The life-cycle, population dynamics and host specificity of a parasite of *Heterodera avenae*, similar to *Pasteuria penetrans*

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SUMMARY

The occurrence of a population of *Pasteuria*, similar to *Pasteuria penetrans*, and possibly a new species, parasitic on *Heterodera avenae* in a cereal-cyst nematode suppressive soil, is reported. The life-cycle was restricted to the *H. avenae* second-stage juvenile, and prevented such infected juveniles from invading plant roots. Between March and July, 38-56 % of the juveniles present in the soil had between one and five spores adhering to their cuticles; microcolonies could be seen in the juveniles as early as April, however, mature spores were not recorded until July. Developing females, and cysts extracted from plant roots were not infected by the bacterium. The suppressive soil also contained juveniles and adults of *Pratylenchus* and *Tylenchorhynchus* infected with *Pasteuria*. Although the spores adhering to, and within, all three nematode species were approximately the same size, between 4 and 5 μm, it was not clear whether or not the *Pasteuria* population was either a single or a mixed population. Host specificity studies with spores extracted from the juveniles of *H. avenae* showed that it adhered to the cuticles of *H. schachtii*, *H. glycines*, *Globodera rostochiensis*, *G. pallida* and *Meloidogyne javanica*. Attempts to bait soil by adding these nematodes failed to result in the infection of females. It is reported the soil contained approximately 7 × 10³ spores per gram as evaluated using differential centrifugation.

RÉSUMÉ

*Cycle, dynamique de population et spécificité d'un parasite d'*Heterodera avenae *semblable à* Pasteuria penetrans

Il est signalé la présence d'une population de *Pasteuria*, semblable à *Pasteuria penetrans* et constituant peut-être une nouvelle espèce, qui parasite *Heterodera avenae* dans un sol « répressif » vis-à-vis des nématodes. Le cycle est limité aux juvéniles de deuxième stade de *H. avenae* et les juvéniles infestés ne peuvent envahir les racines de la plante. Entre mars et avril, 38 à 56 % des juvéniles présents dans le sol portent d'une à cinq spores adhérant à leur cuticule ; des microcolonies peuvent être observées dans les juvéniles dès le mois d'avril, mais les spores mûres ne se rencontrent pas avant le mois de juillet. Les femelles bien formées et les kystes extraits des racines ne sont pas infestés par la bactérie. Le sol « répressif » renferme également des juvéniles et des adultes de *Pratylenchus* et *Tylenchorhynchus* infestés par *Pasteuria*. Bien que les spores, externes ou internes, observées chez ces trois nématodes soient approximativement de la même taille (4-5 μm), il n'a pu être défini si elles appartenaient à une seule population ou à un mélange de populations différentes. Les études de spécificité d'hôte montrent que les spores extraites des juvéniles de *H. avenae* adhèrent à la cuticule de *H. schachtii*, *H. glycines*, *Globodera rostochiensis*, *G. pallida* et *Meloidogyne javanica*. Les essais visant à enrichir le sol en y ajoutant des nématodes n'ont pas conduit à l'infestation des femelles. En utilisant la centrifugation différentielle, il est noté que le sol contient environ 7 × 10³ spores au gramme.

The obligate plant-parasitic nematode hyperparasite *Pasteuria penetrans* (Thorne, 1940) Sayre & Starr, 1985, is known to parasitise root-knot nematodes and to have considerable potential as a biological control agent for these important pests (Mankau, 1975; Stirling, 1984; Brown, Kepner & Smart, 1985). Spore-encumbered juveniles become infected, after they have invaded the root, when the spores adhering to the cuticle germinate. The bacteria produces primary microcolonies which subsequently divide and proliferate throughout the pseudocoelom before maturing into the next generation of spores. By the time the life-cycle is completed the body of the female is filled with these spores and the nematode is unable to reproduce (Sayre & Wergin, 1977). Members of the *Pasteuria penetrans* group produce endospores which adhere to the cuticle of many species of nematodes and some have been observed to parasitise nematodes from both tropical and temperate regions of the world (Sayre & Starr, 1988). However, individual populations of the bacterium have a restricted host range; six populations of spores isolated from *Meloidogyne* only adhered to juveniles from that genus when tested against a wide range of species of nematodes (Davies, Kerry & Flynn, 1988). Also spores may differ in

