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Effects of Fluctuating Temperatures and Different Host Plants on Development of *Pasteuria penetrans* in *Meloidogyne javanica*

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Abstract: Greenhouse and growth room experiments were conducted to investigate the effect of host plant in relation to different nematode inoculum levels, and temperature fluctuations on the development of *Pasteuria penetrans*. Host plant affected the development of *P. penetrans* indirectly through its effect on nematode development. Endospores collected from *Meloidogyne javanica* females reared on different hosts did not show any differences in subsequent attachment and infectivity. The numbers of endospores produced per infected female were reduced with increasing numbers of females parasitizing okra and tomato roots. Fluctuating temperatures retarded the development of *P. penetrans*. The life cycle of the parasite was completed faster at approximately constant temperatures close to 30 °C than when the temperature fluctuated away from 30 °C. The temperature of irrigation water did not affect the duration of life cycle of *P. penetrans*.

Key words: bacterial parasite, biological control, egg plant, *Meloidogyne javanica,* okra, *Pasteuria penetrans,* root-knot nematodes, temperature, tomato.

The gram-positive bacterium *Pasteuria penetrans* has been successfully used for the control of root-knot nematodes in pot experiments (Channer and Gowen, 1988) and microplots studies (Daudi et al., 1990; Trivino and Gowen, 1996) and has been reported to create suppressiveness in soil infested with root-knot nematodes in tobacco and groundnut fields (Weibelzahl-Fulton et al., 1996; Chen et al., 1996). Despite these promising results, a factor that limits its mass production and subsequent release in the field is its dependence on the nematode host to complete its life cycle. Attempts at in vitro production have failed (Bishop and Ellar, 1992; Williams et al., 1989), and so the only possible method of obtaining large numbers of endospores for extensive experiments and small-scale field use is to optimize the system of in vivo mass production. There is insufficient data describing the population dynamics within the parasite-nematode-plant complex. Root-knot nematodes have shown differential ability to invade and reproduce on different plant-hosts or even cultivars of the same host (Hadisoeganda and Sasser, 1982). Since the plant-host influences the dynamics of reproduction of root-knot nematodes, it could also influence the dynamics of parasitism by *P. penetrans.*

Stirling (1981) reported that *P. penetrans* development in *M. javanica* was greatly affected by temperature and that the optimum was 30 °C, whereas for *M. arenaria* the optimum was 35 °C (Hatz and Dickson, 1992). Most experimental studies employed constant temperatures, and Davies et al. (1988) noted that temperature fluctuations in greenhouses might retard development. Temperature seems to play a critical role on the spore attachment and parasitic development of *P. penetrans* (Davies et al., 1988; Giannakou et al., 1997; Stirling, 1981).

The objectives of this study were to investigate effects of various temperature schemes, plant hosts, and nematode inoculum densities on the development of *P. penetrans.*

Materials and Methods

A population of *Meloidogyne javanica* from Malawi and an isolate of *P. penetrans* (designated Pp3) from South Africa were used. The *Pasteuria* spore suspension was prepared using the method described by Stirling and Wachtel (1980). Eggs of *M. javanica* were removed from a tomato root sys-

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tem using the hypochlorite method (Hussey and Barker, 1973), and newly hatched juveniles were collected from modified Baermann trays (Southey, 1986). All plants were placed in a growth room, with 16 hours light, 8 hours dark, and temperatures of 30 and 27 °C, respectively. Plants were harvested after a certain number of degree-days had accumulated (base temperature 17 °C) (Stirling, 1981). The roots were washed and the fresh weights were recorded. The roots were placed in a phloxine B solution for 25 minutes (Southey, 1986) to stain egg masses, rinsed in water to remove excess stain, and then cut into 1- to 2-cm pieces. The number of egg masses and females were counted by direct examination of the roots using a stereoscopic microscope. Percentage infectivity was estimated by excising 40 females from each root system. Each female was placed in a drop of water, squashed with a cover slip, and immediately examined with a light microscope at ×400. The presence of endospores and mycelia (Hatz and Dickson, 1992; Sayre and Wergin, 1977) was used to confirm the infection of females by *P. penetrans.*

