PASTEURIA SP. FOR BIOLOGICAL CONTROL OF THE STING NEMATODE, BELONOLAIMUS LONGICAUDATUS, IN TURFGRASS

1994 USGA TURFGRASS RESEARCH REPORT

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1994 OBJECTIVES

1) Examine ultrastructure with transmission electron microscopy and begin describing new species of Pasteuria that we have discovered parasitizing the sting nematode, Belonolaimus longicaudatus, from Ft. Lauderdale, Florida.

2) Perform host range studies on this new Pasteuria sp.

3) Begin studies to elucidate the population dynamics of this new Pasteuria sp. on sting nematode grown on FX-313 St. Augustinegrass in laboratory pot cultures under controlled conditions.

1994 USGA TURFGRASS RESEARCH EXECUTIVE SUMMARY

We are describing a new species of bacterium in the genus, Pasteuria that we discovered parasitizing the sting nematode, Belonolaimus longicaudatus in Florida. We are hopeful that this obligate bacterial parasite of nematodes (Pasteuria n. sp. [S-1]) will have some potential for inoculative biological control in golf course greens against the sting nematode; a destructive ectoparasite that can reduce the root dr weight of turfgrasses and other crops in sandy soils by as much as 30-50%.

In 1994, we completed ultrastructural studies with transmission electron microscopy (TEM) that show that Pasteuria n. sp. (S-1) is a new species and have helped to elucidate the life cycle. The sporangium and endospore diameters of Pasteuria n. sp. (S-1) are on the average at least 1.0 and 0.5 μm wider than these respective measurements for the other described species of Pasteuria. The epicortical wall of Pasteuria n. sp. (S-1) surrounds the endospore in a sublateral band and the basal cortical wall thins to expose the inner endospore, similar to P. thornei but different from the other two described species. The outer cortical wall thickness at its thickest point is 1/3 the endospore diameter for Pasteuria n. sp. (S-1) compared with 1/4-1/15 for the other described species of Pasteuria. A brief description of the life cycle follows (Fig. 1); after attachment of a mature endospore to the cuticle of the host, penetration ensues via a germ tube through the cuticle into the pseudocoelom of the nematode. A mycelial microcolony is formed which eventually breaks up and is distributed throughout the pseudocoelom (fragmentation). Mycelial filaments are divided by septa and possess double-layered cell walls. Endospores are produced endogenously and the formation sequence (sporogenesis) for Pasteuria n. sp. (S-1) is similar to the three other described species of Pasteuria. A septum is formed within the sporangium, the sporangium cytoplast condenses to form a forespore, the endospore walls form, the endospore matures, and areas adjacent to the endospore give rise to perisporal "attachment" fibers.

Laboratory host attachment studies and field observations done in 1994 on Pasteuria n. sp. (S-1) demonstrate that it is highly host specific and attacks only nematodes in the genus Belonolaimus or within the species, B. longicaudatus. We have initiated population dynamic studies on Pasteuria n. sp. (S-1) in laboratory pot cultures of the sting nematode on the model turfgrass host (FX-313 St. Augustinegrass) under controlled conditions. After 84 days, sting nematode cultures which were inoculated with 10 or 25 sting nematodes with Pasteuria n. sp. (S-1) have not shown suppression or a disease epizootic. The experiment will run for at least 210 days and it may take at least this long for establishment of the bacteria under these conditions. In 1995, we propose to do studies on the effects of temperature on development of Pasteuria n. sp. (S-1), monthly survey work in golf course areas where this bacterium occurs naturally to assess its suppressive effects on sting nematodes, and to begin sampling golf course areas where sting nematode is no longer a problem to try and isolate different species or isolates of antagonists to nematodes of turfgrass.

BACKGROUND

One major problem encountered in southern United States golf courses, athletic fields, and lawns is the destruction of roots by phytoparasitic nematodes. Recent work in the midwestern and western United States has demonstrated the importance of phytoparasitic nematodes to turfgrass culture in these geographical regions as well. The most damaging nematode in the warmseason turfgrass ecosystem in sand soils is the ectoparasitic sting nematode, Belonolaimus longicaudatus. There are many other species of plant-parasitic nematodes that cause below ground root pruning and damage. However, they are not as important as the sting nematode which is recognized as the most pathogenic phytonematode in the state of Florida. Our research has demonstrated 30-50% root weight reductions in controlled inoculation studies with sting nematode on commonly cultivated hybrid bermudagrasses which are used for golf course greens such as 'Tifgreen' and 'Tifdwarf.'

