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Temperature-Dependent Development of *Pasteuria penetrans* in *Meloidogyne arenaria*[1]

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Abstract: *Pasteuria penetrans* is a promising biological control agent of plant-parasitic nematodes. This study was conducted to determine effects of temperature on the bacterium's development in *Meloidogyne arenaria*. Developmental stages of *P. penetrans* were viewed with a compound microscope and verified with scanning electron microscopy within each nematode at 100 accumulated degree-day intervals by tracking accumulated degree-days at three temperatures (21, 28, and 35 °C). Five predominant developmental stages of *P. penetrans* were identified with light microscopy: endospore germination, vegetative growth, differentiation, sporulation, and maturation. Mature endospores were detected at 28, 35, and >90 calendar days at 35, 28, and 21 °C, respectively. The number of accumulated degree-days required for *P. penetrans* to reach a specific developmental stage was different for each temperature. Differences were observed in the development of *P. penetrans* at 21, 28, and 35 °C based on regression values fitted for data from 100 to 600 accumulated degree-days. A linear response was observed between 100 to 600 accumulated degree-days; however, after 600 accumulated degree-days the rate of development of *P. penetrans* leveled off at 21 and 28 °C, whereas at 35 °C the rate decreased. Results suggest that accumulated degree-days may be useful only in predicting early developmental stages of *P. penetrans*.

Key words: bacterium, biological control, degree-days, development, endospore, life cycle, *Meloidogyne arenaria*, *Pasteuria penetrans*, root-knot nematode, scanning electron microscopy, temperature.

Pasteuria penetrans (Thorne) Sayre & Starr is considered among the most promising biological control agents for the management of species of *Meloidogyne* (Dickson et al., 1994; Stirling, 1984). Its obligate parasitism, however, makes it difficult to culture in vitro (Williams et al., 1989). Currently, the production of large quantities of inoculum requires in vivo cultivation in a controlled environment. Temperature is known to affect the development of *P. penetrans* in vivo (Davies et al., 1988; Hatz and Dickson, 1992; Nakasono et al., 1993; Stirling, 1981). Stirling (1981) concluded that all stages of the bacterium's life cycle were favored by temperatures that were optimum for nematode development. Hatz and Dickson (1992) reported that a Florida isolate of the bacterium developed more quickly within the host at 30 and 35 °C than at 25 °C or below, with optimum development at 35 °C. In contrast, a Japanese isolate was favored by a soil temperature of approximately 30 °C when cultured under greenhouse conditions (Nakasono et al., 1993). Because temperature in greenhouses is likely to fluctuate diurnally and seasonally, understanding how temperature affects the development of *P. penetrans* inoculum could aid in forecasting when bacterial cultures have reached full maturity (Stirling, 1981).

Accumulated degree-days provide a physiological-time measurement of the rate of nematode development (Alston and Schmitt, 1988; Ferris et al., 1978) and are calculated by summing the total number of heat units above a base threshold temperature for a 24-hour day (Tyler, 1933). Under laboratory conditions, degree-days have been used to predict the occurrence of particular life stages of nematodes (Tyler, 1933); under field conditions, degree-days have been used to measure the rates of development of several nematode species (Arnold, 1960). However, degree-day accumulation has not been used to predict develop-

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mental stages of *P. penetrans* for mass rearing.

The objective of the current study was to observe the development of *P. penetrans* with light microscopy (LM) and scanning electron microscopy (SEM) to establish stages that could be categorized and to use this information in determining the influence of temperature, as measured by degree-day accumulation, on the rate of development of *P. penetrans* in *M. arenaria*. Preliminary studies revealed *P. penetrans* vegetative fragments that may have been artifacts resulting from the fixation process for light microscopy. This was studied further with SEM.

Materials and Methods

Nematode and bacterium cultures: Single egg-mass cultures of *M. arenaria* race 2 were reared on tomato *Lycopersicon esculentum* Mill. cv. Rutgers and maintained in a temperature-controlled greenhouse between 28 °C (mean daily temperature) and 24 °C (mean night temperature). Eggs were extracted with a sodium hypochlorite solution (Hussey and Barker, 1973). Second-stage juveniles (J2) were allowed to hatch in a modified Baermann funnel (Pitcher and Flegg, 1968) for 2 to 3 days and collected on an autoclaved 26-µm-pore sieve.

An isolate of *P. penetrans* originally obtained from roots of an unknown host infected with root-knot nematode in Pasco County, Florida (Oostendorp et al., 1990), was cultured following the procedure described by Stirling and Watchel (1980). To obtain infected nematode females, the roots were incubated overnight in 1:8 (v/v) aqueous solution of Cytolase PCL5 (Genencor International, Rolling Meadows, IL) and maintained at room temperature (ca. 25 °C). Softened roots were placed on a 600-µm-pore opening sieve nested over a 100-µm-pore opening sieve and sprayed with a vigorous stream of tap water. Dislodged females were examined for *P. penetrans* infection with an inverted compound microscope at ×40.

