UTILIZATION OF IN VITRO PRODUCED PASTEURIA SPECIES ENDOSPORES AS A BIOLOGICAL CONTROL FOR BELONOLAIMUS LONGICAUDATUS

By
JOHN ERIC LUC

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
UNIVERSITY OF FLORIDA
2009
To my mother and father, we turned a dream into reality
ACKNOWLEDGMENTS

I would like to thank my chairman and committee members, William T. Crow, Robin M. Giblin-Davis, Robert McSorley, and Jerry B. Sartain, for their guidance and patience during my pursuit of a Doctorate of Philosophy degree. I have gained a great deal of knowledge from each of them. Their dedication to the advancement and excellence of graduate education is uncompromising. I hope to have the same patience and dedication that they have exhibited during all my endeavors.

Also, I would like to acknowledge Augustus Porter Alden, my grandfather, whose belief in me never wavered. On July 27, 1998, he left this world and continued his journey. His passing was the catalyst for my return to academics.

John T. and Lynda A. Luc, my parents, deserve my sincere appreciation. Their guidance, patience, love, and support have carried me a long way. Without it I, would have never reached this point in my life. I dedicate this degree to them with all my heart.

I would also like to thank, Joseph C. Parker “Buck”, Amy Parker, Joseph C. Parker II, and Sierra Dawn Parker, my best friends and their children, for the love and emotional support they have shown. I have known Buck for 27 years; during that time our friendship has never been broken. I wish him and his family all the love and happiness in the world.

Finally, thanks go out to Wenjing Pang and Frank E. Woods, this degree is as much theirs as it is mine. Their dedication, hard work, and friendship made this project a success. I wish both of them the best that life has to offer.
TABLE OF CONTENTS

ACKNOWLEDGMENTS .......................................................................................................................4
LIST OF TABLES ..............................................................................................................................7
LIST OF FIGURES ...........................................................................................................................8
ABSTRACT .........................................................................................................................................9

CHAPTER

1 INTRODUCTION ..................................................................................................................11

2 LITERATURE REVIEW .......................................................................................................14

   Belonolaimus longicaudatus ..............................................................................................14
      Taxonomy .........................................................................................................................14
      Systematics and Phylogeny ...........................................................................................15
      Morphology and Anatomy ..............................................................................................15
      Biology and Distribution .................................................................................................16
   Pasteuria .............................................................................................................................19
      History and Taxonomy ....................................................................................................19
      Systematics and Phylogeny .............................................................................................21
      Morphology and Anatomy ..............................................................................................23
      Life Cycle .......................................................................................................................24
      Cultivation .......................................................................................................................25
      Ecology ............................................................................................................................25
         Temperature .............................................................................................................25
         Soil moisture ...........................................................................................................26
         pH ...............................................................................................................................27
      Survival ............................................................................................................................27
      Chemical Nematicides ...................................................................................................28
      Biological Control Potential ...........................................................................................29

3 INFECTIVITY OF IN VITRO PRODUCED PASTEURIA ENDOSPORES ON
   BELONO LAIMUS LONGICAUDATUS ................................................................................31

   Introduction ........................................................................................................................31
   Materials and Methods .......................................................................................................32
      Rate Study .......................................................................................................................33
      Sporangium Size Study .................................................................................................34
   Results ...............................................................................................................................35
      Rate Study .......................................................................................................................35
      Sporangium Size Study .................................................................................................36
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-1</td>
<td>Effects of the endospore size of in vitro produced <em>Pasteuria</em> sp. on <em>Belonolaimus longicaudatus</em> populations, percent endospore attachment, and total root length.</td>
<td>39</td>
</tr>
<tr>
<td>4-1</td>
<td>Effect of thatch or wetting agent on movement of topically applied in vitro produced <em>Pasteuria</em> sp. in greenhouse lysimeters planted with ‘Tifdwarf’ bermudagrass at four soil depths.</td>
<td>55</td>
</tr>
<tr>
<td>5-1</td>
<td>Effect of isolates of <em>Belonolaimus longicaudatus</em> and in vitro produced <em>Pasteuria</em> spp. on <em>Belonolaimus longicaudatus</em> populations, percent endospore attachment, and total root length.</td>
<td>69</td>
</tr>
<tr>
<td>6-1</td>
<td>Effect of inoculum level of <em>Belonolaimus longicaudatus</em> and formulation of in vitro produced <em>Pasteuria</em> sp. on <em>Belonolaimus longicaudatus</em> populations and percent endospore attachment.</td>
<td>78</td>
</tr>
<tr>
<td>6-2</td>
<td>Interaction effect of inoculum level of <em>Belonolaimus longicaudatus</em> and formulation of in vitro produced <em>Pasteuria</em> sp. endospores on total root length.</td>
<td>79</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>3-1</td>
<td>Effects of increasing levels of in vitro produced <em>Pasteuria</em> sp. endospores on <em>Belonolaimus longicaudatus</em> on creeping bentgrass during trial one. ........................................40</td>
<td></td>
</tr>
<tr>
<td>3-2</td>
<td>Effects of increasing levels of in vitro produced <em>Pasteuria</em> sp. endospores on <em>Belonolaimus longicaudatus</em> on creeping bentgrass during trial two. .......................................41</td>
<td></td>
</tr>
<tr>
<td>3-3</td>
<td>Effects of increasing levels of in vitro produced <em>Pasteuria</em> sp. endospores on percent endospore attachment to <em>Belonolaimus longicaudatus</em> inoculated onto creeping bentgrass during trial one. ..............................................................................................................43</td>
<td></td>
</tr>
<tr>
<td>3-4</td>
<td>Effects of increasing levels of in vitro produced <em>Pasteuria</em> sp. endospores on percent endospore attachment to <em>Belonolaimus longicaudatus</em> inoculated onto creeping bentgrass during trial two. ..............................................................................................................44</td>
<td></td>
</tr>
<tr>
<td>3-5</td>
<td>Effects of increasing levels of in vitro produced <em>Pasteuria</em> sp. endospores on total root length of creeping bentgrass inoculated with <em>Belonolaimus longicaudatus</em> during trial one. ..............................................................................................................................................46</td>
<td></td>
</tr>
<tr>
<td>3-6</td>
<td>Effects of increasing levels of in vitro produced <em>Pasteuria</em> sp. endospores on total root length of creeping bentgrass inoculated with <em>Belonolaimus longicaudatus</em> during trial two. ..............................................................................................................................................47</td>
<td></td>
</tr>
<tr>
<td>4-1</td>
<td>Effect of increasing irrigation on depth placement of in vitro produced <em>Pasteuria</em> sp. endospores in soil during trial 1. ..............................................................................................................................................56</td>
<td></td>
</tr>
<tr>
<td>4-2</td>
<td>Effect of increasing irrigation on depth placement of in vitro produced <em>Pasteuria</em> sp. endospores in soil columns as determined by bioassay with <em>Belonolaimus longicaudatus</em> during trial 2. ..............................................................................................................................................58</td>
<td></td>
</tr>
<tr>
<td>5-1</td>
<td>Relationships among the two populations of <em>Belonolaimus longicaudatus</em> used in this study, five previously studied <em>B. longicaudatus</em> populations and a representative outgroup taxa based on parsimony analysis of the ITS subunit gene. ..............................................................................................................................................70</td>
<td></td>
</tr>
</tbody>
</table>
Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