their level of attachment to individual populations of the same species of nematode (Stirling, 1985). At present the taxonomy of the hyperparasite remains unclear but it seems likely that the group consists of several species and subspecies of which two have so far been identified; *Pasteuria penetrans sensu stricto* emend. which parasitises root-knot nematodes such as *Meloidogyne incognita* and *M. javanica* and *Pasteuria thornei* observed to parasitise *Pratylenchus brachyurus* (Starr & Sayre, 1988). *Pasteuria* has been implicated in the natural control of root-knot nematodes in vegetables in W. Africa (Mankau, 1980) and on vines in S. Australia (Stirling, & White, 1982); in both, poor nematode reproduction on these susceptible crops was associated with a large proportion of spore-encumbered juveniles in soil. *Pasteuria* has recently been found to suppress populations of *Heterodera elachista* in upland rice and *H. glycines* on soybean (Nishizawa 1986, 1988). It has also been observed to parasitise and complete its life-cycle in *H. avenae*, *H. cajani*, *H. sorghi* and *H. zeae* : the life-cycle is similar to that reported on root-knot nematodes and is completed when young white females, which have failed to tan and have developed a dull yellow-grey tinge on being filled with mature spores, break open releasing spores of the bacterium (Bhattacharya & Swarup, 1988; Sharma & Swarup, 1988). In a similar way to *P. penetrans* on root-knot nematodes, the spores do not germinate if spore encumbered larvae are kept in a suspension of water even after a month (Sharma & Swarup, 1988).

The present study reports the occurrence of a *Pasteuria* population, possibly a new species. It parasitises the second-stage juveniles of *H. avenae* in a similar way to a population observed in Germany (Sturhan, 1985), and therefore has a different life-cycle to that briefly described above. Preliminary investigations with this new population have therefore attempted to study its host range, and to determine the extent of parasitism in an *H. avenae* suppressive soil. Also the number of spores occurring in the soil were estimated and changes in the proportion of *H. avenae* parasited throughout the growing season determined. The results are discussed in relation to the suppression of cereal cyst nematode populations and its subsequent use as a biological control agent.

Materials and methods

Prior to the commencement and following the end of the study the egg population of the soil was assessed following the method of Shepherd (1986).

EXTRACTION OF SECOND-STAGE JUVENILES OF *H. AVENAE* AND THEIR INFECTION WITH *PASTEURIA*

Soil (2 kg) was collected during the first week of each month from March to September, from four plots in a field that had been under continuous barley *(Hordeum*

vulgare) for the last 7 years on Woburn Experimental Farm. The nematodes were extracted from four sub-samples (200 g) per plot using the two-flask technique (Seinhorst, 1956). The nematodes were further concentrated by centrifugation in magnesium sulphate solution (d = 1.116) and collected on a 10 μm sieve (Hooper, 1986). The nematodes were further concentrated by centrifugation in magnesium sulphate (d = 1.116), collected on a 10 μm sieve and counted (Hooper, 1986). A maximum of 25 juveniles per sample were examined further on an inverted microscope for the presence of *Pasteuria* spores adhering to the cuticle and for evidence of infection in the pseudocoelomic cavity. The number of spores adhering to individual juveniles was assessed in May when the proportion of second-stage juveniles with *Pasteuria* spores attached to the cuticle was greatest.

THE EXTRACTION OF DIFFERENT DEVELOPMENTAL STAGES OF *H. AVENAE* FROM THE ROOTS OF BARLEY AND THEIR INFECTION BY *PASTEURIA*

Between April and August the root systems of four barley plants were removed from each plot to a depth of 20 cm. Females and fourth stage juveniles that had broken through the root epidermis were collected by washing the roots of each plant on a sieve (250 μm aperture) and these nematodes were further separated by the use of a fluidising column (Trudgill, Evans & Faulkner, 1972). Fresh root weight was recorded after carefully blotting each washed root system. Developmental stages that had not broken through the root cortex were obtained by digesting the root system in 25 % Pectinex (Novo Enzyme Products, U.K.) on a rotary shaker (156 rpm) at room temperature. After 24 h the slurry was homogenised in a blender for 20 s before the slurry was washed through a sieve (8 mm aperture) and the nematodes collected, together with the root residues, on a lower sieve (10 μm aperture).

