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Effect of Temperature on Attachment, Development, and Interactions of *Pasteuria penetrans* on *Meloidogyne arenaria*[1]

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Abstract: The effect of temperature (10, 20, 25, 30, and 35 C) on attachment and development of *Pasteuria penetrans* on *Meloidogyne arenaria* race 1 was elevated in growth chambers. The greatest attachment rate of endospores of *P. penetrans* occurred on second-stage juveniles at 30 C. The bacterium developed more quickly within its host at 30 and 35 C than at 25 C or below. The development of the bacterium within the nematode female was divided into nine recognizable life stages, which ranged from early vegetative thalli to mature sporangia. Mature sporangium was the predominant life stage observed after 35, 40, 81, and 116 days at 35, 30, 25, and 20 C, respectively. The body width and length of *M. arenaria* females infected with *P. penetrans* were smaller initially than the same dimensions in uninfected females, but became considerably larger over time at 25, 30, and 35 C. This isolate of *P. penetrans* also parasitized and completed its life cycle in males of *M. arenaria.*

Key words: Arachis hypogaea, bacterium, biological control, development, life cycle, *Lycopersicon esculentum,* male, *Meloidogyne arenaria,* nematode, parasitism, *Pasteuria penetrans,* sex reversal, scanning electron microscopy, peanut root-knot nematode.

Pasteuria penetrans is a specific obligate parasite of plant-parasitic nematodes. The development of the parasite begins with the germination of the endospore after it attaches to the nematode cuticle. A germination tube extends through the nematode cuticle and forms vegetative thalli in the nematode body cavity. These vegetative thalli enlarge, branch, and spread throughout the nematode body, and produce sporangia that endogenously form single spores (12). The parasite's development appears synchronized with its host and does not appear to impair the nematode's feeding ability. Root-knot nematode females infected by *P. penetrans* develop to maturity but generally do not produce a functional reproductive system, as the body of the nematode becomes filled with endospores (3). Parasitized females and uninfected females of *Meloidogyne javanica* do not differ in body size early in their development; however, healthy females are larger than infected females by the time the healthy females begin laying eggs (3).

The developmental and sexual stages of the nematode in which the bacterium completes its life cycle may vary with the *Pasteuria* species or isolate. Endospores of a *Pasteuria* spp. parasitizing the cereal cyst nematode *Heterodera avenae* attached to the cuticle and also occurred inside the bodies of second-stage juveniles (J2) (6). In one instance, endospore-filled J2 of *Meloidogyne* spp. were described (9). We have also observed endospore-filled J2 of *M. incognita* (unpubl.). Endospores of *P. penetrans* are usually observed inside bodies of *Meloidogyne* females or attached to the cuticle of J2 and males (1,16). The J2–adult life stages of *Belonolaimus longicaudatus, Hoplolaimus galeatus,* and *Pratylenchus* spp. have been reported filled with endospores (9), although the completion of the life cycle of *P. penetrans* within males of *Meloidogyne* spp. has not been observed (18).

Environmental conditions such as temperature greatly influence the rate of the bacterium's development in *M. javanica,* with an optimum temperature at 30 C (19). Excess watering of experimental host plants, however, slowed down the para-

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site's development in *M. incognita* females (7).

The present study was conducted to determine the effect of temperature on attachment of *P. penetrans* to *M. arenaria,* duration of developmental life stages and reproduction, and the growth rates of healthy and parasitized nematode females. Also, we describe the parasitism of *M. arenaria* males.

Materials and Methods

The root-knot nematode *Meloidogyne arenaria* (Neal) Chitwood race 1 and the bacterium *P. penetrans* Sayre & Starr isolate P-20 used in this study originated from the Fugate farm, Levy County, Florida. The nematode was reared in a greenhouse on tomato (*Lycopersicum esculentum* Miller cv. Rutgers). Eggs of *M. arenaria* were extracted from infected roots by the sodium hypochlorite method (10); J2 were obtained from these eggs on a modified Baermann funnel (2) and used within 2 days. The bacterium was maintained on an *M. arenaria* population in the greenhouse (15).

