Response of Root-Knot Resistant Tobacco to Concomitant Populations of *Meloidogyne* Species

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ABSTRACT

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Mixed populations of *Meloidogyne* species commonly occur in most flue-cured tobacco (*Nicotiana tabacum* L.) fields in the southeastern United States. The interaction of *Meloidogyne arenaria* race 2 (Ma) and *M. incognita* race 3 (Mi) on the Mi-resistant flue-cured tobacco cv. Speight G-28 and on a root-knot susceptible flue-cured tobacco cv. NC 2326 (control) was evaluated under greenhouse conditions. Plants of both cultivars were inoculated with 1,500 or 3,000 eggs of Ma or Mi alone, or with a combination of 1,500 eggs of each species. Root penetration (number of juveniles per g of root) with the mixed population assessed 10 days after inoculation (DAI) was comparable to that of Ma alone on the Mi-resistant tobacco cultivar, and of either nematode species alone on the susceptible cultivar. Also, nematode population density (number of nematodes per g of root, all stages except eggs), numbers of mature females per g of root, root damage (percent root area galled), and nematode reproduction (number of eggs per g of root) on Speight G-28 30 and/or 48 DAI with mixed population were not greater than those attributable to Ma alone. Indeed, inoculation with the mixed population resulted in slightly lower nematode population density (except at 48 DAI), root damage, and nematode reproduction on Mi-resistant cultivar than with Ma alone at the 1,500 egg inoculum level. The concomitant inoculation of Ma and Mi on NC 2326 did not differ from single inoculation with Ma for all variables measured, except reproduction at 48 DAI. These data indicate that Speight G-28 resistance to Mi was not reduced with concomitant infections of Ma and Mi. Fewer Mi than Ma mature females were recovered from the susceptible cultivar in the mixed population, indicating that Ma was more competitive than Mi.

Root knot, caused by nematodes belonging to the genus *Meloidogyne* Goeldi, is a major disease of tobacco in the southeastern United States and in all tobacco-producing countries of subtropical and tropical zones (27). Worldwide, this disease, characterized by the formation of galls on roots of susceptible plants, is responsible for annual yield losses of approximately 15% (27). Annual losses attributed to this disease in flue-cured tobacco are estimated to range from 0.1 to 4.8% in the southeastern United States, and about 1% or more in North Carolina (19, 21).

Release of tobacco cultivars carrying monogenic resistance against the most commonly occurring races (1 and 3) of *M. incognita* (Mi) (Kofoid & White) Chitwood has substantially reduced losses from this disease (21). Over half of the flue-cured tobacco hectarage in the southeastern United States and about two-thirds of the hectarage in North Carolina are planted to these cultivars (19,21). The widespread and continued use of these resistant cultivars, along with changes in the use of nematicides and shortened rotation regimes, have been suggested as causes of population shifts resulting in the increased occurrence of *M. arenaria* (Ma) (Neal) Chitwood, *M. hapla* (Mh) Chitwood, *M. javanica* (Mj) (Treub) Chitwood, and Mi races 2 and 4 (10,25,29). Extensive studies on the pathogenicity, population development, and control of root-knot nematodes on tobacco have shown that these other *Meloidogyne* species and races of Mi severely damage both Mi-resistant and Mi-susceptible cultivars (1,2,29). There are no cultivars with resistance to these other *Meloidogyne* species and races of economic importance (10).

Previous studies indicate that Mi and Ma are now the most frequent species in flue-cured tobacco fields in North and South Carolina (10,25), and that race 2 of Ma is the most common race when this species is detected (30). Mixed field infestations and mixed infections by these species are common in tobacco (10). Nevertheless, information pertaining to the interaction among concomitant populations of *Meloidogyne* species on root-knot resistant tobacco is scarce. In split-root experiments, Eisenback (7) found that plants of Mi-resistant tobacco cv. NC 95 infected with either Ma or Mh lost their resistance to Mi race 1, but otherwise remained resistant when infected with either Mj or Mi race 4. Concomitant and sequential inoculation of NC 95 plants with Mi race 1 and Ma race 2 gave similar results (17). In contrast, mixed population studies in Zimbabwe did not show any reduction in resistance to Mj in Mj-resistant tobacco breeding lines (31).

The objective of the present study was to determine whether concomitant inoculation of tobacco cv. Speight G-28 (resistant to Mi races 1 and 3) with Ma race 2 and Mi race 3 predisposes Mi-resistant tobacco to infection by the latter.