THE DIRECT ESTIMATION OF *PASTEURIA* SPORES OCCURING IN SOIL

Soil samples (1 g) were placed in 10 ml of tap water with one drop of polyoxyethylenesorbitan monolaurate (Tween 20, Sigma) and sonicated for about 2 min using an ultrasound generater fitted with a logarithmic probe. The soil suspension was passed through a series of sieves (150, 63, 63, 63, 53, 10 μm) and the filtrate concentrated on a 1.2 μm filter. The deposit was resuspended in 10 ml water and concentrated by centrifugation (1000 *g* for 10 min) and 9.5 ml of the supernatant was discarded. The remaining 0.5 ml suspension was washed together with a drop of Tween 20 and pipetted onto a one-step 90 % sucrose gradient and spun at 4° C for 30 min at 17 500 *g* in an ultra-centrifuge fitted with a swing-out rotor; the supernatant was discarded. Larger fractions of the soil were removed by resuspending the pellet as before and spinning for 30 min at 4° C and 125 *g* in the

ultra-centrifuge. The supernatant was retained and finally concentrated by spinning for 30 min at 15° C and 100 000 *g*. This pellet was then resuspended in a small volume of water which made it possible to observe and count *Pasteuria* spores using a haemocytometer. The efficiency of this technique was determined by incorporating 50 000 spores of *Pasteuria* per gram of sand/ peat compost and the above technique repeated.

HOST SPECIFICITY AND THE BAITING OF *PASTEURIA* FROM *H. AVENAE* SUPPRESSIVE SOIL WITH OTHER POSSIBLE HOSTS

H. avenae that were observed to contain *Pasteuria* spores were collected as described and stored in water at 4° C. All nematodes containing spores were homogenised and the spores suspended in 0.2 ml tap water. The number of spores was determined using a haemocytometer. An attachment assay was performed by placing the suspension on a cavity slide to which twenty freshly hatched second-stage juveniles of *H. avenae* were added. The slides were placed in a humid chamber and incubated at 18° C. After 24 h the juveniles were individually removed from the suspension and observed on a microscope for adhesion of spores. Other species of nematodes (Table 4) were tested for compatibility to the *Pasteuria* spores from *H. avenae* by repeating the procedure using the same spore suspension. At the end of the test the host, *H. avenae*, was again added to the suspension to ensure enough spores were still present and able to attach. Following the host specificity tests second-stage juveniles of species of nematodes to which spores attached, were added to pots of the cereal-cyst nematode suppressive soil in which the appropriate hosts for the nematodes were grown. The pots were placed in a glasshouse at the optimum temperature for each nematode host. At regular intervals between 2 and 5 months developing females and cysts were extracted, and examined for *Pasteuria* infection.

Results

The density of eggs at the beginning and end of the growing season was not significantly different; 9 (SE = 1.6) and 8 (SE = 1.3) eggs per gram of soil respectively. The greatest density of second-stage juveniles in the soil occurred in May, 35 per 200 g soil, which decreased to only 3 per 200 g soil in August (Tab. 1); on these two sampling occasions 16 and 1 juvenile/200 g were observed with at least one spore respectively. The number of spores adhering to second-stage juveniles at this sampling was between 1 and 5 spores per juvenile but < 10 % had > 3 spores and nearly 40 % had none (Fig. 1). In a number of these second-stage juveniles extracted from the soil, the *Pasteuria* spores adhering to their cuticles had germinated and microcolonies could
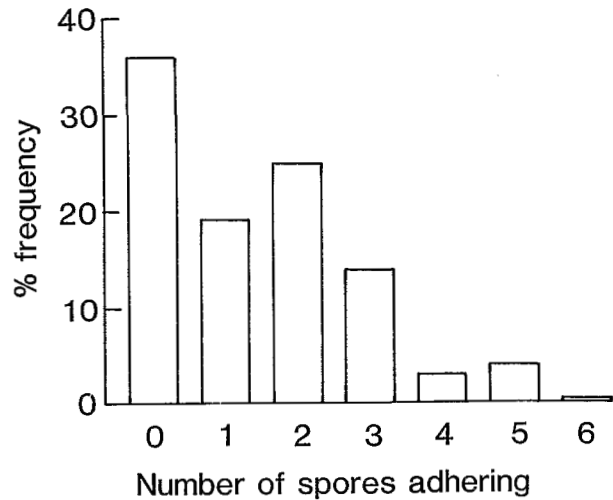