Effect of temperature on attachment to J2: A sandy soil (94% sand, 5% silt, 1% clay; pH 7.5; <1% organic matter) collected from the Fugate farm containing relatively high number of endospores of a field population of P-20 was air dried at 28 C in an incubator. Soil moisture content of the dried soil was adjusted to 50% by weight of the water holding capacity. The endospore viability was screened on freshly hatched J2 of *M. arenaria* incubated at 28 C for 24 hours; the *P. penetrans* population was considered viable when five or more endospores attached per J2 of *M. arenaria.*

A 40 g aliquot of the air-dried soil infested by *P. penetrans* was then added per petri dish (9-cm-d), and 4,000 freshly hatched J2 were added. The J2 were incubated for 24 hours at 10, 20, 30, and 35 C. Each treatment was replicated four times in two trials. At the end of the experiment, the J2 were extracted from the soil with a centrifugal flotation technique (11). Dead and living nematodes were distinguished by staining J2 with a 0.01% solution of Meldola Blue (14) for 17 hours at room temperature. The number of endospores attached to 20 living J2 in each replicate was determined with the aid of an inverted microscope.

The endospore counts were transformed by $\log_e (x + 0.1)$ before the data were subjected to analysis. The data sets from the two trials were first combined to examine differences between the trials. Each data set was subjected to regression analysis using the general linear model procedure in SAS (17) to generate response curves relating endospore numbers to temperature.

Development of P. penetrans in females: Endospores of population P-20 were extracted from *M. arenaria* females inside infected peanut (*Arachis hypogaea* L. cv. Florunner) roots. The roots (100 g) were softened by shaking in 250 ml of Pectinol 59 (Genencor, South San Francisco, CA) at 25 C for 24 hours, decanted onto a 600–150-μm pore sieve series, and subjected to a high-pressure spray of water in order to dislodge female nematodes. The endospore-filled females were caught on the 150-μm-pore sieve, collected by hand with the use of a stereomicroscope, and then macerated in a glass tissue grinder in deionized water to release the endospores.

Approximately 100,000 freshly hatched *M. arenaria* J2 were incubated at room temperature overnight in 150 ml tap water in an Erlenmeyer flask containing 10^5 endospores/ml of P-20. Approximately 600 endospore-ladened J2 were added around each root system of seventy-five 4-week-old tomato plants growing in 200 cm^3 soil in pots. Seventy-five plants each inoculated with 600 healthy J2 were included for comparison. All seedlings were incubated at 25 C. After 48 hours, the seedlings were carefully removed from the soil and their roots were washed to remove J2 from the root surface. The seedlings were then transplanted into autoclaved sand in pots and placed into growth chambers at 10, 20, 25, 30, and 35 C. Fifty plants per treatment

were used at 25 C and below, and 25 plants were used at 30 C and above. The tomato plants were watered when necessary and exposed to a 12-hour-day photoperiod.

Nematodes were dissected from roots after being shaken 24 hours in 100 ml Pectinol 59 at 25 C. The developmental stages of *P. penetrans* in *M. arenaria* were determined weekly, starting 12 days after inoculation at 30 and 35 C, 21 days at 25 C, and 32 days at 20 C. Preliminary studies indicated these intervals were satisfactory for discerning differences in the development of *P. penetrans.*

Preparations of *P. penetrans* life stages for observation with a scanning electron microscope (SEM) were made by squashing selected bacterium-infected female nematode specimens in a drop of water on a silanized 12-mm-d circular cover glass (25). After 20 minutes the specimens were rinsed in 0.1% phosphate buffer (pH 7.2) and placed in a 2% solution of glutaraldehyde in phosphate buffer overnight. After dehydration through a graded ethanol series (25%, 50%, 75%, 95%, and 100%), the specimens were immersed in hexamethyldisilazane for 5 minutes and air dried (13). The cover glass was mounted on an aluminum stub, sputter coated with gold palladium, and examined with a Hitachi S450 or S4000 SEM operated at 5–20 kV.

At the same time intervals listed, 10 randomly selected specimens were squashed on a glass slide, air dried at room temperature, heat fixed, Gram stained (5), and mounted in Permount (Fair Lawn, NJ) for light microscopic observation. The most predominant bacterial life stages were recorded at each sampling time.

The experiment was repeated similarly, except 25 C was substituted for 10 C because of lack of observed development at 10 C. Also, an additional light source was added to the chambers set at 30 and 35 C to improve plant growth. The sample size for determining the developmental stages of *P. penetrans* was increased to a maximum of 20 females per observation date. At the last observation date the total number of endospores per root system was determined for five plants of the *P. penetrans* treatment from each temperature. All females were dissected from each root system, squashed, and dispersed in water, and the number of mature sporangia was counted. The data were subjected to regression analysis.