MATERIALS AND METHODS

Nematode isolates and inoculum preparation. The nematode isolates Ma race 2 (Ma) and Mi race 3 (Mi) (both of North Carolina origin) were used throughout this study. Ma was maintained on Mi-resistant tobacco (cv. NC 95), whereas Mi was maintained on tomato (*Lycopersicon esculentum* Mill cv. Rutgers) in greenhouse pot cultures. Eggs for inocula were collected from galled roots of the respective host plants, using a sodium hypochlorite (NaOCl) technique (16). Egg suspensions were diluted to allow inoculation of plants with 10-ml egg suspension of each nematode species in the desired inoculum levels.

Plant material. Seeds of Mi-resistant cv. Speight G-28 and of the susceptible control cv. NC 2326 were germinated in seedling trays containing Metro-Mix 220 (Grace-Sierra Horticultural Products Co., Milpitas, Calif.), covered with a clear polyethylene film and placed in a growth chamber at 25°C, with a 16-h photoperiod having a light intensity of 400±50 $\mu E\ m^{-2}\ s^{-1}$. Sixty 4-week-old seedlings of each cultivar in 1990 and 1991, and 90 seedlings in 1992, were transplanted into sterilized clay pots containing a 1:1 mixture of steam-sterilized sand and sandy loam soil (vol/vol; 85% sand, 10% silt, 5% clay). Plants were transferred to a greenhouse and allowed to grow for 10 days prior to inoculation.

Thirty plants of each tobacco cultivar grown in both 5- and 15-cm-diameter pots, and an additional 30 plants in 15-cm-diameter pots in 1992, were used in each experiment. Plants in both 5- and 15-cm-

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diameter pots were inoculated with 1,500 or 3,000 eggs of Ma or Mi alone, or with a combination of 1,500 eggs each of Ma and Mi (concomitant population). Noninoculated plants of each cultivar served as checks for any contamination due to extraneous sources of inoculum. Basic nutrients were supplied by periodic watering with either 20:20:20 Peter's solution (Grace-Sierra) at 741 µg/ml nitrogen, or Hoagland's solution (14). Daily mean temperatures in the greenhouse in 1990, 1991, and 1992 were 30±1, 31±3, and 29±2°C, respectively.

Assessment periods and measurements. Plants in the 5-cm-diameter pots for both cultivars were assayed each year for root penetration (number of juveniles per g of root) 10 days after inoculation (DAI). Roots were washed free of soil, cut into 2- to 4-cm segments, and nematodes within random 1-g root samples were stained with acid fuchsin (6). All plants in 15-cm-diameter pots in 1990 and one-half the plants in the 15-cm-diameter pots in 1992 were assayed 30 DAI. Root damage was determined as proportion of total root area galled (3). The root system from each plant was then cut into 2- to 4-cm segments, and separate 1- and 2-g samples were used to determine nematode population density (number of nematodes per g of root, all stages except eggs) and reproduction (number of eggs per g of root), respectively. Nematodes within the 1-g root samples were stained with acid fuchsin (6) to assess population density, and eggs were collected from the 2-g root samples, using a NaOCl extraction technique (16).

All plants in 15-cm-diameter pots in 1991 and the other one-half of the plants in 15-cm-diameter pots in 1992 were assayed 48 DAI. Plants harvested at this period were handled in the same manner as those assayed 30 DAI. In addition, numbers of mature females per g of root were determined. Also, 25 mature females were teased from each root system inoculated with the concomitant population and placed in tubes containing 0.7% NaCl to preserve samples prior to determining species identity by electrophoresis (9). Each female was macerated in 6 µl of extraction solution (20% sucrose, 2% Triton X-100, 0.01% bromophenol blue) and 0.3 µl of each sample was electrophoresed on precast 10 to 15% gradient polyacrylamide gels in a PhastSystem (Pharmacia Limited, Uppsala, Sweden). Single female homogenates of known Ma and Mi were used as standards on each gel. Gels were incubated in the substrate α-naphthyl acetate at 37°C to determine species identity based on esterase phenotypes (9).

Experimental design and statistical analysis. The study utilized a randomized complete block design with five replications within each cultivar. Plants of each cultivar were arranged on a greenhouse bench in a block (i.e., cultivars were not randomized) and were treated as a separate experiment. Each replication consisted of one plant in both 5- and 15-cm-diameter pots, and an additional plant in 15-cm-diameter pot in 1992 for each inoculum. All data were first analyzed using the univariate procedure of SAS (24). Data for root penetration, nematode population density, numbers of mature females, and nematode reproduction were transformed to a logarithmic scale [$\log_{10}(x+1)$], whereas root damage data were arcsin-transformed for further analysis, and transformed back for presentation. For each period of assessment within each cultivar, error mean squares over the appropriate years were found to be homogeneous for all variables, and so data were accordingly pooled for analysis using the general linear models procedure of SAS (24). Contrasts of interest, Ma 1,500 vs. Ma 3,000, Mi 1,500 vs. Mi 3,000, Ma vs. Mi, Ma 1,500 vs. (Ma + Mi) 3,000, Ma 3,000 vs. (Ma + Mi) 3,000, Mi 1,500 vs. (Ma + Mi) 3,000, and Mi 3,000 vs. (Ma + Mi) 3,000, were performed with each analysis within each cultivar. Noninoculated plants were excluded from analysis.

