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# POTENTIAL USE OF *PASTEURIA* SPP. IN THE MANAGEMENT OF PLANT PARASITIC NEMATODES

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**Abstract.** Potentials of *Pasteuria penetrans* and close bacterial nematode parasites are reviewed. Several aspects concerning the identification and recognition of *P. penetrans* are discussed, with description of the bacterium's life cycle, biology, host range and specificity. The application of traditional and molecular taxonomic methods for the identification of isolates and species as well as the available technologies for *in vivo* and *in vitro* mass culture are also reviewed.

## 1. INTRODUCTION

A general, satisfactory control strategy has not yet been developed for plant parasitic nematodes. Host plant resistance, chemicals, rotations and cultural practices can all have a role in nematodes management but for differing reasons they are not widely adopted. Biological control is an attractive practice in theory but, when compared to other available control means, it has not been extensively or sufficiently researched. The success of a biological control agent will depend on its ability to reduce the multiplication of the pest, but this has been often difficult to assess in the field.

Among nematode antagonists, the Gram+ bacteria of the *Pasteuria* group attracted, in the last decades, considerable interest due to several peculiarities of their parasitic behaviour. Research on *Pasteuria* was mainly focused on *Pasteuria penetrans* and its potential as a biological control agent of root-knot nematodes (*Meloidogyne* spp.) (Stirling, 1991).

Although *P. penetrans* is a naturally occurring parasite of root-knot nematodes, it rarely exerts a suppressive effect on populations, when detected by growers or agronomists. However, we consider that the opportunity for exploiting *P. penetrans* is worth the effort.

In this chapter we highlight the positive attributes of this bacterium showing how, through a thorough understanding of its biology, it might be manipulated (within other control strategies) to decrease nematode population densities to an extent resulting in measurable benefits to crop growth.



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## 2. RECOGNISING *PASTEURIA*

Nematode parasites of the *Pasteuria* group are often overlooked because their presence on or within nematodes can only be seen under a microscope at more than 100× magnification. This may be an impediment to their recognition in samples taken to a laboratory. When soil samples are processed for nematode extraction, juvenile or vermiform stages of the nematode species present may be recovered. These may have *Pasteuria* endospores attached to them if the bacterium is present in that soil, but spore attachment will only be seen if nematodes are observed under high power magnification. If a number of root-knot nematode juveniles are endospore encumbered, they may appear to aggregate into clumps: this is often a useful characteristic, that can be noticed at lower magnifications.

Infected female root-knot nematodes can be found in root systems but where the incidence of *P. penetrans* is low, then the chance of detection is small (see section below). Infected females do not produce egg masses, they appear dense and cream coloured in contrast to healthy females which become partially translucent as they mature and produce egg masses.

In summary, we do not disguise the fact that the recognition of *P. penetrans* from the field requires some nematological expertise. This reinforces our consideration that the practical development of this bacterium as a biological control agent will be achieved only with progresses in our understanding of its biology, biochemistry and life cycle.

### *2.1. Life Cycle and Development*

The first observation of a *Pasteuria* from plant parasitic nematodes (*Pratylenchus pratensis*) was provided by Thorne (1940), which considered the organism a microsporidian and named it *Dubosqia penetrans*. The life cycle of *P. penetrans* was first described and illustrated by Mankau (1975), Mankau and Imbriani (1975), Imbriani and Mankau (1977) and Sayre and Wergin (1977).

The initial stage of the life cycle of *P. penetrans* on root-knot nematodes is the chance contact of endospores to the second (infective) stage juvenile, which occurs in the soil as the juvenile seeks a suitable host root. Endospore attachment does not necessarily cause infection, implying that not all endospores may be viable. The extent of endospore viability is difficult to determine, as infection can proceed from the attachment by one single endospore to a juvenile (Trotter, Darban, Gowen, Bishop, & Pembroke, 2005). Greater than 15 endospores may disable the nematode in its movements, and invasion may not take place (Davies, Kerry, & Flynn, 1988). The optimal attachment level should be around 5–10 endospores per juvenile, as enough endospores will initiate infection without reducing the ability of the nematode to invade roots (Davies et al., 1988; Davies, Laird, & Kerry, 1991; Rao, Gowen, Pembroke, & Reddy, 1997). Even so, when plants are infected only with endospore encumbered juveniles, 100% *Pasteuria* infection is not certain (Pembroke, 2007 unpublished).