Ten endospore-filled females per plant were collected by hand and then placed in 1 ml water in a 2.5-cm diam. petri dish. They were squashed, and the resulting endospore suspension was poured into a 50-ml conical flask. The petri dish was rinsed into the flask with 9 ml water to ensure removal of the endospores. The flask was shaken on a mechanical shaker for 15 minutes to homogenize the suspension. Three aliquots per sample were taken to count numbers of mature endospores with the aid of a haemocytometer at ×400.

Infection of M. javanica by P. penetrans endospores as affected by host plant, experiment 1: Sixty milliliters of a nematode suspension containing 20,000 juveniles of *M. javanica* were exposed to 10 ml of *P. penetrans* suspension with a concentration of 1.2×10^5 endospores/ml in trays 17 × 11 cm. The trays were incubated at 28 °C, and endospore attachment was monitored until 80% of juveniles were encumbered with 6 to 12 endospores; then the suspensions were poured through a 20-µm-pore sieve to separate nematodes from spores. The juveniles were washed from the sieve and collected in 150 ml of water. Tomato (cv. Tiny Tim), okra (cv. Lady's Fingers), and egg plant (cv. Money Maker) were grown in loam-based compost in 1-liter plastic pots. Five milliliters of suspension containing 600 encumbered juveniles were used for inoculation. There were five replicates for each treatment.

Infection as affected by host plant, experiment 2: Fifty endospore-filled females of *M. javanica* were collected from each host plant in experiment 1, placed in a conical flask, macerated, and kept refrigerated (4 °C) for 1 month. These three spore suspensions were used as stocks for making new suspensions with the desired concentrations. Five milliliters of these suspensions, each containing 90,000 spores/ml, were added to 40 ml of nematode suspension containing 6,000 juveniles of *M. javanica* extracted from tomato roots. The same procedure as in the previous experiment was followed, and finally 4.5 ml containing 600 encumbered juveniles were used for inoculating 1-month-old tomato plants. Each treatment was replicated five times.

Influence of host plant in which endospores were produced on attachment: Five milliliters of a suspension containing 500 newly hatched juveniles of *M. javanica* reared on tomato plants was pipetted into a 9-cm plastic petri dish, and 1 ml of each of the three *P. penetrans* suspensions containing 90,000 spores was added. The volume in the petri dish was made up to 11 ml by adding tap water. The petri dishes were placed in an incubator at 28 °C and covered to prevent evaporation. Each treatment was replicated six times. Endospore attachment on samples of 20 nematodes was counted after 24 hours. The same procedure was followed once more in which 2 ml of spore suspension containing 10,000 spores/ml was added.

Development of M. javanica and P. penetrans as affected by host plant and inoculation level: Tomato and okra plants grown in a loam-based compost in 1-liter plastic pots were

inoculated with 2-, 6.5-, and 10-ml suspensions containing 1,000, 3,000, and 10,000 endospore-encumbered juveniles, respectively. At the time of inoculation at least 80% of the juveniles were encumbered with 6 to 12 spores/juvenile. After inoculation all plants were placed in a growth room, with 16 hours light and 8 hours dark at temperatures of 30 and 27 °C, respectively. Plants were harvested after 41 days when 495 (Tb 17 °C) degree-days had accumulated above the base temperature. The roots were washed and then cut to 1 to 2 cm pieces. Fifteen to twenty *P. penetrans*-infected *M. javanica* females were picked randomly, and their body lengths and widths were measured at ×100 magnification. After measurements were taken, females were examined to confirm parasitism, and 10 of them were processed through the same procedure as described previously for estimating the number of mature endospores.