The sting nematode has a wide host range and is a major pest on a variety of grasses, vegetables, and perennial crops. It is a relatively large plant-parasite (ca. 2000 μm long) and goes through its life cycle in about 28 days. The sting nematode does best in soils with >80% sand. It is a documented pest in the sandy soils of the Coastal Plains from Florida north to Virginia and along the Gulf Coast into Texas. It also occurs in Arkansas, Kansas, Oklahoma, Missouri, and Nebraska. It has also been recently introduced into southern California where it is causing problems in golf course greens.

Currently, management of phytoparasitic nematodes for perennial crops such as turfgrass relies largely on postplant application of organophosphate pesticides. Nematicides labelled for use on turfgrass in 1994 are nematostatic at the concentrations achieved in the field and usually require multiple applications for short-lived (< 4 weeks) suppression of phytoparasitic nematode populations. Chronic exposure of nematodes and the soil microflora to sublethal doses of nematicides can encourage microbial decomposition of pesticides.

Releases of members of the Pasteuria penetrans group, obligate nematode endoparasitic bacteria, may provide an alternative or supplement to chemical control. These endospore-forming actinomycetes attach to, and infest the nematode host via the cuticle. The parasitized nematode is incapable of reproduction and eventually becomes filled with developing endospores of the bacterium, which are released into the environment upon host disintegration. Some forms of the bacteria attack juveniles and do not sporulate until the nematode becomes an adult, ie. P. penetrans sensu strictu. Other species, such as P. thornei can attack and complete their life cycle before the host reaches the adult stage. The assets of members of the P. penetrans group as biological control agents of turfgrass nematodes are; 1) their ability to persist for long periods of time (> 1 year), 2) host specificity, 3) compatibility with pesticides, and 4) lack of environmental risk to humans and other non-target organisms.

Spores of the Pasteuria penetrans group are resistant to

heat, desiccation, and exposure to nematicides and have been reported adhering to, or infesting, 205 species of nematodes from 51 countries worldwide. Only two species of the P. penetrans group are well characterized, however, and little is known about the ecology of the group in native or managed soil systems.

We have done survey work from 1985-1989 which suggests that isolates of Pasteuria are widely distributed in bermudagrass fairway turf in southern Florida. Five morphometrically distinct isolates of the bacteria were observed on five species of plant-parasitic nematodes. We have done a one year greenhouse study to determine if soil infested with a large-spored isolate of Pasteuria (6.10 μm endospore diameter) was suppressive to populations of the sting nematode on 'Tifgreen II' bermudagrass. Soil containing this isolate was not suppressive to B. longicaudatus in the first six months but caused a significant decrease in sting nematodes after one year with concomitant increases in numbers of Pasteuria sp.-infested sting nematodes.

These results are encouraging because they suggest that the sting nematode isolate of Pasteuria may be valuable in inoculative biological control of the sting nematode in golf course greens, and other turf situations where small amounts of soil infested with the bacteria could be used for inoculation. The purpose of this USGA-funded project is to describe this new species of Pasteuria and see if it can be successfully manipulated in the managed turfgrass ecosystem.

1994 RESEARCH PROGRESS

Description of Pasteuria n. sp. (S-1) from the sting nematode from Ft. Lauderdale, Florida: We are currently working on the description of Pasteuria n. sp. (S-1) from the sting nematode in southern Florida based upon ultrastructure, morphometrics, and host range studies.

a) Transmission electron microscopy (TEM): Belonolaimus longicaudatus filled with the endospores or with different stages of the vegetative phase of Pasteuria n. sp. (S-1) from the Ft. Lauderdale Research and Education Center were cut and fixed in 2.5% glutaraldehyde + 0.1 M sodium phosphate buffer (pH 7.4) overnight at 4 C, embedded in 3% agarose, and cut into small blocks. The glutaraldehyde was rinsed from the blocks with five rinses of phosphate buffer and the tissue was postfixed in 2% osmium tetroxide in phosphate buffer. The tissue was rinsed, dehydrated in an ethanol-acetone series, and infiltrated with Spurr's epoxy resin. The tissue was then placed in molds in a 60-C vacuum-oven (6.8-kg vacuum) for 18 hours for resin polymerization. Thin-sections (80 nm) were cut with glass knives on a LKB ultramicrotome, stained with uranyl acetate and lead citrate, and viewed with a Hitachi 7000 or a Philips 201 TEM (60 kv).