UTILIZATION OF IN VITRO PRODUCED PASTEURIA SPECIES ENDSPORES AS A BIOLOGICAL CONTROL FOR BELONOBLAIMUS LONGICAUDATUS

By

John Eric Luc

December 2009

Chair: William T. Crow
Major: Entomology and Nematology

Management of plant-parasitic nematodes in turf has become increasingly difficult during the last decade due to the limited number of effective post-plant nematicides. The use of in-vitro produced Pasteuria sp. (IVP) as a biological control agent of Belonolaimus longicaudatus (sting nematode) offers an additional management tactic to the chemical and cultural controls currently available. Experiments utilizing IVP endospores were conducted to determine if endospore rate, size, origin, formulation, or placement in the soil profile affect the suppression of B. longicaudatus populations.

Growth room experiments revealed that high levels (280,000 endospores/cm³ of soil) of IVP endospores suppressed B. longicaudatus populations by approximately 75%. Another experiment suggested that sporangium size of IVP endospores did not affect the level of nematode suppression. Furthermore, a bionematicide utilizing IVP endospores is likely to be effective against different populations of B. longicaudatus and the original geographical source of IVP endospores does not appear to affect efficacy. Similarly, B. longicaudatus levels at the time of application, does not appear to affect the ability of IVP endospores to suppress nematode populations. Granular and liquid formulations of IVP endospores suppressed nematode populations by 20% and 63%, respectively, compared to the nontreated control. Greenhouse
experiments indicate that IVP endospore movement into the turf profile is not hindered by thatch and that large irrigation events can move endospores below the turfgrass root zone, possibly reducing effectiveness. In addition, placement of endospores within the root zone (0 to 10 cm soil depth) with one application of 0.6 cm of irrigation indicates that endospore applications can be achieved relatively easy. These trials have provided a great deal of information; however experiments conducted in a controlled environment do not always correspond to field observations and field tests should be conducted in the future to confirm these results. In the future, biopesticides using IVP may be an important component of integrated pest management for B. longicaudatus on turfgrasses.
In Florida, plant-parasitic nematodes of turf are generally root-feeding pests that greatly reduce the development of turf roots. In particular, *Belonolaimus longicaudatus* (sting nematode) is considered the most destructive nematode to turf in Florida (Crow et al., 2003; Crow, 2005). Damage caused by *B. longicaudatus* to bermudagrass root systems can cause decreased water and nutrient uptake, reduced plant growth, and can predispose turf to other adverse conditions such as drought stress, heat stress, malnutrition, insects, pathogens, and weeds which could lead to reduced turf quality, color, and density (Christie, 1959; Johnson, 1970; Lucas, 1982).

*Belonolaimus longicaudatus* is an ectoparasitic nematode with an extensive host range and has been recognized as a pest of many agronomic, horticultural, and ornamental crops (Abu-Gharbieh and Perry, 1970). It is found predominately in sandy costal areas of the southeastern United States. However, it has been observed in localized areas of Arkansas, California, Connecticut, Kansas, Nebraska, New Jersey, Oklahoma, and Puerto Rico (Robbins and Barker, 1974; Rhoades, 1980; Huang and Becker, 1999). Outside the United States, it has been found in Bermuda, and some of the Caribbean islands (Perry and Rhoades, 1982; Mundo-Ocampo et al., 1994).

*Belonolaimus longicaudatus* adults can range from 2000 to 3000 µm long and 29 to 34 µm wide (Rau, 1958; Mai et al., 1996). *Belonolaimus longicaudatus* is a bisexual species, which reproduces exclusively through amphimixis (sexually) with males accounting for 40% of the population. After mating, females lay eggs in pairs as long as a food source is available, with each female laying about 128 ± 13 eggs in 90 days. At 28 °C, they can complete their life cycle within 24 days (Huang and Becker, 1999).
The Food Quality Protection Act of 1996 mandated a review of many pesticides. As a result, in 2000 turfgrass was removed from the label for ethoprop (Mocap), and fenamiphos (Nemacur) was discontinued in 2007. Management of plant-parasitic nematodes in turf has become increasingly difficult over the last decade due to the limited number of effective post-plant nematicides.

In search of new nematode management strategies, researchers have continued to investigate the utility of biological control agents. *Pasteuria* is a gram positive, endospore-forming genus of bacteria having great potential to be developed into biological control agents of plant-parasitic nematodes (Sayre and Starr, 1985; Dickson et al., 1994). *Pasteuria* spp. are most closely related to *Alicyclobacillus* with a life cycle involving five major stages: i) endospore attachment, ii) penetration by germ tube of the cuticle, hypodermis and musculature, iii) vegetative growth within the infected nematode pseudocoel, iv) sporogenesis, and v) release of mature endospores back into the soil. Currently, five species of *Pasteuria* have been characterized: i) *Pasteuria ramosa*, ii) *P. penetrans*, iii) *P. thornei*, iv) *P. nishizawai* (Sayre and Starr, 1989), and v) ‘*Candidatus Pasteuria usgae’* (CPu) (Giblin-Davis et al., 2003). In particular, CPu has been recognized as a naturally occurring biological agent that can suppress *B. longicaudatus* in turf (Giblin-Davis, 2000). Useful characteristics of CPu as a biological control include: endospore durability, pesticide compatibility, and reduced environmental risk to humans and non-target organisms. However, a major obstacle to the commercial development of *Pasteuria* spp. as biopesticides is their limited host range. Previously, CPu was cultivated on *B. longicaudatus* grown in aseptic root culture, greenhouse culture, or collected from suppressive sites in the field (Giblin-Davis et al., 1990; Bekal et al., 2001). Recently, Pasteuria Bioscience LLC, developed an in vitro method of culturing *Pasteuria* spp. that may allow for it to be
commercialized as a biopesticide. Several isolates of *Pasteuria* sp. were collected and cultured from *B. longicaudatus* on turf from South Carolina and Florida. However, these isolates could not be molecularly confirmed as ‘*Candidatus Pasteuria usgae*’, henceforth they will be referred to as in vitro produced *Pasteuria* spp. (IVP) endospores. As development of IVP continues, many questions remain unanswered.