Fig. 1. Percentage frequency of the number of *Pasteuria* spores adhering to the cuticle of second-stage juveniles of *H. avenae* extracted from the soil.
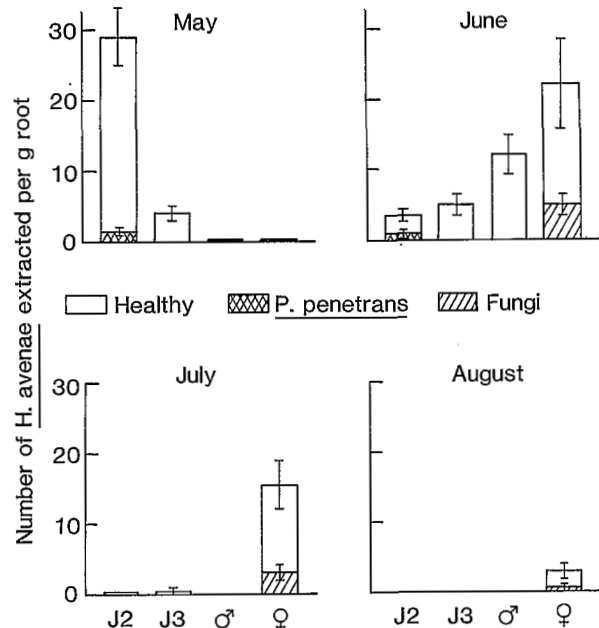

Fig. 2. The mean number of second-stage juveniles, third-stage juveniles, males and females extracted, per gram/root, between May and August and the extent of parasitism by fungi and *Pasteuria* (mean fresh weight of barley root in 200 g soil = 3.44 g).

be observed. These could be seen as early as April, however, the bacterium did not complete its life cycle until July when second-stage juveniles were filled with

spores (Table 1). Approximately, 800 *Pasteuria* spores were produced in an infected second-stage juvenile. Extractions of *Pasteuria* from the peat/sand compost, containing a known density of spores (50×10^3 per gram) by differential centrifugation was difficult and had an average efficiency of only 59 % (Table 3). Using this as a standard level of extraction efficiency the density of spores in the *H. avenae* suppressive soil was estimated at 7×10^3 (Table 3).

Most nematodes were found on the root system in May, with 29 juveniles per gram of root (Fig. 2). Only 3.9 % of these juveniles, extracted from the roots, had *Pasteuria* spores adhering to their cuticle, whereas 45 % of the juveniles, extracted from the soil were encumbered with spores (Table 2). In *H. avenae* extracted from roots there was no evidence of *Pasteuria* in any of the developing juveniles after June (Fig. 2) and the matura-

Table 1

The mean density of : second-stage juveniles (J2) and J2 with *Pasteuria* (PP) spores adhering to the cuticle, and those with spores in the pseudocoelom, per 200 g soil, between March and August (± S.E. of the means in parentheses)

Month	Density of J2	Density J2 with PP on cuticle	Density J2 with PP in pseudocoelom
March	17 (1.3)	7 (0.5)	0.0
April	17 (1.9)	8 (1.0)	M
May	35 (8.4)	16 (3.3)	M
June	28 (3.2)	11 (0.9)	M
July	22 (3.1)	12 (1.5)	0.6 (0.3)
August	3 (0.5)	1 (0.2)	0.4 (0.2)

M = microcolonies observed in one or more juvenile.