The sporangium and endospore diameters of Pasteuria n. sp. (S-1) are on the average at least 1.0 and 0.5 μm wider than these respective measurements for the other described species of Pasteuria or other host isolates of Pasteuria from southern

Florida fairways (Tables 1 and 2). In TEM, the epicortical wall of Pasteuria n. sp. (S-1) surrounds the mature endospore in a sublateral band and the basal cortical wall thins to expose the inner endospore (Fig. 2, Table 2), similar to P. thornei but different from the other two species. The endospore pore diameter, measured from TEM micrographs, is larger than any other described species of Pasteuria (Table 2). The endospore shape in Pasteuria n. sp. (S-1) is an oblate spheroid that is a ventrally flattened ellipse in longitudinal sections (Fig. 2, Table 2). The other species possess endospores which are narrowly or broadly elliptic in longitudinal TEM sections (Table 2). The outer cortical wall thickness at its thickest point is 1/3 the endospore diameter for Pasteuria n. sp. (S-1) compared with 1/4-1/15 for the other described species of Pasteuria (Fig. 2, Table 2).

A brief description of the life cycle of Pasteuria n. sp. (S-1) based upon LM and TEM follows; after attachment of a mature endospore to the cuticle of the host, penetration ensues via a germ tube through the cuticle into the pseudocoelom of the sting nematode. All stages from J2 through adults were observed with attached endospores on the cuticle and with internal infections of vegetative and sporulating Pasteuria n. sp. (S-1). A mycelial microcolony (Fig. 3) is formed which eventually breaks up and is distributed throughout the pseudocoelom (fragmentation). Mycelial filaments are divided by septa and possess double-layered cell walls (Figs. 3). Endospores are produced endogenously and the formation sequence (sporogenesis) for Pasteuria n. sp. (S-1) is typical for the three other described species of Pasteuria. A septum is formed within the sporangium, the sporangium cytoplast condenses to form a forespore, the endospore walls form, the endospore matures, and areas adjacent to the endospore give rise to perisporal "attachment" fibers.

b) Host range studies with Paseuria n. sp. (S-1): Host range studies were conducted. Endospores were harvested from spore-filled sting nematodes recovered by centrifugal-flotation using 1M sucrose from field plots previously determined to be infested. Endospore/water suspensions of 1,000 endospores in 100 µl per 250 µl microfuge tube were quantified using a hemocytometer. A set number of a test nematode host species (ie. 200 each for Meloidogyne incognita, M. javanica, M. hapla, M. arenaria, or 60 each for different isolates of Belonolaimus, Hoplolamius galeatus, or Pratylenchus penetrans) were used in each attachment run in the microfuge. The suspension and nematodes were centrifuged 2 min at 9,500 g in a Beckman microfuge. Nematodes were pipetted from the microfuge tubes onto counting dishes in individual drops of water and 20 randomly chosen individuals were examined for successful spore attachment. The results are summarized in Table 3. Basically, Pasteuria n. sp. (S-1) spores only attach to Belonolaimus longicaudatus. This is consistent with field work that we have done in southern Florida fairways. We only see the Pasteuria n. sp. (S-1) attaching and completing its life cycle in sting nematodes, even when there are many other species of nematodes in the same sample (i.e. Hoplolaimus galeatus, Tylenchorhynchus

annulatus, Meloidogyne spp., Helicotylenchus microlobus, Hemicriconemoides annulatus, Criconemella ornata, Trichodorus proximus, and several freeliving nematode species).