RESULTS

Analyses of variance revealed inoculum-by-year interaction effects for some variables. For Speight G-28, interactions ($P < 0.05$) with inoculum-by-year occurred with nematode population density, root damage, and nematode reproduction recorded 30 DAI only. For NC 2326, inoculum-by-year interactions ($P < 0.05$) were observed for root penetration 10 DAI, and root damage assessed 30 DAI only. However, these interactions were due to variation in the magnitude of these variables rather than rank order changes. The inoculum-by-year interaction was, therefore, not considered to be of major importance, and so data for all variables were pooled over the appropriate years for each assessment period within each cultivar.

Root penetration. Root penetration assessed 10 DAI increased ($P < 0.05$) on Speight G-28 with increased inoculum level of Mi alone (Fig. 1A). However, Ma penetration was greater ($P < 0.05$) than Mi penetration on this cultivar when each nematode species was inoculated singly. Penetration was greater ($P < 0.01$) with the concomitant population than with Mi alone at either inoculum level. For NC 2326, increased ($P < 0.05$) penetration was observed with increased inoculum level of Ma alone (Fig. 1B). No differences ($P > 0.05$) in penetration were detected between the nematode species when inoculated individually. Also, root penetration of the concomitant population was not different ($P > 0.05$) from that for either nematode species alone at any inoculum level.

Nematode population density. Inoculum level of either Ma or Mi did not affect ($P > 0.05$) nematode population density on Speight G-28 30 DAI (Fig. 2A). However, the Ma population density was higher ($P < 0.01$) than Mi when each species was inoculated singly. For Mi alone, no nematodes per g of root were detected at the 1,500 eggs level and only 2 were found at the 3,000 eggs level. Nematode population density on this cultivar was higher ($P < 0.05$) with the concomitant population than with Mi inoculation at either inoculum level. As with Speight G-28, inoculum level of either Ma or Mi had no effect ($P > 0.05$) on nematode population density on NC 2326 30 DAI (Fig. 2B). The difference between the two nematode species for this variable was not significant ($P > 0.05$) for

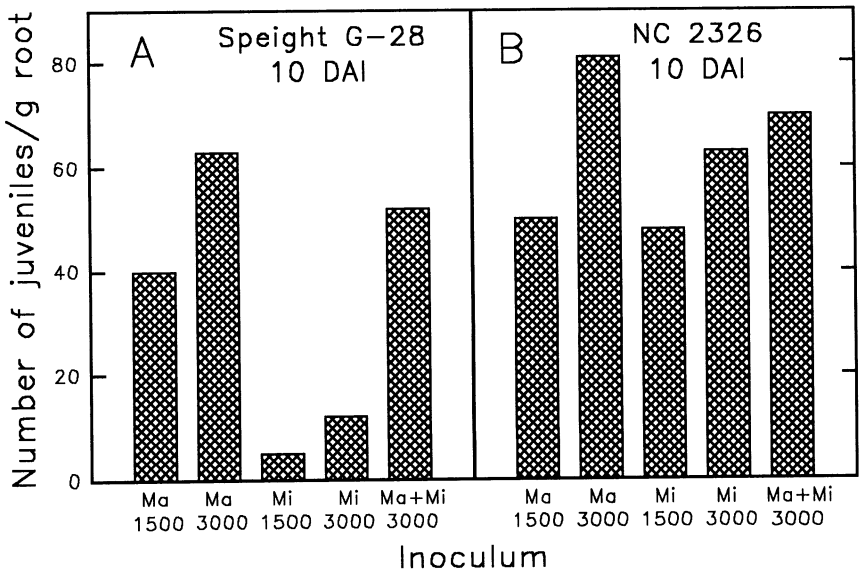

Fig. 1. Root penetration (number of juveniles per g of root) on **(A)** *Meloidogyne incognita*–resistant tobacco cv. Speight G-28 and **(B)** root-knot susceptible tobacco cv. NC 2326, 10 days after inoculation (DAI) with 1,500 or 3,000 eggs of *M. arenaria* race 2 (Ma) or *M. incognita* race 3 (Mi) alone, or combined (1,500 eggs of each species). Data represent means over 3 years (1990 through 1992).

NC 2326. Also, the concomitant inoculation of Ma and Mi resulted in a higher ($P < 0.05$) population density than with the single inoculation of Mi at the lower inoculum level.