For developmental studies of *P. penetrans* the bacterium was cultured on *M. arenaria* race 2 growing on dwarf cherry tomato cv. Florida Petite. Seeds of the tomato were surface-sterilized in an aqueous solution of 2.5% NaOCl for 5 minutes, rinsed twice with sterile deionized water, and germinated in vermiculite at 23 °C to 28 °C. Ten days later, 20 seedlings were transplanted into individual 15-cm-diam. pots containing pure silica sand. Plants were maintained in a greenhouse for 21 days before nematode inoculation. All seedlings were watered twice daily with a hydroponic nutrient solution (Schuerger and Mitchell, 1992).

Suspensions of 1×10^5 endospores per ml were prepared by disrupting endospore-filled females in distilled water with a glass tissue grinder and preparing a serial dilution with deionized water. Endospore suspensions were stored before use in glass test tubes at 4 °C for no longer than 5 days. At inoculation, approximately 30,000 J2 were transferred to a 250-ml Erlenmeyer flask containing the endospore suspension, and the flask was placed in a Lauda RMS-20 recirculating water bath (Brinkman Instruments, Westbury, NY) for 24 hours at 25 °C to ensure that greater than 95% of J2 became encumbered with endospores. To estimate the number of endospores attached to J2, a subsample from the flask was placed in a plastic petri dish. Twenty J2 were observed with an inverted microscope at ×100. Endospore numbers ranged from 15 to 20 endospores/J2. Approximately 500 endospore-encumbered J2 were pipetted around the roots of each of six tomato plants on two successive occasions at 12-hour intervals; thus, each plant received approximately 1,000 spore-encumbered J2. All inoculations were performed at 25 °C, and plants were maintained at 25 °C for 2 days to allow nematodes to invade the roots. After 2 days, the inoculated plants were removed from the sand cultures, washed free of sand with a gentle stream of water, and then transferred to 2.5-liter opaque plastic containers filled with hydroponic nutrient solution. The stock nutrient solution was composed of the following inorganic salts (Sigma Chemical St. Louis, MO): 4.51 mM $Ca(NO_3)_2 \cdot 4H_2O$, 3.48 mM KNO_3, 1.0 mM KH_2PO_4, 1.65 mM

$MgSO_4 \cdot 7H_2O$, 0.037 mM H_3BO_4, 7.28 μM $MnSO_4 \cdot H_2O$, 4.59 μM $ZnSO_4 \cdot 7H_2O$, 3.5 μM $CuCl_2 \cdot 2H_2O$, 0.0074 μM $(NH_4)_6 MoO_{24} \cdot 4H_2O$, and $Fe_2(SO)_3$ mixed in equivalent molar concentrations (89.9 μM) with diethylenetriamine-pentaacetic acid (DTPA) as a chelating agent (Schuerger and Mitchell, 1992). The nutrient solution in each container was aerated with compressed air flowing through carbon air filters (Gelman Versaflow Filter, Gelman Science, Ann Arbor, MI) to ensure clean air.

Each plant was secured in the container by holding the stem with a 28-mm-diam. open-celled polyurethane foam plug (Dispo Plug, Baxter Healthcare, Stone Mountain, GA). Before use, the polyurethane foam plugs were heated to 70 °C for 24 to 48 hours and then autoclaved at 121 °C (1.1 kg/cm^2) for 30 minutes as recommended by Wheeler et al. (1985) to eliminate phytotoxic compounds. The pH of the nutrient solution was monitored daily with a portable pH meter and was maintained between pH 5.5 to 5.8 by addition of 0.5 M HNO_3 and 0.02 M KOH. Containers were refilled with nutrient solution as needed to keep the tomato roots completely submerged.

Life stages of P. penetrans—Light and scanning electron microscopy: At designated sampling times bacteria-infected nematode females were collected. Five galls from each of six tomato plants were randomly selected and excised from the roots, and the females were separated from the root tissue by incubation overnight in a 1:8 (v/v) aqueous solution of Cytolase PCL5 at room temperature (ca. 25 °C).

A numerical rating index based on the work by Hatz and Dickson (1992), Sayre and Starr (1985), Williams (1960), and our own observations of the developmental stages of *P. penetrans,* was assigned to each nematode infected with *P. penetrans.* The index corresponded to the predominant developmental stage (greater than 50% of the particular developmental stage in each nematode) as follows: 1 = germination stage—endospores attached to the cuticle with clearly observable germ tubes penetrating the cuticle and hypodermis of J2; 2 = vegetative growth stage—the formation of mycelial colonies and thalli disseminated throughout the pseudocoelom; 3 = differentiation stage—thalli fragmentation and detection of quintets, quartets, triplets, and doublets; 4 = sporulation stage—developing endospores with distal swollen ends and appearance of the endospore coat; 5 = mature stage—free mature ellipsoidal endospores with the sporangium wall clearly visible.

A total of 30 *P. penetrans*-infected female nematodes were rated at each sampling time. Nematodes were mounted in lactophenol and 1% methyl blue stain (v/w) (Sigma Chemical, St. Louis, MO) on glass microscope slides. Nematodes were crushed by applying pressure to cover slips placed over the nematodes and lactophenol stain. The developmental stages of *P. penetrans* were determined by examining the contents from the female bodies with bright-field LM at ×100. Photographs of the bacterium's life stages were recorded on Kodak Professional Tungsten film (E.I. = 160, Eastman Kodak Company, Rochester, NY).

