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# Dynamics of *Meloidogyne incognita* Virulence to Resistance Genes *Rk* and *Rk*$^2$ in Cowpea

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*Abstract:* The virulence index of three *Meloidogyne incognita* field isolates to the resistance gene *Rk* in cowpea was 0%, 75%, and 120%, with the index measured as reproduction on resistant plants as a percentage of the reproduction on susceptible plants. Continuous culture of the 75% virulent isolate on susceptible tomato for more than 5 years (about 25 generations) resulted in virulence decline to about 4%. The rate of the decline in virulence was described by exponential decay, indicating the progressive loss of virulence on a susceptible host. The 120% virulent isolate declined to 90% virulence during five generations on susceptible cowpea. Following virulence decline, the two isolates were compared over 5 years in inoculated field microplots both separately and as a mixture on susceptible, gene *Rk*, and gene *Rk*$^2$ cowpea plants. At infestation of the plots, the two isolates were 1.2% and 92.0% virulent, respectively, to gene *Rk* and 0.2% and 8.1% virulent, respectively, to gene *Rk*$^2$. Virulence to gene *Rk* in the two isolates and in mixture increased under 5 years of continuous *Rk* cowpea plants to 129% to 172% and under *Rk*$^2$ cowpea plants to 113% to 139 % by year 5. Virulence to gene *Rk*$^2$ increased during continuous cropping with *Rk* cowpea plants to 42% to 47% and with *Rk*$^2$ cowpea plants to 22% to 48% by year 5. Selection of *Rk*$^2$-virulence was slower in the isolate with low *Rk*-virulence. The virulence to both genes *Rk* and *Rk*$^2$ in the mixed population was not different from that in the highly virulent isolate by year 5 of all cropping combinations. Selection of *Rk*$^2$-virulence on plants with *Rk*, and vice versa, indicated at least partial overlap of gene specificity between *Rk* and *Rk*$^2$ with respect to selection of nematode virulence. This observation should be considered when resistance is used in cowpea rotations.

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

The root-knot nematode, *Meloidogyne incognita*, is a serious pathogen of cowpea (*Vigna unguiculata*) and is part of a disease complex with Fusarium wilt (Roberts et al., 1995). A dominant single gene (*Rk*) for resistance to *M. incognita* and other root-knot nematodes (Fery and Dukes, 1980) has been bred into several cowpea varieties that are grown as grain, fresh pod, and cover crops (Roberts et al., 1995; Ehlers et al., 2002; Hall et al., 2003). Although gene *Rk* is effective in reducing reproduction in most *M. incognita* populations, several cowpea fields in central California have *M. incognita* populations that reproduce on, and cause yield loss in, cowpea blackeye bean cultivars with gene *Rk* (Roberts and Matthews, 1995; Roberts et al., 1995). The cowpea fields with the virulent isolates have a history of growing resistant cowpea cultivars (Roberts et al., 1995, 1997), so it is likely that the virulent isolates resulted from selection on resistant cowpea plants. Field isolates virulent to *Rk* prompted a search for new cowpea resistance to *M. incognita* and *M. javanica*, and a stronger, broader-based resistance was discovered, based on a major gene, *Rk*$^2$ (Roberts et al., 1996). The *Rk*$^2$ gene confers a higher level of resistance to *Rk*-avirulent and *Rk*-virulent *M. incognita* isolates and also to *M. javanica* compared to gene *Rk* (Roberts et al., 1997). The *Rk*$^2$ gene was determined to be either allelic to gene *Rk* or a tightly linked locus within 0.17cM of *Rk* (Roberts et al., 1996), and it is currently being bred into new cowpea cultivars for California and elsewhere.

Greenhouse stock cultures of the *Rk*-virulent *M. incognita* isolates maintained on susceptible tomato showed a decline in virulence to gene *Rk* in our standard tests. In addition, the original source of gene *Rk*$^2$ appeared to be slightly less resistant to *Rk*-virulent isolates than to *Rk*-avirulent isolates. Therefore, we assessed both the in-field stability of the *Rk*-virulence in an *M. incognita* isolate when cultured in the absence of gene *Rk* and whether cross-selection for virulence to the two genes would occur under simulated field conditions. In work related to the current study, we have made in-depth analyses of these *Rk*-(a)virulent *M. incognita* isolates, using isofemale lineages from the isolates to determine *Rk*-virulence selection and stability (Petrillo and Roberts, 2005a) and relative fitness components (Petrillo and Roberts, 2005b) under controlled greenhouse culture conditions.