Female body size comparisons: The body widths and lengths of up to 20 *P. penetrans*-infected and uninfected *M. arenaria* females recovered from tomato roots grown at 20, 25, 30, and 35 C were measured with a microcomputer data acquisition system (Southern Micro Instruments, Atlanta, GA). Measurements of females of the untreated control were discontinued when females reached their peak of growth—after 30 days at 30 C, 35 days at 25 C, and 40 days at 20 C. After measurements were taken, females from the *P. penetrans* treatment were examined for parasitism. Except for data from uninfected nematodes (which was discarded), all data were subjected to regression analysis by fitting quadratic response curves for body width and length to each treatment and temperature level.

Parasitism of males by P. penetrans: Several times during the studies on development of *P. penetrans,* males of *M. arenaria* infected with the bacterium were extracted from roots. Infection of males appeared to be an unusual event (19). One mechanism by which males could become infected would be through sex reversal (24). Under normal conditions J2 (assumed to be sexually undifferentiated) parasitized by *P. penetrans* develop into females with two ovaries. If feeding conditions for such J2 are favorable during their early stages of development, but later change to unfavorable, than such J2 undergo sex reversal resulting in males with two testes (24).

An experiment was set up to determine the extent of *P. penetrans* infection of males and to determine if infected males had undergone sex reversal. Approximately 600 J2 of *M. arenaria,* each with an average attachment of 10 endospores/J2, were inoculated around the roots of one 4-week-old tomato plant/pot growing in 100 cm^3 soil at

25 C. A control treatment with 600 healthy J2 was included. Each treatment contained five replicates, and the experiment was repeated once. After 48 hours at 25 C the temperature was increased to 30 C, because high numbers of males had been found inside root systems between the 18th and 25th day postinoculum at 30 C in preliminary tests. After 21 days, plants from each treatment were removed from the soil. The roots were washed, stained (4), and stored in glycerin:lactic acid:water 2:1:1 (v:v:v). Males were extracted from soil with the centrifugal flotation technique. All nematodes within each root system were extracted from the roots under a stereomicroscope. The number of testes were counted in a minimum of 20 males per replicate from the treatment without *P. penetrans;* the males from the treatment with *P. penetrans* were observed for parasitism by the bacterium.

Results

Effect of temperature on attachment to J2: The number of endospores of *P. penetrans* that adhered to the cuticle of *M. arenaria* J2 was affected by temperature (Fig. 1). Because data from the two trials differed ($P \leq 0.05$), response curves were fitted separately for each trial. Both tests showed a similar trend, however. More endospores attached as the temperature was increased to 30 C ($P \leq 0.001$), with the greatest number at 30 C. Above 30 C, the number of endospores attached per J2 declined.

Development of P. penetrans in females: Average endospore attachment in these experiments was seven endospores per J2 with 60% of the J2 having five or more endospores attached. Because of the suboptimum light intensities of the first experiment, which affected plant growth and influenced host–parasite relationships, only data from the second test were used for analysis. The developmental period of *P. penetrans* was greatly affected by temperature ($P \leq 0.05$). Observations by light and scanning electron microscopy revealed continuous development from the early mycelial stage to the mature sporangium. The life cycle of *P. penetrans* was