Once a spore-encumbered juvenile has invaded a root, it will establish a feeding site and apparently normal development will continue. Stirling (1991), quoting the

life cycle as described by Imbriani and Mankau (1977), states that germination of the spore(s) and production of the germ tube does not occur until approximately 8 days after nematode invasion. Sayre and Wergin (1977) suggested that germination of the endospore is initiated by the onset of the nematode feeding activity, however endospores on second stage juveniles have been observed to germinate in the absence of a host plant (Davies, personal communication).

The germ tube (infection peg) emerges through the central opening of the basal ring of the endospore and penetrates the nematode cuticle entering the hypodermal tissue. No deformation of the nematode cuticle occurs, suggesting that there is no appreciable force exerted on the nematode during this process. Rhizomorphs are first observed close to the site of penetration. However, mycelial colonies (microcolonies) up-to 20 μm are ultimately found in the pseudocoelom (Sayre & Wergin, 1977).

The exponential growth phase of the bacterium in the infected host is not altogether clear, but recently rod-shaped bacillus-like cells have been observed (Davies et al., 2004). These are likely to be the vegetative growth stages of the bacterium, but their identity awaits confirmation.

Sporogenesis is triggered in a manner similar to other *Bacillus* spp. and involves a phosphorylation pathway (Kojetin et al., 2005). It results initially in the production of microcolonies that mature to contain fewer but larger cells. These fragments lead to quartets and then doublets, each eventually developing a single sporangium which gives rise to a single, true endospore (Stirling, 1991). The external development of the nematode remains unaltered, undergoing normal moults and only microscopic examination (higher than 200× magnification) would reveal the intensification of the hyperparasite within the nematode. The resultant swollen adult female is almost devoid of eggs and may contain greater than 2 million spores (Stirling, 1981; Stirling, 1991; Darban, Pembroke, & Gowen, 2004).

The developmental cycles of *Pasteuria* spp. can be different with respect to the duration and the nematode stages that are capable of being infected. Although detailed studies in this area have not been undertaken, preliminary observations show that the length of life cycles differs and that the development of *Pasteuria* within different life stages of the nematodes also differs. For example, the life cycle of *P. thornei* was shorter than that for *P. penetrans* and all forms of the life cycle were observed in one or other of the migratory stages of the nematode (Starr & Sayre, 1988). Similarly, all stages of the life cycle have been observed in the pseudocoelom of second stage juveniles of *Heterodera avenae* (Davies, Flynn, Laird, & Kerry, 1990) whereas this has not been seen in *Meloidogyne* spp.

### *2.2. Morphology*

There are two major developmental structures that have been used to characterise the species: the sporangial shape and the structures and dimensions of the endospore, as viewed by brightfield scanning and electron microscopy (Sayre, Wergin, Schmidt, & Starr, 1991). Although the dimensions of mature endospores are robust features on which to characterise different isolates of the bacterium, developmental structures are more problematic to use, as these will often be age

related, continually undergoing changes and therefore difficult to assess. Also, the early stages of development are often fleeting and difficult to observe in some specimens and therefore do not represent reliable structures useful for taxonomic purposes.

The mature endospore itself is probably the best morphological structure to use to characterise an isolate. Measurements of the width and height of the endospores and of the sporangium can be made with a high degree of accuracy in order to compare different populations. In undertaking such studies it should be born in mind that specimen fixation, staining and orientation can all play a part in generating the observable variation encountered and the only valid comparisons are those made between populations that have been processed following the same method.