Laboratory time-course study of sting nematode with or without Pasteuria n. sp. (S-1): We have designed a laboratory pot assay to study the population dynamics of the sting nematode and compare the ability of Pasteuria to suppress the establishment of B. longicaudatus on FX-313 St. Augustinegrass [Stenotaphrum secundatum]. Washed aerial stolons of FX-313 St. Augustinegrass were planted in autoclaved 60-mesh sand in 26 X 52 mm plastic trays kept on a raised bench for rooting. Stolons were 6-8 cm long terminal cuttings with 2-3 nodes. After 28 days, sprigs were transplanted to square tapered pots (80 mm wide at the top, 60 mm wide at the bottom, 75 mm deep). Sprigs had one strong terminal with two to three nodes and four to six basal roots at transplanting. Pots were filled with 250 ml (378 g) of moist, autoclaved Margate fine sand. Treatments were applied five days after transplanting. Those pots receiving nematodes were inoculated with 100 B. longicaudatus without spores of Pasteuria as described below and placed in 0.26 mls of water into a small depression near the base of each plant. The nematode inoculum was obtained by centrifugal flotation from a stock culture maintained on FX-313 St. Augustinegrass.

Treatments involved a harvest factor (to be harvested 42, 84, 126, 168, and 210 days after inoculation) and a Pasteuria encumbrance factor. The treatments were 1) no nematodes, 2) 100 healthy sting nematodes without spores, 3) 100 healthy sting nematodes plus 10 B. longicaudatus with about 5 spores each, and 4) 100 healthy sting nematodes plus 25 B. longicaudatus with about 5 spores each. Spore encumbered B. longicaudatus were harvested from a Ft. Lauderdale, FL, field site with Pasteuria n. sp. (S-1). The resulting 20 combinations were arranged in a randomized complete block design with 9 replications.

Pots are watered twice weekly to bring soil moisture content up to just below saturation. Pots are situated on a laboratory bench under flourescent lights with a 16 hr photoperiod (photosynthetic photon flux: 138 μmole m^{-2} s^{-2}). Soil temperatures are maintained between 22 and 25°C.

At harvest, the soil has been washed from the root ball and nematodes are extracted from the entire soil volume by centrifugal flotation. Cohorts of 15-25 nematodes are stained with crystal violet and examined for infestation with the vegetative and/or spore phase of Pasteuria n. sp. (S-1). Following nematode extraction, roots are separated from stolons and leaves, dried at 60°C for 72 hr, and weighed.

Preliminary results from the first two harvest dates (42 and 84 days after inoculation) are presented in Figure 4. These results demonstrate that the extra nematodes inoculated with spores of Pasteuria n. sp. (S-1) have contributed to sting nematode population growth. The original thought was that these encumbered nematodes would die and release spores that would begin to infect the healthy population and that they would not add to the reproductive fitness of the nematode population. At

84 days, we are just starting to see Pasteuria n. sp. (S-1) attached to less than 1% of the nematodes harvested in the high spore treatment. This suggests that Pasteuria n. sp. (S-1) is slow to cycle under these conditions and that more time is needed to draw conclusions. It also suggests that further experimentation with soil temperature might be beneficial.

FUTURE RESEARCH (1994-1995)

In 1995, we propose to do studies on the effects of temperature on development of Pasteuria n. sp. (S-1), to do monthly survey work in 'Tifgreen' bermudagrass areas where this bacterium occurs naturally to assess its suppressive effects on sting nematodes, to begin sampling golf course areas where sting nematode is no longer a problem to try and isolate different species or isolates of antagonists to nematodes of turfgrass, to finish disease time-course study with Pasteuria n. sp. (S-1), and finish ultrastructural work with Pasteuria n. sp. (S-1) and publish description.