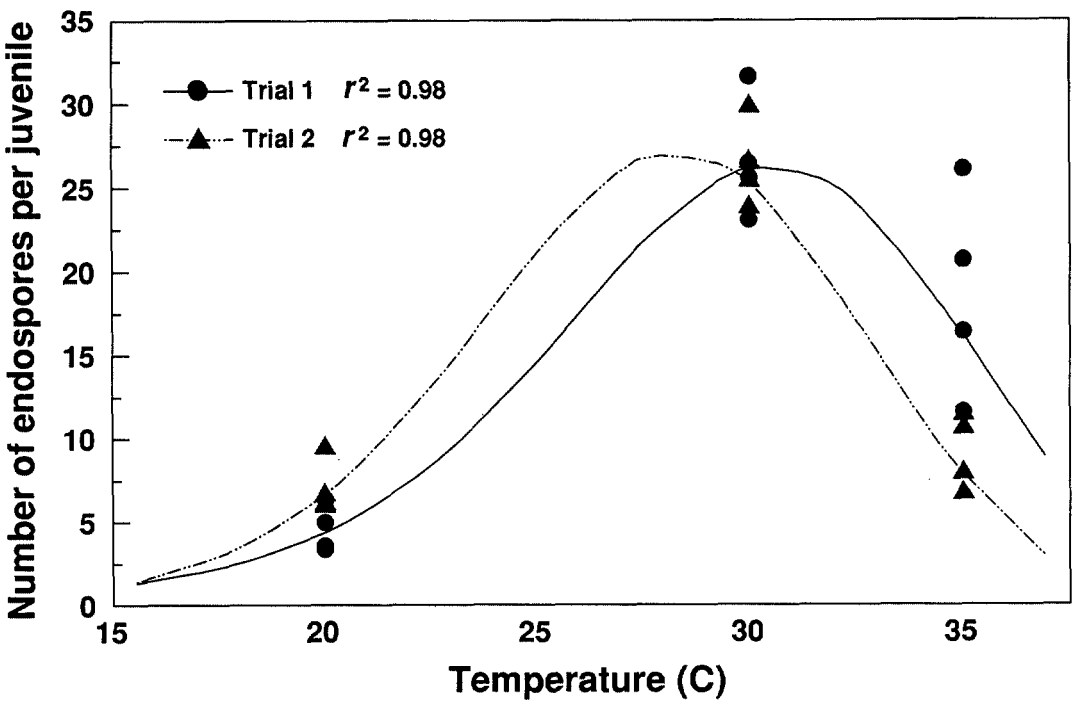

FIG. 1. Attachment of endospores of *Pasteuria penetrans* on second-stage juveniles (J2) of *Meloidogyne arenaria* race 1 after 24 hours incubation in *P. penetrans*-infested soil at 10, 20, 30, and 35 C. The lines are the predicted response from a model fitted separately to the data from each trial. The data points are means from 20 observed J2 per replicate.

divided into nine developmental stages that ranged from early vegetative thalli to mature sporangia (Fig. 2). Within the first 18 days after inoculation, at 35 C the mycelial colonies became irregularly shaped and enlarged rapidly, and mycelial