Concurrently and under similar experimental conditions, plants were inoculated with *Pasteuria*-free J2s and numbers of eggs per egg mass were recorded. Two batches of 10 egg masses each randomly taken from every plant were placed in a glass tube each containing 4 ml water. One milliliter of NaOCl was added and left for 5 to 10 minutes and then another 5 ml of tap water was added. Eggs in two 1-ml aliquots were counted using a stereoscopic microscope. The mean number of four counts (two sets of 10 egg masses) was used in the statistical analysis.

Effect of temperature fluctuations on development of P. penetrans in M. javanica, experiment 1: Sixty 45-day-old (13 to 15 leaves) tomato plants were each inoculated with 5 ml of suspension containing 600 second-stage juveniles (J2). At least 80% of them were encumbered with 6 to 11 endospores per J2. Immediately after inoculation, plants were placed in a growth room. After 48 hours, half of the plants were placed in the greenhouse (25 to 40 °C) and half remained in the growth room (28 to 31 °C). Throughout the experiment, half of the plants in each treatment (greenhouse or growth room) were watered with warm water (27.1 ± 0.7 °C for the growth room and 21.6 ± 0.4 °C for the greenhouse) while the other half were watered with cold water (16.3 ± 0.8 °C).

The development of *P. penetrans* was recorded based on the following key: 1 = microcolonies the predominant stage; 2 = microcolonies and quartets; 3 = quartets the predominant stage; 4 = quartets and immature endospores; 5 = immature endospores the predominant stage; 6 = immature and mature endospores; 7 = mature endospores the predominant stage (Sayre and Wergin, 1977). Plants were harvested after 315, 410, and 500 degree-days. At each sampling five plants of each treatment and watering regime were harvested.

Effect of temperature fluctuations, experiment 2: One hundred sixty 30-day-old (8 to 10 leaves) tomato plants in small plastic pots (100 cm^3) were inoculated with 2 ml of nematode suspension containing approximately 600 endospore-encumbered J2s. Eighty-five percent of the J2s were encumbered with 6 to 12 endospores per J2. Immediately after inoculation they were placed in a growth room where they were kept for 48 hours to ensure good invasion. After this period, the tomato roots were washed free of soil and transplanted back to 640-cm^3 plastic pots containing fresh soil. After watering, the plants were divided into four groups of 40 plants each. Each group of plants was placed in a different environment: a growth cabinet, a growth room, or two different greenhouses. The temperature in the growth cabinet was a constant 26 °C, while in the growth room it was higher with a limited fluctuation (16 hours at 31 °C and 8 hours at 28 °C). In the two greenhouses the temperatures fluctuated, with ranges of 25 to 40 °C and 15 to 40 °C. However, only a small proportion of the accumulated degree-days was recorded at temperatures between 35 and 40 °C. Four plants were harvested each time, starting after 180 degree-days had accumulated (Tb = 17 °C) up to 570 degree-days, and *Pasteuria* development was monitored according to the key described above.

TABLE 1. Effects of different host plants on nematode development, egg mass production, incidence of *Pasteuria penetrans* infection, production of endospores, and fresh root weights of plants inoculated with 600 *Meloidogyne javanica* juveniles encumbered with *P. penetrans.*

Host plant	Females per root system	Egg masses per root system	Percent infected females	Endospores per female	Fresh root weight (g)
Eggplant	181 ± 37 a	78 ± 26 a	69.5 ± 3 a	473,870 ± 146 b	2.3 ± 0.5 b
Okra	156 ± 28 a	67 ± 10 a	57.4 ± 7 a	939,230 ± 63 a	16.2 ± 2.8 a
Tomato	162 ± 66 a	55 ± 29 a	65.9 ± 14 a	461,910 ± 66 b	4.3 ± 1 b

Numbers are the means of five replications ± SD; means in columns followed by different letters are different at $P = 0.001$.

RESULTS

Infection of M. javanica by P. penetrans spores as affected by host plant, experiment 1: There were no differences ($P > 0.05$) for the numbers of females, egg masses, and incidence of *P. penetrans* infection among the three host plants (Table 1). However, significantly more ($P < 0.001$) endospores were produced by females reared in okra roots (Table 1). Parasitized females in okra roots contained twice as many endospores as those in tomato and eggplant roots. The fresh root weights of okra were much greater ($P < 0.001$) than those of tomato and egg plant.