The objectives of the research reported here were to determine the stability of field-detected virulence during long-term greenhouse culture on susceptible tomato and to determine the potential for virulence selection and cross-selection in response to two cowpea resistance genes, *Rk* and *Rk*$^2$, under field-microplot conditions. A preliminary report on the virulence of the *M. incognita* greenhouse cultures is available (Roberts and Matthews, 1995).

## Materials and Methods

*Plant material:* Tomato (*Lycopersicon esculentum* 'Tropic') plants susceptible to *M. incognita* were used in long-term greenhouse culture experiments. Cowpea genotypes CB3, CB5, CB46, UCR430, H8-9, and 8685 were used to test for virulence and in the field plot experiments. CB5 and CB46 carry the single dominant gene *Rk* that confers resistance to avirulent *M. incognita* populations (Fery and Dukes, 1980) and here are termed *Rk*-plants. UCR430 carries the single dominant gene *Rk*$^2$ (Roberts et al., 1996) that confers resistance to both *Rk*-avirulent and *Rk*-virulent *M. incognita* populations and here is termed the *Rk*$^2$-plant. CB3, H8-9,

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and 8685 are not resistant to *M. incognita* and are termed susceptible plants.

*Nematode isolates:* An isolate of *M. incognita* race 3 was collected from a cotton field near Tipton, Tulare Co., California, and cultured for about 10 yr in the greenhouse on susceptible tomato Tropic. Two *M. incognita* race 1 isolates were collected 6 yr apart from a field site near Denair, Stanislaus Co., California, a location known to be infested with *Rk*-virulent *M. incognita* (Roberts et al., 1995) and where *Rk*-cowpea cultivars had been planted frequently. The two isolates were characterized as *Rk*-avirulent or *Rk*-virulent, respectively. The *Rk*-avirulent isolate was initially virulent when isolated from the field in 1989, with a virulence index on resistant cowpea of about 75%. As reported in Experiment 1, before the field plot study was initiated, the *Rk*-avirulent isolate declined to about 1% virulence during 6 yr of continual culturing on susceptible tomato, representing ≥ 25 generations. For the purposes of comparison (Experiment 2), a sub-culture of this isolate was cultured on resistant CB46 cowpea for 1 yr. The *Rk*-virulent isolate, collected in 1995, declined in virulence index from 120% to 92% during 9 mon (five generations) of culture on susceptible cowpea prior to this research. The *Rk*-avirulent (1% virulence) and *Rk*-virulent (92% virulence) isolates are referred to as the "Avirulent" and "Virulent" isolates, respectively, as used in the experiments described here.

*Greenhouse culture experiment 1:* The original isolate of *M. incognita* race 1 collected from the cowpea field site near Denair, California, was assayed for virulence to gene *Rk* during continuous culturing on tomato Tropic plants. The initial inoculum was collected by extracting eggs from cowpea roots collected from the plant row at several locations within the field during late season. Eggs were recovered from nematode-infected roots by maceration of the total root system in 0.5% NaOCl solution (Hussey and Barker, 1973). Tomatoes were grown in 1-liter fiber pots containing steam-sterilized sand and were inoculated 3 wk after transplanting as young seedlings with a suspension of eggs and J2 in water to four holes around the root system. Tomato plants were provided slow-release fertilizer as needed, watered daily, and maintained at an ambient temperature of 22°C to 28°C. A minimum of 10 tomato plants were maintained as cultures, and eggs were extracted from roots for use as inoculum, as described, when plants were 20- to 25-wk-old. Ten new tomato plants were inoculated to maintain the stock culture, while the remaining inoculum was used for a virulence assay. Assays were also performed on inoculum from a subset of tomato culture plants at additional times during the 6-mon re-culturing intervals. Virulence to gene *Rk* was assayed on 22 occasions on CB46 and 17 occasions on CB5, during 2,061 d of culture on tomato.

