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Host-Parasite Relationships in Root-Knot Disease of White Mulberry

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ABSTRACT

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Severe infections of white mulberry feeder roots and heavy soil infestations by *Meloidogyne arenaria* race 2 were found in southern Spain. This is the first record of *M. arenaria* on white mulberry in Europe. Morphometric observations, analysis of the esterase electrophoretic pattern, and artificial inoculations of race differentials were used to characterize nematodes. Nematode-induced mature galls were spherical and usually contained one or more females, males, and egg masses with eggs. Feeding sites were characterized by the development of giant cells that contained granular cytoplasm and many hypertrophied nuclei. Giant cell cytoplasm was aggregated along a thickened cell wall. Vascular tissues within galls appeared disorganized. The relationship between the initial nematode population density (*Pi*) in a series from 0 to 1,024 eggs and juveniles/cm^3 soil and growth of white mulberry seedlings was tested in the greenhouse. A Seinhorst model was fitted to plant height and top fresh weight. Tolerance limits of white mulberry to *M. arenaria* race 2 for plant height and top fresh weight were, respectively, 1.1 and 1.38 eggs and juveniles/cm^3 soil. The minimum relative values for plant height and top fresh weight were 0 at $Pi \geq 64$ and $Pi \geq 128$ eggs and juveniles/cm^3 soil, respectively. Maximum nematode reproduction rate was 435-fold at the lowest *Pi*.

Additional keywords: histopathology, *Morus alba*, pathogenicity, threshold limit

Severe feeder-root infections of garden grown white mulberry trees and heavy soil infestations by *Meloidogyne arenaria* (Neal) Chitwood were found recently at two localities in Córdoba and Sevilla provinces in Andalucía, southern Spain. This is the first recorded infection of white mulberry by this root-knot nematode in Europe. The highly devastated root system and the abundance of characteristic spherical galls suggested that specialized nematode-plant relationships might occur.

White mulberry (*Morus alba* L.) is a perennial tree of the Moraceae family that originates from the lower slopes of the Himalayas (17). This tree can grow under climatic conditions ranging from temperate to tropical, and is economically important in China, Egypt, and India, where the foliage serves as food for the monophagous silk worm (*Bombix mori* L.) (2,25,26). In addition, mulberry plantings play a role in a sustainable strategy for soil and water conservation in north China (26).

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Attacks by *M. arenaria* may be a constraint for mulberry cultivation; however, little information exists on the host-parasite relationships between this root-knot nematode and white mulberry. *M. arenaria* is often found in greenhouses in northern Europe, and is common in woody fruit crops in European countries (12,14). The taxonomic identification of the *M. arenaria* population infecting these crops, including white mulberry, is of concern because this species is morphologically and geographically very close to *M. hispanica* Hirschmann. *M. hispanica* was first described parasitizing peach (*Prunus persica* L.) grown in Sevilla (8). In addition, populations of *M. arenaria* can be differentiated into two pathogenic races. Race 1 reproduces on peanut (*Arachis hypogaea* L.) and is distributed mainly in peanut-growing areas. Race 2 is widespread and can reproduce on many hosts but not on peanut and pepper (*Capsicum annuum* L.) (18). Thus, accurate identification of *M. arenaria* populations and their pathogenic race characterization are needed for designing effective control measures in the context of sustainability and integrated pest management. This is especially important in root-knot nematodes, since host-plant resistance to reduce the initial nematode population density is scarce among crop plants (18). Furthermore, the extent of crop growth impairment by the nematode is influenced by nematode population density, with a minimum population density determining the threshold for measurable yield loss (the tolerance limit) (19). The objectives of this study were to determine: first, the taxonomic identity of the *M. arenaria* population infecting white mulberry; second, the histopathology in nematode-feeding sites on white mulberry roots; and third, the relationship between initial population density of the nematode and growth of white mulberry seedlings under greenhouse conditions.

