Reproduction of *Pasteuria penetrans* in a Tissue-Culture System Containing *Meloidogyne javanica* and *Agrobacterium rhizogenes*-Transformed Roots

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ABSTRACT

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A three-component tissue-culture system for the study of *Pasteuria penetrans* biology and for gnotobiotic production of spores is described. *Meloidogyne javanica* juveniles with or without *P. penetrans* spores were added to plates in which *Agrobacterium rhizogenes*-transformed tomato or potato roots were growing on solid Gamborg's B5 medium. After 39 days, both parasitized and nonparasitized females were observed on *P. penetrans* cultures. Although at least one spore was attached to the cuticle of each juvenile added to such root cultures, only one third of the adult females became infected with *P. penetrans*. The bacterium infected similar numbers of females in potato- and tomato-root cultures. Nematode reproduction (eggs/female) and spore production (spores/infected female) were much greater on tomato- than potato-root cultures. Trends toward lower numbers of eggs/culture and increased numbers of eggs/healthy female were observed in cultures inoculated with *P. penetrans*.

Additional keywords: biological control, *Lycopersicon esculentum*, root-knot nematode, *Solanum tuberosum*.

The spore-forming bacterium *Pasteuria penetrans* (Thorne) Sayre & Starr is a pathogen of many species of plant-parasitic nematodes. *Meloidogyne* spp. is the host most frequently used in studies of parasitism by *P. penetrans*, and populations of this nematode have been suppressed in greenhouse, microplot, and field experiments by this bacterium (1,3,6,8). *Meloidogyne* females infected with *P. penetrans* produce few or no eggs (5).

The interaction between *P. penetrans* and plant-parasitic nematodes has been difficult to study because both the bacterium and nematodes are obligate parasites. A three-component, tissue-culture system involving tomato-root explants, *M. incognita*, and *P. penetrans* has been developed (10). Plant roots genetically transformed by *Agrobacterium rhizogenes* were recently used to culture and propagate *M. javanica* monoxenically (11). *A. rhizogenes* induces a genetic transformation of higher plants by inserting a fragment of its plasmid DNA (T-DNA) into the plant genome. Roots so transformed grow faster than nontransformed roots and are highly branched (9).

In this paper, a tissue-culture system that makes use of *A. rhizogenes*-transformed root cultures and provides reproducible infections of *M. javanica* by *P. penetrans* is described. The effect of the parasite on development and reproduction of a single generation of the nematode also is reported.

MATERIALS AND METHODS

Roots. Roots of *Lycopersicon esculentum* Mill. (tomato cultivar South Australian Early Dwarf Red) and *Solanum tuberosum* L. (potato) transformed by *A. rhizogenes* strain A4 were grown on Gamborg's B5 medium (4) (Gibco, Grand Island, NY) plus vitamins (11) in 9-cm-diameter petri dishes. The medium was solidified with 1% agar. Root cultures were incubated for 5 days at 25 C before inoculation with nematodes.

Pasteuria penetrans. Females of *M. javanica* infected with *P. penetrans* were obtained from stock cultures of the nematode and bacterium maintained on tomato plants in a greenhouse. Surface-sterilized females (10) were transferred to plates containing 1% water agar and stored at room temperature until needed. Spores were released by crushing infected females in a drop of sterile distilled water. Suspensions (200–500 μl) containing 5×10^5 spores were added to the surface of plates containing 1% water agar (5 cm diameter). Plates were left half-open on a laminar flow bench until the excess water had evaporated from the agar surface.

Meloidogyne javanica. Second-stage juveniles from monoxenic cultures were obtained by placing egg masses in a sterile vial with 2 ml of sterile distilled water. Two-day-old juveniles were added to a water agar plate with *P. penetrans* spores or to a plate without spores. Plates were left half-open on a laminar flow bench until the water had evaporated from the agar surface. Juveniles were recovered within 2–4 hr by flooding the plates with sterile distilled water. The number of spores attached per juvenile was determined by examining a random sample of 40 nematodes with a compound microscope at 400×. Each juvenile added to the *P. penetrans* treatments had at least one spore attached to its cuticle.