All virulence assays were performed using a modified growth-pouch technique (Ehlers et al., 2000). Pouches consisted of a 12.5- by 15.0-cm plastic pouch containing a paper insert, folded into a trough near the top. Cowpea seeds were placed with the hymen facing down directly into the trough for germination and watered with 13 ml of tap water; the pouches were placed in a manila folder (two pouches per folder) held in an upright vertical position. Pouches were transferred to a controlled-environment growth chamber maintained at a constant temperature of 26.7°C ± 0.6°C and 16 hr light/d.

Reproduction bioassays for virulence were performed using eggs recovered from infected roots by macerating the root system in 0.5% NaOCl solution (Hussey and Barker, 1973) and hatched in a modified Baermann plate (Barker and Niblack, 1990) at 26°C ± 1.0°C. Eggs were allowed to hatch for 3 to 5 d prior to inoculation. Root systems were inoculated with a mean of 1,210 motile J2 12 to 14 d post-plant. Following inoculation, pouches were watered once or twice per day and fertilized with Hoagland's solution (Hoagland and Arnon, 1950) every 4 d. A complete randomized design was used with 10 replicates each of CB3 (susceptible), CB5, and CB46.

To determine reproduction, each pouch was inundated with 75 mg erioglaucine/$L^{-1}$ (Sigma Chemical Co., St. Louis, MO) solution to stain eggs masses, for at least 1 hr 28 d post-inoculation. Pouches were drained and root systems visibly evaluated for numbers of egg masses produced using an illuminated desk magnifier and light table. The virulence index was calculated as a percentage obtained by the number of egg masses produced on resistant plants divided by the number of egg masses produced on susceptible plants, times 100.

*Greenhouse culture experiment 2:* After 1,330 d in culture on tomato, the original *Rk*-virulent isolate was compared to its sub-culture maintained on resistant CB46 for 1 yr, and to the *M. incognita* race 3 isolate. Each culture was assayed for egg mass production and virulence as described in Experiment 1 with some modifications. Each isolate was tested on CB3, CB5, CB46, and UCR430. Root systems were inoculated with a mean of 1,516 motile J2. Each isolate x genotype combination was replicated 10 times, and inoculated plants in pouches were arranged in a randomized complete block design. The experiment was conducted twice.

*Field microplot experiment:* Small field microplots were established at the Agricultural Experiment Station, UC-Riverside, and were planted with H8-9, CB46, or UCR430 in each cowpea growing season (May through August) for 5 yr. Each plot was 0.64 $m^2$ and contained 7.2 $m^3$ of loamy sand (87:2:11 sand:silt:clay) surrounded by a concrete barrier 1 m deep. Each plot was planted with the same cowpea genotype over the five seasons. Each plot was planted with 12 plants in late May and harvested in late August. Plots were maintained as a weed-free fallow after harvest until planting the next year. Each plot was infested with 160,000 eggs

3 wk after planting in the first year with either the Avirulent, the Virulent, or an 80,000:80,000 eggs combination of the Avirulent:Virulent isolates (mixed isolate). Each of the nine nematode x cowpea combinations was represented by a single plot arranged in a completely randomized design.

The Avirulent, Virulent, and mixed isolates in plots planted repeatedly with H8-9 are referred to as the AS, VS, and MS populations, respectively. The Avirulent, Virulent, and mixed isolates in plots planted repeatedly with CB46 are referred to as the AR, VR, and MR populations, respectively. The Avirulent, Virulent, and mixed isolates in plots planted repeatedly with UCR430 are referred to as the AR2, VR2, and MR2 populations, respectively.

Virulence to *Rk* and *Rk*$^2$ was assayed at infestation and at harvest in years 3, 4, and 5. All virulence assays were performed as described for the greenhouse culture experiments with the following modifications. Virulence assays were performed using eggs of the Avirulent and Virulent isolates at inoculation or sub-samples of the field plot populations collected from roots at harvest in years 3, 4, and 5. Roots systems in pouches were inoculated with 900 motile J2. The mixed isolate inoculum was not tested at the time of inoculation, and an estimate of its percent virulence to genes *Rk* and *Rk*$^2$ was calculated as follows: [(% Virulence of Avirulent isolate)(% Hatch of Avirulent isolate) + (% Virulence of Virulent isolate)(% Hatch of Virulent isolate)]/(Sum of % Hatch of Avirulent and Virulent isolates). Inoculum from the Avirulent and Virulent isolates used to inoculate the field plots in year 1 was assayed for virulence using 1,000 J2/pouch and cowpea 8685 as the susceptible genotype.

