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Partial characterization of a *Pasteuria* sp. attacking the citrus nematode, *Tylenchulus semipenetrans*, in Florida [1]

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Summary – Juveniles and males of the citrus nematode, *Tylenchulus semipenetrans*, infected with a *Pasteuria* sp. were detected in soil samples collected from a citrus grove in Central Florida. In laboratory studies, three to sixteen endospores attached to cuticles of individual nematodes. Mature endospores were observed within the nematode body 18 days after endospore attachment. Parasite development was asynchronous within individual nematodes. In males, the region of the body filled with spermatocytes was not colonized. Infected second stage juveniles and males each contained 320-400 endospores 18 days after endospore attachment at 25 °C. Adult females infected by *Pasteuria* sp. were not detected nor were endospores observed attached to the cuticles of adult females. Endospores of the *Pasteuria* sp. did not attach to the cuticles of *Meloidogyne incognita, M. javanica, Radopholus citrophilus*, or *R. similis*.

Résumé – ***Caractérisation partielle de* Pasteuria *sp. attaquant le nématode des citrus*, Tylenchulus semipenetrans, *en Floride.*** - Des juvéniles et des mâles du nématode des citrus, *Tylenchulus semipenetrans*, infestés par *Pasteuria* sp. ont été détectés dans des échantillons de sol de vergers de citrus de la partie centrale de la Floride. Au cours d'essais au laboratoire, il a été observé que trois à seize endospores s'attachent à la cuticule du nématode. Des endospores mûres sont présentes à l'intérieur du nématode 18 jours après l'attache des endospores. Le développement du parasite est asynchrone, dépendant du spécimen du nématode. Chez les mâles, la région du corps contenant les spermatocytes n'est pas colonisée. Les juvéniles de deuxième stade et les mâles infestés contiennent de 320 à 400 endospores (18 jours après l'attache des endospores, à 25 °C). Les femelles ne sont pas parasitées et aucune endospore n'a été détectée sur leur cuticule. Les endospores de *Pasteuria* sp. ne s'attachent pas à la cuticule de *Meloidogyne incognita, M. javanica, Radopholus citrophilus* ou *R. similis*.

Key-words : bacteria, biological control, Citrus, parasitism, *Pasteuria, Tylenchulus*, nematodes.

Pasteuria penetrans (Thorne) Sayre & Starr have been associated with *Tylenchulus semipenetrans* Cobb in France (Corsica), Florida, Iraq, and Samoa (Sturhan, 1985, 1989; Fattah *et al.*, 1989; Walter & Kaplan, 1990). To date, the only published detailed report of a *Pasteuria* sp. attacking *T. semipenetrans* characterized the endospores within the nematode's pseudocoelom and illustrated endospore attachment to the nematode body wall of second-stage juveniles in Iraq (Fattah *et al.*, 1989). A *Pasteuria* sp. was among a diverse group of predators, parasites, and antagonists of the citrus nematode identified in a survey of Florida citrus groves (Walter & Kaplan, 1990). The purpose of this paper is to further characterize the *Pasteuria* sp. detected in that survey.

Materials and methods

Life cycle and development

Second-stage citrus nematode juveniles (J2) and adult males were retrieved from 50 cm^3 soil samples collected from a citrus grove using modified Baermann funnels. Approximately 250 females were collected by either mechanically removing them from untreated roots or by enzymatically treating roots to facilitate release of intact females prior to dissection of females from the roots (Kaplan & Davis, 1990). The grove was located in Ona, Florida, where a *Pasteuria penetrans*-like organism attacking citrus nematodes was previously detected (Walter & Kaplan, 1990).

Citrus nematodes were examined using an inverted light-microscope (200 ×) and those with *Pasteuria* sp. endospores attached to their cuticles were transferred to individual drops of sterile distilled water on microscope slides and observed at 400 × with a light microscope. Three J2 or males containing *Pasteuria* sp. endospores were individually transferred to watch glasses (BPI) containing 200 µl of sterile distilled water. A dissecting needle was used to break open and crush the parasitized nematodes in order to release the *Pasteuria* sp. endospores.

