

# Temperature Effects on the Attachment of *Pasteuria penetrans* Endospores to *Meloidogyne arenaria* Race 1<sup>1</sup>

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**Abstract:** *Pasteuria penetrans* is a gram positive bacterium that prevents *Meloidogyne* spp. from reproducing and diminishes their ability to penetrate roots. The attachment of the endospores to the cuticle of the nematodes is the first step in the life cycle of the bacterium and is essential for its reproduction. As a preliminary study to a field solarization test, the effects of temperature on the attachment of *P. penetrans* on *Meloidogyne arenaria* race 1 were investigated. Preexposing second-stage juveniles (J2) of *M. arenaria* to approximately 30 °C in water before exposing them to endospores increased their receptivity to endospore attachment when compared to treating J2 at 25 °C or 35 °C. In tests with soil, highest attachment occurred when J2 were incubated in soil infested with endospores and maintained at 20 °C to 30 °C for 4 days. Heating J2 in soil to sublethal temperatures (35 °C to 40 °C) decreased endospore attachment. Incubating *P. penetrans* endospores in soil at 30 °C to 70 °C for 5 hours a day over 10 days resulted in reductions of endospore attachment to nematodes as temperatures of incubation increased to 50 °C and higher.

**Key words:** attachment, bacteria, biological control, cuticle, endospore, *Meloidogyne arenaria*, nematode, *Pasteuria penetrans*, root-knot nematode, temperature

*Pasteuria penetrans* is an obligate parasite of plant-parasitic nematodes that has great potential as a biological control agent (Bird and Brisbane, 1988; Brown et al., 1985; Stirling, 1984). Upon contact, the endospores of *Pasteuria* spp. attach to the cuticle of their nematode hosts. The adhesion of the endospores is the first step in the life cycle of the bacterium and is essential for its reproduction. Parasitism of *Meloidogyne* spp. by *P. penetrans* reduces the number of females in roots (Davies et al., 1991) and their fecundity (Bird, 1986; Bird and Brisbane, 1988); second-stage juvenile mobility and host-finding ability are reduced when heavily encumbered with endospores (Davies et al., 1991; Mankau and Prasad, 1977).

Few studies have been conducted on the effects of temperature on the attachment of endospores of *Pasteuria* spp. to the nematode cuticle. Dutky and Sayre (1978) studied the effect of temperature on endospores of *P. penetrans* by heating soil infested with endospores at different temperatures and sub-

sequently introducing nontreated, second-stage juveniles of *Meloidogyne incognita* into the cooled soil to test for attachment. There was no attachment of endospores after they were exposed to 130 °C for 1 hour. Attachment occurred after exposure in soil heated at 80 °C for 30 minutes, but *P. penetrans* did not penetrate and develop inside the nematode. The percentage of *M. javanica* encumbered with endospores increased as temperature increased from 15 °C to 30 °C and as time increased from 24 to 72 hours (Stirling, 1981). Attachment of *Pasteuria* sp. on *Heterodera cajani* increased from 15 °C to 25 °C, but decreased at 35 °C (Singh and Dhawan, 1990). Hatz and Dickson (1992) determined that 30 °C was the optimum temperature for attachment when *M. arenaria* race 1 was incubated for 24 hours in soil infested with *P. penetrans*.

Although the attachment process is not well understood, it most likely involves proteins and carbohydrates that are temperature-dependent (Davies and Danks, 1993; Persidis et al., 1991). Also, the greater movement of root-knot nematodes at 20 °C and 25 °C than at lower temperatures (Bird and Wallace, 1965) increases the chance of contact between endospores and nematodes, which may result in increased attachment.

*Pasteuria penetrans* has great potential for integration with other nematode management practices (Stapleton and Heald, 1991),

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including soil solarization (Stirling, 1981; Tzortzakakis and Gowen, 1993; Walker and Wachtel, 1988). As a preliminary study to a field solarization test, the effects of temperature on the attachment of *P. penetrans* endospores to second-stage juveniles of *M. arenaria* race 1 were studied at temperature ranges normally achieved during soil solarization at different soil depths. Our objectives were to determine endospore attachment following the preexposure of second-stage juveniles and endospores to different temperatures in a water bath, and endospore attachment following the preexposure of endospores to different temperatures in soil.