The objectives of these studies were: i) to determine a level of IVP endospores that suppress *B. longicaudatus* populations below damaging numbers, ii) to determine if sporangia size effects efficacy of IVP endospores, iii) to determine the effect of thatch and wetting agent on movement of IVP endospores into the soil profile, iv) to determine if IVP endospores were subject to leaching from large irrigation or rainfall events, v) to determine the intraspecific host specificity of IVP on efficacy against *B. longicaudatus*, vi) to determine if the efficacy of IVP as a biopesticide is affected by *B. longicaudatus* population density at the time of application, and vii) to compare the efficacy of a clay granule formulation with a liquid spore suspension.
CHAPTER 2
LITERATURE REVIEW

*Belonolaimus longicaudatus*

**Taxonomy**

Steiner (1949) first established the genus *Belonolaimus* with the discovery and description of *Belonolaimus gracilis*. This first observation of *B. gracilis* was from the rhizosphere of slash pine (*Pinus elliottii* Engelm) and longleaf pine (*Pinus palustris* P. Mill.) in the Ocala National Forest near Ocala, FL. Over the next few years, *B. gracilis* was reported to damage crops such as celery (*Apium graveolens* L.), corn (*Zea mays* L.), sorghum (*Sorghum bicolor* [L.] Moench), millet (*Sorghum halepense* [L.] Pers.), peanut (*Arachis hypogaea* L.), cotton (*Gossypium hirsutum* L.), soybean (*Glycine max* [L.] Merr.), and cowpea (*Vigna unguiculata* [L.] Walp.) (Owens, 1951; Christie, 1952; Christie et al., 1952; Christie, 1953).

Rau (1958) subsequently added a second species to the genus *Belonolaimus* by describing *Belonolaimus longicaudatus*. The major morphological differences separating these species are that *B. longicaudatus* has a longer stylet and shorter tail than *B. gracilis*. *Belonolaimus longicaudatus* was initially found on crops such as bermudagrass (*Cynodon dactylon* [L.] Pers.), corn, citrus (*Citrus* spp.), soybean, peanut, and other crops (Rau, 1958). Furthermore, Rau stated that *B. longicaudatus* was the more commonly encountered species. Rau (1963) described three additional species of *Belonolaimus*: *Belonolaimus euthychilus*, *Belonolaimus maritimus*, and *Belonolaimus nortoni*. The current taxonomic placement of *Belonolaimus longicaudatus* within the domain Eukaryota is: kingdom Animalia, subkingdom Metazoa, branch Eumetazoa, division Bilateralia, subdivision Protostomia, section Pseudocoelomata, superphylum Aschelminthes, phylum Nematoda, class Secernentea, subclass Tylenchia, order Tylenchida, suborder
Tylenchina, superfamily Dolichodoroidea, family Belonolaimidae, subfamily Belonolaiminae, genus Belonolaimus, species longicaudatus (Siddiqi, 2000).

Systematics and Phylogeny

Research from the 1950’s to mid 1970’s, revealed differences in morphology and host specificity of different B. longicaudatus populations from Florida and other states (Owens, 1951; Perry and Norden, 1963; Good, 1968; Adu-Gharbieh and Perry, 1970; Rau and Fassuliotis, 1970; Robbins and Barker; 1973; Duncan et al., 1996), this work suggested that phylogenetic differences may exist. Han et al., (2006) revealed that isolates of B. longicaudatus from Gainesville, FL; Hastings, FL; Lake Alfred, FL; Tifton, GA; and Scotland County, NC exhibited morphological differences in regards to: body length and width; stylet length; stylet cone and shaft lengths; knob shape and length; distance of excretory pore to anterior end; head, esophagus, and tail lengths; as well as a, b, and c ratios. However, phylogenetic analysis of the ITS1 region of rDNA did not reflect these morphological differences. Gozel et. al. (2006) examined 33 populations of B. longicaudatus obtained throughout Florida to infer the molecular phylogeny of the species. Similar to the previous study, differences in morphological characteristics were observed within species. However, phylogenetic analysis in this study indicated three clades of B. longicaudatus were observed with overlapping geographical regions moving east to west across the peninsula of Florida.

Morphology and Anatomy

Belonolaimus longicaudatus adults are 2000 to 3000 µm long and 29 to 34 µm wide (Rau, 1958; Mai et al., 1996). The lip region has four major lobes with two smaller lobes with amphids. A constriction sets the lip region off from the rest of the body. The lateral field has one incisure running most of the body length. The stylet is thin and flexible ranging in size from 100 to 140 µm long with rounded knobs. When retracted, the stylet causes the esophageal tube to be
convoluted. The median bulb is well developed and elongated. Esophageal glands overlap the anterior end of the intestines on the ventral side. The intestine can be found slightly posterior of the median bulb extending almost to the terminus. Lateral canals are prominent and serpentine along the intestine, becoming visible near the esophageal glands and extending to the terminus. The vulva is a transverse slit found near the middle of the female, with lips not protruding. The vagina nearly always has two sclerotized pieces that can be observed in lateral view. The reproductive system is didelphic, amphidelphic, and outstretched. Spermathecae are present which store sperm after copulation for fertilization of eggs over time. The male reproductive system is found posteriorly, with testis prodelphic and outstretched. Spicules and gubernaculum are well-developed averaging 44 µm and 16 µm long, respectively. The female tail is 115 to 189 µm long with a rounded terminus. The male tail tapers to a more pointed terminus and is enveloped by a long and narrow bursa, which may aid during copulation. (Robbins and Barker, 1973; Mai et al., 1996). It should be noted, however that populations of *B. longicaudatus* from North Carolina and Georgia have been shown to exhibit differing morphological characteristics such as stylet length, stylet cone and shaft length, knob shape and length, distance of excretory pore to anterior end, tail length, and spicule length. Interpopulation mating of these two populations resulted in a few offspring, which were sterile, while intrapopulation offspring reproduced normally. All populations investigated possessed eight haploid chromosomes suggesting they are closely related, but may be different species (Robbins and Hirschmann, 1974).

**Biology and Distribution**

*Belonolaimus longicaudatus* is an ectoparasitic nematode with an extensive host range and has been recognized as a pathogen of many agronomic, horticultural, and ornamental crops (Abu-Gharbieh and Perry, 1970). Feeding by *B. longicaudatus* involves the insertion of a long
stylet into root tips and subsequent injection of digestive enzymes. Feeding can cause root tips to stop growing; this can be devastating to the developing root system of young plants (Perry and Rhoades, 1982; Giblin-Davis et al., 1992; Huang and Becker, 1997; Crow et al., 1997). Similarly, root damage caused by *B. longicaudatus* to newly or well-established bermudagrass root systems can cause decreased water and nutrient uptake, increased nitrate leaching, and reduced plant vigor (Johnson, 1970; Luc et al., 2006).