MATERIALS AND METHODS

Nematode diagnosis and pathogenicity. The root-knot nematode infecting white mulberry was identified by means of microscopic observations and isozyme characterization of specimens (13). Samples of white mulberry feeder roots together with bulk soil were taken with a shovel from the upper 20 cm of soil from each of two home gardens at La Carlota (Córdoba province) and Utrera (Sevilla province) in southern Spain. Second-stage juveniles and males extracted from roots and soil (1), and females recovered from infected root tissues, were mounted in glycerin. Nematode anatomy of glycerin infiltrated specimens were examined by light microscopy. Single- or five-specimen groups of young egg-laying females were studied by isozyme electrophoretic methods (13).

For pathogenicity studies, the population of root-knot nematodes collected from infected white mulberry and a population of *M. hispanica* infecting peach in Sevilla, Spain, were used for a comparative study of *M. arenaria*-race differentials. Inoculum of *M. arenaria* consisted of eggs and second-stage juveniles collected with sodium hypochlorite (9). Inoculum of *M. hispanica* was first increased in tomato (*Lycopersicum esculentum* Mill. 'Rutgers') and extracted as that for *M. arenaria*. Twenty-day-old seedlings of cotton (*Gossypiun hirsutum* L. 'Delta Pine'), peanut 'Florunner', pepper 'Early California Wonder', tobacco (*Nicotiana tabacum* L. 'NC 95'), tomato 'Rutgers', and watermelon (*Citrullus vulgaris* Schad. 'Charleston Grey'), were transplanted (one per pot) into 1-liter clay pots filled with autoclaved field soil. Two days later individual seedlings were inoculated by adding 10 ml of a suspension

containing 10,000 eggs and juveniles of the *M. arenaria* or *M. hispanica* populations. Plants that served as controls received the same amount of water. Plants were incubated in a greenhouse adjusted to 26 ± 2ºC. There were four plants per nematode population-race differential combination. Fifty days after inoculation plants were uprooted and their roots gently washed, examined, and rated both for galls and egg masses developed.

Histopathology. Galled roots from naturally *M. arenaria*-infected white mulberry plants sampled at La Carlota (Córdoba) and from plants artificially infected in the inoculum-density plant-growth experiment were selected for histopathological studies. Roots were gently washed free of soil and debris, and individual galls selected. Root segments of uninfected seedlings served as control. Galled and healthy root tissues were fixed in formaldehyde chromoacetic solution for 48 h, dehydrated in a tertiary butyl alcohol series (40-70-85-90-100%), and embedded in 58ºC-melting point paraffin for histopathological observations. Embedded tissues were sectioned with a rotary microtome. Sections 10 to 12 µm thick were mounted on glass slides, stained with safranin and fast-green, mounted permanently in dammax xylene, examined microscopically, and photographed (10).

Inoculum-density plant-growth impairment relationship. Inoculum of *M. arenaria* was extracted (9) from white mulberry roots collected at La Carlota. This inoculum was increased in tomato 'Rutgers' inoculated as before and incubated in a greenhouse adjusted to 26 ± 2ºC. Two months after inoculation, at the time that egg masses were well formed in the tomato roots, the inoculated plant roots were washed free of soil and finely chopped. To estimate the amount of eggs and juveniles formed in the chopped tissue, 10 5-g aliquots of infected chopped roots were suspended in 1% aqueous solution of sodium hypochlorite in 100-ml jars for 4 min (9). For inoculation, chopped infected roots were thoroughly mixed with 3 kg of steam-sterilized sandy soil and the mixture was used as inoculum within a range of inoculum densities. Appropriate amounts of this inoculum were mixed with a potting mixture of 97.5% steam-sterilized sandy soil (sand 88%, silt 5%, clay 7%) and 2.5% organic matter, to reach a population

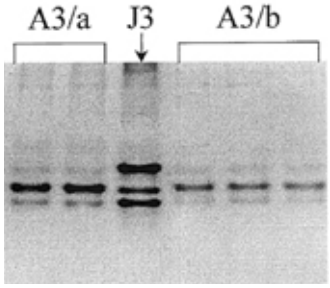

Fig. 2. *Meloidogyne arenaria* race 2. Esterase electrophoresis pattern of protein homogenates from five (A3/a) and single young egg-laying females (A3/b). J3 = *M. javanica* (reference population).