#### MATERIALS AND METHODS

*Nematode maintenance and collection:* *Meloidogyne arenaria* race 1 was originally isolated from the Green Acres Research Farm of the University of Florida in Alachua County. The nematode was cultured on tomato (*Lycopersicon esculentum* cv. Rutgers), and perineal patterns were examined periodically to ensure that the culture was free of contamination by other *Meloidogyne* spp. The nematode population was allowed one generation per year on peanut (*Arachis hypogaea* cv. Florunner). Eggs were collected from galled roots by means of a hypochlorite method (Hussey and Barker, 1973). Second-stage juveniles (J2) were hatched from eggs in a modified Baermann (Pitcher and Flegg, 1968) for 2 to 3 days and collected on a sieve with 25- $\mu$ m openings that had been autoclaved to eliminate alien *Pasteuria* endospores.

*Pasteuria penetrans* maintenance and collection: Isolate P25 of *P. penetrans* was originally obtained from females of *M. arenaria* race 1 parasitizing roots of peanut in a field at the Green Acres Research Farm. Galled peanut roots were collected from the field and spread on greenhouse benches to dry for 3 weeks. The roots were cut into pieces 2 to 5 cm long and incubated in an 8:1 (v/v) aqueous solution of commercial Cytolase PCL5 (Genecor International, Rolling Meadows, IL) at room temperature (ca. 24 °C). Soft-

ened roots were placed on a sieve with 600- $\mu$ m-pore openings nested over a sieve with 180- $\mu$ m-pore openings and sprayed with a vigorous stream of water. The dislodged females together with root debris were collected. The females were separated from the debris with a Pasteur pipette under a stereoscope and examined for *P. penetrans* infection with an inverted compound microscope. Infected females were differentiated from healthy females by their dense and opaque milky color. Endospores of *P. penetrans* were obtained by rinsing infected females with distilled water for 3 minutes and then macerating their bodies in sterile deionized water in a 15-ml tissue grinder (Pyrex Tenbroeck). Endospore suspensions were stored in glass test tubes at 5 °C.

*Infested soil:* Soil containing endospores of isolate P25 of *P. penetrans* was collected from the Green Acres Research Farm. The soil was collected 0 to 30 cm deep from randomly selected sites, mixed, and stored in a 150-liter plastic container in the shade until used in laboratory experiments.

*Endospore attachment as affected by preexposing second-stage juveniles to different temperatures:* New, 1.5-ml polypropylene centrifuge tubes were washed with detergent, and 65 1- to 4-day-old J2 of *M. arenaria* were added to each tube in 1 ml of water. Four tubes were immersed for 10 minutes in water in each of six water-bath tanks adjusted to 25, 30, 35, 40, 45, or 50 °C. After incubation, the tubes were removed from the tanks, shaken, and opened. Five microliters of a water suspension of *P. penetrans* at  $5.2 \times 10^6$  endospores/ml were added to each tube. The tubes were closed and placed in an automatic shaker (Eberbach, Ann Arbor, MI) for 8 hours at 24 °C. After this period, the tubes were maintained at 5 °C until attachment was assessed. The number of endospores attached per J2 on 20 randomly selected J2 per tube was counted with an inverted compound microscope at  $\times 400$  magnification. The experiment was repeated once with the same methodology, except that the treatments were replicated five times.

*Endospore attachment as affected by preexposing endospores to different temperatures:* A sus-

pension of endospores was adjusted to  $5.2 \times 10^6$  endospores/ml of distilled water, and 10 ml of the suspension was placed into each of 30, prewashed 15-ml polystyrene centrifuge tubes. Five water-bath tanks were adjusted to a daily cycle of 5 hours at 30, 40, 50, 60, or 70 °C, followed by 19 hours at room temperature (23 °C). In another treatment, five glass tubes, each with 10 ml of the endospore suspension, were heated in boiling water (100 °C) for 5 hours a day and were then maintained at room temperature (23 °C) for the remaining 19 hours each day. After 10 days of heat treatment, all tubes were removed from the water, shaken, and opened.

One hundred eighty 1- to 4-day-old J2 of *M. arenaria* in 1 ml of water were placed in each of 30, prewashed conical bottom microcentrifuge tubes. Endospores subjected to heat treatment were added to the nematode suspensions to produce concentrations of  $1 \times 10^5$  endospores/tube. The microcentrifuge tubes, with suspensions of nematodes and endospores, were placed on the shaker for 24 hours at 24 °C. The tubes were then stored at 5 °C until attachment was assessed. The number of endospores per J2 on 20 randomly selected J2 per tube was counted with an inverted compound microscope at  $\times 400$  magnification.