*Statistical analysis:* An analysis of variance (ANOVA) was performed using Fisher's Protected LSD 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 performed using Minitab Release 13 statistical software.

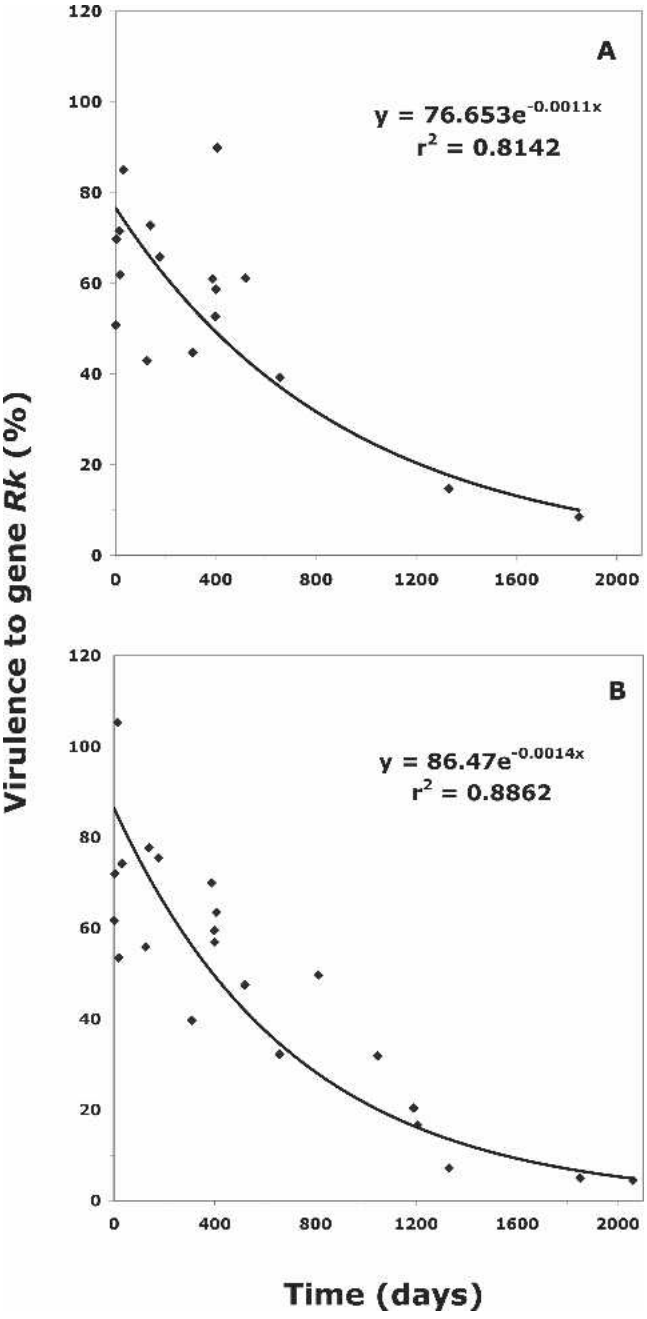


FIG. 1. The effect of continuous culturing on susceptible tomato for several years on the virulence to resistance gene *Rk* in cowpea of a *Meloidogyne incognita* population isolated from a cowpea field showing breakdown of *Rk*-resistance. Percent virulence was determined on cowpea genotypes CB5 (A) and CB46 (B) carrying gene *Rk*, based on egg-mass production on these resistant genotypes as a proportion of that on susceptible cowpea CB3.