After endospore release, 200 µl of water containing 100 J2 and males were added to each of the BPI watch glasses containing *Pasteuria* sp. endospores. These were

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subsequently placed on a shaker in moist chambers in the dark at 25 °C. The citrus nematodes added to the endospore suspension were hatched from eggs isolated from a citrus grove located in Ocoee, Florida, where *Pasteuria* sp. had not been detected. To attain adequate numbers of freshly hatched citrus nematodes, infested fibrous roots of sour orange (*Citrus aurantium* L.) were cut into small pieces (*ca* 5 mm) and 250 ml of water was added to create a slurry of root pieces. This was vigorously shaken for 5 min and then passed trough a series of sieves (2000-, 354-, 90-, 63-, and 30-μm opening) to separate the eggs from root pieces, soil particles, and debris. Nematode eggs and fine soil debris were collected on a 20-μm opening nylon-mesh sieve. Citrus nematode eggs were then isolated by adding 10.0 ml of 1.103 specific gravity $MgSO_4 - 7H_2O$ beneath 20 ml of the egg-debris suspension and centrifuged for 10 min (2000 *g*). Eggs were collected again on the 20-μm opening sieve. The eggs were then rinsed with distilled water. The egg suspension was placed in a vial fitted with 20-μm nylon mesh over the open end. The vial was inverted for 20 min into a watch glass filled with water to allow males and J2 that had previously hatched to migrate from the eggs. Nematodes that migrated through the mesh-fitted end of the vials were discarded. The vials were then turned upright, sealed, and placed on a shaker in a dark incubator at 25 °C. Citrus nematode J2 were subsequently isolated from the egg suspensions after 48 h by collecting nematodes that migrated through the 20-μm nylon mesh as before. These nematodes were visually inspected at 400 × with a light microscope to ensure that neither *Pasteuria* sp. nor any other organisms were present prior to their addition to the aqueous endospore suspension.

Three watch glasses were removed from the moist chamber at 3-day intervals over a 30-day period and the nematodes fixed in TAF (Courtney *et al.*, 1955). Nematodes in each of the watch glasses were then examined using an inverted light-microscope (200 ×). Nematodes examined for the presence of endospores or other *Pasteuria* sp. life cycle stages on/in the nematodes at 1000-1600 × with a Zeiss Photomicroscope II using Normarsky optics fitted with a DAGE MTI CCD72 video camera and viewed on a DAGE MTI-HR 1000 high resolution monitor. Developmental stages of the parasite, morphometrics (diameter of endospore and central core) of endospores attached to the nematode cuticle and within the nematode host, the location of endospores within the nematode body, and the number of endospores within each of 20 J2 and males were determined with a Hamamatsu Argus-10 Image processor and recorded with a Panasonic Time Lapse Video Cassette Recorder AG-6750A. Images recorded for publication were either photographed off the screen or produced with a Sony Color Video Printer UP 3000.

Host specificity

An experiment was conducted to determine if the *Pasteuria* sp. would attach and/or parasitize *Meloidogyne incognita, M. javanica* and *Tylenchulus semipenetrans* collected from *Pasteuria* sp.-free greenhouse pot cultures. The burrowing nematodes, *Radopholus citrophilus* and *R. similis*, retrieved from carrot disk cultures (Kaplan & Davis, 1990) were also evaluated. Then, 200 cm^3 soil samples from the Ona site were placed in beakers. A 5.0-ml aqueous suspension containing 1000 nematodes was added to each soil sample. The samples were incubated at 25 °C for 5 days and then placed on Baermann funnels. Nematodes retrieved from each of five samples per nematode species were examined using an inverted light-microscope (200 ×) for the presence of attached endospores. Individuals with endospores attached to their bodies were transferred to BPI watch glasses containing distilled water and incubated in a dark moist chamber at 25 °C. Nematodes were examined for the presence of endospore development 20 days after placement in incubators.