At 48 DAI, population density of Ma alone increased ($P < 0.05$) on Speight G-28 as inoculum level increased (Fig. 2C). For Mi, results followed the same pattern as observed 30 DAI. As before, nematode population density found with the concomitant population was higher ($P < 0.01$) than that observed with single inoculation of Mi at either inoculum level. For NC 2326, population density of Mi alone increased ($P < 0.05$) with increased inoculum level (Fig. 2D). The difference in population density between the two nematode species was not significant ($P > 0.05$) for this cultivar. Furthermore, the concomitant population was not different ($P > 0.05$) from either nematode species alone for this variable.

Mature nematode females. Numbers of mature Ma females in roots of Speight G-28 48 DAI were greater ($P < 0.05$) for the 3,000 than the 1,500 egg inoculum level, whereas Mi concentration had no significant effect (Fig. 3A). Also, higher ($P < 0.01$) numbers of mature Ma than Mi females were found in roots of Speight G-28 when each species was inoculated singly. There were greater ($P < 0.05$) numbers of mature females for Ma alone at the higher inoculum level than for the concomitant population. Inoculation with Mi alone at either inoculum level resulted in lower ($P < 0.01$) numbers of mature females than inoculation with the concomitant population. For NC 2326, numbers of mature females 48 DAI increased ($P < 0.05$) with greater inoculum level of Mi alone (Fig. 3B). Nevertheless, no differences ($P > 0.05$) in numbers of mature females were found between the two nematode species. Inoculation with Mi alone at the higher inoculum level resulted in more ($P < 0.05$) female development than inoculation with the concomitant population. Based on esterase phenotypes, only four of 238 mature females extracted from Speight G-28 and 63 of 241 from NC 2326 in the concomitant population were identified as Mi; the remainder were identified as Ma (Table 1).

Root damage. Inoculum level of either Ma or Mi did not affect ($P > 0.05$) root damage on Speight G-28 30 DAI (Fig. 4A). Greater ($P < 0.01$) root damage occurred with single inoculation of Ma than with Mi. Mi alone induced no galls on this cultivar regardless of inoculum level. Root damage due to the concomitant population was not different ($P > 0.05$) from that caused by Ma alone at either inoculum level. As with Speight G-28, no differences ($P > 0.05$) in root damage were observed on NC 2326 due to inoculum level for either Ma or Mi (Fig. 4B). Also, no differences ($P > 0.05$) in root damage were detected between the two nematode species when inoculated individually. Root damage induced by either Ma or Mi alone at any inoculum level was not different ($P > 0.05$) from that due to the concomitant population.

At 48 DAI, results for root damage on Speight G-28 followed the same pattern as with 30 DAI (Fig. 4C). However, the concomitant population resulted in less ($P < 0.05$) root damage, compared with Ma alone at the higher inoculum level. Similarly, root damage data for NC 2326 also followed the same pattern as with 30 DAI (Fig. 4D). Root damage due to concomitant inoculation of Ma and Mi was slightly lower than that induced by either species alone at either inoculum level.

Nematode reproduction. Inoculum level of either Ma or Mi did not influence ($P > 0.05$) reproduction on Speight G-28 30 DAI (Fig. 5A). However, inoculation with Ma resulted in higher ($P < 0.01$) reproduction than inoculation with Mi. The few eggs found on Speight G-28 when inoculated with Mi alone were from a single pot for each inoculum level in 1990 only. The concomitant population induced limited fecundity compared with Ma alone at either inoculum level, even though the differences were not significant ($P > 0.05$). Likewise, reproduction on NC 2326 30 DAI was not affected ($P > 0.05$) by inoculum level for either species alone (Fig. 5B). Furthermore, no differences ($P > 0.05$) in fecundity were observed between the two nematode species when inoculated individually. Inoculation with the concomitant population did not result in different ($P > 0.05$) reproduction than inoculation with either species alone at either inoculum density.

At 48 DAI, reproduction on Speight G-28 increased ($P < 0.05$) as inoculum level of Ma increased (Fig. 5C). No eggs were produced on this cultivar irrespective of Mi inoculum level. The concomitant population induced a lower ($P < 0.01$) reproduction than Ma alone at the higher inoculum level. Reproduction found with