SUMMARY OF PERSONNEL TIME AND EXPENDITURES MADE DURING 1994

Dr. Robin M. Giblin-Davis (Project P.I.).....30%
Mr. Frank Bilz (State line Biologist).....30%
Mrs. Barbara J. Center (USGA-grant paid laboratory assistant).....50%

Dr. Don W. Dickson (Project Co-P.I.).....07%
Mr. Tom Hewlett (State line Biologist).....20%
Mr. Ross Robinson (USGA-grant paid laboratory assistant).....25%

Dr. John L. Cisar (Project Co-P.I.).....05%
Ms. Karen Williams (State line Biologist).....05%

Salaries and wages:
Laboratory assistant: Barbara J. Center (Ft. Lauderdale R.E.C. 1/2 time, $12.00/hr).....$11,100

Laboratory assistant (University of Florida 1/4 time, $10.00/hr).....$ 5,200

Operating expenses:
Materials and supplies.....$ 500
Overhead:
16%.....$ 3,200

1994 total.....$20,000

TABLE 1. Summary of the pooled light microscope (LM) measurements of sporangium and endospore diameter of different nematode-host isolates of *Pasteuria* from southern Florida fairways of 'Tifgreen' bermudagrass.

Nematode species	Sporangium diameter (μm)			Endospore diameter (μm)			
	Mean	SD	Range	Mean	SD	Range	N
Hoplolaimus galeatus (HFL-1)	7.26 a	0.36	6.53-8.05	3.54 a	0.24	2.72-3.54	80
Belonolaimus longicaudatus (S-1)	6.10 b	0.39	4.71-7.00	2.93 b	0.24	2.33-3.78	413
Tylenchorhynchus annulatus (TP-1)	4.55 c	0.33	3.98-5.38	2.53 c	0.17	2.21-2.95	41
Helicotylenchus microlobus	3.87 d	0.39	2.78-4.73	1.97 d	0.22	1.39-2.50	225
H. galeatus (HFL-2)	3.87 d	0.33	3.19-4.64	1.95 d	0.22	1.45-2.45	92
Meloidogyne spp.	3.42 e	0.27	2.91-4.19	1.58 e	0.16	1.22-1.98	195

Means in a column followed by the same letters were not different ($P < 0.01$) with a Student Newman-Keuls multiple-range test.

TABLE 2. Comparisons of morphometric, ultrastructural, and developmental attributes of described species of nematode-associated *Pasteuria* and the S-1 isolate of *Pasteuria* sp. from *Belonolaimus longicaudatus* from Ft. Lauderdale, Florida.

Trait	*P. penetrans*	*P. thornei*	*P. nishizawa*	*Pasteuria* n. sp. (S-1)
Colony shape:	Spherical, to cluster of elongated grapes	Small, elongate clusters	Spherical, to cluster of elongated grapes	Elongate clusters
Sporangium:				
shape (dorsal)	Convex	Cone-shaped	Convex	Convex
diameter (μm)				
LM	4.5 ± 0.3	3.5 ± 0.2	5.3 ± 0.3	6.1 ± 0.4
TEM	3.4 ± 0.2	2.4 ± 0.2	4.4 ± 0.3	5.6 ± 0.1
exosporium	Present, smooth	Present, smooth	Present, hairy	Present, smooth
Endospore:				
shape	Oblate spheroid, narrowly elliptic in longitudinal section	Oblate spheroid, broadly elliptic in section	Oblate spheroid, narrowly elliptic in section	Oblate spheroid, ventrally flattened ellipse in section
orientation of major axis to sporangium base	Horizontal	Horizontal	Horizontal	Horizontal
diameter (μm)				
LM	2.1 ± 0.2	1.6 ± 0.1	2.1 ± 0.2	2.9 ± 0.2
TEM	1.4 ± 0.1	1.3 ± 0.2	1.3 ± 0.3	1.7 ± 0.1
epicortical wall	Surrounds endospore in lateral band	Surrounds endospore in sublateral band	Entirely surrounds endospore	Surrounds endospore in sublateral band
outer cortical wall thickness (at thickest point)	1/4 endospore diam.	1/15 endospore diam.	1/9 endospore diam.	1/3 endospore diam.

TABLE 2. (continued).