Infection as affected by host plant, experiment 2: Numbers of females and infected females in tomato roots did not differ significantly among treatments with *P penetrans* produced on different host plants (Table 2).

Influence of host plant in which endospores were produced on attachment: The spore attachment of *P. penetrans* was similar ($P > 0.05$) for both trials. Means of 13.0 ± 2 and 4.4 ± 0.7 spores/J2 were recorded for the first and second attachment tests, respectively.

Effect of host plant and inoculation level on development of M. javanica and P. penetrans: Infected and uninfected females obtained from okra roots were larger than those from tomato roots (Table 3). On okra more endospores were produced by infected females than on tomato roots (Table 4), and more eggs per egg mass were produced by uninfected females (Table 4). The body size of healthy and *P. penetrans*-infected females and healthy females was larger in the treatments with the low inoculation level (Table 3).

Effect of temperature fluctuations on development of P. penetrans in M. javanica, experiment 1: The data of this experiment were subjected to a factorial analysis of variance. Differences in development of *P. penetrans* were found between the two treatments (greenhouse vs. growth room) ($P = 0.05$) but not between watering treatments (warm vs. cold water) ($P = 0.05$) (Table 5). The difference in development between greenhouse and growth room treatments was more evident at the early than at the second and last sampling dates.

Effect of temperature fluctuations, experiment 2: Development of *P. penetrans* was greatly affected by temperature (Fig. 1). At constant temperatures the development of *P. penetrans* began earlier. After 300 degree-days had accumulated, the females parasitizing tomato plants at a constant temperature were filled with immature endospores, while those parasitizing tomato plants in highly fluctuating temperatures in the greenhouse were mainly filled with microcolonies. The highest developmental stage (numerical index 7) was recorded after 420 degree-days

TABLE 2. Numbers of *Meloidogyne javanica* females and egg masses, and percentages of females infected with *Pasteuria penetrans* in tomato roots 40 days after inoculation with 600 juveniles encumbered with endospores produced in *M. javanica* previously reared in three different host plants.

Origin of endospores from nematodes	Females	Percent infected females
Reared in tomato	207 ± 49	71.5 ± 4.7
Reared in okra	203 ± 47	80.6 ± 12.2
Reared in eggplant	298 ± 56	72.5 ± 11.8

Means within columns do not differ. Numbers are the means of five replications ± SD.

TABLE 3. Body length and width (μm) of *Pasteuria penetrans*-infected (Pp+) and uninfected (Pp−) *Meloidogyne javanica* females extracted from tomato and okra roots at three inoculum levels.

		Inoculum (juveniles per plant)					
		1,000		3,000		10,000	
Host	Pp	Length[a]	Width[b]	Length	Width	Length	Width
Okra	+	888 ± 26	700 ± 8	834 ± 31	667 ± 26	728 ± 37	586 ± 24
Tomato	+	775 ± 33	616 ± 29	748 ± 34	571 ± 64	700 ± 20	504 ± 10
Okra	−	820 ± 33	593 ± 20	788 ± 23	584 ± 24	736 ± 4	517 ± 35
Tomato	−	764 ± 19	539 ± 32	761 ± 36	555 ± 11	707 ± 13	537 ± 48

Numbers are the means of four replications ± SD.
[a] Treatment effects for length: host, $P < 0.001$, S.E.D. = 13.59; inoculation level, $P < 0.001$, S.E.D. = 16.65; interaction, $P > 0.05$.
[b] Treatment effects for width: host, $P < 0.001$, S.E.D. = 14.51; inoculation level, $P < 0.001$, S.E.D. = 17.77; interaction, $P > 0.05$. S.E.D. = standard error of the difference between the means.

had accumulated in the growth room at a temperature around 30 °C. The slowest developmental rate of *P. penetrans* was recorded in the greenhouse with a high temperature fluctuation.