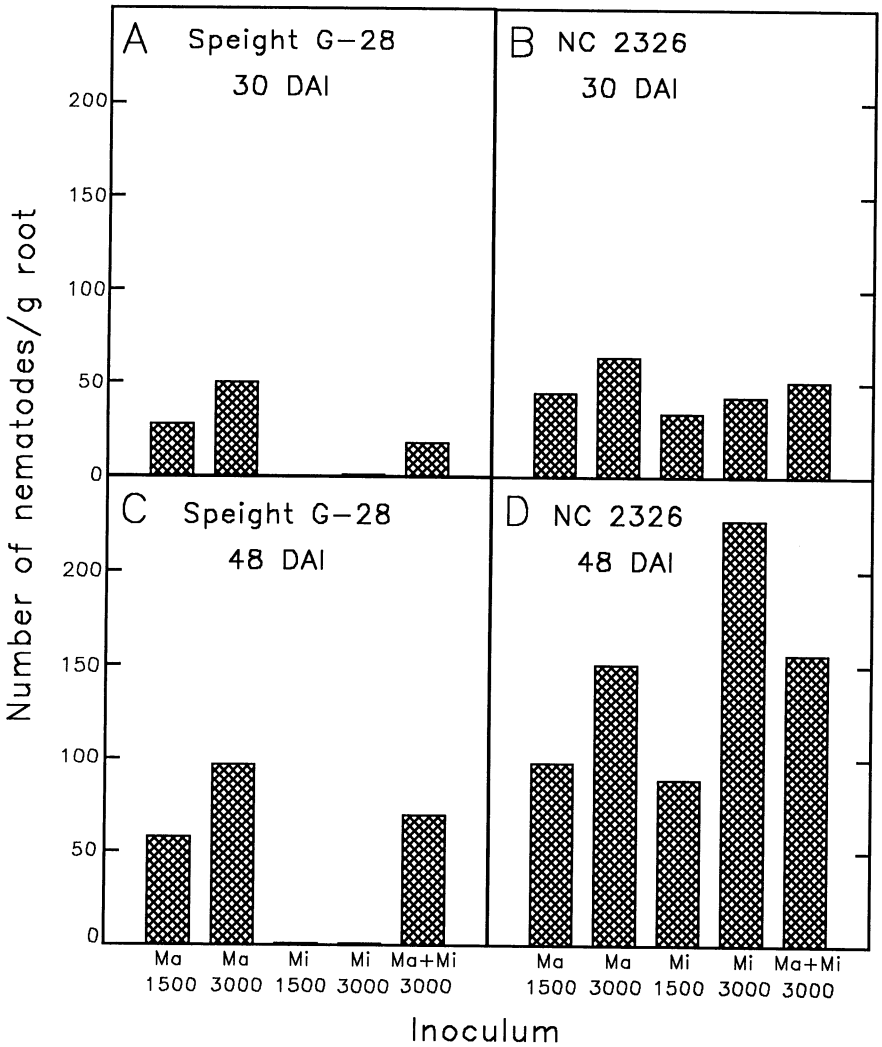

Fig. 2. Nematode population density (number of nematodes per g of root) on **(A, C)** *Meloidogyne incognita*-resistant tobacco cv. Speight G-28 and **(B, D)** root-knot susceptible tobacco cv. NC2326 30 and 48 days after inoculation (DAI) with 1,500 or 3,000 eggs of *M. arenaria* race 2 (Ma) or *M. incognita* race 3 (Mi) alone, or combined (1,500 eggs of each species). Data represent means over 2 years (1990 and 1992 for 30 DAI, and 1991 and 1992 for 48 DAI).

the concomitant population was slightly less than that with Ma alone at the lower inoculum level, although the differences were not significant ($P > 0.05$). For NC 2326, reproduction increased ($P < 0.01$) with inoculum density of either nematode species alone (Fig. 5D). The difference between the two nematode species for this variable was not significant ($P > 0.05$) for this cultivar. The concomitant population resulted in more ($P > 0.05$) reproduction than either nematode species alone at the lower inoculum level. Conversely, greater ($P < 0.01$) reproduction was observed with Ma alone at the higher inoculum level than with the concomitant population.

DISCUSSION

Our results did not support previous reports (7,17) that concomitant inoculation of Ma and Mi would result in loss of resistance to Mi in Speight G-28. Nematode population density, numbers of mature females, root damage, and nematode reproduction for Speight G- 28 inoculated with a concomitant population of Ma and Mi were not greater than those attributable to Ma alone. The concomitant inoculation resulted in slightly lower nematode population density (except at 48 DAI), root damage and reproduction on the resistant cultivar than with Ma alone at the lower inoculum level. Concomitant inoculation of Ma and Mi also did not differ from single inoculation with Ma on NC 2326 for all variables measured except for reproduction at 48 DAI. Further evidence against the loss of resistance hypothesis was provided by esterase phenotype analysis of mature females recovered from roots inoculated with the concomitant population. The presence of a few Mi females developing on Speight G-28 is not evidence that Ma negated resistance in this cultivar against the former species, because, occasionally, a few mature females also developed with single inoculation of Mi.