## RESULTS

*Greenhouse culture experiment 1:* Virulence to gene *Rk* declined during continuous culture of the 75% *Rk*-virulent *M. incognita* isolate on susceptible tomato (Fig. 1). Periodic assays of virulence on both the *Rk*-carrying cowpea genotypes CB5 (Fig. 1A) and CB46 (Fig. 1B) revealed similar trends of decreasing *Rk*-virulence with time. Virulence to *Rk* declined from a range of 77% to 86% at isolation from the field to 4.5% on CB46 after 2,061 d on susceptible tomato (Fig. 1B). The virulence decline on both CB5 and CB46 fit exponential decay curves (Fig. 1A and 1B). There was no correlation of numbers of egg masses produced on susceptible CB3 plants with length of culture on tomato; a mean of 157.7 egg masses were produced per CB3 plant across all assays.

*Greenhouse culture experiment 2:* Analysis of variance indicated no significant effect of experiment, so the data from the two experiments were combined, and analyses are presented for the combined data. The natural avirulent *M. incognita* race 3 isolate produced a mean of < 1

TABLE 1. Numbers of egg masses per root system and percent virulence to genes *Rk* and *Rk*$^2$ of *Meloidogyne incognita* isolates on susceptible and resistant cowpea genotypes.

| Cowpea genotype | Natural *Rk*-avirulent isolate | | *Rk*-virulent isolate (after 1,330 days on tomato) | | *Rk*-virulent isolate (from cowpea with *Rk*) | |
|---|---|---|---|---|---|---|
| | Egg masses/ root system | % virulence | Egg masses/ root system | % virulence | Egg masses/ root system | % virulence |
| CB3 (susceptible) | 123.6 a | 100.0 a | 189.5 a | 100.0 a | 138.8 ab | 100.0 a |
| CB5 (*Rk*) | 0.7 b | 0.6 b | 28.3 b | 14.9 b | 166.1 a | 119.8 a |
| CB46 (*Rk*) | 0.5 b | 0.4 b | 21.5 b | 11.5 b | 119.3 b | 88.6 b |
| UCR430 (*Rk*$^2$) | 0 b | 0 c | 2.7 c | 1.4 c | 10.7 c | 7.4 c |

Values are means of 20 replicates combined from two experiments. Values in columns followed by the same letter do not differ ($P = 0.05$) according to Fisher's Protected LSD.

egg mass/root system on *Rk* plants and no egg masses on *Rk*$^2$ plants (Table 1). The *Rk*-virulent isolate cultured on susceptible tomato for 1,330 d had a moderate level of virulence (average 13.2%) on both CB5 and CB46 plants. This isolate produced a mean of 2.7 egg masses/root system on *Rk*$^2$ plants (1.4%) (Table 1). In contrast, a sub-culture of this isolate maintained for 1 yr on *Rk*-cowpea plants produced similar numbers of egg masses per root system on susceptible CB3 and resistant (gene *Rk*) CB5 and CB46 plants, indicating a high level of virulence (88.6% and 119.8%, respectively) (Table 1). Egg mass production and virulence of this subculture were higher on CB5 plants than CB46 plants ($P < 0.05$) in both experiments (Table 1), and this subculture had a higher level of egg mass production and *Rk*$^2$-virulence (7.4%) on UCR430 plants ($P < 0.05$) than the other two isolates.

*Field microplot experiment:* At the time of microplot infestation, virulence to *Rk* was higher ($P < 0.05$) in the Virulent isolate (92%) than in the Avirulent isolate (1.2%), and virulence to *Rk*$^2$ was higher ($P < 0.05$) in the Virulent isolate (8.1%) than in the Avirulent isolate (0.2%). The mixed population was not tested at infestation, but percent virulence was calculated to be 66% to *Rk* and 5.8% to *Rk*$^2$ from the virulence of the component isolates.

Differences ($P < 0.05$) in virulence to *Rk* (Table 2) and *Rk*$^2$ (Table 3) were observed at all assay dates among the populations. At each assay date, populations AS, VS, and MS from plots planted with susceptible cowpea were less virulent ($P < 0.05$) to *Rk* and *Rk*$^2$ compared to populations from plots planted with cowpeas carrying *Rk* or *Rk*$^2$. For example, population VS was less virulent to *Rk* and *Rk*$^2$ compared to populations VR or VR2.