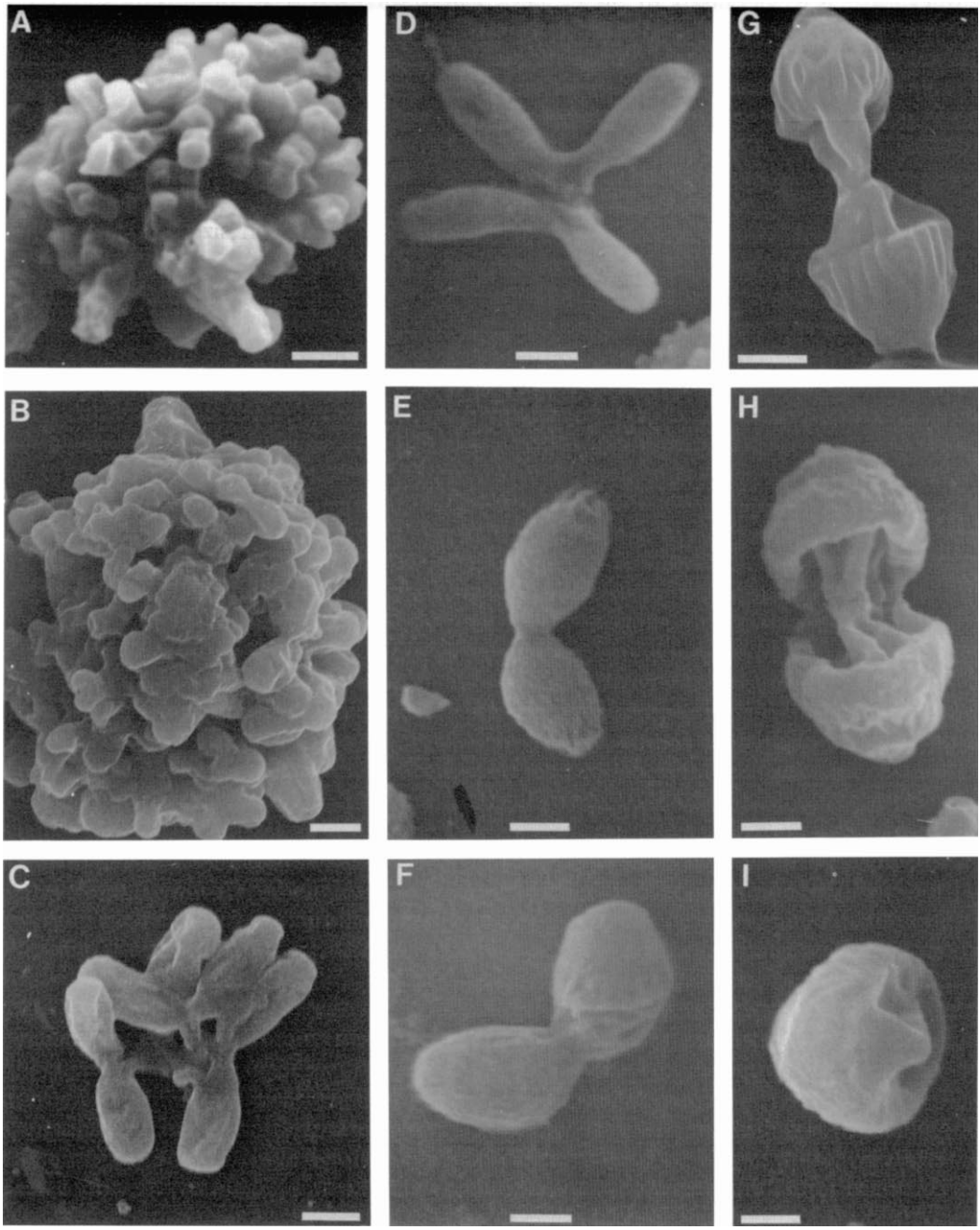

FIG. 2. Scanning electron micrographs of the developmental stages of *Pasteuria penetrans*. Scale bars = 1 µm. A) Early vegetative stage of a mycelial colony. B) Later stage of a mycelial colony, irregularly shaped and enlarged. C) Mycelial branches after breaking apart from the thallus cluster. D) Enlargement of mycelial branches and fragmentation into quartets. E) Early sporangial development, doublets beginning to form. F) Sporangium size increases and doublets become predominant. G) Endospore development within the sporangium continues. H) Nearly mature sporangia beginning to separate. I) Single mature sporangium with endospore inside.

branches broke apart from the thallus clusters forming quartets (Fig. 2A–D). Also, within this timeframe early sporangial development was observed. The sporangium size increased, and doublets became the predominant stage of development (Fig. 2E,F). The developmental study did not include endospore attachment to or penetration of the J2 cuticle.

With increasing temperature, the development of *P. penetrans* began earlier and the final developmental stage (mature sporangium) was also observed earlier (Fig. 3). Development of mycelial colonies of the vegetative stage was first observed after 12 days at 30 and 35 C, 25 days at 25 C, and 45 days at 20 C. At 35 C mature sporangia were the predominant stage present 35 days after inoculation (Fig. 3). At 20 and 25 C, the development of *P. penetrans* progressed much more slowly than at 30 and 35 C. Mature sporangia became the predominant stage after 81 days at 25 C and 116 days at 20 C.

Numbers of mature sporangia per root system were also related to temperature ($P \leq 0.001$) (Fig. 4). At temperatures of 20, 25, 30, and 35 C, the average number of endospores per root system was 12.5×10^6, 14.7×10^6, 11.5×10^7, and 11.3×10^7, respectively.

Female body size comparisons: During early development at 25, 30, and 35 C, the uninfected females were slightly larger and reached maturity sooner than infected females, but over time *P. penetrans*-infected females increased in size and their body dimensions became greater than those of uninfected females ($P \leq 0.01$) (Fig. 5). At 20 C, however, the body size of infected females did not reach the size of healthy females until the end of our examinations (data not shown).

Parasitism of males by P. penetrans: In the first experiment, no males were extracted from soil by the centrifugal-flotation technique, but an average of 53 males was observed from root systems of the treatment