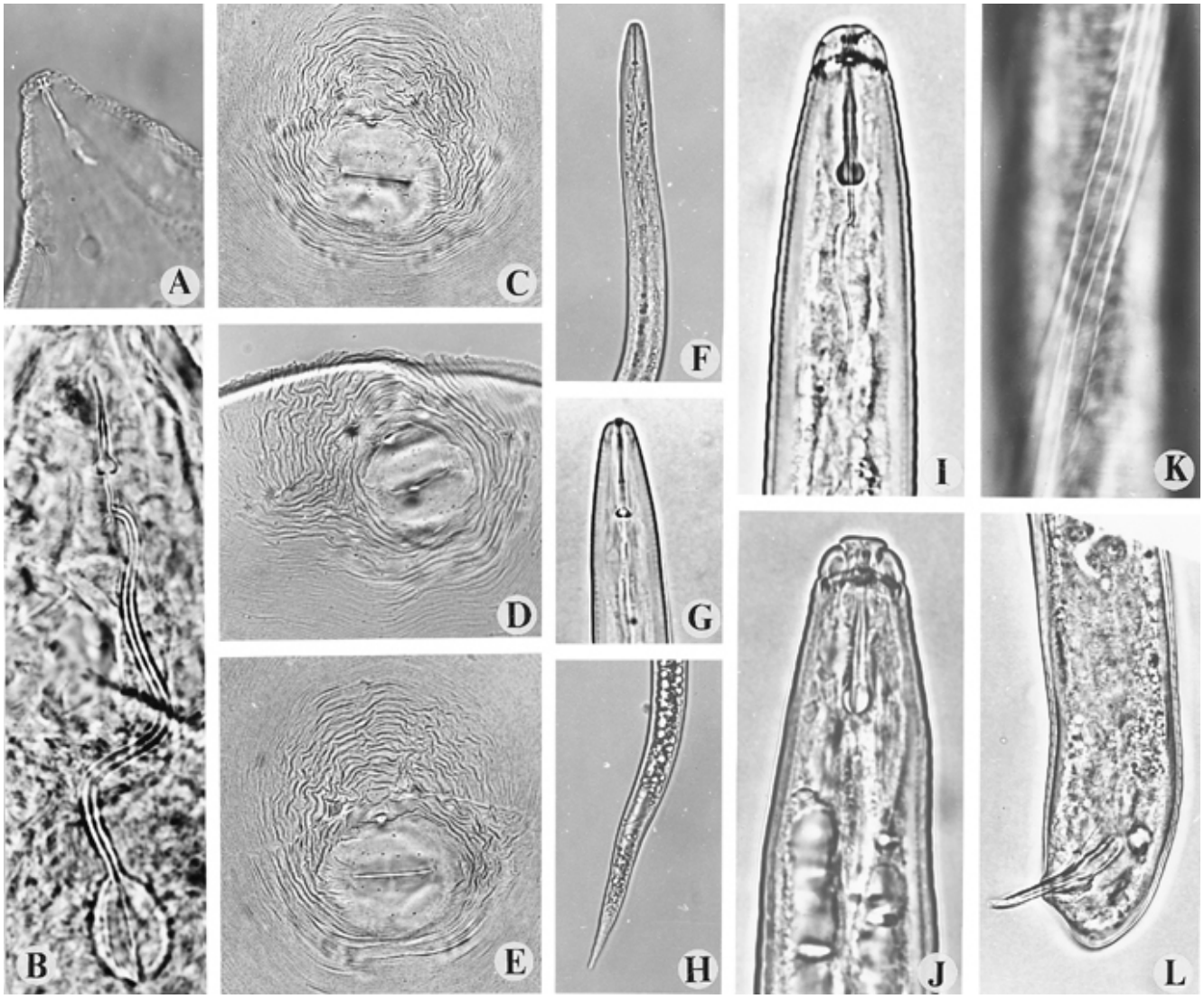

Fig. 1. Diagnostic features of *Meloidogyne arenaria*. **A** and **B,** Female anterior regions. **C, D,** and **E,** Female perineal patterns. **F, G,** and **H,** Second-stage juvenile. **I** and **J,** Male anterior region. **K,** Lateral fields of male at mid-body. **L,** Male tail region; ep = excretory pore.