The differential root penetration by both species on the resistant cultivar and the subsequent decline in nematode population density and numbers of mature females of Mi detected in roots of the same cultivar at all harvest periods were in

Table 1. Numbers of *Meloidogyne arenaria* race 2 (Ma) and *M. incognita* race 3 (Mi) mature females from concomitant infection of roots of Mi-resistant tobacco cv. Speight G-28 and root-knot susceptible cv. NC 2326 48 days after inoculation as identified by esterase phenotypes

	Numbers of mature females[a]		
Cultivar	**Ma**	**Mi**	**Total**
Speight G-28	234	4	238
NC 2326	178	63	241

[a] Twenty-five mature, egg-laying females from each root system were used; 21 to 25 female samples could be identified. Data represent totals over 2 years (1991 and1992).

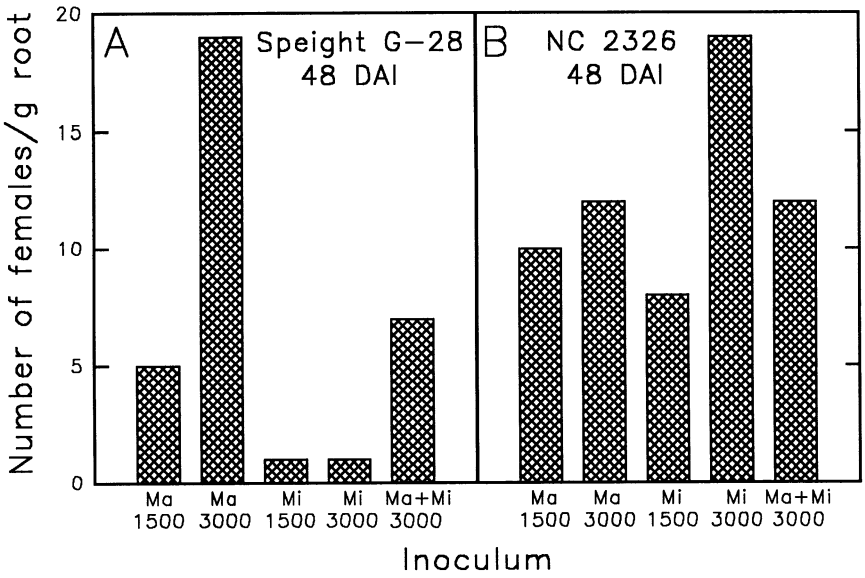

Fig. 3. Numbers of mature females per g of root of **(A)** *Meloidogyne incognita*-resistant tobacco cv. Speight G-28 and **(B)** root-knot susceptible tobacco cv. NC 2326 48 days after inoculation (DAI) with 1,500 or 3,000 eggs of *M. arenaria* race 2 (Ma) or *M. incognita* race 3 (Mi) alone, or combined (1,500 eggs of each species). Data represent means over 2 years (1991 and 1992).