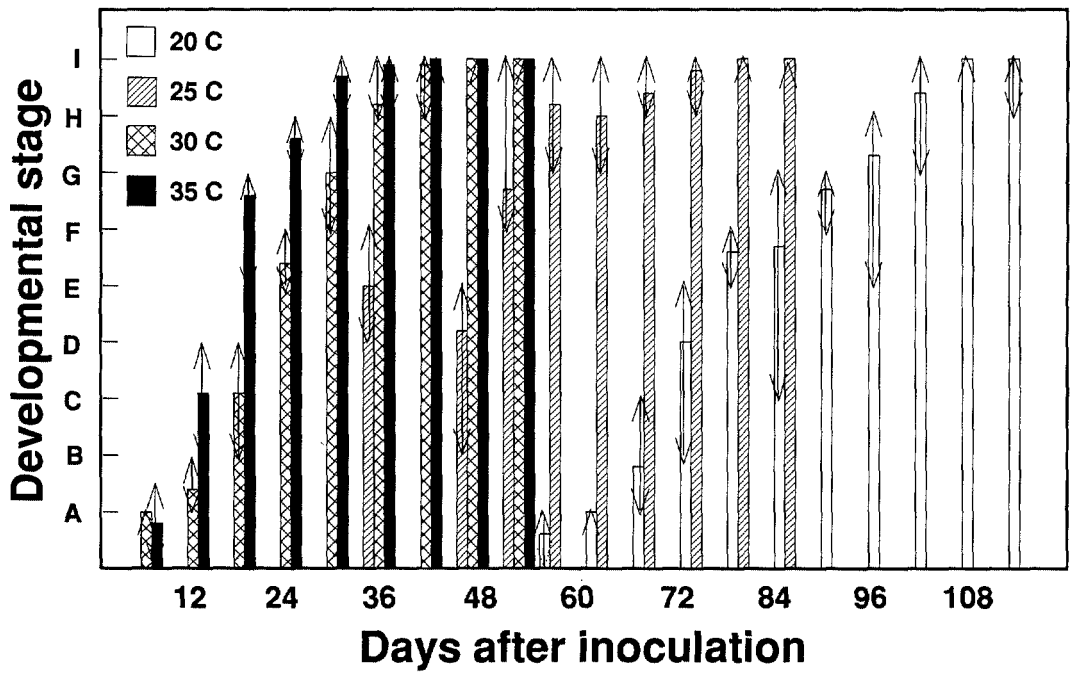

FIG. 3. Development of *Pasteuria penetrans* in females of *Meloidogyne arenaria* on *Lycopersicon esculentum* at 20, 25, 30, and 35 C over time. After inoculation refers to inoculation of tomato with second-stage juveniles of *M. arenaria* with endospores attached. The developmental stages are listed in the legend of Figure 2. Bars represent the means of more than 10 females observed at each sampling time. Vertical arrows show the range of developmental stages present at each sampling time.

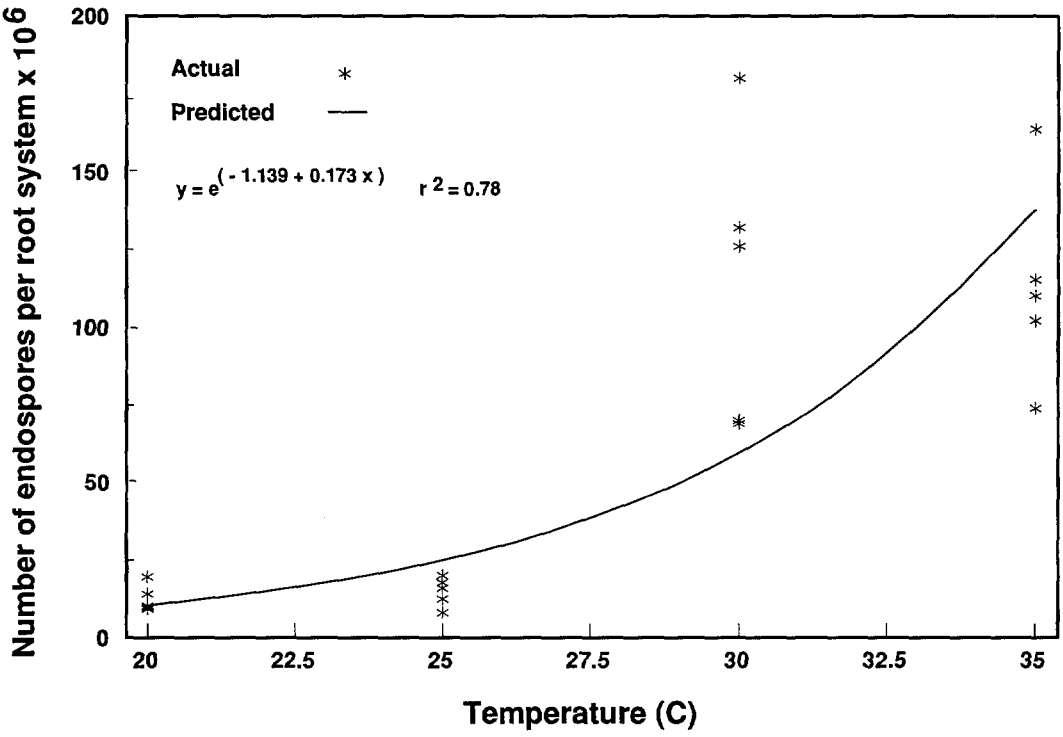

FIG. 4. Number of mature sporangia of *Pasteuria penetrans* (produced in *Meloidogyne arenaria*) per root system of *Lycopersicon esculentum* at 20, 25, 30, and 35 C. Plants had been inoculated with 600 second-stage juveniles of *M. arenaria* that had five or more endospores of *P. penetrans* attached to their cuticle. Roots were harvested 123, 88, 45, and 40 days after inoculation at 20, 25, 30, and 35 C, respectively. Single data points are observed values per root system.