Table 2

Total number of second-stage juveniles observed and the percentage with *Pasteuria* spores adhering to their cuticle which were extracted from the soil and from the roots of barley

Month	Soil		Root	
	Total juveniles	% juveniles with spores	Total juveniles	% juveniles with spores
March	267	38.9	—	—
April	275	44.8	—	—
May	561	45.0	508	3.9
June	454	37.3	162	9.2
July	344	56.3	10	0.0
August	48	26.7	0	0.0
Mean	325	41.5	170	6.5

Table 3

Efficiency of *Pasteuria* spore extraction by differential centrifugation on a one-step sucrose gradient from a peat/sand compost and the density of *Pasteuria* spores present in a *H. avenae* suppressive soil

Treatment	Mean spores observed (S.E.)	Efficiency %	Spore density per gram soil
Peat/sand compost	29×10^3 (5×10^3)	59	50×10^3
Suppressive soil	4×10^3 (1×10^3)		$7 \times 10^{3*}$

* Calculated using the extraction for the peat/sand compost.

Table 4

Attachment of *Pasteuria* spores, in a spore suspension extracted from juveniles of *H. avenae* and, by baiting Woburn soil, to second-stage juveniles (J2) of a number of plant-parasitic nematode species, and their subsequent infection of developing females and cysts

Nematode spp.	% J2 with spores		Infection of females and cysts
	Spore suspension	In soil	
Heterodera avenae	30	—	—
H. glycines	30	7	0
H. schachtii	60	0	—
Globodera rostochiensis	100	8	0
G. pallida	100	NA	NA
Meloidogyne arenaria	0	—	—
M. incognita	0	—	—
M. javanica	70	6	0
Pratylenchus crenatus	0	—	—
P. neglectus	0	—	—

NA = not available.
— = untested.

tion of spores was only observed from second-stage juveniles extracted from soil. The life-cycle of this population of *P. penetrans* was completed in the second-stage juvenile. Well over 1000 young females of *H. avenae*, extracted from the roots of barley and individually dissected between May and August, did not contain any evidence of *Pasteuria* infection (Fig. 2). A small number of second-stage juveniles extracted from the roots did have the occasional spore adhering to the cuticle but this was not commonly observed. There was no evidence of *Pasteuria* in either third- or fourth-stage

juveniles or in adult males and females (Fig. 2). The life-cycle therefore appears to be : (1) spores come into contact and adhere to second-stage juveniles as they migrate towards roots after hatching (Fig. 3 A). (2) The spores germinated soon afterwards and lead to the production of primary microcolonies which subsequently divide and proliferate throughout the pseudocoelom of the juvenile. On infection the juvenile becomes less mobile and unable, in the majority of cases, to invade the root system. (3) The microcolonies fragment during sporogenesis producing quartets and doublets before immature spores are produced (Fig. 3 B, C). No mature *Pasteuria* spores were observed in second-stage juveniles before July (Table 2). (4) Ultimately mature spores develop (Fig. 3 D) and are liberated into the soil when the juvenile breaks up. (5) Once in the soil they remain dormant until they are able to adhere to a passing second-stage juvenile. Those juveniles which invade the root system and to which *Pasteuria* spores adhere, either, fail to develop, or develop and do not become infected.

During the extraction of *H. avenae* second-stage juveniles from the soil a limited number of *Pratylenchus* spp. and *Tylenchorhynchus* spp. (Fig. 3 E) were extracted and found to be infected with *Pasteuria*, both juvenile and adult stages were parasitised. Mature spores from all of these three types of plant parasitic nematode were between 4 and 5 µm in diameter. In the host specificity studies spores extracted from *H. avenae* attached to all cyst nematodes tested, however, their levels of attachment did differ; of the root-knot nematodes tested the spores attached only to *M. javanica* (Tab. 4). Spores did not attach to either of the *Pratylenchus* spp. tested. In the baiting of the suppressive soil, with second-stage juveniles of species to which spores adhered in the attachment tests, there was no evidence of any parasitism by *Pasteuria* in either developing females or cysts. Whether or not any of the second-stage juveniles in the soil were parasitised and prohibited from invading the roots is not known. The population of the bacterium has therefore proved difficult to increase in *in vivo* culture.