### *2.3. Traditional and Molecular Taxonomy*

Six species of *Pasteuria* have been identified to date. Five of these parasitize plant parasitic nematodes: *P. penetrans* (parasitic on *Meloidogyne* spp.); *P. thornei* (parasitic on *Pratylenchus penetrans)*; *P. nishizawae* (parasitic on *Heterodera* and *Globodera* spp.); Candidatus P. usage (parasitic on *Belonolaimus longicaudatus*), and *P. hartismeri* (parasitic on *M. ardenensis)*. *Pasteuria ramosa* parasitic on the Cladoceran *Daphnia magna*, a water flea, was first described by Metchnikoff in 1888 (Metchnikoff, 1888). Identification and characterisation of *Pasteuria* spp. have been based on a number of features that include morphology, life cycle and development, host range and more recently DNA sequences (Sayre & Starr, 1985; Sayre, Starr, Golden, Wergin, & Endo, 1988; Sayre et al.,1991; Giblin-Davis et al., 2003; Bishop, Gowen, Pembroke, & Trotter, 2007).

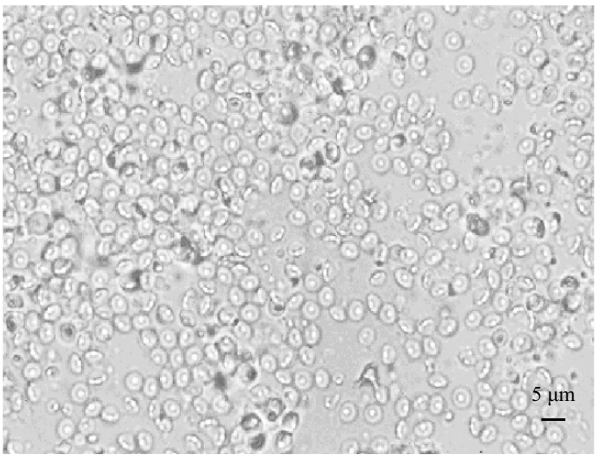


*Figure 1. Endospores of* Pasteuria penetrans *at life-cycle completion, 35 days after nematode infection (circa 650 degree days).*

*Pasteuria* endospores have an ellipsoidal characteristic shape, and with experience they should always be recognisable at 150–200× magnifications. The endospores are 3–5 µm wide, non-motile and have two distinct components: a central refractive core, surrounded by a peripheral matrix. These are readily recognised when an infected nematode is squashed and the endospores are released (Fig. 1).

Recognition of the early development stages after the endospore has germinated in a nematode is difficult. The identification of this stage requires careful examination of squashed nematodes at high magnification (Fig. 2). As the colonies develop into sporangia, the enlarging mycelia assume characteristic forms initially displaying branches of four (quartets) and then two (doublets) cells. The new endospore eventually develops from the terminal part of the sporangium.

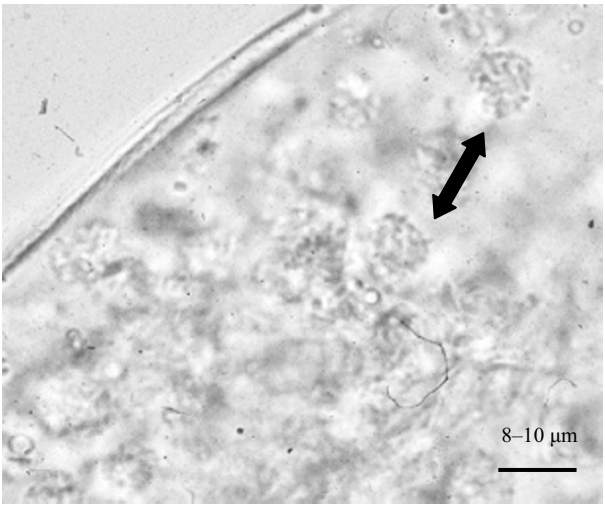


*Figure 2. Microcolonies of* Pasteuria penetrans *inside a developed second stage juvenile of root-knot nematode.*

### *2.4. DNA Approaches*

As DNA replicates it undergoes mutations with a given likelihood. Therefore closely related organisms sharing a recent common ancestor will have less sequence divergence than organisms that have a very distant common ancestor. As a rule of thumb it is accepted that a DNA reassociation value higher than 70% is the threshold for delineating a bacterial species (Wayne et al., 1987). However, recent research data suggest that a DNA similarity lower than 97% can be regarded as the threshold for delineating a new bacterial species (Amann, Ludwing, & Schleifer, 1995; Hagström et al., 2002).