Results and discussion

Males and J2 of *T. semipenetrans* infected with *Pasteuria* sp. were detected in soil samples collected from a citrus grove in Central Florida (Fig. 1 A). Three to sixteen endospores were attached to the cuticles of J2 and males six days after the *Pasteuria* sp-free citrus nematodes were added to the *Pasteuria* sp-endospore suspension. The diameter of endospores attached to the nematode cuticles were 2.7 ± 0.3 μm and their central cores were 1.4 ± 0.2 μm. The morphology of the *Pasteuria* sp. detected in the Florida citrus grove was not significantly different from that reported from Iraq (Fattah *et al.*, 1989) where the endospore diameter was 2.6 μm. From published photographs, the central core diameter of that isolate was estimated to be 1.6 μm (± 0.1).

Mature endospores (" fried-egg " shaped) appeared within the bodies of *T. semipenetrans* 18 days after endospore attachment (Fig. 1 B, C). In contrast to *P. penetrans* which developed synchronously in *Meloidogyne* (Sayre & Starr, 1985), development of *Pasteuria* sp. was asynchronous within a single citrus nematode (Fig. 1 D). In contrast to the *Pasteuria* sp. isolated from Iraq (Fattah *et al.*, 1989), the Florida *Pasteuria* sp. isolate produced sporangia and endospores within males (Fig. 1 E). The endospores probably attached to males after they had reached the adult stage (Fig. 1 F). Infected second-stage juveniles and males contained 320-400 endospores 18 days after attachment.

Adult female specimens of many sedentary and several migratory species of plant parasitic nematodes have also been found filled with endospores of members of the *P. penetrans* group (Davies *et al.*, 1990; Giblin-Davis, 1990). However, endospores were not detected on the cuticles or within mature citrus nematode females.