Tissue-culture experiments. Tomato- and potato-root cultures were inoculated with 80 ± 3 ($\overline{X}\pm$SD) juveniles with 16 ± 10 spores per nematode or without spores. Numbers of juveniles per plate were counted with a dissecting microscope after the nematode inoculations. Treatments with or without *P. penetrans* were replicated (one culture/replicate) nine or seven times, respectively. Cultures were incubated at 28 C and examined periodically. After 39 days, the agar in each culture was melted in a microwave oven and the root removed, blotted, and weighed. The numbers of healthy females, *P. penetrans*-infected females, and egg masses were determined by dissecting the entire root culture under a microscope. Infected females were distinguishable because of their characteristic milky white color. "Healthy hosts are by contrast of a dull color and more or less translucent" (12). Infection was confirmed by crushing white females that lacked egg masses in a drop of water and observing the spores at 400×. Translucent females with egg masses were considered healthy. Thirty infected females (one to five females per replicate, five replicates for tomato, and seven replicates for potato) were collected, and the number of spores within each female was determined as follows: Individual females were crushed, and the spores were dispersed in 1 ml of distilled water and counted with a hemacytometer. We were unable to examine more infected females because they were particularly fragile, and many could not be recovered intact. Eggs

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were dispersed from egg masses by incubation in 0.525% sodium hypochlorite for 5–10 min. Both hatched (empty egg shells) and unhatched eggs were counted. Galls containing more than one female were termed coalesced galls (2).

In a second experiment, tomato- and potato-root cultures were inoculated with the same number of juveniles (± *P. penetrans* spores) as in the first experiment, but juveniles with *P. penetrans* had an average of 10 ± 8 spores per nematode. In this experiment, spores were obtained from *M. javanica* infected females grown gnotobiotically on transformed potato-root cultures. Seven replicated cultures of each treatment were prepared (± *P. penetrans* on tomato or potato cultures). The number of spores produced by 56 infected females (three to five females per replicate, seven replicates each for tomato and potato) was determined. Reproduction of both *M. javanica* and *P. penetrans* was assessed after 35 days as before.

Data were analyzed by the general linear model procedure; experiments were treated as blocks. For factorial analyses of the effects of *P. penetrans* and two species of root host, main effects of the bacterium and root species were compared after analyses indicated the absence of interaction.

Greenhouse experiments. A spore suspension was prepared as above and spores added to plates containing 1% water agar. *M. javanica* juveniles from monoxenic cultures were added to the plates and recovered 24 hr later by a modified Baerman funnel technique. Nematodes (1,300 juveniles, each with 4 ± 4 *P. penetrans* spores) were added to each of four whole tomato plants growing in 250 cm^3 of autoclaved soil. Plants were maintained in a greenhouse for 7 wk and the number of healthy and *P. penetrans* infected females per gram of roots determined. Plants infested with *M. javanica* minus *P. penetrans* were not included as controls because we maintain *M. javanica* on tomato in the same soil and have not detected *P. penetrans* in such cultures.

RESULTS

Spores placed on the surface of water agar plates adhered rapidly to passing juveniles. All juveniles on these plates had at least one spore attached to their cuticle in about 2 hr.

M. javanica and *P. penetrans* developed and reproduced on *A. rhizogenes*-transformed tomato- and potato-root cultures. Both *P. penetrans* parasitized females and nonparasitized females were observed in cultures inoculated with *P. penetrans*. Many females (parasitized or nonparasitized) were partially exposed on the root surface, and the presence of *P. penetrans* within such females could be predicted by the white color of the female and absence of an egg mass.

More roots, females, egg masses, and eggs per culture were produced on transformed tomato- than on potato-root cultures (Table 1). Coalesced galls containing several females were observed frequently on transformed potato roots. Such galls had large amounts of callus tissue and females were located deep within the gall. Most nematodes on tomato roots were found in discrete galls having a single female, and callused tissue was infrequent.

P. penetrans parasitized similar numbers of females in both root cultures (11 and 10 females/culture in tomato and potato roots, respectively). However, twice as many spores were found in females developing on tomato than potato roots (151,897 and 79,884 spores/infected female, respectively; $P = 0.01$). Although each juvenile had attached spores when added to these cultures, only 13 and 14% of the juvenile inoculum developed into infected females on potato and tomato roots, respectively. Because fewer total females developed on potato cultures (Table 1), the percentage of females infected with *P. penetrans* was higher ($P = 0.01$) on potato- (45%) than tomato-root cultures (29%).

Partially parasitized females (with body cavities not filled with spores) were found on roots of both species but more often on potato cultures. *P. penetrans* occurred in localized zones in the body cavities of such females; consequently, both white and translucent areas were observed.

