and one SDL from IFL Av-06S due to individual selection. This result confirms that virulence can be actively selected either from a mixture of lineages or from the progeny of an avirulent female. A fourth profile was of virulent IFL that decreased in virulence when cultured for 17 to 21 generations in the absence of the selection pressure on susceptible plants. This decrease in virulence frequency suggests that there is an associated fitness cost with virulence in at least some virulent lines. However, the stable virulence of some lineages indicates that not all virulent forms will diminish without selection from resistance gene exposure. We reported the significant reduction, but not total loss, of virulence to gene *Rk* in the Avirulent isolate used in this study, when cultured in the absence of gene *Rk*. Those observations suggested that virulence was associated with a reduced fitness as part of a stabilizing selection (Roberts and Matthews, 1995). Indeed, lower relative fitness of virulent compared to avirulent individuals in mixed populations would explain the gradual loss of virulence. We found lower reproductive fitness on susceptible cowpea, especially in fecundity levels, in some virulent compared to avirulent lineages in a related study (Petrillo and Roberts, unpubl. data). Loss of 46% of the IFL derived from the Virulent isolate when establishing the IFL on susceptible cowpea, compared to no loss of Avirulent IFL, further supports a fitness cost associated with virulence in some individuals, although extinction rates of the IFL of these isolates did not differ in later generations.

A virulence profile of increased virulence on susceptible cowpea, i.e., in the absence of the resistance gene, was also found, indicating random variation in virulence under no selection pressure. The virulence was either maintained (two IFL) or lost (one IFL) over multiple generations. Jarquin-Barberena et al. (1991) observed a similar event, when number of galls and egg mass production on resistant tomato increased in a previously selected IFL despite culturing on susceptible tomato for nine successive generations. They proposed the possibility of autoamplification of virulence after its induction. In sexual organisms, random drift is often associated with random changes in overall gene frequency countering the tendency toward genetic homogenization, but no such mechanism is known for obligate mitotic parthenogens.

This study of *M. incognita* virulence-cowpea resistance interaction with field-collected isolates has revealed considerable genetic variation for virulence in a mitotic parthenogenetic nematode population. Furthermore, the profiles of virulence dynamics that characterize the component lineages provide some insight into the processes over multiple generations that maintain the genetic constitution of the population in which virulence is present. The constant recruitment and extinction of lineages and individuals would result at any point in time in a composite of lineages that have stable virulence or avirulence, and lineages that change from low to high virulence frequency and vice versa, in the presence or absence of selection on resistant plants. These events would maintain both avirulent and virulent genotypes in the population.

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