To confirm the categorization of the bacterial developmental stages as determined by LM, specimens were subjected to SEM. Nematodes infected with *P. penetrans* were collected and extracted as described for light microscopy and then processed for SEM. Nematode specimens were rinsed three times in deionized water and placed on 1-cm pieces of moist Whatman No. 1 filter paper (Whatman International Limited, Springfield Mill, Kent, England). Nematodes were ruptured with fine forceps and smeared on to the filter paper before transferring the samples to chilled (4 °C) 2% glutaraldehyde in 0.07 M phosphate buffer (pH 6.8). After 24 hours, the samples were rinsed in chilled buffer (4 °C) four times at 20-minute intervals and post-fixed with similarly buffered and chilled 2% osmium tetroxide for 2 hours. Samples were washed three times in chilled buffer, three times in chilled deionized water, and then allowed to stabilize at room temperature (25 °C). Samples were dehydrated through a 25, 50, 75, 95, and 100% ethanol series. After a second rinse in 100% ethanol, the samples

were immersed in hexamethyldisalazane (Alltech Associates, Deerfield, IL) and maintained at room temperature for 5 minutes. Specimens of *P. penetrans* were then air-dried overnight, mounted on double-sided sticky tape attached to aluminum SEM stubs, sputter coated with gold palladium, and examined with an Hitachi F-530 scanning electron microscope (Hitachi Instruments, Danbury, CT) operating at 20 kV. Photographs of life stages of *P. penetrans* were recorded on Professional Polaroid 10- × 12.7-cm Instant Film No. 55 (Polaroid Corporation, Cambridge, MA).

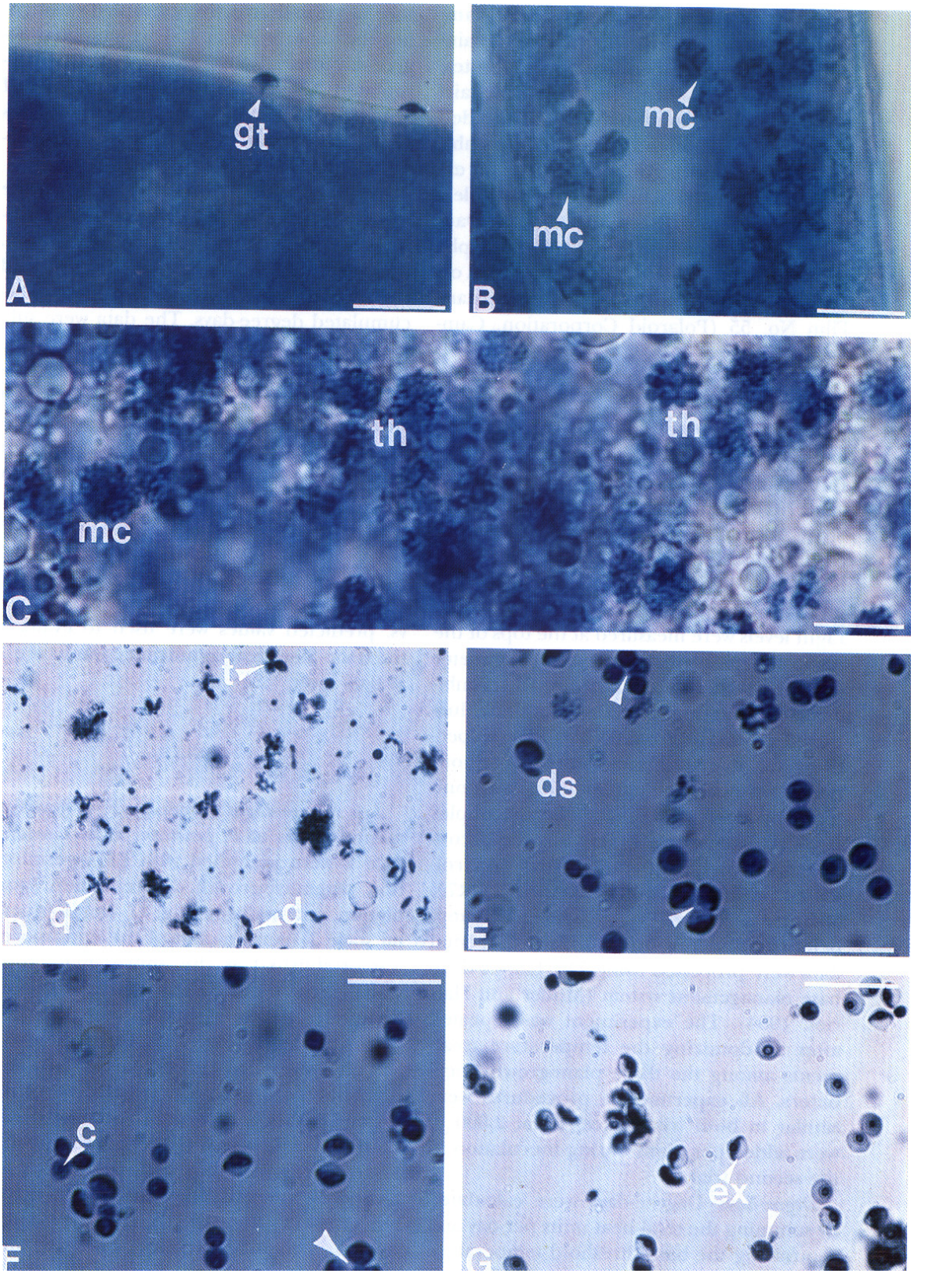
Temperature study: Six of the hydroponically grown *M. arenaria P. penetrans*-infected tomato plants were randomly placed inside each of three plant-growth incubators (Model 23L, Rheem Manufacturing, Asheville, NC). Incubators were set at 21, 28, or 35 °C. Plants were exposed to 14 hours of continuous light per day from nine 20-watt fluorescent light bulbs providing 120 µmol/m^2/s of photosynthetically active radiation. Light levels were measured at the tops of the plant canopies with a portable light meter (Model L1-189, Li-Cor, Lincoln, NE). Ambient temperature and relative humidity fluctuated ± 2 °C and from 60% to 75%, respectively, for all growth incubators throughout the experiment. Soil temperature was monitored by inserting bi-metal thermocouples (Omega, Stamford, CT) into two plastic containers for each temperature. Data were collected with a Campbell Scientific CR7X Measurement and Control System (Campbell Scientific, Logan, UT). Environmental data were processed using The Land's database management system (Muller and Harriot, 1984). The experiment was repeated after randomizing the temperature treatments among the three plant-growth incubators. All experimental procedures were similar in both trials, except that 2,000 J2 were added per plant during inoculation in the second trial.