DNA based techniques are now being routinely applied to populations of *Pasteuria*: endospores are collected from infected nematodes and then bead beating

is used to release the DNA. PCR is then employed with primers that recognise the 16S rDNA ribosomal subunit (Ebert, Rainey, Embley, & Scholz 1996; Anderson et al., 1999; Atibalentja, Noel, & Domier, 2000; Bekal, Borneman, Springer, Giblin-Davis, & Becker, 2001; Sturhan, Shutova, Akimov, & Subbotin, 2005; Bishop et al., 2007). There are at present 58 sequences from *Pasteuria* 16S rDNA genes submitted to GenBank (*http://www.ncbi.nlm.nih.gov/Genbank*), which can be used to characterise the different populations.

However, it has been recently suggested that even greater stringency should be applied to species definitions and that an average nucleotide identity higher than 99% should be applied, because several bacteria show minimum differences of their (well characterised) genes (Konstantinidis & Tiedje, 2005). There are few available studies to date which used genes other than the 16S rDNA to characterise *Pasteuria* spp. (Trotter & Bishop, 2003; Schmidt, Preston, Nong, Dickson, & Aldrich, 2004; Charles et al., 2005). Where these studies have been undertaken the results tended to be consistent, showing that *Pasteuria* lies deep within the *Bacillus-Clostridium* clade, with species designation being related to the host from which the bacterium had been isolated.

### *2.5. Host Range*

Spores of *Pasteuria* spp. represent the resting stage and can remain viable for several years (Giannakou, Pembroke, Gowen, & Davies, 1997). These propagules are non-motile. They are responsible of transmission, since the host infection process starts when they passively adhere to the nematode cuticle on contact. The encounter with the nematode occurs as the host migrates through the soil in search of a plant root. Therefore, the endospore adhesion is a crucial step in the infection process.

Numerous studies have been carried out testing the ability of different populations to adhere to different nematode populations (Stirling, 1985; Davies et al., 1988; Channer & Gowen, 1992; Sharma & Davies, 1996a, 1996b; Español, Verdejo-Lucas, Davies, & Kerry, 1997; Mendoza de Gives, Davies, Morgan, & Behnke, 1999; Davies et al., 2001; Wishart, Blok, Phillips, & Davies, 2004). Data showed a range of variation, varying from endospore populations whose attachment is highly restricted to one population of nematodes but not to any others (either within a species or between species), to those having a much broader host range and adhere not only to the population from which they were originally isolated, but even to nematodes of a different genus. This aspect is particularly important for the use of *Pasteuria* spp. as biological control agents, because spores must be targeted to the nematode species that is occurring as a pest.

The endospores ability to adhere to different host life stages is also variable, with recent research showing that they can sometimes adhere to the cuticle of males, but sometimes they cannot (Carneiro, Randig, Freitas, & Dickson, 1999; Davies & Williamson, 2006). Endospores adhesion to the nematode cuticle cannot necessarily be interpreted as conducive to an infection, since not all adhering endospores will germinate and lead to the development of parasitism.

## 3. MASS PRODUCTION

### *3.1. In vitro Culture*

The need for an environmentally benign method to control plant parasitic nematodes as an alternative for chemical pesticides, combined with the fact that *Pasteuria* is associated with nematode suppressive soils and has been shown to unequivocally act as a biological control agent (Stirling, 1984), has focused research on *in vitro* production methods. However, the obligate nature of the bacterium life cycle has proved difficult to overcome and initial attempts at *in vitro* mass production have proved illusive (Reise, Hackett, & Huettel, 1991; Bishop & Ellar, 1991). As early as 1992 a patent was submitted showing that endospores could be cultured *in vitro* in a media that contained explanted tissues from nematodes (Previc & Cox, 1992), however this achievement has not been developed further.

Media have also been developed whereby one was able to sustain very small amounts of vegetative growth, while another led to sporulation and the development of endospores (Bishop & Ellar, 1991), however exponential growth was not obtained. More recently research has focused on the possibility of other bacteria being necessary for the development of *Pasteuria* and experiments have been undertaken in which *P. penetrans* was co-cultured with another bacterium, *Enterobacter cloacae* (Dupponis, Ba, & Mateille, 1999).