*Effect of temperature on J2 and endospores in soil:* Seventy grams of air-dried soil infested with endospores of *P. penetrans* and 7 ml of water were placed into a pre-washed, 50-ml polystyrene centrifuge tube. The water was allowed to diffuse throughout the soil for 30 minutes before 440 J2 in 5 ml of water were introduced into each tube. The experiment had five treatments (incubation at 10, 20, 30, 40, or 50 °C) and five replicates per treatment. One set of tubes was maintained in a cool room with a constant temperature of 10 °C, and another set was maintained in an incubator at 20 °C. Three water-bath tanks were adjusted to have a daily cycle of 5 hours at 30, 40, or 50 °C followed by 19 hours at room temperature (23 °C), and a set of tubes was maintained for each temperature cycle. The J2 in the soil were incubated for 4 days to allow them to move throughout the soil and to come into contact with *P. penetrans*.

After the incubation period, the J2 were extracted with a centrifugal flotation method (Jenkins, 1964). The experiment was repeated once. The J2 used in inoculations in the first trial were 1 to 4 days old. Second-stage juveniles used in the second trial were from the same batch as used in the first test but were stored in 500 ml of water at 5 °C for 40 days. After this period, the nematodes in water were moved to room temperature (23 °C) and subjected to bubbling air for 1 hour before they were used in the experiment.

Because a difference in the level of attachment of endospores occurred among J2 stored in water for 40 days and the 1- to 4-day-old J2, a bioassay was conducted to investigate if the long-term storage in water at low temperature caused the difference via some mechanism other than nematode mobility. The experiment consisted of two treatments and four replicates. The treatments were 1- to 4-day-old J2 and J2 stored in water for 45 days at 5 °C. Second-stage juveniles of *M. arenaria* race 1, originally from the same field population and kept under the same conditions in a greenhouse, were used for both treatments. Ninety J2 were added per pre-washed microcentrifuge tube in 1 ml of water. Endospores of *P. penetrans* were added to the nematode suspensions to produce concentrations of  $1 \times 10^5$  endospores/tube. The microcentrifuge tubes, with the suspension of nematodes and endospores, were placed on a shaker for 24 hours at 24 °C. The number of endospores per J2 on 20 randomly selected J2 from each tube was counted with an inverted compound microscope at  $\times 400$  magnification. All experimental data were subjected to regression analyses with SAS software (SAS Institute, Cary, NC).

## RESULTS

*Endospore attachment as affected by preexposing second-stage juveniles to different temperatures:* Attachment of endospores to J2 that were preexposed to different temperatures in two trials initially increased and then decreased with increasing incubation temperatures (Fig. 1). Higher numbers of endospores were attached to J2 treated at 30 °C