Degree-days: Degree-days were calculated by summing the total heat units per day and subtracting the base threshold temperature of 10 °C reported for *M. arenaria* (Ferris et al., 1978). Each plant from each temperature was non-destructively sampled throughout the experiment at 100 accumulated degree-day intervals until each treatment accumulated 1,000 degree-days. Sampling of nematodes infected with *P. penetrans* was performed in an identical manner as described above (see section for life stages).

Experimental design and data analysis: The experimental design was a repeated-measures design that was blocked for trial. The main plot factor was temperature, and the subplot factor was time-measured by accumulated degree-days. The data were subjected to analysis as a split-plot after checking that the assumptions of equal correlation between all time points were not violated. Data from both trials were combined between 100 and 600 degree-days for each temperature and subjected to analysis by linear regression with PROC GLM of the Statistical Analysis System (SAS/STAT User's Guide, SAS Institute, vol. 2, 1990, Cary, NC). The coefficient of determination (R^2), the value of the slopes, and plots of residuals vs. predicted values were used to evaluate the data. Statistical significance was at the $P \leq 0.05$ level.

Results

Life stages—Light microscopy: Five developmental stages of *P. penetrans* in *M. arenaria* race 2 were identified with LM—germination, vegetative growth, differentiation, sporulation, and maturity (Fig. 1). Developmental stage 1 (germination of the endospore) was established as the approximate time when germ tubes of attached endospores penetrated the cuticle and the nematode hypodermal layer (Fig. 1A). Developmental stage 2 (vegetative growth), occurred when mycelial colonies and vegetative thalli formed within the pseudocoelom of developing juvenile stages (Fig. 1B). Eventually, mycelial colonies that appeared to be attached by intercalary hyphae became swollen clusters (Fig. 1B,C). Developmental stage 3 (thalli differentiation) occurred with the elongation of distal cells and the fragmentation of vegetative thalli (Fig. 1D) into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quintets, triplets, and doublets. These fragmented clusters were observed throughout the body of the nematode. During developmental stage 4 (sporulation), oval-shaped immature sporangia with the visible coat were observed attached by intercalary mycelial threads, and the longitudinal walls of the sporangia appeared thickened (Fig. 1E,F). Developmental stage 5 (maturity) was observed after the endospores had acquired an ellipsoidal shape, and the sporangium wall was observed on individual endospores. At stage 5, mature endospores (Fig. 1G) usually filled the entire body cavities of nematodes.

Scanning electron microscopy: Life-stages of *P. penetrans* observed with LM were confirmed with SEM (Fig. 2). Prominent bifurcation of the mycelial colonies (stage 2) were observed (Fig. 2A–C). Distal thalli cells (stage 3) appeared to fragment into filamentous fragments of quintets, quartets, triplets, and doublets; these fragments often appeared attached near the cell center. Swelling of terminal cells led to the formation of endospores, followed by a change in the developing sporangia from oval to elliptical shape. Endospores still appeared connected by hyphal strands at this stage (stage 4) before the appearance of the spore coat and the exosporium (Fig. 2H). After formation of the external membranes, the immature sporangia appeared swollen at one polar point, and several longitudinal lines or ridges were observed in the endospore wall (Fig. 2I,J). The mature endospore (stage 5) eventually detached from the central hyphae but remained ensheathed by the sporangial membranes and a wrinkled exosporium (Fig. 2K).