Research by Pasteuria BioScience LLC in Florida, USA has focused on the possibility that *Pasteuria* was an acidophile (Gerber & White, 2001; Hewlett, Gerber, & Smith, 2004). Pasteuria BioScience LLC is clearly making progress and *in vitro* cultured endospores produced in a fermentation vessel have recently been tested in the field (Hewlett, Griswold, & Smith, 2006).

### *3.2. In vivo Culture*

This technique is based on a system first described by Stirling and Wachtel (1980) in which a plant host is inoculated with spore-encumbered root-knot nematode juveniles. The root systems, containing infected (spore-filled) female nematodes, are harvested after an appropriate period of time and then dried for long term storage as a fine powder (Pembroke, Darban, Gowen, & Karanja, 2005).

To achieve the best possible mass production of *Pasteuria* spores, it is important to consider all the organisms involved in the system: host plant, nematodes and *Pasteuria* propagules, giving particular attention to the conditions under which they are grown. The overall objective should be to harvest root systems containing as many *Pasteuria*-infected females as possible. Temperature and the time of harvest are the most critical factors. The optimum temperature for *P. penetrans* development is around 28–30°C, and spores can be found in females after 35 days. Greater endospore numbers are obtained if the plants are left to grow for longer periods (Darban et al., 2004).

The host origins and species composition appear very important. If the host nematode is a field population proceeding from a tropical location, it could include a mixture of species e.g. *M. incognita, M. javanica* or *M. arenaria*, which may differ

in their susceptibility to *P. penetrans*. Considering that the nematode life cycle from egg to egg is about 3 weeks at 28–30°C, the production system may appear unsuitable. The uninfected females, and the consequent egg production and secondary infections (due to uninfected nematodes) can increase the stress on the host plant and thus may reduce its potential to sustain the *Pasteuria*-infected females, thus affecting the numbers of endospores produced.

*Pasteuria* is a hyperparasite, dependant on the well being of its nematode host which in turn requires a thriving host plant. Tomato is often the preferred plant host because it is easy to grow and is highly susceptible to root-knot nematodes. However, tomato grows best in a diurnally fluctuating temperature regime. A constant temperature of 28–30°C may be suitable for nematode reproduction and *Pasteuria* development, but cannot result as an optimum for the plant host. The number of endospores in an infected female nematode will hence increase as long as the nematode is receiving sufficient nutrients from the plant.

In the fluctuating conditions of a glasshouse (20–32°C), infected female nematodes were observed to increase in weight for up to 88 days, contained 2.3 million endospores and numbers of endospores in each female had not peaked (Darban et al., 2004). Although Stirling (1981) showed that endospores can be found after 700-degree days at 30°C, his data also show that the highest number of endospores per female was achieved at 20°C.

Attention should be given to the growth habit of the variety used in an *in vivo* production system, if tomato is the chosen host plant. At temperatures around 30°C tomato plants senesce early, perhaps hastened by nematode induced stress. An indeterminate variety may live for longer and may produce more roots, thus providing more nutrients to the nematodes than a determinate variety. This may be an advantage for endospore production, if host vigour and longevity are important. No data from such studies have yet been reported.

Also the size of the host plant at inoculation is another important parameter when *in vivo* system is chosen for endospores mass production. In general, the larger the plant, the greater can be the initial inoculum of endospore encumbered nematodes. However, a compromise is required because of the expected longevity of the host plant, as described above. If the host plant is too old at inoculation then the maximum possible endospore production may not be achieved.

Finally, the longevity of a female infected by *P. penetrans* is unknown.

### *3.3. Distribution in Natural Systems*

Records of *Pasteuria* endospore attachment on nematodes have been made from many countries and all continents, apart from the Antarctic. Most records are for *P. penetrans* on the major tropical root-knot species in warmer climates. There are relatively fewer records on root-knot nematodes from temperate regions (Bishop et al., 2007) although these areas show a greater number of *Pasteuria* endospore attachment records, on a diversity of nematodes (Sturhan, 1988; Subbotin, Sturhan, & Ryss, 1994). It cannot be excluded that this possibly occurs, because more nematologists (particularly taxonomists) observe specimens under high power magnification in these regions.