*Belonolaimus longicaudatus* is a bisexual species, reproducing exclusively by amphimixis with males accounting for 40% of the population (Huang and Becker, 1999). Experiments conducted on axenic corn root cultures at 28 °C by Huang and Becker (1997; 1999) have given a detailed description of the life cycle of *B. longicaudatus*. After mating, each female lays about 128 ± 13 eggs in 90 days, when an adequate food source is available. Following the egg laying (day 0), the first stage juvenile (J1) molted in the egg during day 4 and the second stage juvenile (J2) hatched on day 5. The J2 must quickly find root tips to feed on or die. Once a food source was found, the J2 fed for 12 to 24 hours before becoming immobile and the second molt began during day 7 and ended during day 9. Third stage juveniles (J3) began feeding again and then entered the third molt on day 12 which lasted 2 days. Fourth stage juveniles (J4) began feeding once more, but depending on the final sex of the nematodes molting began at different times. Males entered the fourth molt during day 18 and by day 20 were fully functional males and began feeding again. Females entered the fourth molt during day 19, and by day 22 were considered virgin females ready for mating. The life cycle from J2 to J2 took 24 days at 28 °C (Huang and Becker, 1999).

Predominately located in the sandy coastal areas of the southeastern United States, *B. longicaudatus* is most frequently found in soils consisting of >80% sand and <10% clay with
minimal organic matter (Robbins and Barker, 1974; Rhoades, 1980). Nevertheless, localized infestations have been observed in Arkansas, California, Connecticut, Kansas, Oklahoma, Nebraska, New Jersey, and Texas (Robbins and Barker, 1974; Rhoades, 1980; Huang and Becker, 1999). *Belonolaimus longicaudatus* has also been found outside the United States in Bermuda, Puerto Rico, and some of the Caribbean islands, frequently in resort areas where sod or sprigs were sent from the southeastern United States to establish fairways, greens, tees, or commercial turf (Perry and Rhoades, 1982; Mundo-Ocampo et al., 1994).

Soil texture, soil particle size, soil moisture, root depth, and movement of nematodes are factors which influence the distribution of nematodes within the soil profile as well as geographically. As the diameter and length of the nematode increases, the pore and particle size of the soil must be increased or movement can be hindered (Wallace, 1971). Conversely, if pore diameter increases dramatically, lateral movement can be hindered (Brodie, 1976). Optimum soil moisture for *B. longicaudatus* is about 7% above oven dry weight, however populations have been recovered from soils ranging from 2 to 30% soil moisture (Robbins and Barker, 1974). Saturated soils replenish oxygen much slower than well-drained soils, which can reduce activity and ultimately be detrimental to certain nematode species. Conversely, soils with excessively low soil moisture hinder movement by reducing the water films encircling soil particles used by nematodes to move through the soil. Soil texture, particle size, moisture, and aeration are interrelated factors, which effect movement. Movement is essential for species that reproduce exclusively by amphimixis (*B. longicaudatus*) because they must search for food as well as a mate to reproduce, otherwise the population will die (Robbins and Barker, 1974). Root depth of the host plant also influences where *B. longicaudatus* will occur, generally they are found within the top 75 cm of the soil profile, with greatest population densities found in the top 30 cm of the
soil profile (Brodie, 1976; Todd, 1989; McSorley and Dickson, 1990). In sites where turf is the 
host crop, greatest population densities occur within the top few cm of soil (Giblin-Davis et al., 
1991). Conversely, in corn and citrus, which possess deeper root systems, high populations can 
be found below 30 cm depth when other soil conditions are favorable for nematode movement 
(Brodie, 1976; Todd, 1989; Noling, 1993). Nematode populations are frequently erratically 
distributed at standard sampling depths even within the same fields site (Todd, 1989). Erratic 
distribution of nematodes can be a result of previously mentioned factors, as well as the lack of 
active movement of nematodes from one location to another. When consideration is given to the 
previously discussed factors, golf greens represent an ideal habitat for *B. longicaudatus* due to a 
high content of fine to medium sand that hold approximately 12 to 15% water at field capacity 
(Anonymous, 1993).

*Pasteuria*

**History and Taxonomy**

Metchnikoff (1888) first established the genus *Pasteuria* with the discovery and 
description of *Pasteuria ramosa* infecting water fleas, *Daphnia magna*. However, the 
relationship of *P. ramosa* (Metchnikoff 1888) to bacterial parasites of nematodes was not known. 
Since that time, *Pasteuria*-like organisms have been reported from 323 species of soilborne 
nematodes belonging to 116 genera, from 79 countries worldwide (Sturhan, 1985; Sayre and 
Starr, 1988; and Chen and Dickson, 1998). Host nematodes for *Pasteuria* spp. include free-
living, predacious, plant-parasitic, and entomopathogenic nematodes. Endospores of *Pasteuria* 
spp. show remarkable size variations, ranging from approximately 1.5 to 8.0 μm (Chen and 
Dickson, 1998). This wide range of host nematodes, endospore dimensions, and host specificity 
of *Pasteuria* spp. has caused considerable confusion in their taxonomy. Currently, five species of 
*Pasteuria* have been described in detail. These are; i) *Pasteuria ramosa* (*Daphnia magna*, water
flea) (type species of the genus), ii) *P. penetrans* (*Meloidogyne* spp., root knot nematode), iii) *P. nishizawae* (*Heterodera* and *Globodera* spp., cyst nematodes), iv) *P. thornei* (*Pratylenchus* spp., lesion nematode) (Sayre and Starr, 1989), and v) ‘*Candidatus* Pasteuria usgae’ (*Belonolaimus longicaudatus*, sting nematode)(Giblin-Davis et al., 2003).

Since *Pasteuria* was first discovered, it has undergone various name changes and scientific placements. In 1906, a Pasteuria-like organism was observed infecting *Dorylaimus bulbiferus*, but it was believed to be a parasitic sporozoan (Cobb, 1906). In 1925, the suggested placement of this parasitic sporozoan was within the genus *Duboscqia* (Micoletzky, 1925). Later in 1940, a parasite that is now considered as *P. thornei* was reported as a sporozoan parasite of *Pratylenchus penetrans* (Thorne, 1940). Mankau and Imbriani (1975) reviewed the description by Thorne and considered the organism described as a prokaryote. Detailed examination of a similar bacterial parasite of root-knot nematodes with an electronic microscope established that structural features of the spores were similar to endospores of *Bacillus* spp. This led to their designation of the organism as *Bacillus penetrans* (Mankau, 1975a). *Bacillus penetrans* differed from other members of the genus *Bacillus* with respect to spore shape and vegetative growth (Mankau, 1975b). Accordingly, *B. penetrans* was never accepted for placement in Bergey’s Manual and the taxonomic position of this organism remained uncertain (Skerman et al. 1980).