without *P. penetrans* and an average of 11 males was observed from the treatment with *P. penetrans* (Table 1). In the treatment without *P. penetrans,* the number of testes could be determined in each male examined (an average of 10% and 90% of the males had one testis or two testes, respectively). The number of testis could be determined in only 50% of the specimens examined in the treatment with *P. penetrans,* because the presence of the developing bacterium within the male bodies made it difficult to see the gonads (Fig. 6). When it was possible to determine the number of testes, the infected males had two testes. Twelve percent of the males that showed no evidence of the bacterium had two testes. In the second experiment, again no males were extracted from soil. The total number of nematodes that had entered the root systems was low, with only 49 nematodes observed per root system in the treatment without *P. penetrans* and 62 nematodes in the treatment with *P. penetrans.* An average of 0.3 males per root system, all with two testes, was dissected from roots of the treatment without *P. penetrans;* an average of 1.3 males per root system was found in the treatment with *P. penetrans.* In the treatment with *P. penetrans,* 80% of the males were infected with *P. penetrans.* Each of these specimens had two testes, whereas the remaining 20% showed no indication of parasitism and had only one testis.

DISCUSSION

The increased rate of attachment of endospores of *P. penetrans* to J2 of *M. arenaria* with increasing temperature is consistent with attachment studies done with other *Meloidogyne* spp. (20) and is probably caused by the greater mobility of nematodes at higher temperatures (27). The de-

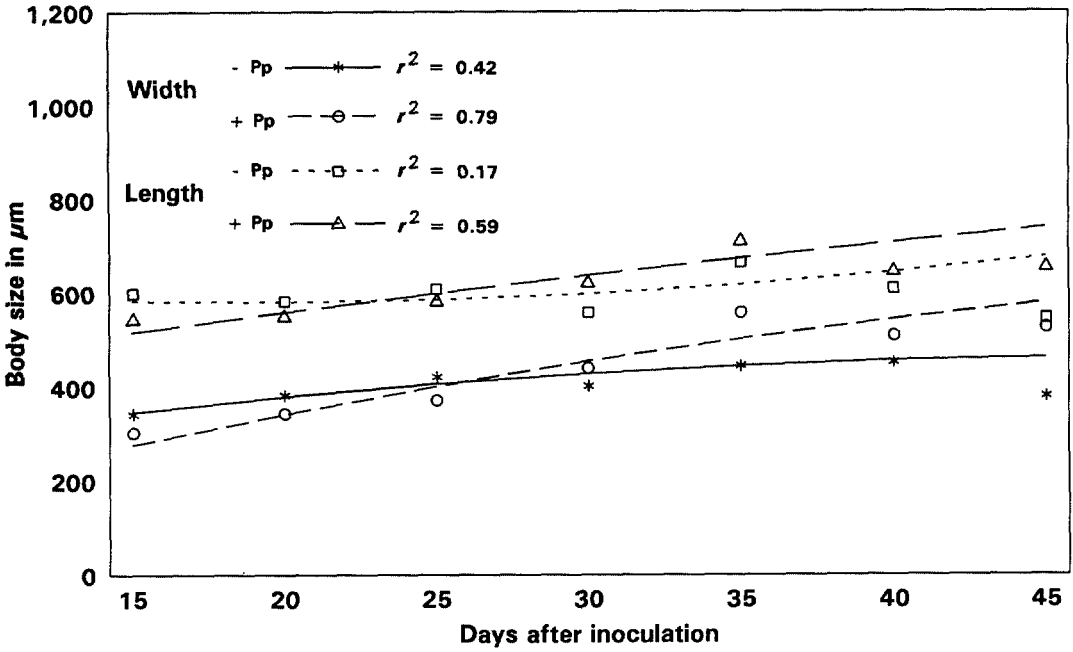

FIG. 5. Regression models (lines) and observed data points (from at least 10 observations) for width and length of *Meloidogyne arenaria* females uninfected or infected with *Pasteuria penetrans* at 30 C. Width, untreated: $y = 215.89 + 10.43x - 0.11x^2$, $r^2 = 0.42$. Width, treated: $y = 45.56 + 17.28x - 0.12x^2$, $r^2 = 0.79$. Length, untreated: $y = 631.5 - 5.28x + 0.14x^2$, $r^2 = 0.17$. Length, treated: $y = 380.37 + 9.8x - 0.04x^2$, $r^2 = 0.59$.

creased attachment rate above 35 C strengthens the theory that attachment is related to nematode mobility. *Meloidogyne* spp. have little activity above 40 C or below 5 C (21). Temperature may also modify the ability of endospores to adhere to and infect *Meloidogyne* J2. Attachment of endospores to *M. javanica* J2 was not markedly affected by heating, although infectivity was reduced (26).