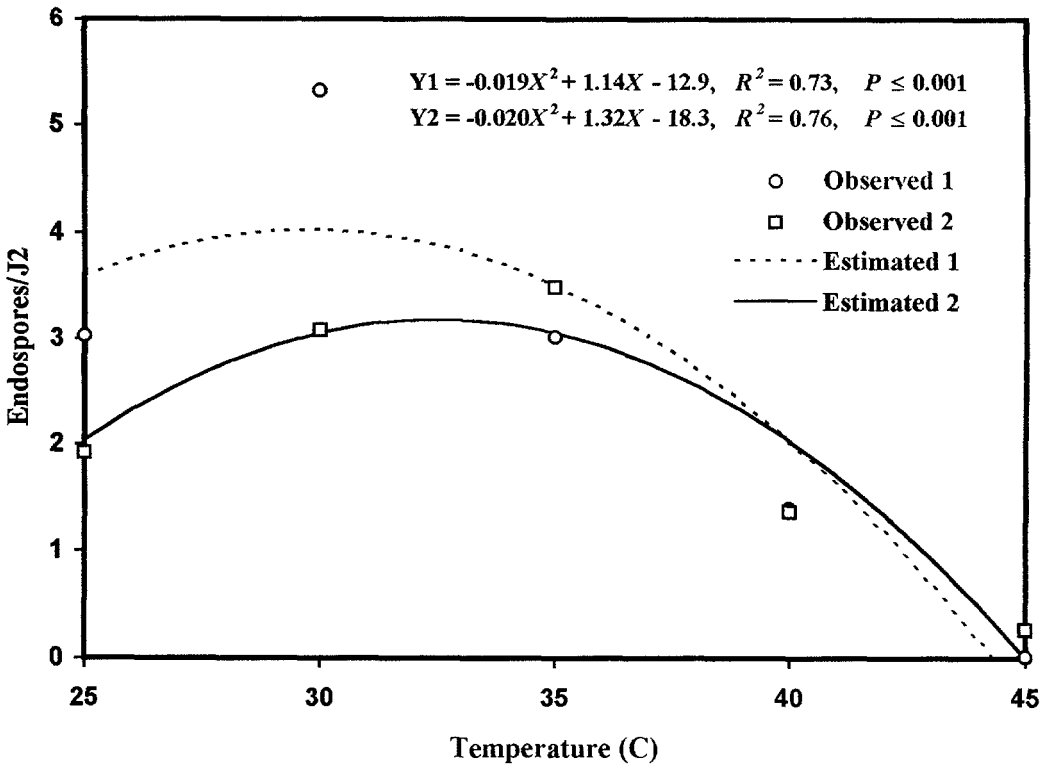


FIG. 1. Regression of the number of endospores attached per second-stage juvenile (J2) of *Meloidogyne arenaria* race 1 to the incubation temperature of *M. arenaria* J2 for 10 minutes in water. Equation Y1 and the estimated curve 1 are for the first trial, and equation Y2 and the estimated curve 2 are for the second trial.

than to those treated at 25 °C in both tests; however, attachment was highest with pre-exposure at 30 °C in the first trial, and at 30 °C and 35 °C in the second trial. Temperatures higher than 35 °C were deleterious to attachment in both experiments, and little or no attachment occurred when nematodes were heated to 45 °C and above; thus, regression equations were calculated for the range of temperatures of 25 °C to 45 °C.

*Endospore attachment as affected by preexposing endospores to different temperatures:* Exposure of endospores to temperatures higher than 30 °C resulted in decreased attachment (Fig. 2). An average of eight or fewer endospores per J2 resulted from exposure of endospores from 60 °C to 100 °C; therefore, data above that point were considered meaningless and were not included in the regression analysis.

*Effect of temperature on J2 and endospores in soil:* Only a low level of endospore attach-

ment occurred at 40 °C and above; therefore, regression equations were calculated for the range of temperatures of 10 °C to 40 °C. Based on these regression analyses (Fig. 3), maximum attachment would occur when J2 and endospores in soil were incubated at approximately 25 °C. Regression curves for both tests estimated that attachment would be near zero in soils treated at temperatures near 10 °C and 40 °C. Although both curves were similar (Fig. 3A,B), the highest average number of endospores attached per J2 in the first trial was 63, whereas in the second trial the maximum was only 2.5.

This large difference in attachment levels led to a supplementary bioassay to determine if long-term storage of J2 in water before exposing them to endospores resulted in low attachment. One- to four-day-old J2 had an average attachment of 59 endospores per J2, whereas J2 stored for 45 days had an average of 0.2 endospore per J2. The