Temperature and development of P. penetrans: Temperature, as measured by both calendar-days and accumulated degree-days, affected duration of the *P. penetrans* life cycle (Table 1). Germination and penetration of the cuticle and hypodermis (stage 1) occurred 9 to 10, 6, and 4 to 5 calendar days after inoculation at 21, 28, and 35 °C, respectively. Vegetative growth (stage 2) was the predominant stage observed at 27 to 28 calendar days after inoculation at 21 °C; however, vegetative mycelia was observed earlier at 28 and 35 °C than at 21 °C. Thalli differentiation (stage 3) was most numerous between 45 to 49, 17, and 20 to 24 days after inoculation at 21, 28, and 35 °C, respectively. Sporulation (stage 4) was detected after 50 calendar days at 21 °C but occurred sooner at 28 and 35 °C. Mature endospores (stage 5) were detected as early as 34 and 28 calendar days after inoculation at 28 and 35 °C, respectively. However, no nematodes were detected at 35 °C by day 40. Few endospores were observed after 65 calendar days (data not shown) at 21 °C, and they were never the predominant stage before 90 calendar days.

Accumulated degree-days required to reach each developmental stage were generally different for each temperature (Table 1). Germination of the endospores (stage 1) required 100 degree-days for all temperatures. Vegetative growth (stage 2) was predominant after 300 degree-days at 21 °C or at 200 to 300 degree-days at 28 and 35 °C. Differentiation of thalli and detection of fragments (stage 3) including quintets, triplets, quartets, and doublets occurred around 500 degree-days at 21 °C, and 400 degree-

FIG. 1. Light microscopy of developmental stages of *Pasteuria penetrans* P100 parasitizing *Meloidogyne arenaria* race 2 cultured on tomato plants cv. Florida Petite growing in a hydroponic system. Developmental stage 1 (germination of the endospore) was established as the approximate time when germ tubes (gt) of attached endospores penetrated the cuticle and the nematode hypodermal layer (A). Developmental stage 2 (vegetative growth) = mycelial colonies (mc) and vegetative thalli (th) formed within the pseudocoelom of developing juvenile stages (B). Eventually, mycelial colonies that appeared to be attached by intercalary hyphae became swollen clusters (B,C). Developmental stage 3 (thalli differentiation) = elongation of distal cells (ds) and the fragmentation of vegetative thalli (D) formed quintets (q), triplets (t), and doublets (d). These fragmented clusters were observed throughout the body of the nematode. Developmental stage 4 (sporulation) = oval-shaped immature sporangia with the visible cortex (c) attached by intercalary mycelial threads (arrow), and the longitudinal walls of the sporangia appeared thickened (E,F). Developmental stage 5 = mature endospores (G) filled the entire body cavities of nematodes. Stage 5 was observed after the endospores had acquired an ellipsoidal shape, and the exosporium (ex) was observed on individual endospores. Bars = 20 μm.