Sayre and Wergin (1977) examined the life cycle of the bacterial endoparasite of root-knot nematodes by electron microscopy and found it to have more in common with Actinomycetales and the bacterium *P. ramosa* than with members of the genus *Bacillus*. A detailed analysis of this bacterial parasite followed and the organism was renamed *Pasteuria penetrans* because of its similarity to *P. ramosa* (Sayre and Starr, 1985). *Pasteuria penetrans* was accepted for placement in the Bergey’s manual and is currently the accepted name.
Between 1985 and 1990, a survey of bermudagrass in south Florida was conducted for isolates of *Pasteuria* spp. on plant-parasitic nematodes. Five distinct isolates of *Pasteuria* spp. were observed on *B. longicaudatus*, *Hoplolaimus galeatus*, *Helicotylenchus microlobus*, and *Tylenchorhynchus annulatus*. The ultrastructure of the large spore isolates from *B. longicaudatus* and *Hoplolaimus galeatus* appeared to be distinct from known *Pasteuria* species. In the following years considerable work on morphology, ultrastructure, and host range was conducted. Giblin-Davis et al. (2003) suggested the naming of the S-1 strain of *Pasteuria* sp. from *B. longicaudatus* in south Florida (Fort Lauderdale Research and Education Center, Davie, Florida). However, in vitro culturing of *Pasteuria* spp. was not yet possible and a definitive type strain could not be maintained, a new requirement for placement in the Bergey’s manual (De Vos and Trüper, 2000; Labeda, 2000). Currently the strain S-1 of *Pasteuria* sp. from *B. longicaudatus* is a ‘*Candidatus* Pasteuria usgae’ sp. nov. (Giblin-Davis et al., 2003).

**Systematics and Phylogeny**

Initial, *Pasteuria* spp. identification was accomplished using morphology, ultrastructure, host range, and life cycle (Chen and Dickson, 1998). However, the reliability of these methods for accurate identification has been challenged (Cianco, 1995). More than 300 nematode species have been reported with *Pasteuria* spp. endospores attached to their cuticle, but only four *Pasteuria* spp. have been described in detail. Many of these undescribed *Pasteuria* spp. may have overlapping morphometrics and host ranges, making accurate species identification increasing difficult (Chen and Dickson, 1998). Currently, endospore-forming bacteria are placed in 13 genera, which are based on morphological, physiological, and genetic diversity. The 16S rDNA sequences for *Pasteuria* spp. were obtained from endospores of *P. ramosa* (Ebert et al., 1996), *P. penetrans* (Anderson et al., 1999), *Pasteuria* sp. from soybean cyst nematode (Atibalentja et al., 2000), and CPu from sting nematodes (Bekal et al., 2001; Giblin-Davis et al,
The 16S rDNA sequences of CPu were compared to *P. penetrans*, *P. ramosa*, and an unknown strain of *Pasteuria* sp., and indicated that CPu is a similar but distinct species of *Pasteuria* (Bekal et al., 2001). Conversely, Duan et. al. (2003) examined numerous isolates of *Pasteuria* spp. to infer phylogenetic relationships. Certain isolates of *Pasteuria* spp. obtained from *B. longicaudatus* were shown to have a closer relationship to isolates of *Pasteuria* spp. from *Hoplolaimus* spp., *Meloidogyne* spp., and *Heterodera* spp. than to other isolates from *B. longicaudatus*. Despite the weakness of the branches formed in this phylogenetic tree, due to small amplification products, this suggests genetic diversity in the *Pasteuria* spp. endospores that attach to *B. longicaudatus*. Phylogenetic analysis suggested that *Bacillus tusciae*, *Alicyclobacillus cycloheptanicus*, and *A. acidocaldarius* are the nearest relatives (Ebert et al., 1996; Anderson et al., 1999), and showed that *P. ramosa* does not belong to the Actinomycetales, as had been previously suggested (Sayre and Wergin, 1977; Bird, 1986). The endospore morphology and sporogenesis of *Pasteuria* spp. was similar to that of a typical bacterium, except that *Pasteuria* has a more complex life cycle. Based on these characteristics, *Pasteuria* spp. belongs with members of the true bacteria (Ebert et al., 1996; Chen et al., 1997). The DNA sequences of conserved genes encoding sporulation transcription factors sigE and sigF from *P. penetrans* biotype P-20 show different phylogenetic relationships to other endospore forming bacteria, supporting the idea that better loci may be available for differentiation of *Pasteuria* spp. and biotypes (Preston et al., 2003). Charles et al. (2005) examined the phylogenetic relationships of *Pasteuria* spp. using forty housekeeping gene sequences, indicating that the genus *Pasteuria* is an ancient member of the Bacillus group and is firmly within the Bacillus class of gram positive, low G-C content eubacteria. The systematics and phylogeny of
*Pasteuria* spp. will be better informed as methods of culture are refined and more loci can be sequenced and examined for use in molecular phylogenetic inference.

**Morphology and Anatomy**

*Pasteuria* spp. differentiation based on the Linnean species concept is accomplished using morphology and ultrastructure. The following will compare and contrast morphological and ultrastructure differences between *Pasteuria* spp. Mean diameters for the core, central body, and endospore are 1.54 ± 0.24 μm, 3.08 ± 0.24 μm, and 6.05 ± 0.36 μm for CPu, while other described *Pasteuria* spp, have mean diameters for central body and endospore ranging from 1.3 to 2.7 and 2.9 to 5.6, respectively. ‘*Candidatus* Pasteuria usgae’ central body is rounded-rectangular to rounded-trapezoid contrasting with other species that are round, horizontally elliptical, or rounded-square in shape. ‘*Candidatus* Pasteuria usgae’ central body size and shape differences appear to be due to the outer spore coat, which is thinner on the top and bottom and wider laterally than *P. ramosa*, *P. penetrans*, *P. thornei*, and *P. nishizawae*. The lateral thickening appears to be due to densely packed fibrous micro-projections that are directed outwardly toward the peripheral fibers in contrast to *P. ramosa*, *P. penetrans*, *P. thornei*, and *P. nishizawae* that lay perpendicular to the inner spore coat or are not present. *Pasteuria penetrans*, *P. thornei*, *P. nishizawae*, and CPu possess peripheral fibers required for attachment to the cuticle of a nematode, while they are absent in *P. ramosa*. This may be related to the mode of parasitism by *P. ramosa*, which is believed to be ingested by its host. ‘*Candidatus* Pasteuria usgae’ and *P. thornei* both possess epicortical wall remnants in mature endospores that encircle the cortex sublaterally, which is lacking in *P. ramosa*, *P. penetrans*, and *P. nishizawae*. At the ventral midpoint of the endospore, a pore can be observed measuring 0.3 μm in diameter similar to *P. penetrans*, but larger than *P. thornei* and *P. nishizawae*. No basal ring is observed in *P. ramosa*, *P. thornei*, *P. nishizawae*, or CPu, but is present in *P. penetrans* (Giblin-Davis, 2000;
Giblin-Davis et al, 2001). These characteristics may aid identification of *Pasteuria* spp., when molecular identification is not available or corroborate molecular differences that are identified.