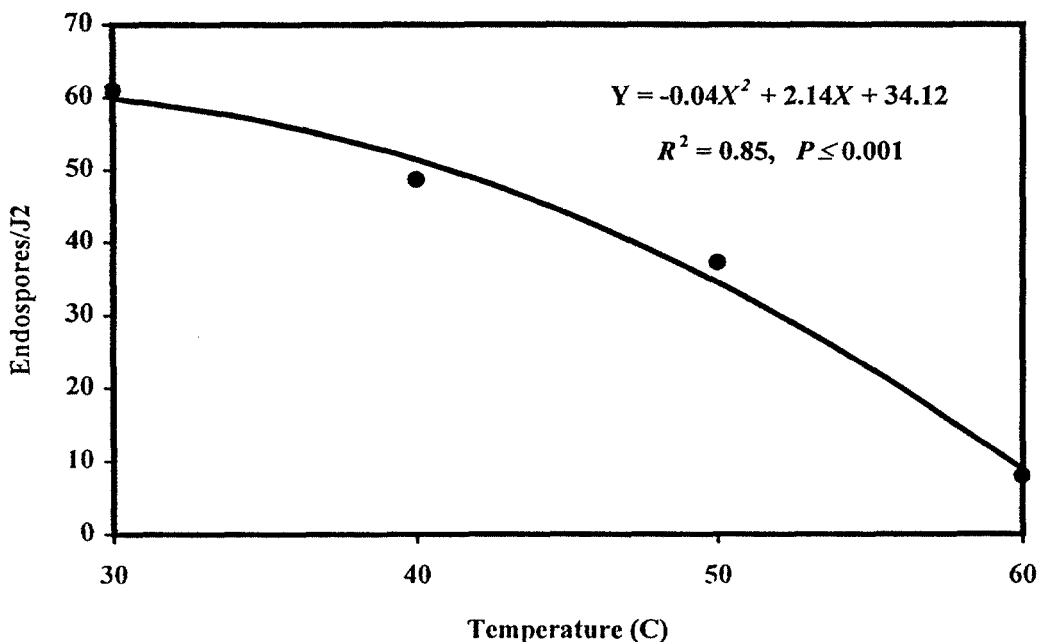


FIG. 2. Number of endospores of *Pasteuria penetrans* attached per second-stage juvenile (J2) of *Meloidogyne arenaria* race 1 after incubation of endospores in water for 5-hour periods each day over 10 days at different temperatures.

difference was statistically significant ( $P \leq 0.01$ ).

#### DISCUSSION

The effectiveness of soil solarization for the management of *Meloidogyne* spp. has been highly variable (Gaur and Dhingra, 1991; Katan and DeVay, 1991; Walker and Wachtel, 1988) primarily because of differences in available solar radiation and resulting soil temperatures, soil characteristics, and nematode distribution in the soil (Madulu and Trudgill, 1994). Since the endospores of *P. penetrans* are considered to be tolerant of higher temperatures, the combination of solarization with the bacterium has been considered for integrated management of *Meloidogyne* spp. Tzortzakakis and Gowen (1993), as an example, demonstrated that the efficacy of *P. penetrans* may be enhanced by solarization. Maximum average daily temperatures in solarized soil in tropical and subtropical countries typically range between 45 °C and 50 °C in the top 15 cm but are lower, often 40 °C to 45 °C, at depths of 15 to 30 cm (Freitas, 1997; Katan

and DeVay, 1991). Thus, temperatures may be attained in the top layers of solarized soil that kill nematodes and reduce the survival of endospores of *P. penetrans* or their attachment to J2, but lower temperatures attained in deeper levels of soil may have beneficial effects on the efficacy of the bacterial parasite. In our study, incubating *P. penetrans* endospores for 10 days at 40 °C and higher resulted in subsequent reductions in attachment when treated endospores were mixed with J2. The greatest receptivity of J2 of *M. arenaria* to endospore attachment occurred when J2 were treated in water at 30 °C to 35 °C and then exposed to spores. The highest endospore attachment levels occurred when nematode J2 were incubated in soil infested with endospores at 20 °C to 30 °C for 4 days. Thus, the bacterium is more tolerant than J2 of temperatures attained in surface layers of soil under solarization, but temperatures of 50 °C or more may also be detrimental to the bacterium. Although the conditions of our study do not simulate solarized soil, the tests allowed the differentiation of the effects of temperatures attained under solarization on endospores of *P. penetrans*, on J2