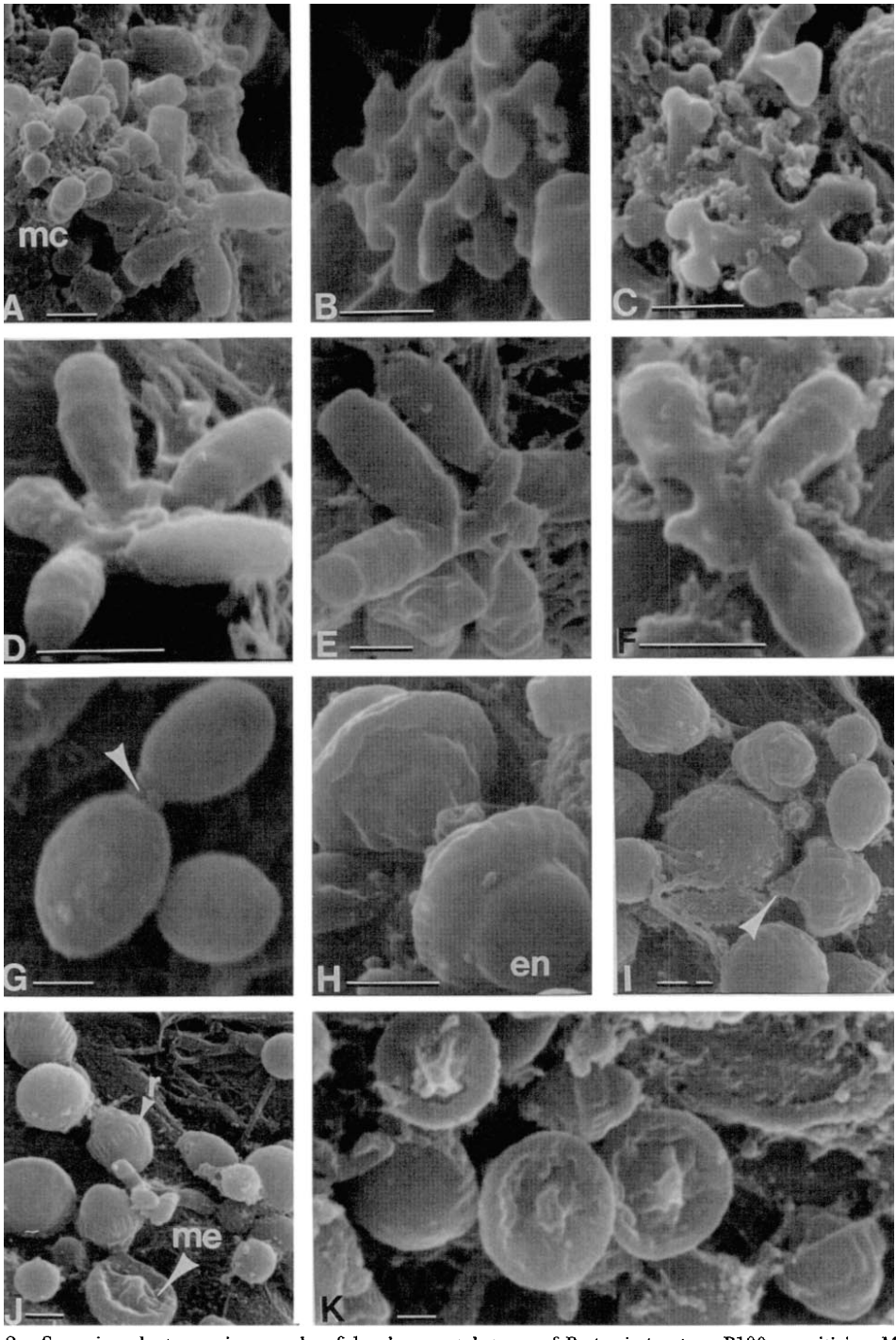

FIG. 2. Scanning electron micrographs of developmental stages of *Pasteuria penetrans* P100 parasitizing *Meloidogyne arenaria* race 2 cultured on tomato plants cv. Florida Petite growing in a hydroponic system. Life-stages of *P. penetrans* observed with light microscopy were confirmed (see Fig. 1). Prominent bifurcation of the mycelial colonies (mc) (stage 2) were observed (A–C). Distal thalli cells observed (stage 3) appeared as filamentous fragments of quintets (D), quartets (E), triplets (F), and doublets (G); these fragments often appeared attached. Swelling of terminal cells led to the formation of the endospores (en), followed by a change in the developing sporangia from oval to elliptical shape. Endospores still appeared connected by hyphal strands at this stage (stage 4) before the appearance of the spore coat and the exosporium (H). After formation of the external membranes, the immature sporangia appeared swollen at one polar point, and several longitudinal lines or ridges (r) were observed in the endospore wall (I,J). The mature endospore (stage 5) eventually detached from the central hyphae but remained ensheathed by the sporangial membranes and a wrinkled exosporium (K). Bars = 1 μm.

TABLE 1. Calendar days and accumulated degree-days required to detect predominant developmental stages of *Pasteuria penetrans* P100 in *Meloidogyne arenaria* race 2.

Developmental stage[c]	Calendar days[a]			Accumulated degree-days[b]		
	21 °C	28 °C	35 °C	21 °C	28 °C	35 °C
				Trial 1		
Germination	9	6	4	100	100	100
Vegetative growth	27	11	12	300	200	300
Differentiation	49	17	24	500	400	400
Sporulation	62	28	28	600	500	600
Maturity stage	90	35	28	1,000	600	900
				Trial 2		
Germination	10	6	5	100	100	100
Vegetative growth	28	11	9	300	200	200
Differentiation	45	17	20	500	400	400
Sporulation	56	28	24	700	500	500
Maturity stage	90	34	30	1,000	600	700

[a] Calendar days are equivalent to Julian days.
[b] Degree-days calculated with base temperature of 10 °C of *M. arenaria* reported by Ferris et al. (1978).
[c] The predominant developmental stages of *P. penetrans* over time were estimated. For details see text.

days at 28 and 35 °C. It appeared that sporulation (stage 4) required at least 600 degree-days at 21 °C, but this stage was first detected at 500 accumulated degree-days at 28 °C and became the predominant stage thereafter. At 35 °C, mature endospores also were first observed at 600 accumulated degree-days. The number of nematodes available declined, and no nematodes were found by 1,000 accumulated degree-days. Although some endospores (stage 5) were detected before 1,000 degree-days at 21 °C, the most numerous stages estimated before 1,000 degree-days were not fully developed.