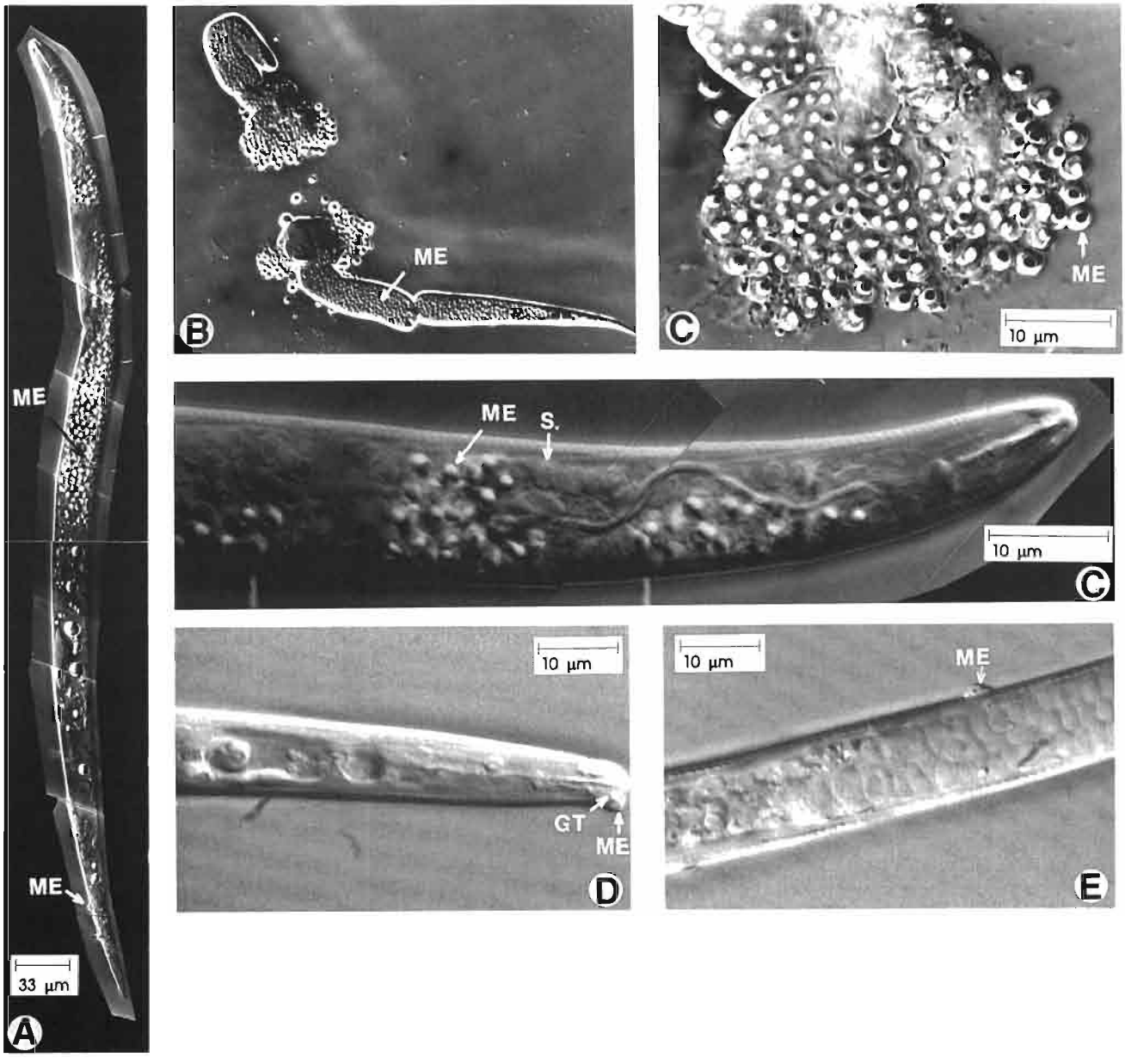

Fig. 1. *Second-stage juveniles (J2) and males of* Tylenchulus semipenetrans *parasitized by* Pasteuria *sp. A : J2 with mature endospore (ME) attached to tail and containing mature endospores and sporangia; B :* Pasteuria *sp.-filled J2 broken to release mature endospores (ME); C : Detail of mature endospores released from broken J2; D : Asynchronous development of* Pasteuria *sp. within the J2 as evidenced by sporangia (S) and mature endospores (ME); E : Mature endospore (ME) attached to head region of male* T. semipenetrans *with germ tube (GT); F : Mature endospore (ME) attached to male cuticle in vicinity of spermatocytes.*

Examination of citrus nematode females is complicated by the parasitic habit of the citrus nematode; intracellular invasion of the root accompanied by the enlargement of the anterior half of the female makes it difficult to attain entire females without breaking them at the point where they emerged from roots (semi-endoparasite). That is, the anterior end of the nematode generally remains in the root when the posterior portion and body contents are lost. However, it is likely that if present, endospores would have been observed when females

were broken away from the root surface. In an attempt to circumvent this, tissue-softening enzymes were used to release females from root tissue, but none of the females retrieved had endospores attached to their cuticles or within their bodies.

Citrus nematode J2 and males remain in soils for extended periods of time. This, in conjunction with the relatively short life cycle of *Pasteuria* sp. (18 days), would be conducive to the development of mature endospores in the migratory stage of the nematode. Isolates of other *Pasteuria* sp. that complete their life cycles in vermiform stages of some other sedentary plant parasitic nematodes have also been noted to have a rapid life cycle (Sayre & Starr, 1988; Giblin-Davis, 1990).

Pasteuria sp. did not attach to cuticles of *Meloidogyne incognita, M. javanica, Radopholus citrophilus,* or *R. similis*. However, *Pasteuria* sp. did become attached to *T. semipenetrans* J2 and males that migrated through the soil. After 20 days incubation, endospore development was observed in several *T. semipenetrans* J2 and males. This suggests that *Pasteuria* sp. is specific for the citrus nematode.

Pot cultures of the Florida isolate of *Pasteuria* sp. have been established using *T. semipenetrans* on citrus trees growing in *Pasteuria* sp.-infested soil and by direct infestation with *Pasteuria* sp.-infected J2 and males. However, it has been difficult to recover enough *Pasteuria* sp.-colonized nematodes to determine the effect of *Pasteuria* sp. on citrus nematode population dynamics. Failure to detect females filled with endospores suggests that previously described techniques for *P. penetrans* culture (Stirling & Wachtel, 1980) are not useful for this citrus nematode parasite since *Pasteuria* sp.-infected stages occur in the soil. Although the objective of this study was not directed toward development of a culture method for *Pasteuria* sp. on citrus nematode, the ability of *Pasteuria* sp. to attach, penetrate, and produce endospores under laboratory conditions suggests that this may provide a means of *Pasteuria* sp. population increase. We are exploring this possibility.

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