**Life Cycle**

The life cycle of CPu involves four major stages: i) endospore attachment, ii) germination of the endospore and penetration by germ tube of the cuticle, epidermis and musculature, iii) development within the infected nematode’s pseudocoel, and iv) release of mature endospores. Endospores are the final developmental stage of the bacterium. Endospores attach to the cuticle of all life stages from second-stage juveniles to adults of *B. longicaudatus* as they move through the soil searching for a host plant. Germination of endospores occurs when the protoplasm becomes granular in appearance. A germ tube forms and exits the pore and punctures the cortical layer of the cuticle. The advancing germ tube then moves parallel within the median zone before penetrating the basal layer of the cuticle, muscles, and enters the pseudocoelom. After entering the nematode pseudocoelom, the germ tube develops into a cauliflower-like microcolony consisting of a dichotomously branched and septate mycelium, stage 0 of sporogenesis. Daughter colonies form when the intercalary cells in the microcolony lyse (Sayre and Starr, 1989). Due to unknown triggers, the colony breaks up. In stage I, the terminal cells of the fragments swell and become fully septate. Stage II, is characterized by the formation of a transverse septum separating the forespore from the mother cell. The protospore enlarges during stage III, but remains elliptical in shape, the forespore is engulfed and peripheral fibers emerge. The spore continues to widen in stage IV, and the cortex forms, peripheral fibers lengthen, and the exosporium develops. In stage V, microfibrillar layers appear on the peripheral fibers, the epicortical layer surrounds the cortex, the inner spore coat is observed, and the outer spore coat appears dorsally and engulfs the spore core. As sporegenesis continues, the inner spore coat matures, the outer spore coat thickens dorsally and laterally, while the epicortex recedes.
Throughout stages VI and VII, the cytoplasm between the spore and exosporium is diminished, with a mature endospore being formed during stage VII. Eventually, developing sporangia dominate in the nematode body cavity and finally mature endospores are released into the soil when the nematode decomposes. Observations of field samples, suggest that the most opportune times to obtain endospore filled cadavers of *B. longicaudatus* in Florida appears to be in April and October. This is most likely related to declining soil temperatures and reduced decomposition of nematode cadavers.

**Cultivation**

Current methods of mass-producing *Pasteuria* spp. rely on in vivo reproduction of the pathogen in its nematode host on greenhouse-grown plants (Stirling and Wachtel, 1980). The production systems might be improved by culturing the nematode and pathogen in excised or transformed root cultures (Verdejo and Mankau, 1986; Verdejo and Jaffee, 1988; Bekal et al., 2001). Various media have been tested for artificial cultivation of *Pasteuria* spp., but have been unsuccessful (Reise et al., 1988; Williams et al., 1989; Bishop and Ellar, 1991). Studies focusing on chemical and physiological changes in the pseudocoelom of the female nematodes as it matures may provide insights into the critical requirements needed for the formulation of an artificial medium. Recently, Pasteuria Bioscience LLC has developed an in vitro method for culturing *Pasteuria* spp. that may allow for it to be commercialized as a biopesticide. However, their in-vitro culturing method remains proprietary knowledge.

**Ecology**

**Temperature**

The development of *P. penetrans* in nematodes is temperature dependent (Stirling, 1981; Hatz and Dickson, 1992; Freitas, 1997; Serracin et al., 1997; Talavera and Mizukubo, 2003). Development of *P. penetrans* within females of *Meloidogyne javanica* and *M. arenaria* was not
observed at 10 °C (Hatz and Dickson, 1992). The minimal developmental temperature was determined as 17 °C (Chen and Dickson, 1997), with optimal growth temperature between 28 °C and 35 °C (Hatz and Dickson, 1992; Serracin et al., 1997). Based on these temperature requirements, *P. penetrans* can be defined as a mesophilic bacterium. However, different temperature requirements exist for various isolates of the bacterium due to its cosmopolitan distribution. An Indian isolate of *Pasteuria* spp. that infects both *Heterodera* spp. and *Meloidogyne incognita* completed its life cycle in *M. incognita* within 49 days at 10 °C to 17 °C (Bhattacharya and Swarup, 1988). Furthermore, temperature affects endospore production (Chen and Dickson, 1997, 1998; Hatz and Dickson, 1992), endospore attachment (Ahmed, 1990; Singh and Dhawan, 1990; Stirling, et al., 1990; Hatz and Dickson, 1992), germination (Sayre and Wergin, 1977; Serracin et al., 1997), and pathogenicity (Stirling, 1981).

**Soil moisture**

Little is known about the effect of soil moisture on endospore attachment and development of *Pasteuria* spp. It has been reported that the proportion of J2 with attached *P. penetrans* endospores was greater in moistened soil than in dry soil (Brown and Smart, 1984). The rate of development of *P. penetrans* in infected females was reduced when soils were maintained at or near field capacity (Davies et al., 1991). Although the reasons for these effects are not certain, it is known that oxygen depletion in saturated soils inhibits plant respiration (Taiz and Zeiger, 1998), which may inhibit the development of both the nematode and bacterial parasite.

Water is the basic method of bacteria dispersal throughout the soil profile and is a major reason for *Pasteuria* spp. endospore losses from the cultivated soil horizon (Gammack et al., 1992). Golf course greens are constructed predominately of sand to have high percolation rates and as a result have high leaching potential. Once in the mineral soil, increased percolation due to rainfall or irrigation following treatment may affect the distribution of endospores or cause
endospores to be moved below the turf root zone (upper 10 cm of soil), reducing effectiveness (Cetintas and Dickson, 2005; Dabiré et. al., 2005).

**pH**

Endospore attachment is affected by pH. The highest attachment occurred at pH 9 (Ahmed and Gowen, 1991) and decreased at low pH values (Ahmed, 1990). Davies et al. (1988) observed that the attachment was higher at pH 7 than pH 4 or pH 9 in tap water, but lower at pH 7 than pH 4 or pH 9 in distilled water. Studies have revealed that the endospore surface has a net negative charge, which was greater at neutral pH and was reduced with a change of pH away from neutral (Afolabi et al., 1995). Electrostatic forces between the nematode cuticle and the endospore surface oppose attachment because the charges on nematode cuticle were also negative. Reasons for the pH effects remain uncertain (Afolabi et al., 1995).