Trait	*P. penetrans*	*P. thornei*	*P. nishizawa*	*Pasteuria* n. sp. (S-1)
Endospore (continued):				
pore	Basal annular opening formed from thickened outer cortical wall	Basal cortical wall thins to expose inner endospore	Basal cortical wall tapers gradually to pore opening	Basal cortical wall thins to expose inner endospore
diameter (μm)				
TEM	0.3 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	0.4 ± 0.1
Perisporium:				
fibers, origin, and orientation	Fibers arise from cortical wall arching ventrally to to form attachment layer of numerous shorter fibers	Same as *P. penetrans*	Same as *P. penetrans*, but additional layer is formed on surface of endospore	Same as *P. penetrans*
Host(s):	Root-knot nematodes *Meloidogyne incognita*	Lesion nematode *Pratylenchus brachyurus*	Cyst nematodes *Heterodera* spp.	Sting nematode *Belonolaimus longicaudatus*
Nematode life stage where sporogenesis occurs:	Adult only	All larval stages and adult	Adult only	All larval stages and adult
Location in host:	Pseudocoelom	Pseudocoelom	Pseudocoelom	Pseudocoelom

TABLE 3. Laboratory attachment studies with *Pasteuria* n. sp. (S-1) from Ft. Lauderdale, Florida.

Nematode	% Attachment		Average number of spores / nematode	
	Test 1	Test 2	Test 1	Test 2
Belonolaimus longicaudatus				
Gainesville, Florida	30	26	2.2	6.5
Sanford, Florida	28	40	1.6	1.9
North Carolina	90	70	3.5	5.7
Hoplolaimus galeatus				
Gainesville, Florida	0	0	0	0
Meloidogyne species				
arenaria	0	0	0	0
javanica	0	0	0	0
incognita	0	0	0	0
Pratylenchus penetrans	0	0	0	0

FIGURE 1

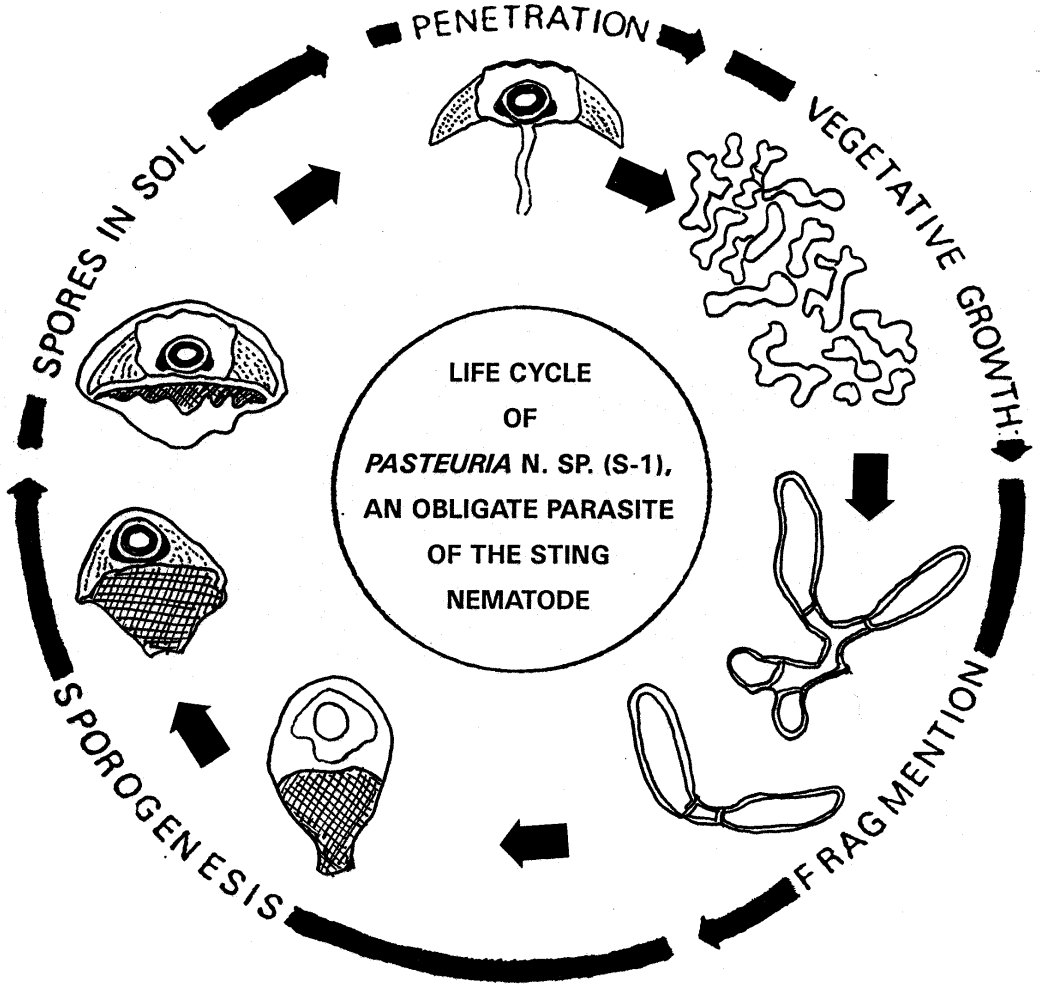
PENETRATION
VEGETATIVE GROWTH
FRAGMENTION
SPOROGENESIS
SPORES IN SOIL
LIFE CYCLE
OF
PASTEURIA N. SP. (S-1),
AN OBLIGATE PARASITE
OF THE STING
NEMATODE