DISCUSSION

The same rate of development was reported for *P. penetrans* in root-knot nematodes parasitizing five plants, but more endospores were produced in females reared on okra than another plants (Giannakou and Gowen, 1996). Okra produced a much bigger root system than the other plants. The densities observed were 78, 38, and 9 females/g of root of tomato, eggplant, and okra, respectively, suggesting that in okra roots there is less competition for food resources among females. Better conditions for development of female nematodes should provide a better medium for development of the bacterium. This conclusion is supported by the data of Hatz and Dickson (1992), who showed that there is coincident development by host (nematodes) and parasite (*Pasteuria*).

The ability of endospores to attach to juvenile root-knot nematodes has been positively correlated with the nematode in which the spores were produced (Davies et al., 1988). This study shows that plant does not affect the compatibility of the endospores with the nematode.

The coincident development of the host and the parasite was demonstrated in okra and tomato plants inoculated at three different inoculum levels. The length and width of the infected and uninfected females reared in okra roots were consistently greater than those of females in tomato roots. This difference in female body size in okra roots was reflected in the total number of endospores, which was up to twice as great as in females from equivalent treatments with tomato. A similar difference was

TABLE 4. Bacterial endospores (in thousands) and nematode eggs obtained from *Meloidogyne javanica* females that were infected (Pp+) or uninfected (Pp−) by *Pasteuria penetrans;* nematodes were obtained from tomato and okra roots that had been inoculated with second-stage juveniles (J2) of *M. javanica* at three inoculum levels.

		Inoculum (juveniles per plant)					
		1,000		3,000		10,000	
Type of propagule	Pp	Okra	Tomato	Okra	Tomato	Okra	Tomato
Endospores[a]	+	660 ± 86	466 ± 126	540 ± 82	354 ± 122	299 ± 59	141 ± 33
Eggs[b]	−	730 ± 108	528 ± 84	742 ± 78	533 ± 120	579 ± 55	447 ± 227

Numbers are the means of four replications ± SD.
[a] Treatment effects for endospores: host, $P < 0.05$, S.E.D. = 38.04; inoculation level, $P < 0.001$, S.E.D. = 46.59; interaction, $P > 0.05$.
[b] Treatment effects for eggs: host, $P < 0.05$, S.E.D. = 50.95; inoculation level, $P > 0.05$; interaction, $P > 0.05$. S.E.D. = standard error of the difference between the means.

TABLE 5. Developmental index[a] of *Pasteuria penetrans* in *Meloidogyne javanica* as affected by accumulated heat units and irrigation water temperature in two environments (growth room and greenhouse) that differed significantly in diurnal temperature fluctuation.

Irrigation water temperature[b] (°C)	Accumulated heat units (degree-days)	Developmental index	
		Growth chamber (21–38 °C)	Greenhouse (25–40 °C)
Warm	315	4.87	2.10
	410	6.41	5.77
	500	6.87	6.21
Cold	315	4.73	1.60
	410	6.40	5.58
	500	6.85	6.16

Statistically significant treatment effects: location effect, $P < 0.001$, SE = 0.09; time effect, $P < 0.001$, SE = 0.11; location × time, $P < 0.001$, SE = 0.15.

[a] Developmental index was rated on a 1-to-7 scale: 1 = microcolonies the predominant stage; 2 = microcolonies and quartets; 3 = quartets the predominant stage; 4 = quartets and immature endospores; 5 = immature endospores the predominant stage; 6 = immature and mature endospores; 7 = mature endospores the predominant stage (Sayre and Wergin, 1977).

[b] Warm water was 27.1 ± 0.7 °C in the growth room and 21.6 ± 0.4 °C in the greenhouse; cold water was 16.3 ± 0.8 °C in both environments.

shown in egg production. Body dimensions also were affected by inoculum level. Lengths and widths of infected females decreased as the inoculum level was increased from 1,000 to 10,000 juveniles, while in uninfected females there was a decrease of body dimensions only at the highest inoculum level. The differences in the body size of infected females presumably influenced the number of endospores through the lower level of food resources but, curiously, the egg production of uninfected females was not affected by the different inoculum levels.