**Survival**

The natural enemies and long-term survival of *Pasteuria* endospores in soil have not been well defined. In a peanut field in Florida, *P. penetrans* endospores maintained suppressive levels for *M. arenaria* over 10 years (Dickson et al., 1994). In a laboratory study, endospores of *Pasteuria* spp. were reported viable for a period of more than one year (Mani, 1988). Storage of endospores for 5 years and 11 years did not affect their rate of attachment, but decreased their rate of infection (Giannakou et al., 1997). Endospores of *P. penetrans* have resisted various chemicals and environmental conditions (Mani, 1988; Williams et al., 1989). However, endospores of *Pasteuria* spp. were killed by autoclaving, but were only slightly affected by microwaving or heat treatment at 47 °C for 72 hours (Weibelzahl-Fulton, 1996; Giblin-Davis, 2000). Long term survival may be related to lack of Adenosine-5’-triphosphate (ATP) and Nicotinamide adenine dinucleotide (NAD+/NADH); high levels of divalent cations; enzyme dormancy; dehydration of protoplasm; or a thick spore coat (Setlow, 1994). Resistances to high
temperatures may be linked to sporulation temperature, cortex size, and protoplasm mineral and moisture content (Gerhardt and Marquis, 1989). Chemical resistance is most likely related to lack of membrane impermeability and endospore coat layers (Setlow, 1994).

**Chemical Nematicides**

The use of *Pasteuria* spp. as a biological control agent in combination with other management practices, especially nematicides, is an important issue (Freitas, 1997). Stirling (1984) reported that infection of *M. javanica* by *P. penetrans* after treatment in vitro was not affected by nematicides: 1,3-dichloropropene (1,3-D) or 1,2-dibromo-3-chloropropane (DBCP). Brown and Nordmeyer (1985) demonstrated a synergistic reduction of root galling by *M. javanica* by carbofuran or aldicarb combined with *P. penetrans*. A plausible explanation for the synergism was that low concentrations of the carbamate nematicide stimulated nematode movement increasing the likeliness of nematode-endospore contact. Organophosphates and carbamate nematicides at high concentrations have been shown to decrease nematode mobility, reducing the probability of nematode-endospore contact and infection. Chloropicrin and methyl bromide + 33% chloropicrin were highly detrimental to *P. penetrans* in field and greenhouse experiments. Treatments with 1,3-D + 17% chloropicrin, 1,3-D + 25% chloropicrin, and 1,3-D + 35% chloropicrin had moderate effects on the bacterium. However metam sodium did not have a harmful effect on the bacterium (Freitas, 1997). Freitas (1997) suggested and Kariuki (2007) confirmed that chloropicrin is responsible for the bactericidal effect. The fact that 1,3-D did not have an adverse effect on the percentage infected females supports the hypothesis that 1,3-D can be used alongside *P. penetrans* to manage *M. arenaria*. Further field studies are necessary to determine whether other pesticides are detrimental to *Pasteuria* spp.
Biological Control Potential

Field plots inoculated with 900 cm$^3$ of soil laden with in vivo CPu endospores (ca. 5,000 endospores/cm$^3$) have been shown to effectively suppress *B. longicaudatus* in south Florida after 12 months (Giblin-Davis, 2000). Chen (1996b) incorporated 0, 1,000, 3,000, 10,000 and 100,000 endospores of *P. penetrans* per gram of soil in microplots infested with *M. arenaria* race 1, and then planted peanut. Following harvest (125 days), root and pod galls were reduced by 60 and 95%, respectively, in soil inoculated with 100,000 endospores/g of soil compared to untreated control plots. The following year, peanut were replanted into the same microplots without additional endospore inoculum, root and pod galls were reduced by 61% and 82% and by 81% and 90% respectively, in the 10,000 and 100,000 endospores/g treatments compared to untreated control plots. Increased effectiveness in the treatment with 10,000 endospores/g indicates possible natural amplification occurring in the soil. No difference was observed in J2 populations at harvest in either year. Spore attachment to the nematode most likely inhibits root penetration which reduced root and pod galls. While high levels of endospore inoculum (100,000 endospores/g of soil) may be necessary to reduce damage by an endoparasitic nematode, which is only exposed to endospores in the soil for 2 to 3 days, ectoparasitic nematodes such as *B. longicaudatus* could require lower levels of endospores to reduce root damage. In addition, *P. penetrans* and CPu have shown they can both increase soil suppressiveness to their nematode hosts. However, the time required to achieve suppressive levels will mostly be different since CPu only produces about 2,500 endospore per cadaver, while *P. penetrans* produces approximately 2,000,000 per cadaver (Giblin-Davis et al, 2001; Preston et al. 2003).

Bekal et al. (2001) conducted an attachment test of in vivo CPu endospores (Strain S-1) and revealed the number of endospores attached per nematode and the percent of the nematode population with spores attached to the cuticle were lower (61% and 27% respectively) in
nematode populations from North Carolina, Arkansas, and Lake Alfred, FL., when compared to populations of nematodes from California, Georgia, Fort Lauderdale, and Gainesville, FL. 

Recently, Gozel et. al. (2006) examined 33 populations of *B. longicaudatus* obtained throughout Florida to determine the phylogeny of the species. Three clades of *B. longicaudatus* were observed with overlapping geographical regions moving east to west across the peninsula. The genetic diversity among *B. longicaudatus* populations in Florida and other locations may explain the reduced number of endospores attached per nematode and percent of the nematode population with spores attached (Bekal et al., 2001). Similarly, the diversity of *Pasteuria* endospores that attach to *B. longicaudatus* may show varying degrees of attachment and pathogenicity on *B. longicaudatus* (Davies et al., 1994). ‘Candidatus Pasteuria usgae’ shows great potential as a biological control agent of *B. longicaudatus*. However, more research is needed to elucidate the relationship of CPu with *B. longicaudatus* in its soil environment.
CHAPTER 3
INFECTIVITY OF IN VITRO PRODUCED PASTEURIA ENDOSPORES ON
BELONOLAIMUS LONGICAUDATUS

Introduction

In recent years, environmental awareness has focused attention on heavy users of water, fertilizers, and pesticides (Haydu and Hodges, 2002). Lately, nematode management has been perceived by the turf industry as a growing problem due to the limited number of effective post-plant nematicides currently available. The most damaging nematode on turfgrasses in Florida is Belonolaimus longicaudatus (sting nematode) (Crow, 2005).

In search of new and novel management strategies, researchers have continued to investigate the utility of biocontrol agents. Among biocontrol agents studied, Pasteuria spp. have been recognized as having great potential for the biological control of nematodes (Dickson et al., 1994). Field plots inoculated with ‘Candidatus Pasteuria usgae’ (CPu) demonstrated a significant reduction of B. longicaudatus populations after 13 months (Giblin-Davis, 2000). Research conducted on peanut showed that root and pod galls from Meloidogyne arenaria were reduced by 60 and 95%, respectively in soil inoculated with Pasteuria penetrans at 100,000 endospores/g of soil compared to nontreated control plots. The following year, peanut were replanted into the same microplots without additional endospore inoculum, root and pod galls were reduced by 61% and 82% and by 81% and 90%, respectively in the 10,000 and 100,000 endospores/g treatments compared to untreated control plots. (Chen et. al., 1996b).