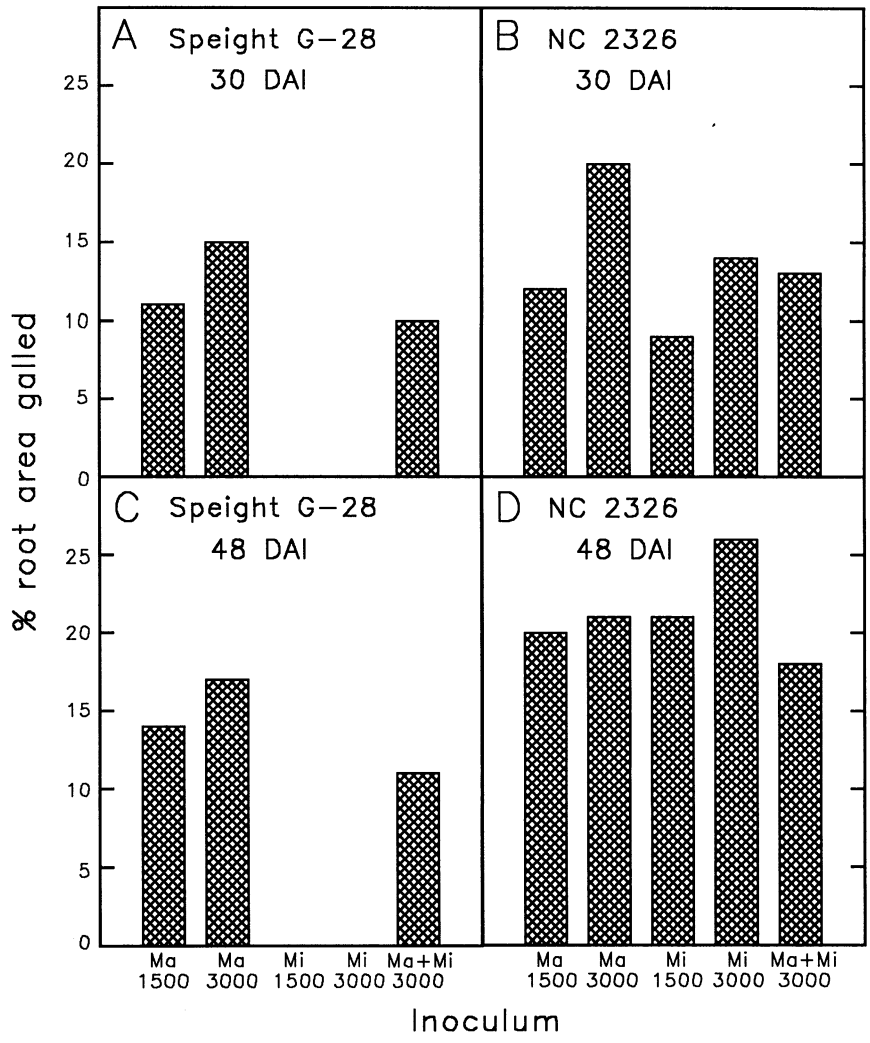

Fig. 4. Root damage (percent root area galled) on **(A, C)** *Meloidogyne incognita*–resistant tobacco cv. Speight G-28 and **(B, D)** root-knot susceptible tobacco cv. NC 2326 30 and 48 days after inoculation (DAI) with 1,500 or 3,000 eggs of *M. arenaria* race 2 (Ma) or *M. incognita* race 3 (Mi) alone, or combined (1,500 eggs of each species). Data represent means over 2 years (1990 and 1992 for 30 DAI, and 1991 & 1992 for 48 DAI).

agreement with the findings of Sosa-Moss et al. (29). Previous studies have shown that second-stage juveniles of *Meloidogyne* species generally penetrate roots of resistant cultivars as readily as roots of susceptible cultivars of most crop species, precluding the possibility of a barrier to penetration as a common form of resistance (15). Schneider (26) reported that higher numbers of Mi juveniles penetrated roots of Mi-resistant tobacco cv. K 399 than roots of susceptible cv. NC 2326, 24 h after inoculation. However, numbers of Mi juveniles remaining in roots of the resistant cultivar declined rapidly following a peak reached 4 DAI (26). Similar observations for *Meloidogyne* species have been reported on alfalfa (11,23), cotton (22), soybean (13) and tomato (12). The observation of differential penetration rates could be attributed to death or emigration of nematodes from roots due to failure by nematodes to establish a feeding site (11, 12,23,26,29), rather than absence of specific nutrients (15).

The mechanism of resistance to Mi in tobacco has been shown to involve a necrotic or hypersensitive reaction (28). When juveniles penetrate roots of resistant plants, the cells surrounding the nematode become necrotic and die. A feeding relationship cannot be established and resistance is expressed. The hypersensitive reaction against Mi may be working against Ma and thereby reducing nematode population density, root damage, and reproduction as observed with the concomitant population on the resistant cultivar (29). The apparent suppression of reproduction by the concomitant inoculation of Ma and Mi on the resistant cultivar also was observed by Ibrahim (17).

Our data indicate that competition occurs between the two *Meloidogyne* species. The apparent decline in nematode population density, related host damage, and reproduction when Ma and Mi were in a mixed population rather than in a monospecific condition, is suggestive of an antagonistic interaction on the resistant cultivar (8). An antagonistic interaction can be caused by spatial competition, physical alteration or destruction of feeding sites, or a decrease in the suitability of the host caused by a physiological change, and can be further complicated by such factors as temperature and other climatic conditions (8). Interactions between sedentary endoparasites, which establish a complex relationship with hosts, are generally mutually suppressive because they compete for the available feeding sites and cause similar histopathological and physiological alterations in the host (8). In our experiments, more mature females of Ma than of Mi were recovered from roots of the susceptible cultivar when the two species were in mixed population, suggesting that Ma was dominant over Mi. With equal inoculum level of Ma and Mi (1,500 eggs each) in the concomitant inoculation, a nearly equal proportion of mature females would be expected in the absence of competition, especially on the susceptible cultivar, rather than the approximately 3:1 ratio of Ma to Mi observed in this study. Similar observations of dominance of one species of *Meloidogyne* over the other have been reported in tobacco (18) and tomato (20).

Contrary to what has been reported (7, 17), results obtained in our study provide no evidence that infection of Mi-resistant tobacco cv. Speight G-28 by Ma reduces its resistance to Mi. Differences in results may be attributed in part to differences in temperature regimes among the studies. It is known that resistance to Mi breaks down at high temperatures (28), but Eisenback (7) did not report the temperatures used in his study. Another possible explanation for the discrepancy lies in the techniques used, especially those used for species characterization in mixed populations. Ibrahim (17) used the subjective perineal pattern analysis to determine species identity, which is less accurate (9) than the biochemical technique used in our study. Furthermore, Ibrahim (17) interpreted the fact that neither root-gall development nor reproduction occurred with Mi race 1 alone on NC 95 in his study as evidence that Mi females did not develop in roots of the cultivar. However, Mi females could have developed on NC 95 without inducing root-galling or reproducing. Examination of roots with single inoculations of Mi race 1 as done in our study through acid-fuchsin staining provides valuable information because some second-stage juveniles are able to establish feeding sites and continue to develop even in resistant cultivars (26). Our results are in agreement with those of Way (31), who reported no reduction in resistance to Mj in Mj-resistant tobacco breeding lines following inoculation with either race 1 or 3 of Mi in split-root experiments. Also, our results are further strengthened by the findings of Baum et al. (4,5) who conducted a similar