*Effects on virulence of susceptible cowpea plantings:* Population AS was less virulent ($P < 0.05$) to *Rk* at all assay dates compared to populations VS and MS, ranging from 1.2% (at infestation) to 4.4% virulent over 5 yr (Table 2). Population VS declined in virulence to *Rk* from 92% at infestation to 26% in year 3, then increased to 58% in year 5. Population MS was similar to VS in virulence to *Rk* by years 4 and 5 (Table 2).

Population AS was highly avirulent to *Rk*$^2$ during 5 yr on susceptible cowpea (Table 3). Populations VS and MS retained a similar, low level of virulence to *Rk*$^2$ that was higher ($P < 0.05$) than that of AS in years 4 and 5 (Table 3).

*Effects on virulence of Rk-gene cowpea plantings:* Populations AR, VR, and MR increased in virulence to *Rk* from infestation to harvest in year 3 (Table 2). The three populations remained highly virulent to *Rk* in years 4 and 5, with virulence ranging from 122% to 190%. AR and VR did not differ in any year, whereas MR was more *Rk*-virulent ($P < 0.05$) than AR and VR in year 4 and than AR in year 5 (Table 2).

Virulence to *Rk*$^2$ increased in the AR, VR, and MR populations after 3 yr of *Rk*-cowpea cropping and remained at moderately high levels during years 4 (19%–60%) and 5 (42%–47%) (Table 3). AR was less *Rk*$^2$-virulent ($P < 0.05$) than MR in years 3 and 4. Similarly, AR was less *Rk*$^2$-virulent ($P < 0.05$) than VR in year 3 but not in year 4. MR was more *Rk*$^2$-virulent ($P < 0.05$) than

TABLE 2. Percentage virulence to gene *Rk* of *Meloidogyne incognita* populations in inoculated field plots planted with susceptible, *Rk*, or *Rk*$^2$ cowpea over 5 yr.

| Cropping history and population | Year in cowpea rotation | | |
|---|---|---|---|
| | Year 3 | Year 4 | Year 5 |
| On susceptible plants | | | |
| AS | 4.4 a | 1.5 a | 2.4 a |
| VS | 26 b | 40 b | 58 b |
| MS | 68 c | 39 b | 57 b |
| On *Rk*-plants | | | |
| AR | 175 de | 122 c | 129 cd |
| VR | 198 de | 127 c | 153 de |
| MR | 116 d | 190 de | 172 e |
| On *Rk*$^2$-plants | | | |
| AR2 | 55 c | 128 cd | 139 cde |
| VR2 | 217 e | 205 e | 124 cd |
| MR2 | 180 de | 125 c | 113 c |

Population codes are A = avirulent, V = virulent, and M = mixed (A plus V) in field plots planted with susceptible (S), *Rk*-resistant (R), or *Rk*$^2$-resistant (R2) cowpea plants. Plots were infested after planting in year 1 and planted with the respective cowpea genotype in each of 5 yr. Values are means of seven replicates. Values within a column followed by the same letter are not different ($P \leq 0.05$) using Fisher's Protected LSD. At infestation, virulence to gene *Rk* of the Avirulent and Virulent isolates was 1.2% and 92%, respectively. Virulence to gene *Rk* of the mixed population was estimated to be 66%.

TABLE 3. Percent virulence to gene $Rk^2$ of *Meloidogyne incognita* populations in infested field plots planted with susceptible, *Rk*, or $Rk^2$ cowpea over five seasons.

| Cropping history and population | Year in cowpea rotation | | |
|---|---|---|---|
| | Year 3 | Year 4 | Year 5 |
| On susceptible plants | | | |
| AS | 0.8 a | 0.0 a | 0.1 a |
| VS | 0.8 a | 5.2 c | 4.6 b |
| MS | 5.8 b | 2.8 b | 6.6 b |
| On *Rk*-plants | | | |
| AR | 8.4 b | 19 de | 42 d |
| VR | 19 c | 26 ef | 43 d |
| MR | 46 d | 60 g | 47 d |
| On $Rk^2$-plants | | | |
| AR2 | 3.9 b | 15 cd | 22 c |
| VR2 | 99 e | 60 g | 34 d |
| MR2 | 69 de | 41 fg | 48 d |