P. penetrans did not affect the number of *M. javanica* eggs/culture produced after a single generation of the nematode ($P = 0.39$) even though 37% of the females were parasitized (Table 1). The bacterium limited the number of fertile females (females producing eggs) per culture but did not affect the number of eggs produced by fertile females (= eggs/egg mass). Similar percentages of infection (juveniles that became adult females/juvenile inoculum × 100) occurred in cultures with or without the parasite (Table 1). Only 3% of the females in *P. penetrans* cultures that did not have egg masses did not contain spores of *P. penetrans*.

Healthy females appeared to develop faster than diseased ones. Exposed females with egg masses were first seen after 19 and 24 days on tomato- and potato-root cultures, respectively, whereas exposed infected females were seen after 24 and 29 days on the same cultures. The presence of egg masses, however, made healthy females easier to detect. Most *P. penetrans* females were observed on roots close to where juveniles were added, and healthy females were more frequently seen distant from the site of addition. Males occurred infrequently and mainly on potato-root cultures; there was never more than one male per culture.

Many *M. javanica* juveniles with *P. penetrans* spores from transformed potato-root cultures became infected when grown on whole tomato plants in a greenhouse. We found 1,428 ± 645 females per root system, and more than half (57%) of those females contained mature spores.

DISCUSSION

The tissue-culture system described here will be useful for the study of the influence of plant or nematode host, bacterial isolate, temperature, nutrition, and other factors on parasitism of nematodes by *P. penetrans*. Advantages of this system compared

TABLE 1. Reproduction of *Meloidogyne javanica* and *Pasteuria penetrans* on *Agrobacterium rhizogenes* transformed root cultures[a]

Treatments	Root wt (mg)	Total females /culture	Fertile females /culture	Eggs /egg mass	Eggs/culture	P_f/P_i[b]	Infection (%)[c]	Females parasitized by *P. penetrans* /culture	Parasitism (%)[d]
Roots									
Tomato	1,349	36	28	633	16,032	201	45	...	...
Potato	496*	22*	12*	367*	3,951*	50*	28*	...	...
P. penetrans									
+	953	30	17	462	9,309	116	38	10	37
−	906	28	23*	430	10,923	138	36	0*	0*

[a] The statistical interaction between root culture and *P. penetrans* was not significant. Thus, data for *P. penetrans* inoculated and uninoculated juveniles were pooled for comparison of root cultures. Similarly, data for tomato and potato were pooled for comparison of *P. penetrans* levels. Values are means of two experiments, seven to nine replications per experiment. In comparison of root cultures or levels of *P. penetrans*, means followed by an asterisk are significantly different, ($P \leq 0.05$).

[b] Final population/initial population of *M. javanica*.

[c] Percentage of nematode inoculum that developed into adult females.

[d] Females parasitized by *P. penetrans*/total females × 100.

to a greenhouse system include the easy control of variables (e.g., temperature, pH, and nutrient status of the host), the ability to make nondestructive observations, the small number of spores required, and the exclusion of other organisms. However, all components must be free of contamination before addition to the cultures and rigorous aseptic technique is required. Because three living organisms are involved in the system, variation among cultures is great and highly replicated experiments are required. The use of freshly hatched nematodes and young, actively growing root cultures reduces variability and favors parasitism of *M. javanica* by *P. penetrans*. The three component tissue-culture system also will be useful for providing pure spore inoculum for laboratory studies.

Almost twice the number of spores/infected female were produced on tomato roots as on potato roots. This large difference in spore production probably reflects differences in suitability of the roots for *M. javanica* development and reproduction (many more eggs/healthy female were produced on tomato).

In contrast, the numbers of females infected by *P. penetrans* were equal on tomato- and potato-root cultures. Although at least one spore was attached to each juvenile and the isolates of bacterium and nematode used were compatible (5), the bacterium infected only one-third of the nematodes. Reasons for the low level of infection are not known but may include poor germination of spores (7,8).

P. penetrans did not suppress *M. javanica* population density (eggs/culture) after a single generation of the nematode even though fewer fertile females developed on cultures with *P. penetrans*. However, trends toward lower numbers of eggs/culture and increased numbers of eggs/healthy female were observed in cultures inoculated with *P. penetrans*. Reproduction of root-knot nematodes may not be greatly affected by *P. penetrans* if the percentage of parasitism is low and the reduction in number of fertile females is compensated by an increase in egg production by those females (1).

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