A difficulty in computing the accumulated degree-days for predicting growth and development is the accurate estimation of the minimum (Tb) and maximum (Tm) temperatures. The rate of development does not follow a linear relationship and decreases as the temperature approaches the thermal maximum (Tm) (Trudgill, 1995). Data presented in this study support the hy-

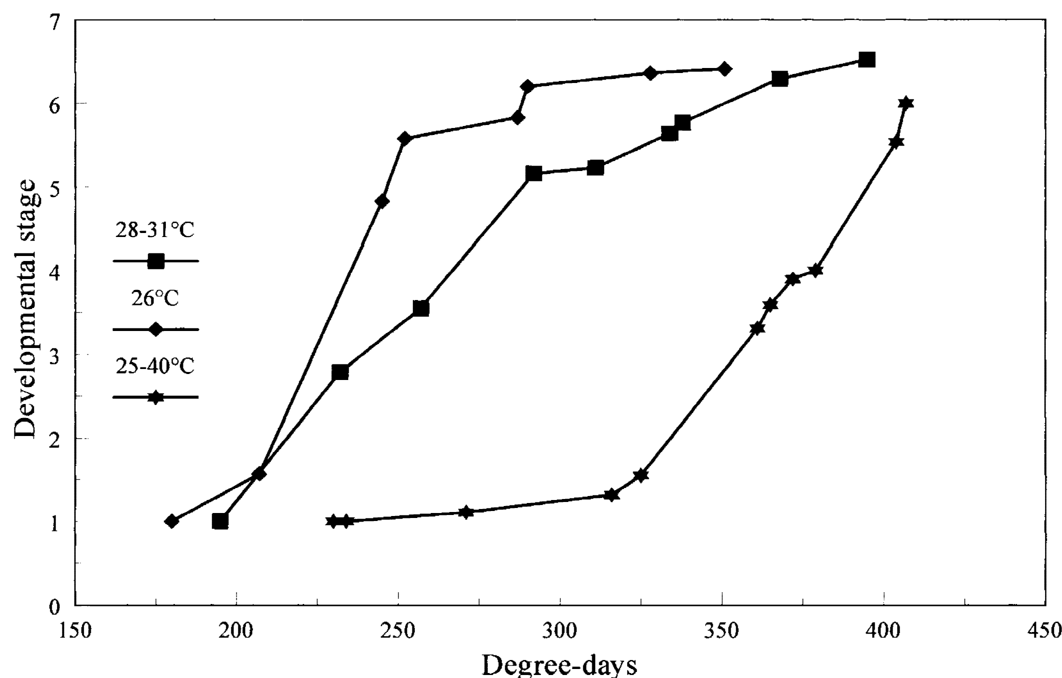

FIG. 1. Development of *Pasteuria penetrans* in *Meloidogyne javanica* in three different temperature regimes. Developmental stages: 1, microcolonies the predominant stage; 2, microcolonies and quartets; 3, quartets the predominant stage; 4, quartets and immature endospores; 5, immature endospores the predominant stage; 6, immature and mature endospores; 7, mature endospores the predominant stage.

pothesis that not only the absolute sum of heat-units but also their quality has an effect on the development of *P. penetrans*. The method of summing heat-units may be useful only for temperatures at or close to optimum. This is suggested by the variability of the development curves of *P. penetrans* in the four different temperature regimes. Although there is no difference between the curves in the growth room (26 °C) and cabinet (28 to 30 °C), there are differences between these two and the other curve obtained under fluctuating temperatures. Hatz and Dickson (1992) reported that with increasing temperature the development of *P. penetrans* and the formation of mature endospores began earlier. Our data suggest that not only the low temperature but also the fluctuation can retard development and formation of endospores. However, differences in temperature of the irrigation water was not sufficient to cause any significant retardation of development by lowering soil temperature.

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