Population codes are A = avirulent, V = virulent, and M = mixed (A plus V) in field plots planted with susceptible (S), *Rk*-resistant (R), or $Rk^2$-resistant (R2) cowpea plants. Plots were inoculated after planting in year 1 and planted with the respective cowpea genotype in each of 5 yr. Values are means of seven replicates. Values within a column followed by the same letter are not different ($P \leq 0.05$) using Fisher's Protected LSD. At inoculation, virulence to gene $Rk^2$ of the Avirulent and Virulent isolates was 0.2% and 8.1%, respectively. Virulence to gene $Rk^2$ of the mixed population was estimated to be 5.8%.

VR in years 3 and 4 (Table 3). The populations did not differ in $Rk^2$-virulence in year 5 (Table 3).

*Effects on virulence of $Rk^2$-gene cowpea plantings:* Populations AR2, VR2, and MR2 in plots planted with $Rk^2$ plants increased in virulence to *Rk* between infestation and harvest in year 3 (Table 2). Population AR2 was less *Rk*-virulent ($P < 0.05$) than VR2 in years 3 and 4 and less *Rk*-virulent ($P < 0.05$) than MR2 in year 3. After 5 yr of cropping to $Rk^2$-plants, the three populations were highly *Rk*-virulent (113%–139%) and did not differ (Table 2). The *Rk*-virulence levels of the AR2, VR2, and MR2 populations were similar to those of the AR, VR, and MR populations from plots planted with *Rk*-cowpeas in years 4 and 5 (Table 2).

Population AR2 increased in virulence to $Rk^2$ between infestation and harvest in year 3 and increased again in year 4 and year 5 (Table 3). However, the $Rk^2$-virulence of AR2 was lower ($P < 0.05$) than that of VR2 and MR2 in years 3, 4, and 5. Populations VR2 and MR2 did not differ on any assay date. By year 5, populations AR, VR, MR, VR2, and MR2 did not differ in $Rk^2$-virulence (34% - 48%). Only population AR2 differed, being less virulent (22%, $P < 0.05$) than these other populations (Table 3).

## DISCUSSION

Virulence has been studied in other long-term culturing experiments with *Meloidogyne* populations, including virulence to gene *Mi* in tomato, and variable results have been obtained concerning selection and stability of virulence (Castagnone-Sereno et al., 1996). Selection for virulence in other nematodes also has been reported, e.g., in *Globodera pallida* to resistance in wild potato hybrids of *Solanum vernei* (Turner and Fleming, 2002), in *Heterodera schachtii* to resistance in sugarbeet derived from *Beta procumbens* (Mueller, 1992), and in *H. avenae* to resistance in oat (Lasserre et al., 1996).

The long-term culture experiment with *M. incognita* showed that virulence to a resistance gene can decline under standard culture of *Meloidogyne* on susceptible hosts, an expected consequence of stabilizing selection in the absence of the resistance gene (Caswell and Roberts, 1987). Similar observation of virulence loss during culture on susceptible host plants has been made for *Meloidogyne* populations virulent to resistant grapevines (M. V. McKenry, unpub. data). However, this type of virulence loss has not been clearly established in other nematode-host resistance interactions. In the case of *Mi* resistance in tomato, both stable maintenance of *M. incognita* virulence to *Mi* on susceptible tomato over many generations (Riggs and Winstead, 1959; Bost and Triantaphyllou, 1982; Castagnone-Sereno et al., 1993) and decline in virulence or loss of host range of virulent isolates have been reported (Jarquin-Barberena et al., 1991; Castagnone-Sereno et al., 1996).

Although the observed decrease in virulence was gradual and required several years in the present study, this finding has important implications for resistance deployment. Virulence decline in the absence of the resistance gene indicates that rotation to susceptible crops between resistant cowpea may reduce the virulence potential of the field population, assuming rotations of sufficient duration. However, the rapid directional selection of virulence by culture on resistant *Rk*-gene plants, as found in the sub-culture maintained for 1 yr on resistant cowpea, suggests that even one season of a resistant crop will result in increased virulence in the *Meloidogyne* population. The change in virulence frequency under greenhouse culture raises concerns for the use of cultures in experiments and screening for resistance. To maintain cultures of *Rk*-virulent lines for inoculum for screening sources of resistance effective against *Rk*-virulent populations, it is necessary to maintain cultures on resistant cowpea, such as the subculture maintained on *Rk*-cowpea in this study.