Temperature is one of several environmental factors that influence the growth of microorganisms. Therefore, a positive relationship between temperature and the rate of development of *P. penetrans* was not unexpected. The length of time for development of *P. penetrans* in *M. arenaria* is markedly different at 30 to 35 C than at 20 C, with developmental time over threefold greater at 20 C. These results are similar to

TABLE 1. Total number of nematodes and males of *Meloidogyne arenaria* (per root system) infected and uninfected with *Pasteuria penetrans* and the percentage of infected and sex-reversed males 21 days after inoculation at 30 C.

Treatment	Number of nematodes per root system		Males in root system	
	Total	Males	Percentage with two testes	Percentage with visible *P. penetrans*
		Experiment 1		
Infected	261	11	†	88
Uninfected	284	53	90	0
		Experiment 2		
Infected	62	1.3	†	80
Uninfected	49	0.3	100	0

All data are means of five replications.
† Testis number determination was interfered with by *P. penetrans*.

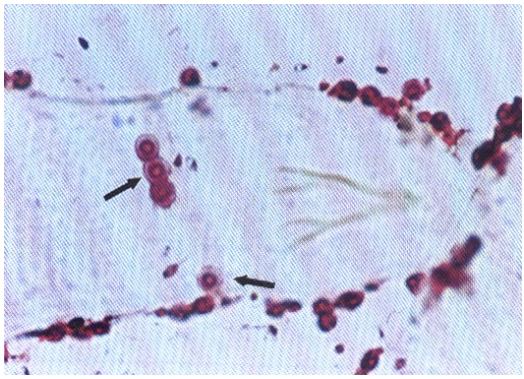

FIG. 6. Photomicrograph of the tail region of a squashed male of *Meloidogyne arenaria* parasitized by *Pasteuria penetrans*. Arrows mark mature, Gram-stained sporangia (×920).

previous experiments with *M. javanica* (19). Our isolate, however, required a slightly longer period of time until mature sporangia were first observed. Similarly, healthy *M. arenaria* females started egglaying after a longer developmental time than *M. javanica*. This further demonstrates the synchronization of development between host and parasite. The optimum temperature for development of P-20 was 35 C, characteristic of a mesophilic bacterium (23). This observation was supported by the greater numbers of endospores per root system obtained at 30 and 35 C than at 20 and 25 C. Because we did not observe development of *P. penetrans* at 10 C, its minimum developmental temperature lies between 10 and 20 C. The maximum developmental temperature was not determined but probably coincides with the maximum temperature range for the growth of the nematode. Although the upper temperature limit reported for growth and reproduction of a *M. arenaria* population from California was 32.5 C (22), we observed considerable development and reproduction of our population of *M. arenaria* at 35 C.

Pasteuria penetrans parasitism affects the body size of *M. arenaria* females. The measurements for the uninfected female bodies were within the normal ranges for *M. arenaria* (8). Females infected with *P. penetrans* grew at a slower rate initially than uninfected females; however, at 25, 30, and 35 C, the body volume of infected females eventually surpassed that of healthy individuals. This slower rate of growth of infected females may be caused by the initial infection process by the parasite. Given the slow development rate at 20 C, the infected females at this temperature likely would also have become larger than the uninfected specimens if we had continued our experiment.

Our isolate of *P. penetrans* completed its life cycle inside the bodies of males of *M. arenaria*. This is the first report of *P. penetrans* development inside *Meloidogyne* males, although attachment has been reported (1,16). Because only attachment and not parasitism was reported previously, we propose that attachment be defined as the adhesion of the endospore to the outside cuticle of a nematode and that parasitism refer to the observation of developmental structures of *P. penetrans* within the nematode body after penetration by the bacterial endospore.

We believe there may be a connection between parasitism of males of *M. arenaria* by *P. penetrans* and sex reversal. In each of the few cases where it was possible to observe the gonad of parasitized males, two testes were present. The number of males that we observed was too small to relate parasitism to sex reversal; however, the ratios between sex-reversed males in the uninfected treatment and parasitized males in the infected treatment could indicate a positive relationship.

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