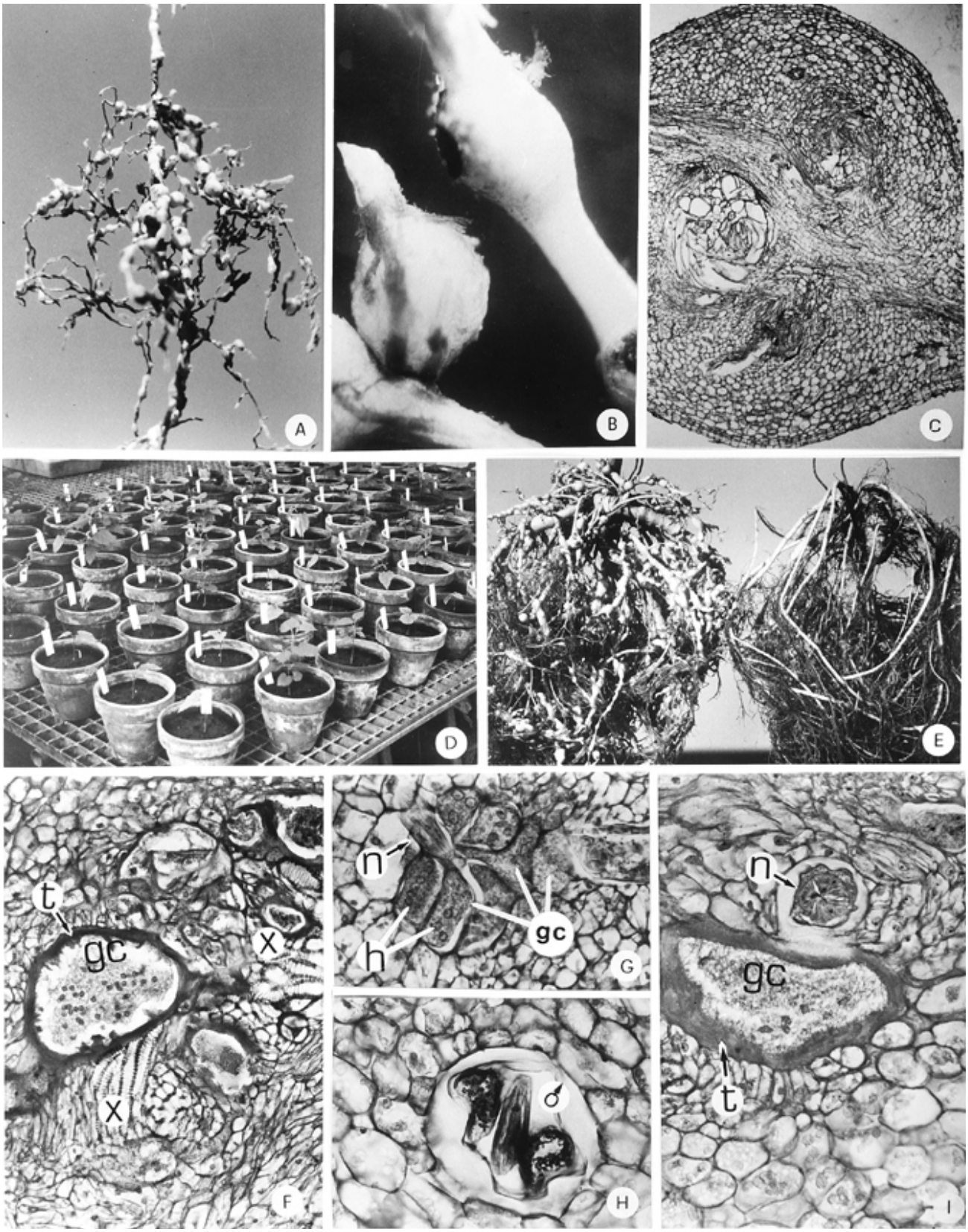

Fig. 3. Host-parasite relationships between *Meloidogyne arenaria* race 2 and white mulberry. **A,** and **B,** Galled roots from naturally infected plants, showing spherical galls. **C,** Longitudinal section of naturally infected roots showing giant cells and galling induced by *M. arenaria*. **D,** Display of plants artificially inoculated with *M. arenaria* showing marked reduction in shoot growth. **E,** Root systems of plants artificially inoculated with 1,024 eggs + J_2/cm^3 of soil of *M. arenaria* (left) and uninoculated control (right), respectively. **F, G, H,** and **I,** Transverse sections of roots from plants artificially inoculated with *M. arenaria*. Abbreviations: gc = giant cell; h = hypertrophic nucleus; n = nematode; t = thickened cell wall; x = xylem.