Discussion

The life-cycle of this population of *Pasteuria* which parasitises *H. avenae* is very different from those reported previously on either *Heterodera* spp. (Bhattacharya & Sharma, 1988; Nishizawa, 1988; Sharma & Swarup, 1988), or *Meloidogyne* spp. (Starr & Sayre, 1988). It does have some similarities with *Pasteuria thornei*, a parasite of *Pratylenchus brachyurus* (Starr & Sayre, 1988); it has rhomboidal sporangia (Fig. 3 B, C) and completes its life-cycle in the juvenile (Fig. 3 D), however, it differs from *P. thornei* in that mature spores were larger, 4-5 µm as opposed to 2-3 µm. This population of *Pasteuria* appears to behave similarly to one observed in India, where, although spores adhered to the

second-stage juvenile cuticle, cysts of *H. avenae* and *H. sorghi* did not show any infection (Sharma & Swarup, 1988); however no infection of second-stage juveniles of these nematodes was reported. Although *Pasteuria* has been observed to parasitise second-stage juveniles of *H. avenae* before (Sturhan, 1985), no observations were reported on the infection of developing females and cysts.

Unfortunately the number of parasitised *Pratylenchus* and *Tylenchorhynchus* individuals was very small and therefore it was not possible to obtain enough spores from these nematodes to undertake attachment tests. Populations of *P. penetrans* vary greatly in their attachment to different populations of *Meloidogyne* with some levels of attachment being very low (Stirling, 1985). The observations here, obtained in a relatively dilute suspension of spores, demonstrated that spores of *Pasteuria* from *H. avenae* second-stage juveniles did not attach to the *Pratylenchus* species tested (Table 4). However, this may not, therefore, exclude them from attaching to other species of *Pratylenchus*. It is not clear whether or not the *Pasteuria* in the soil at Woburn is either a single population parasitising *H. avenae*, *Pratylenchus* and *Tylenchorhynchus* or a mixed population, each with its own host range. It is possible that this is a new species or strain but, until methods of culture are available to obtain sufficient spores for further studies, this is unlikely to be elucidated.

The occurrence of *P. penetrans'* spore populations in soil have been determined by bioassays in which soils containing the bacterium have been baited with known hosts (Oostendorp, Dickson & Mitchell, 1988). Indirect methods of estimating spore levels in soil require a compatible host/parasite relationship, and it is difficult to assess the efficiency of such a method with unknown and possibly mixed populations of bacteria. Differential centrifugation overcomes these problems but is very laborious to use and would not make a good routine technique. The number of spores occurring in the soil was estimated at 7×10^3. This density resulted in up to 64 % of *H. avenae* second-stage juveniles having between one and five spores adhering to their cuticles.