FIGURE 4

Belonolaimus longicaudatus population growth

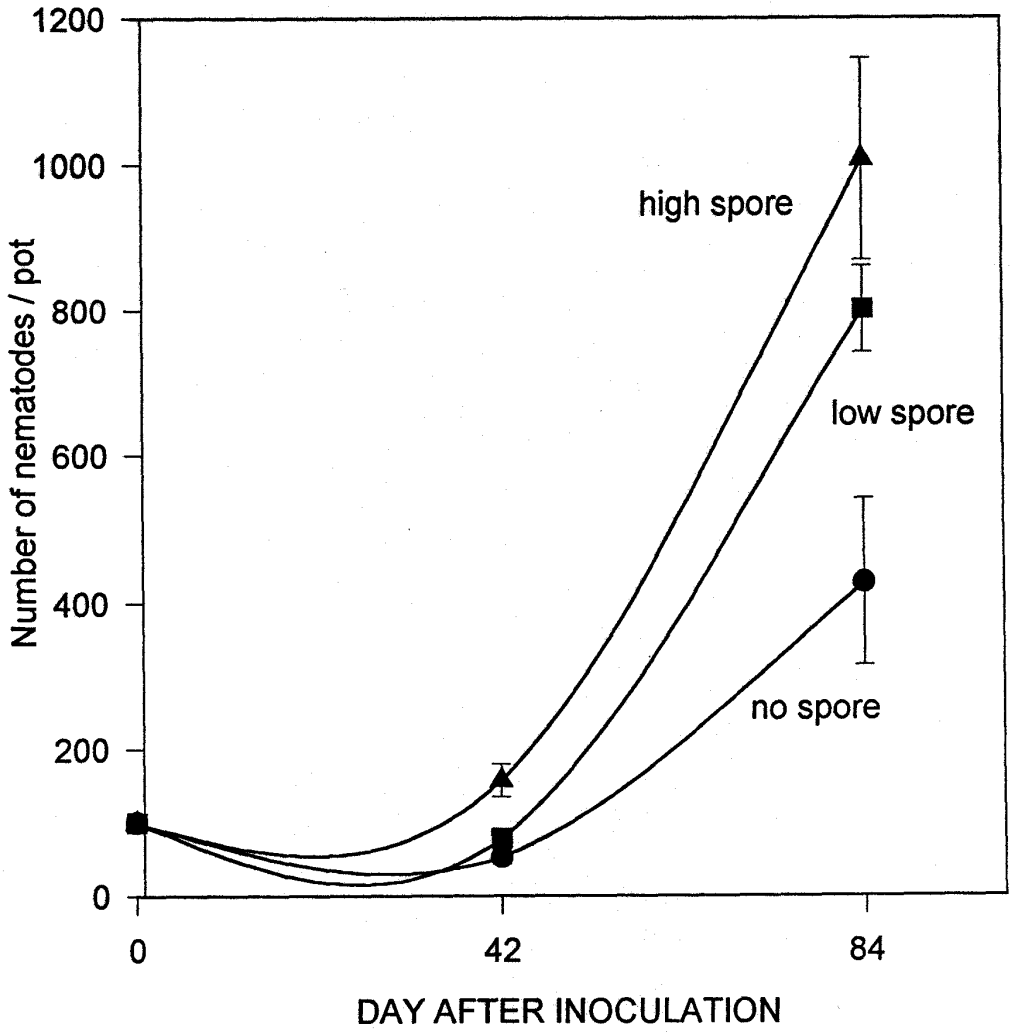