A detailed examination of *Rk*-virulence in these and other *M. incognita* isolates by isofemale line analysis revealed that the population was a mixture of virulent and avirulent lineages (Petrillo and Roberts, 2005a) and that some virulent lineages had a lower relative fitness than avirulent lineages (Petrillo and Roberts, 2005b). The differential fitness of the lineages provides an explanation for our observed changes in virulence in greenhouse cultures and the field plots. The substantial genetic variability within *M. incognita* isolates also may be involved. For example, a hierarchical analysis of mtDNA variable number tandem repeats of these same and other *M. incognita* isolates revealed only 7% diversity among the isolates, but 60% and 30% of the total genetic diversity occurred within individuals and within

isolates, respectively (Whipple et al., 1998). The consistently higher egg mass production on CB5 plants than CB46 plants indicated that reproduction on resistant plants is influenced to some extent by factors other than the resistance alleles, although the overall virulence trends were the same for the two *Rk*-genotypes.

In greenhouse experiments, the isolate that had declined in *Rk*-virulence on tomato was quickly reselected for *Rk*-virulence by sub-culturing on resistant cowpea, presumably because of differential reproduction of the *Rk*-virulent lineages within the isolate. Those experiments also indicated that the *Rk*-virulent sub-culture was moderately virulent to gene $Rk^2$. This apparent co-virulence to genes *Rk* and $Rk^2$ was confirmed in the field plot study, wherein cross-selection of virulence to genes *Rk* and $Rk^2$ was observed in both directions. The AR, VR, and MR populations during five seasons on *Rk*-cowpea became moderately virulent to gene $Rk^2$. Likewise, in the reverse direction, all populations (AR2, VR2, MR2) exposed to $Rk^2$-cowpea plantings for five seasons became highly virulent to gene *Rk*. This reciprocal cross-selection of virulence suggests that the *Rk* and $Rk^2$ genes share some commonality or overlap with respect to the signaling and recognition pathways involved in resistance. Gene $Rk^2$ is associated with *Rk* as an allele at the *Rk* locus or tightly linked to *Rk* within 0.17 map units (Roberts et al., 1996). Thus these genes have features of complex resistance loci, as found for other plant-pathogen resistance systems (Hulbert et al., 2001).

$Rk^2$ expresses a broader and stronger resistance than *Rk*, being effective against diverse *M. incognita* populations including ones virulent to *Rk*, and also against *M. javanica* (Roberts et al., 1996; Ehlers et al., 2002). Although cross-selection for virulence to *Rk* and $Rk^2$ was observed, it was not reciprocally uniform. By year 5, the levels of virulence to $Rk^2$ were much lower (34%–48%) than the virulence to *Rk* (113%–190%), regardless of whether virulence selection occurred on *Rk*-cowpea or $Rk^2$-cowpea plantings. Nevertheless, cross-selection could be a problem for effective deployment of resistance using $Rk^2$-cowpea cultivars, due to selection of virulence against both genes.

The mixed field study population was intended to simulate what we suspected and later confirmed (Petrillo and Roberts, 2005a), namely, that the *Rk*-virulent field populations were a natural mixture of avirulent and virulent lineages. The mixed population on susceptible plants retained virulence over five seasons and had a similar virulence profile to the virulent population (VS), suggesting that in-field virulence decline on susceptible hosts might be slower than what occurs in greenhouse cultures. On the *Rk*- and $Rk^2$-cowpea plantings, the mixed population (MR, MR2) was indistinguishable from the virulent population (VR, VR2) in each case. In the field, the virulent population apparently had greater fitness on the resistant plants.

We have demonstrated the decline of virulence during greenhouse culture on susceptible host plants and that rapid selection for virulence is possible by culturing nematodes on resistant plants. In addition, cross-selection for virulence to genes *Rk* and $Rk^2$ in both directions was found, with important implications for nematode management based on deployment of these resistance genes in root-knot nematode-infested cowpea fields.

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