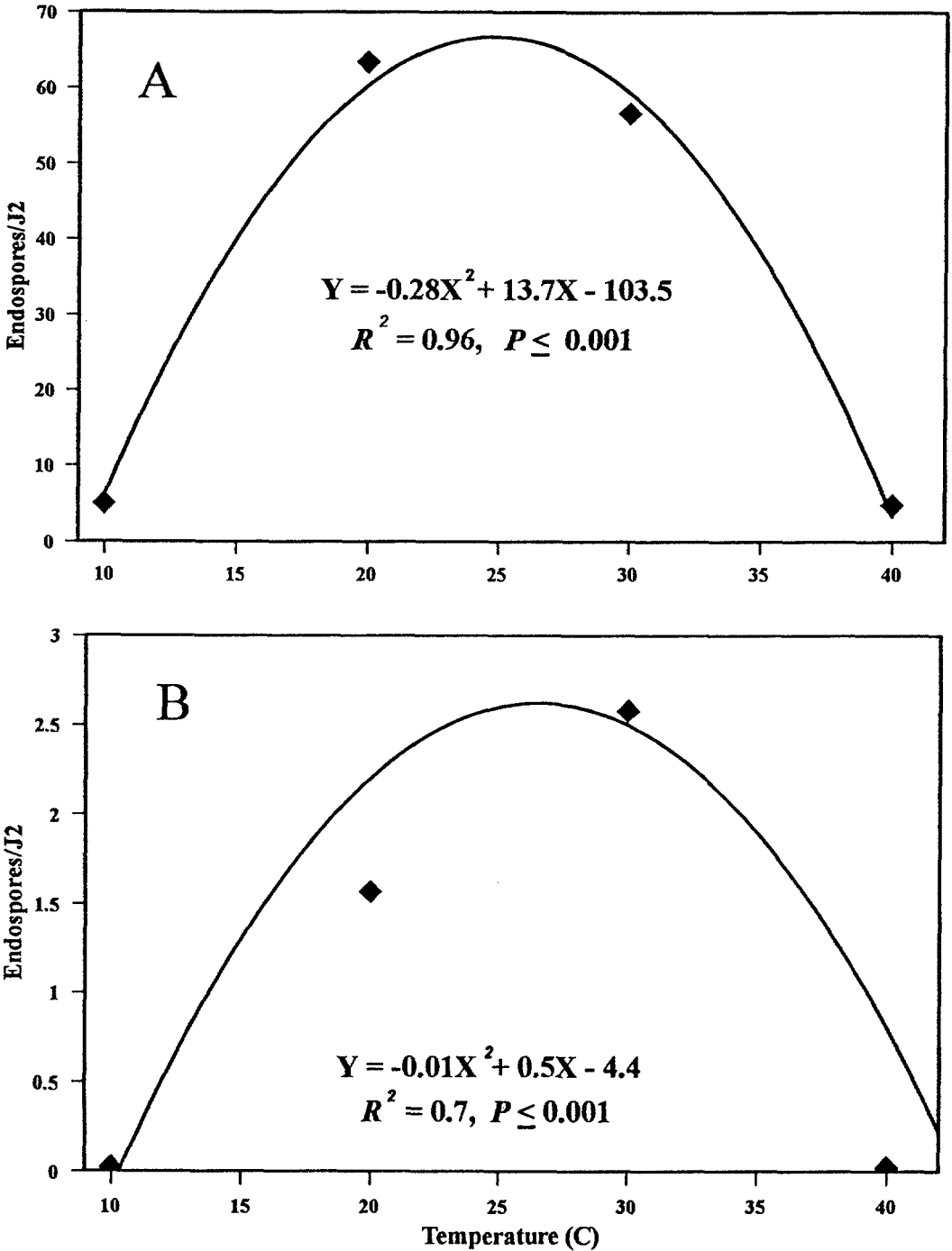


FIG. 3. Endospores of *Pasteuria pentrans* attached per second-stage juvenile (J2) of *Meloidogyne arenaria* race 1 after 4 days of incubation of J2 in soil infested with bacterial endospores at different temperatures. The curves represented by the solid lines resulted from regression equations, and the black dots represent the attachment observed at different temperatures in the first (A) and second (B) tests.

of *M. arenaria*, and on the attachment of endospores to J2.

Although attachment of endospores of *P. penetrans* after treatment of J2 of *M. arenaria* for 10 minutes in water at temperatures above 25 °C initially increased, both the level of endospore attachment and survival of J2 declined with increasing temperatures. Approximately 50% of the J2 were killed at 35 to 40 °C, and attachment at these temperatures was reduced to about 50% to 70% of that at 25 °C. Similarly, Stirling et al. (1986) observed that the attachment of endospores to nematodes killed after exposure to 60 °C for 5 minutes was 67% of the rate of attachment on living nematodes. Thus, the viability of the J2 directly influences endospore attachment.