*3.3.1. Finding Pasteuria*

There is no universally established method for the direct recovery of *Pasteuria* endospores from soil. Spore attachment on nematodes has been the most frequent means of detection. However, recent studies looking at the interaction between plant parasitic nematodes in a natural sand dune system has successfully employed immunological techniques to quantify the number of endospores in this ecosystem (Costa, Kerry, Bardgett, & Davies, 2006).

In general, suggested methods required to find *Pasteuria* spp. rely on:

1. Collection of root-knot nematode infested plants, drying the roots and then re-hydrating root segments, carefully looking for females in the roots. *Pasteuria*-infected females are characterised by a porcelain colour with no transparent regions in the body (Fig. 3).
2. Grinding roots and making a suspension which is then probed with juveniles, leaving them in the suspension for 24 hrs and looking for endospore attachment. If endospores are present, encumbered juveniles may clump together.
3. Direct observations of endospores in suspensions may be made from ground roots at high power (higher than 200×). However, recognition of endospores in such suspensions is difficult, particularly if their concentations are low. The problem with the grinding roots method is that at low densities, the chances of missing infected females are high (Pembroke et al., 2005).
4. Collecting soil samples from around infested plants, extracting nematodes and looking for juveniles encumbered with spores. The examination of samples taken from crops where root-knot nematode damage is less than expected may be more effective in perennial crops. Stirling and White (1982), found that numbers of root-knot nematodes were lower in 25 year old vineyards where *P. penetrans* was widely distributed. Dabiré, Chotte, Fardoux, and Mateille, (2001) developed techniques for direct microscopic observation of spores, following their dispersion in soil aggregates.
5. Immunological techniques can be used for recognition and quantification of *Pasteuria* endospores in soil (Fould, Dieng, Davies, Normand, & Mateille, 2001; Costa et al., 2006).

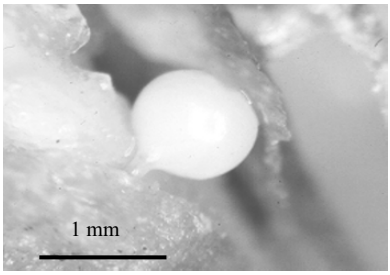


*Figure 3. Mature root-knot nematode female infected with* Pasteuria penetrans *dissected from re-hydrated roots.*

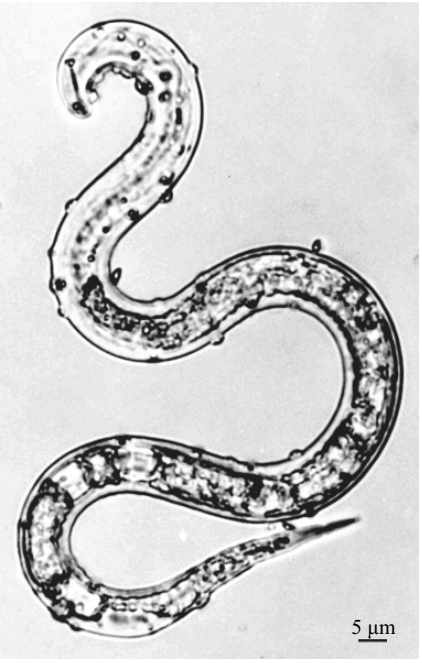


*Figure 4. Root-knot nematode second stage juvenile encumbered with spores of* Pasteuria penetrans.