FIGURE 2

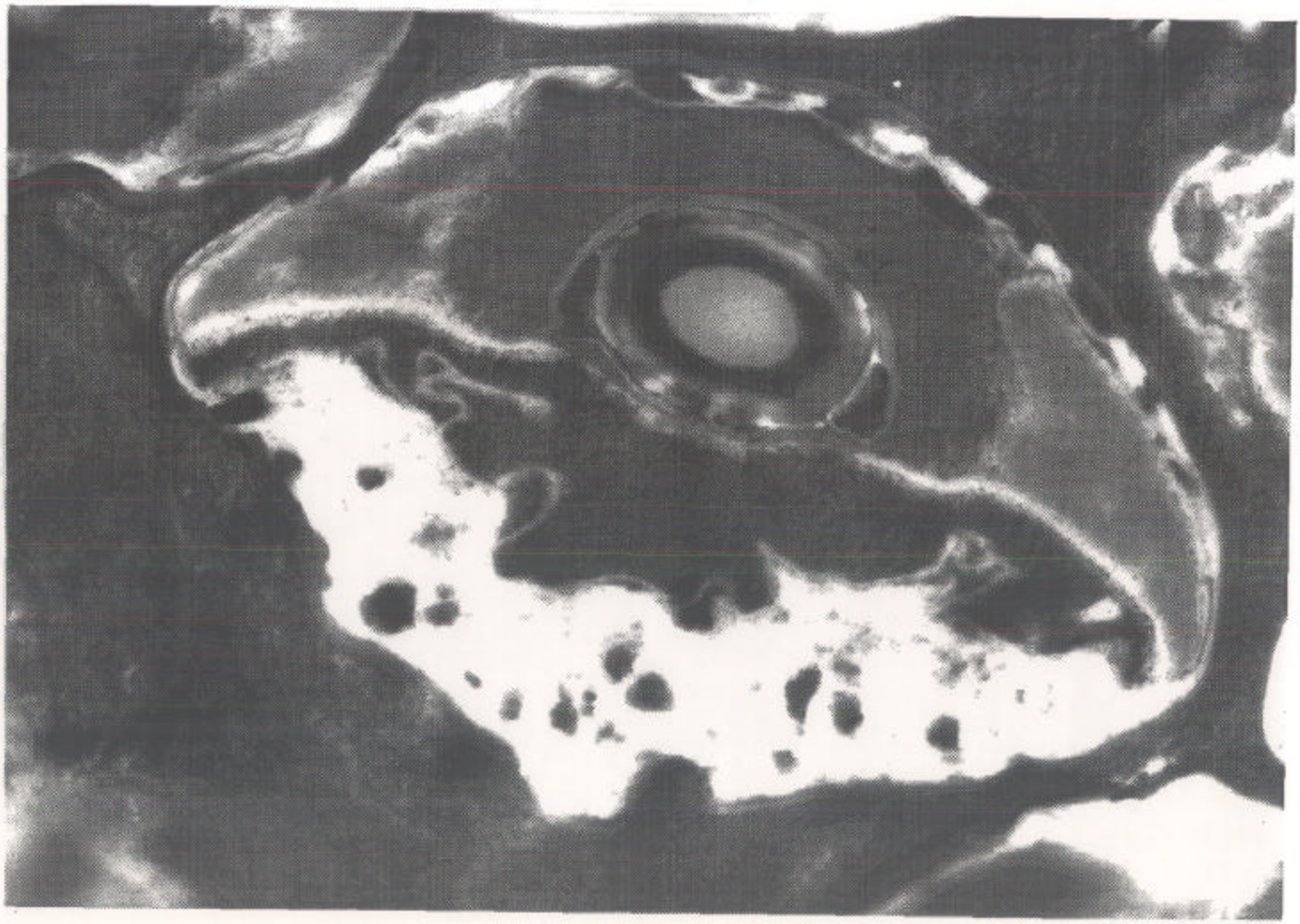

FIGURE 3