When endospores were treated for 10 days in water, attachment to J2 decreased with increasing temperatures above 30 °C. Attachment was minimal at 60 °C or higher, but it was not completely prevented even at 100 °C for 5 hours per day over 10 days. Endospores used in this investigation were in high concentrations in water when they were induced to attach through agitation for 24 hours at 24 °C. This method was chosen to reduce the variation in attachment encountered in soil, but it increased the contact of endospores and J2 during the agitation period. This may explain why attachment occurred with endospores exposed to 100 °C for 10 days. Williams et al. (1989) also reported that attachment of endospores of *P. penetrans* to J2 of *M. javanica* occurred even after heating at 121 °C if endospores treated in water were subsequently mixed with J2 and shaken for 24 hours. Conversely, attachment was not observed by Dutky and Sayre (1978) after soil with endospores was heated at 130 °C for 1 hour. Although procedures for inducing endospore attachment based on shaking in water differed from those employing soil (Bird et al., 1989; Dutky and Sayre, 1978), it is clear that attachment of endospores to J2 is prevented or severely limited at temperatures greater than 60 °C.

In soil with *M. arenaria* and bacteria ex-

posed to treatments together, temperature may affect the capability of endospores to attach to the nematode, the J2 cuticle's receptivity to endospores, and the incidence of contact of J2 with endospores. The increase in attachment observed from 10 °C to 20 °C, followed by optimum attachment between 20 °C and 30 °C, and decreased attachment above 30 °C agreed with previous studies (Hatz and Dickson, 1992; Singh and Dhawan, 1990; Stirling, 1981). Little or no attachment occurred when endospores and J2 were maintained together in soil at 10 °C for 4 days, which is assumed to have been caused by the limited movement of J2. As in the low-temperature treatment, a possible reduction in movement of the nematode, which prevented contact among J2 and endospores, also occurred above 40 °C. In addition, about 50% of the J2 extracted after 4 days at 40 °C were dead. Some J2 had cuticle deformities characteristic of exposure to high temperature. The absence of attachment at 50 °C may be best explained by the lethality of high temperature to *M. arenaria*.

Our results indicate that high temperatures may cause solubilization or denaturation of protein recognition sites, or ligands, on the surface of the nematode cuticle that may be involved in attachment of endospores to J2. Proteins and carbohydrates present on the nematode surface coat (or glycocalyx) are secreted by the nematode via excretory pore, mouth, anus, amphids, and phasmids, and spread over the nematode surface (Bird and Bird, 1991; Bird et al., 1989; Bird and Zuckerman, 1989). The proteins recognize certain carbohydrates and are thought to be responsible for attachment because they bind to N-acetylglucosamine on the surface of endospores (Chen et al., 1997; Davies and Danks, 1993). Decreased attachment resulting from exposure of endospores to heat also suggests that N-acetylglucosamine or similar moieties on the surface of the endospores responsible for attachment may have become solubilized or denatured. High temperature may denature proteins or peptidoglycans in the bacterial cell wall, or break the bonds that link

N-acetylglucosamine moieties to them. Further biochemical studies are required to understand the mechanisms that govern the decline of attachment with increasing temperature.

Low temperatures during the winter in Florida caused a decrease in nematode reproduction in the greenhouse, and the lack of J2 for the repeated trial resulted in the use of stored J2 instead of freshly hatched ones. Although the trends of the curves for attachment in both trials were similar, the average number of endospores attached per J2 in the first trial was almost 25 times higher than in the second trial. Proteins of the surface coat of J2 are released into water during incubation, indicating that they may be transitory (Lin and McClure, 1994). According to Spiegel and McClure (1991), the surface coat, when removed from the J2 cuticle, is replaced in 24 hours, but coat production is a metabolic process and is dependent on nematode age and environment. The loss of surface coat in water has been observed (Lin and McClure, 1994), and it is probable that the J2 used in the bioassay had lost much of their entire surface coat after the long period of incubation in water. Since the J2 were stored at 5 °C, it is assumed that the J2 were in anabiosis and, therefore, incapable of restoring lost surface coats. Thus, the degree of attachment was probably low at the time the J2 were introduced into soil. This hypothesis is supported by the differential attachment observed when 1- to 4-day-old J2 and 45-day-old J2 stored at 5 °C were subjected to attachment in endospore suspensions under agitation. A new surface coat should have been secreted by the J2 during the subsequent 4 days in the soil; however, attachment on 1- to 4-day-old J2 was 255 times higher than on the J2 stored at 5 °C. Although the J2 of *M. arenaria* stored in water at 5 °C recovered their mobility after 1 hour at 23 °C under bubbling air, their metabolism may have been altered by long-term storage at low temperature and could have resulted in reduced surface coat replacement. Second-stage juveniles extracted from soil in the first trial were much more active than those from the second trial.

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