density of 0, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, and 1,024 eggs and juveniles/cm^3 soil, and 600-ml clay pots were filled with the infested soil mixture. A single, 2-month-old seedling of white mulberry 'Superior' was transplanted into each pot. There were eight pots for each inoculum density, arranged in a randomized complete block design in a greenhouse at 26 ± 2ºC. Three months after transplanting, top fresh weight and height of plants were recorded. Plants were uprooted, the roots washed free of adhering soil and weighed, and eggs and juveniles in the egg masses in roots were extracted by the sodium hypochlorite method (9). Nematodes in soil were extracted by the modified Coolen's method (1,4). The final nematode population densities were calculated as the total of that from roots and soil. The relationship between plant growth (indicated by the top fresh weight and height of plants) and the initial nematode population density was determined by fitting the data to the Seinhorst model: $y = m + (1 - m)z^{P-T}$ when $P \geq T$, and $y = 1$ when $P < T$ (19,20). In this model, y = relative value of the plant growth parameter; m = minimum y value (y at a very large initial nematode population density); P = the initial nematode population density; T = a tolerance limit (initial population at which plant growth is not impaired) and z is a constant >1 reflecting nematode damage, with $z^{-T} = 1.05$ (19). The Seinhorst equation was fitted using the SeinFit program (21). The coefficient of determination (R^2) and the residual sum of squares were used to indicate goodness-of-fit of data to the model.

RESULTS AND DISCUSSION

Nematode diagnosis and pathogenicity. Detailed morphometric observations based on the sampled second-stage juveniles, shape of male stylet knobs and features of the female perineal pattern (Fig. 1) agreed with those that characterize *M. arenaria* (15). The isozyme electrophoretic analyses of single- and five-specimen groups of young egg-laying sampled females (Fig. 2) revealed the esterase pattern that is characteristic of *M. arenaria* (6). Inoculations of the *M. arenaria* race differentials indicated that the population of the root-knot nematode from white mulberry in southern Spain is not virulent to cotton, peanut, and pepper, but is virulent to tobacco, tomato, and watermelon. Therefore, this population was identified as *M. arenaria* race 2. The pattern of disease reactions induced by the population of *M. hispanica* in the differentials host was the same than that induced by *M. arenaria*. Therefore, these two species of root-knot nematodes are closely related both taxonomically and biologically.

Histopathology. Galls occurred either singly or in clusters which encircled the entire root perimeter. In this latter case the root diameter was 2 to 6 times larger than that of uninfected roots (Fig. 3). More than 45% of individual galls selected at random contained an egg mass. Usually, galls contained more than one nematode female. Observations of stained root sections revealed both tissue hypertrophy and hyperplasia, as well as disorganization and disruption of xylem elements and primary phloem cells. Nematode feeding sites comprised 3 to 8 giant cells that surrounded the lip region of a single female. Undersized feeding cells were associated with pre-adult males. Active multinucleated giant cells contained granular cytoplasm, thickened cell wall, and numerous hypertrophied nuclei and nucleoli. Dense giant cell cytoplasm lined deeply stained thick walls. The histological and anatomical changes induced by *M. arenaria* to mulberry roots were similar to those described for other root-knot nematode species infecting fruit trees (11,14,22).

Inoculum-density plant-growth impairment relationship. The inoculum densities of the nematode included in the study impaired growth of mulberry plants (Fig. 3). The relationship between the top fresh weight and height of plants and the initial nematode population density was appropriately described by the Seinhorst equation (Fig. 4). Symptoms of attack by *M. arenaria* race 2 and reduction of plant top growth were evident 10 days after inoculation even with an initial population density of Pi = 16 eggs and juveniles/cm^3 soil. Reduction of plant top growth was also evident at lower initial population densities 20 days later. The white mulberry tolerance limits (T) to *M. arenaria* race 2 were 1.1 and 1.38 eggs and juveniles/cm^3 soil for height plant and top fresh weight,