## 4. *PASTEURIA* ASSOCIATION WITH NEMATODE SUPPRESSIVE SOILS

The goal of any biological management strategy is to develop suppressiveness of the pest population to a level which is less damaging to the host crop. Among nematode antagonists, *P. penetrans* has the attributes that would make it appropriate for such a strategy. However, there are few reports of instances where it established to the extent that root-knot nematode populations were suppressed. In intensively grown vegetable crops on light textured soils close to Dakar (Senegal), Mankau (1980) found that production was high and root-knot nematodes appeared not to be damaging. Upon examination, 80–98% of root-knot nematode juveniles were found to be encumbered with endospores of *P. penetrans*. Stirling and White (1982) found that numbers of root-knot nematodes were lower in vineyards more than 25 years old, where *P. penetrans* was widely distributed. Success was demonstrated in contriving an increase in *P. penetrans* endospore densities in field microplot experiments with *M. incognita/M. javanica* in Ecuador (Triviño & Gowen, 1996) and Tanzania (Trudgill et al., 2000). Similarly, positive results have been reported in a peanut-based cropping system in Florida where the pest was *M. arenaria* (Oostendorp, Dickson, & Mitchell, 1991; Chen, Dickson, Mc Sorley, Mitchell, & Hewlett, 1996) and in a 7-year tobacco monoculture where *M. incognita/M. javanica* were the principal species (Weibelzahl-Fulton, Dickson, & Whitty, 1996). Also,

populations of *P. penetrans* have been shown to increase when nematode susceptible crops are grown continuously (Chen & Dickson, 1998; Cetintas & Dickson, 2004).

The texture of soil could be important in developing suppression: *P. penetrans* occurs more frequently in sand and sandy loam soils than in those with greater amounts of loam and clay (Spaull, 1984; Chen & Dickson, 1998). However, in the localities where suppression has been demonstrated, soils varied in texture from 94% sand in Florida (Chen & Dickson, 1998) to silty loam soil (50% silt, 39% clay, 11% sand) in Ecuador (Trudgill et al., 2000).

### *4.1. Biological and Ecological Features of* Pasteuria penetrans

Endospores of *Pasteuria penetrans* are resistant to desiccation, as neither natural nor laboratory-induced drying affects the survival of the spores. Spores require a 3-day period of re-hydration (Brown & Smart, 1984) before maximum attachment ability is restored. Dried endospores can remain viable for long periods: Giannakou et al. (1997) showed that dried root powder that was stored in a laboratory drawer for 11 years contained viable spores. Though attachment did not differ from a freshly produced population, there was evidence to show that the pathogenicity may have declined. However, a criticism of this work is that the "new" population to which the original *Pasteuria penetrans* population was compared had been generated on a nematode population different from the original *Pasteuria*-infected one. Therefore it could be argued that the genetic make-up of the "new" population could have changed (Cook, personal communication), and that the two populations, though similar, could not categorically be described as identical.

There are no experimental data on the long-term survival of endospores in soils. Similarly, there is no published information on natural enemies that may parasitise or ingest endospores (Chen & Dickson, 1998). More information would be useful on the dynamics of populations of endospores in soil in the absence of hosts, if predictive studies on endospore densities and epidemiology are to be progressed.

## 5. CONCLUSIONS

For *Pasteuria* endospores to be applied inundatively, high quantities of propagules are needed. Mass production of endospores by *in vivo* techniques could under certain circumstances be sufficient for practical use, but there are few documented instances from field experiments where soil inundation has led to satisfactory root-knot nematode control. The main issues to consider concern the need for an immediate effect (in a nematicide-like manner) or if the endospores are applied with the expectation that their densities will increase over a number of crop cycles (Triviño & Gowen, 1996).

Like many biological control agents, it might be expected that greater success with *Pasteuria* will be achieved in the relatively smaller areas of protected crops, rather than in open fields (Pembroke, Gowen, & Giannakou, 1998). There are two reasons supporting this view: beds cultivated under glass or plastic are generally permanent and may be cropped with perennials (flowers) or annuals vegetables. In such intensively cropped systems the opportunities for integrating a soil applied

microbial agent should be greatest. Also, the natural build-up of endospore densities in soil may be greater than in open fields. Finally in protected cropping systems with controlled irrigation, the movement of endospores to deeper soil layers may be less than in fields receiving natural rainfall.

In conclusion, the data produced since the rediscovery of these bacteria show that *P. penetrans* and other *Pasteuria* spp. have potentials for application in biological control of nematodes. However, several aspects of their biology and application remain yet to be investigated, with particular concern for the availability of low cost, mass production technologies and of isolates covering the broad range of host nematode diversity.

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