The development of *P. penetrans* was estimated with linear regression by plotting temperature against accumulated degree-days. Development appeared to level off beyond 600 accumulated degree-days at 21 and 28 °C, but at 35 °C the number of nematodes available for sampling decreased. Only data between 100 and 600 degree-days were combined from both trials and subjected to analysis with regression (Fig. 3A). Developmental of *P. penetrans* exhibited a linear trend at 21 and 28 °C (Fig. 3B). However, data from trials 1 and 2 at 35 °C were variable. The estimated regression equations are as follows: 21 °C, $Y = 0.0045X + 0.850$ ($R^2 = 0.78$; $P = 0.0001$); 28 °C, $Y = 0.0072X + 0.492$ ($R^2 = 0.94$; $P = 0.0001$); and 35 °C, $Y = 0.0060X + 0.650$ ($R^2 = 0.72$; $P = 0.0001$), in which Y represents the developmental stages of *P. penetrans* and X represents time measured by accumulated degree-days. Slope values of linear regression equations for all temperatures indicated that there were significant differences among the rates of development of *P. penetrans* at 21 °C compared to 28 °C ($P = 0.0001$), 21 °C compared to 35 °C ($P = 0.0001$), and 28 °C compared to 35 °C ($P = 0.01$).

DISCUSSION

Pasteuria penetrans development in *Meloidogyne* spp. has received little study. The most detailed studies on development to date (Hatz and Dickson, 1992; Sayre and Starr, 1985) reported that vegetative fragments, namely quintets, quartets, triplets, and doublets, were identified readily, implying that such fragments were specific stages of the *P. penetrans* life cycle. However, LM observations in the current study show that clusters of vegetative thalli and developing endospores remain attached by intercalary strands. The individual vegetative fragments may be artifacts of the fixation process as a result of fractured vegetative clusters of *P. penetrans* during sample preparation. These observations suggest that single mature endospores could develop in clusters and not necessarily through sequential fragmenta-