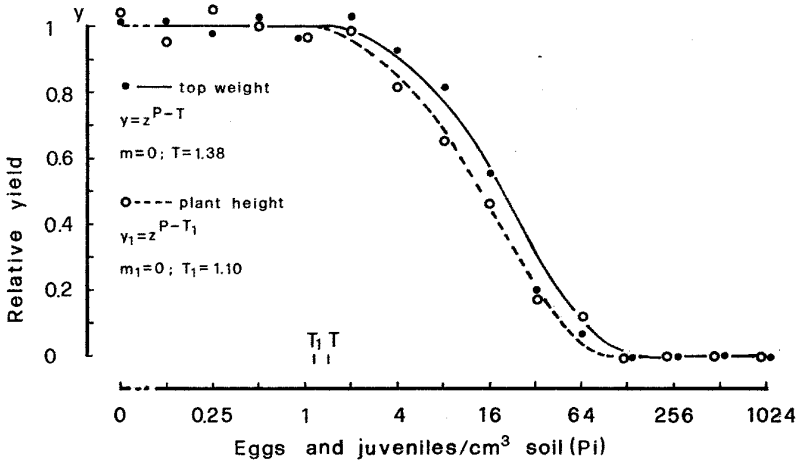

Fig. 4. Relationship between initial population densities (*Pi*) of a population of *Meloidogyne arenaria* race 2 from Spain and relative top fresh weight and height of white mulberry plants grown in pots at 26 ± 2°C in the greenhouse for 3 months. Actual data are presented for top weight (•) and plant height (○). Solid and dashed lines represent the predicted model. Statistics for fitted models of top and plant height were: $R^2 = 0.84$, Sum of squares = 16.31; and $R^2 = 0.90$, Sum of squares = 154.16, respectively.

Table 1. Relationship between initial population density of *Meloidogyne arenaria* race 2 (*Pi*, eggs and juveniles per cm^3 soil) and final population density (*Pf*) and reproduction rate (*Pf/Pi*) in white mulberry seedlings[a]

Initial population density (*Pi*)	Final population density (*Pf*)	Reproduction rate (*Pf/Pi*)
0.125	20.6	164.8
0.25	108.7	434.8
0.5	135.2	270.4
1	99.2	99.2
2	91.8	45.9
4	68.4	17.1
8	86.7	10.8
16	42.2	2.6
32	20.8	0.6
64	15.1	0.2
128	50.6	0.4
256	21.0	0.1
512	26.9	0.1
1,024	23.1	0

[a] Two-month-old white mulberry seedlings were transplanted (one per pot) into a potting mixture infested with the appropriate Pi. Plants were grown in a greenhouse adjusted to 26 ± 2ºC for 3 months.

respectively (Fig. 4). The minimum relative value (*m*) for plant height and top fresh weight was 0 at $Pi \geq 64$ and $Pi \geq 128$ eggs and juveniles/cm^3 soil, respectively. The maximum nematode reproduction rate (*Pf/Pi; Pi* = initial population density, *Pf* = final popuation density) was 434.8 at *Pi* = 0.25 eggs and juveniles/cm^3 of soil. This reproduction rate decreased as the initial nematode population increased (Table 1). Reduction of nematode reproduction rate with increasing initial nematode inoculum density has been reported associated with infections of several crops by *M. incognita* (3–5). Our findings could be a consequence of nematode competition for nutrients or root tissue availability (feeding sites), as a result of which a smaller proportion of the inoculum would develop succesfully.

Results demonstrated that *M. arenaria* race 2 has the potential to severely impair growth of white mulberry. The tolerance limit of this plant to the nematode is as low as approximately 1 egg/cm^3 soil. An initial population density of this parasite exceeding 64 eggs/cm^3 soil had a lethal result for white mulberry. Our results on pathogenicity of *M. arenaria* on white mulberry agreed with those of other researchers in China (23,24) and India (7,16), who found that *M. arenaria* and *M. incognita* (Kofoid et White) Chitwood significantly reduced the number of leaves and plant growth of white mulberry trees. Therefore, control measures need to be implemented in order to guarantee production of nematode-free planting stocks and to avoid spread of the nematode to areas not infested.

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