Although sufficient females were produced to ensure a substantial multiplication of *H. avenae*, there was no significant difference in the pre and post cropping egg populations in soil. Such a failure of *H. avenae* to multiply as susceptible crops has been well documented at this site (Kerry, Crump & Mullen, 1982*a, b*) and appeared to be related to loss of females on the root system during July and August. Such losses have been attributed to parasitism by nematophagous fungi. The levels of infestation of second-stage juveniles by *Pasteuria* were not sufficient to prevent the development of all females. The results reported here suggest that considerably more than 7×10^3 spores per gram of soil would be required to give effective control. Stirling (1988) has estimated that at least 10^5 spores per gram soil
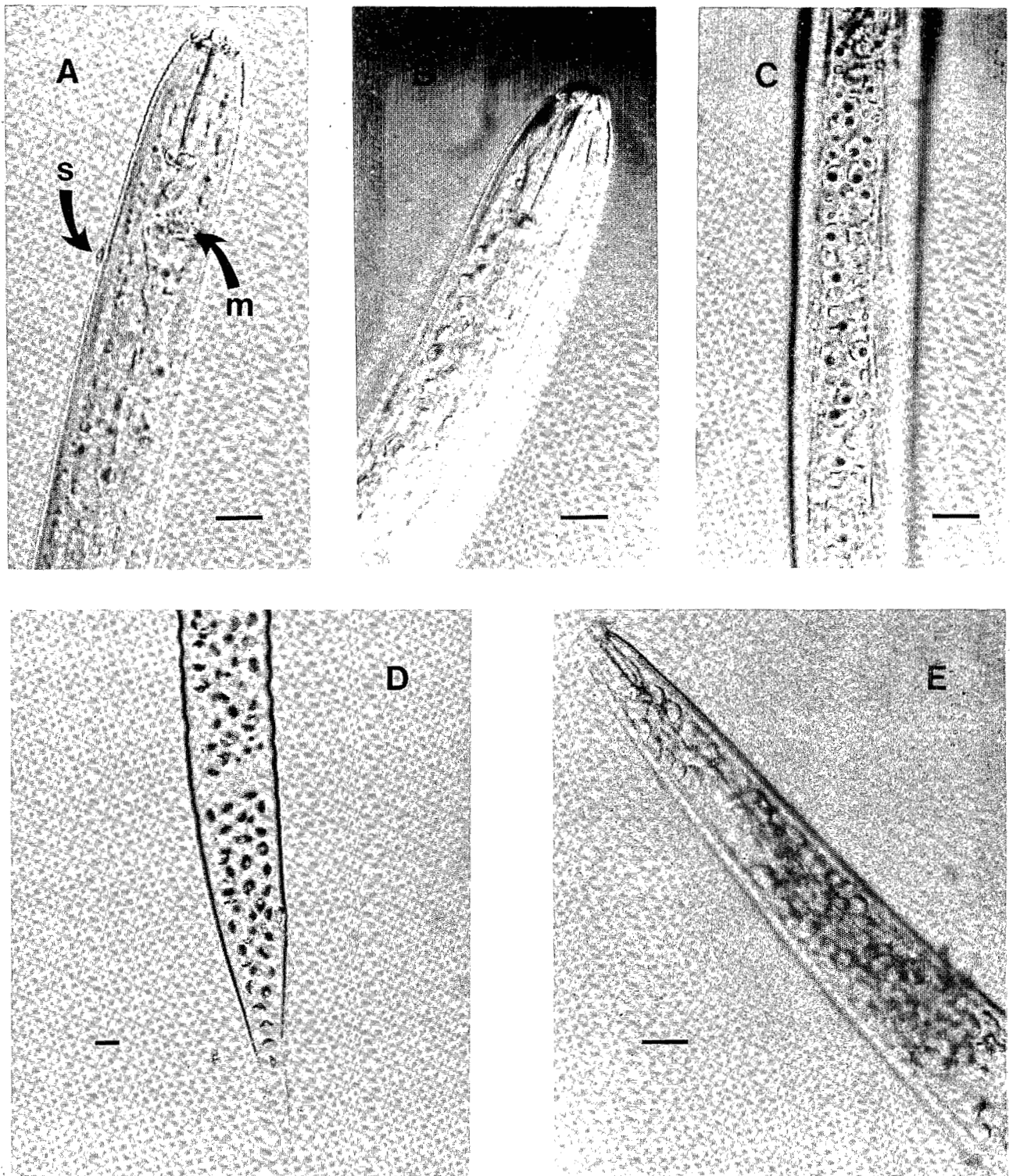
Fig. 3. A : Head of *H. avenae* second-stage juvenile with a spore (s) attached to the cuticle and a primary microcolony (m) infecting the oesophageal region; B : Immature *Pasteuria* spores contained within rhomboidal sporangia in the oesophageal region; C : in pseudocoelom; D : Mature spores in the tail; E : Oesophageal region of *Tylenchorhynchus* sp. containing *Pasteuria* spores *(Bars = 10 μm).*

would be required to reduce invasion of root-knot juveniles and significant levels of population control. So far the bacterium reported here has not been grown *in vitro* and as relatively few spores are produced in second-stage juveniles attempts at *in vivo* production have proved much more difficult than those with other populations of the bacterium which infect developing females. However, if this organism could be cultured and the spores added to soil, this bacterium has potential as a biological control agent because it appears to prevent invasion of infected second-stage juveniles and this would have a significant effect in reducing plant damage.

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