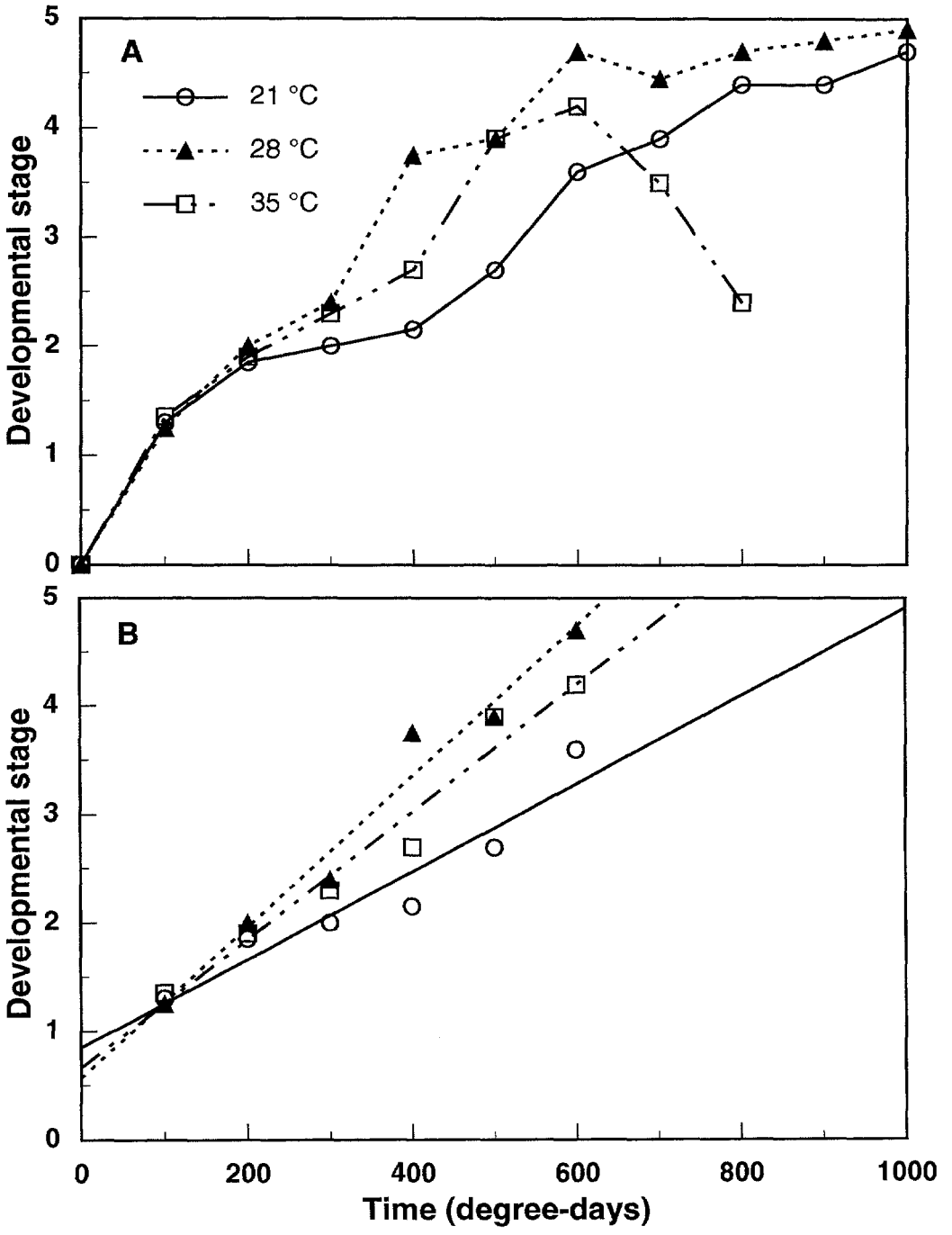

FIG. 3. A) Development of *Pasteuria penetrans* P100 infecting *Meloidogyne arenaria* race 2 cultured on tomato plants (cv. Florida Petite) growing in a hydroponic system at three different temperatures. The predominant developmental stage of *P. penetrans* was estimated at different times after root penetration by endospore-encumbered J2. A numerical rating index corresponding to the predominant developmental stage observed was assigned to each nematode infected with *P. penetrans* as follows: 1 = germination; 2 = vegetative growth; 3 = differentiation; 4 = sporulation, and 5 = maturation. The numbers of nematodes examined (at indicated temperature and accumulated degree-days) were 52 to 60 infected nematodes (at 21 and 28 °C from 100 to 1,000 degree-days), 50 to 60 infected nematodes (at 35 °C from 100 and 600 degree-days), and 6 to 30 infected nematodes (at 35 °C from 700 to 900 degree-days). No nematodes were recovered at 1,000 degree-days at 35 °C in either trial. B) Relationship between accumulated degree-days and rate of development of *Pasteuria penetrans* P100 at three different temperatures. Coefficient slopes of the linear regression equations for all temperatures indicated differences between the rates of development of *P. penetrans* at 21 °C compared to 28 °C ($P = 0.0001$), 21 °C compared to 35 °C ($P = 0.0001$), and 28 °C compared to 35 °C ($P = 0.015$).

tion. The use of low-temperature SEM (Wergin et al., 1993) could improve the interpretation of the *P. penetrans* life cycle and perhaps allow analysis of three-dimensional images inside the nematode body.

The methyl-blue and lactophenol stain was superior to the conventional gram stain for visualization of *P. penetrans* life stages and internal structures. This staining method works particularly well for identification of microcolonies inside nematode bodies. Microcolonies are usually difficult to detect and quantify because the gram stain requires repeated heating and washing of samples during which most nematodes and *P. penetrans* life stages are washed off the slides.

The differences in the rate of *P. penetrans* development between 100 to 600 accumulated degree-days indicate that early development is influenced significantly by temperature. The variability of the development curves after 600 degree-days at 21 and 28 °C suggests that accumulated degree-days may be useful only in predicting early developmental stages of *P. penetrans;* however, the leveling off may be an artifact of the type of data collected due to *P. penetrans* approaching the maximum value in the rating scale.

In the present report the highest rate of development of *P. penetrans* occurred at 28 °C, but a dramatic reduction in numbers of nematodes was observed at 35 °C. These data are in contrast to the report (Hatz and Dickson, 1992) that mature endospores were detected 35 to 45 days after inoculation at 30 and 35 °C; the optimum temperature for development of *P. penetrans* in *M. arenaria* race 1 was 35 °C.

The optimum cultivation of *P. penetrans* in vivo may depend upon well-defined growing conditions for the host plant and the nematode. Because temperature has been reported to affect the partitioning of photosynthates within plants (Wolf et al., 1991), it is likely that at high temperatures metabolic root dysfunction may negatively affect the ability of the nematodes to obtain photosynthates from the plant, hence affecting the development of *P. penetrans.* Thomason and Lear (1961) suggested that the upper threshold for development of *M. arenaria* species in soil was approximately 32 °C. Although plant parameters such as fresh and dry weights of the shoots and roots of plants were not tested in this study, plants were more vigorous at 21 and 28 °C than at 35 °C (data not shown). Plants at 35 °C were stunted, chlorotic, and senesced earlier than plants grown at 21 or 28 °C.

Reports from Australia (Stirling, 1981) and England (Davies et al., 1988) with different isolates of *P. penetrans* suggest that development of *P. penetrans* may be favored by temperatures optimum for the nematode. Davies et al. (1988) reported that the *P. penetrans* life cycle varied between 60 to 80 days at 20 °C; but at 30 °C, mature endospores of *P. penetrans* formed 20 to 30 days after inoculation. The high genetic diversity among different isolates of *P. penetrans* has been addressed (Gowen and Channer, 1988) and also may explain why different temperatures have diverse effects on *P. penetrans.*

The differences between the present study and that of Hatz and Dickson (1992) may be due to different experimental conditions including the plant culture system, light quality, temperature measurement procedures, sampling error, and techniques for detecting *P. penetrans* inside the nematode. For example, in the current study plants were grown in a hydroponic system maintained in plant-growth incubators in which the photosynthetic photon flux between 400 and 700 nm was approximately 120 $\mu mol/m^2/s$. Thus, the tomato plants may have grown better in the current study than plants in the previous study (Hatz and Dickson, 1992), in which plants were maintained in soil-filled pots with light levels of approximately 70 $\mu mol/m^2/s$ (Dickson, unpubl.). The effects of light quality and plant nutrients on the development of *P. penetrans* were not tested in the present study.

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