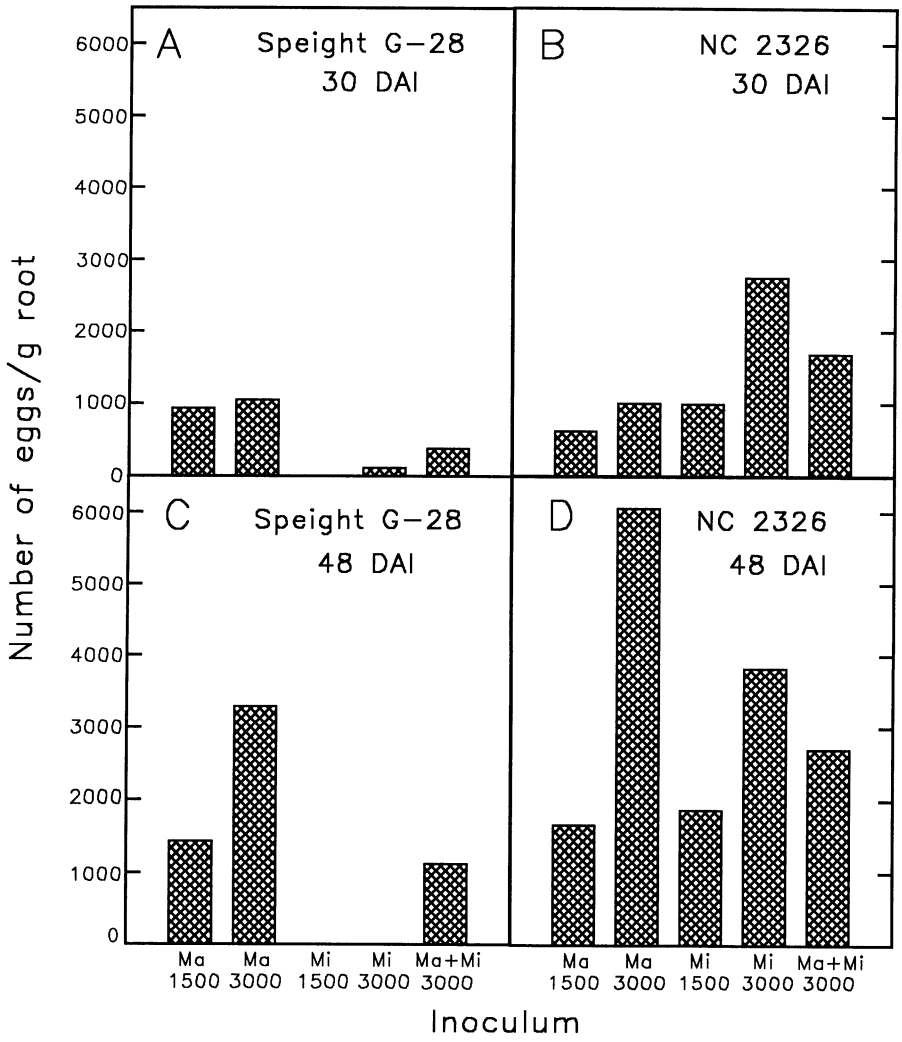

Fig. 5. Nematode reproduction (number of eggs per g of root) on **(A, C)** *Meloidogyne incognita*-resistant tobacco cv. Speight G-28 and **(B, D)** root-knot susceptible tobacco cv. NC 2326 30 and 48 days after inoculation (DAI) with 1,500 or 3,000 eggs of *M. arenaria* race 2 (Ma) or *M. incognita* race 3 (Mi) alone, or combined (1,500 eggs of each species). Data represent means over 2 years (1990 & 1992 for 30 DAI, and 1991 & 1992 for 48 DAI).

study almost at the same time as ours. They reported no loss of resistance to Mi in Mi-resistant tobacco cultivars following inoculation with Ma in greenhouse split-root and field experiments.

The increased prevalence of Ma, coupled with no available commercial cultivars highly resistant to this species (10) and the low efficacy of nonfumigant nematicides against the species (2) suggest a need for further research aimed at identifying and transferring host resistance against this species into cultivars with acceptable agronomic characteristics.

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