Previously, CPu was cultivated on B. longicaudatus grown in aseptic root culture, greenhouse cultures, or collected from suppressive sites in the field (Giblin-Davis et al., 1990; Bekal et al., 2001). Recently, Pasteuria Bioscience LLC developed an in vitro method of culturing Pasteuria spp. that may allow for it to be commercialized as a biopesticide. In vitro produced Pasteuria spp. endospores have more variable spore diameters than those occurring in
vivo. Giblin-Davis et al., (2001) described mature CPu endospores produced in vivo having a mean core diam. of 1.54 ± 0.24 µm and a mean sporangium diam. of 6.05 ± 0.36 µm. In vitro produced endospore (IVP) measurements indicate that mean core diam. is consistent with these published measurements, while mean sporangium diam. is variable. In vitro sporangium diam. variability may be due to nutrient availability and duration of the fermentation process (Smith, 1998). In vitro sporangium diam. differences appear to be due to peripheral fiber development and stage of sporogenesis of each endospore, when the in vitro process is halted. The ability of IVP endospores to be used as an inundative control for _B. longicaudatus_ populations warrants further investigation. The objective of this research was to determine a level of IVP endospores that suppress _B. longicaudatus_ populations below damaging numbers and to determine if sporangia size effects efficacy of IVP endospores.

**Materials and Methods**

Two experiments were conducted and replicated simultaneously in a growth room on the campus of the University of Florida in Gainesville, FL from February to May 2008. Both experiments used in-vitro endospores produced from an isolate of _Pasteuria_ spp. that was collected and cultured from _B. longicaudatus_ on turf from Sebring, Florida. The endospores were obtained from Pasteuria Bioscience LLC (Alachua, FL) and refrigerated at 4 °C for 3 d to allow time to quantify endospores/ml and determine endospore core and sporangia size. In-vitro endospore measurements indicate that mean core diam. is consistent with previously published measurements for CPu, however mean sporangium diam. is variable (Giblin-Davis et al., 2001). Furthermore, molecular identification of the _Pasteuria_ isolate was unsuccessful. Data for these experiments were collected over an 84 d period for each trial.
**Rate Study**

Sixty clay pots (15-cm-diam.; 20-cm-high; 1500-cm$^3$-volume) were cleaned and autoclaved. This experiment utilized a randomized complete block design consisting of four endospore levels (0, 28,000, 56,000, 140,000, and 280,000 endospores/cm$^3$ of soil) at three observation times (28, 56, and 84 d) with four replications. The respective endospore treatments were prepared as a liquid suspension (100 ml) of water, growth media, and endospores. Each endospore treatment was added to a plastic bag containing 1430-cm$^3$ nematode-free United States Golf Association (USGA) specification sand (Anonymous, 1993), gently hand mixed for two minutes, and then potted. ‘Penncross’ creeping bentgrass (*Agrostis palustris* Huds.) was seeded at 98 kg/ha (0.14 g/pot) and allowed to germinate and establish a root system for 13 d before being inoculated with nematodes. Experimental units were keep in a growth room and maintained at 27 °C ± 3 °C with a light period of 14 hr/d.

Following turf establishment, *B. longicaudatus* were extracted using the decant and sieve method (Cobb, 1918) from pure nematode populations maintained on ‘FX313’ St. Augustine grass (*Stenotaphrum secundatum* [Walt.] Kuntze), an excellent host for this nematode (Busey et al., 1993). Nematode population density was determined by counting the *B. longicaudatus* in 1-ml aliquots on a counting slide (Hawksley and Sons Limited, Lancing, Sussex, UK.) Nematode counts were replicated five times. Nematode inoculum was pipetted into four holes (1-cm-diam. x 2.5-cm-deep) in the soil at 450 ± 35 mixed-life stages nematodes/pot (31.5 ± 2.5 nematodes/100 cm$^3$ of soil).

Turf was watered twice/d with 25 ml of water. Turf was fertilized every 2-wk with Peters® 20-20-20 (N-P$_2$O$_5$-K$_2$O) fertilizer (United Industries Corp., St. Louis, MO). Nutrient inputs were 10.9 kg/ha N, 4.9 kg/ha P, 9.1 kg/ha K (0.020 g/pot N, 0.009 g/pot P, and 0.017 g/pot K), and trace amounts of essential micronutrients. Turf was trimmed to 3-cm once/wk.
Nematode populations and root lengths were assessed with destructive sampling 28, 56, and 84 d after nematode inoculation. Nematode and root samples were obtained from a single core sample (5.08-cm-diam.) from the middle of each pot. The sample extended from the soil surface to the bottom of the pot (15.0-cm deep). Each sample was placed onto a 135-µm sieve. The roots were rinsed with water and the sand and nematodes collected. Rinsates were agitated and nematodes extracted by centrifugal-flotation (Jenkins, 1964) using a 25-µm sieve to catch *B. longicaudatus* that were present. Nematodes were collected and counted using an inverted light microscope x40 magnification. Subsequently, 20 nematodes were randomly selected from each sample and the number of attached endospores were counted (Chen et al., 1996a). Roots were collected, placed into a 50 ml conical polypropylene tube, submerged in water, and refrigerated at 4 °C for 1 to 2 days until the samples could be scanned. Unfortunately, prior to being scanned, the 56 d root samples were thrown out by staff. Root samples were placed into a clear plastic tray and scanned with Epson Perfection 4990 Photo Desktop Scanner (Epson, America Inc., Long Beach, CA) to obtain bitmap images of the root system (Pan and Bolton, 1991). The bitmap images were imported into the WinRhizo (Regent Instruments, Chemin Sainre-Foy, Quebec) software program for analysis. This program is designed to determine root length in millimeters. All data were tested for normality and homoscedasticity without issue. Data were subjected to regression analysis using SAS (SAS Institute, Cary, NC).

**Sporangium Size Study**

*Pasteuria* sp. endospores produced from two in vitro batches obtained from Pasteuria Bioscience were the experimental treatments in this experiment. Each treatment was shaken for 20 sec., a subsample obtained, and five permanent slide mounts were made for each treatment. Subsequently, 20 endospores from each slide were randomly selected to measure core and sporangium diam. at x1000 magnification. Individual endospores were designated large or small.
based on sporangium diameter. Endospores with sporangium diameters \( \geq 4 \mu m \)-diam. were considered large and \(< 4 \mu m\)-diam. small. One batch was determined to have 25% large endospores; the second had 45% large endospores.

Experimental design was a randomized complete block consisting of three endospore treatments (Nontreated control; 25% large endospores; and 45% large endospores) with five replications. Endospore treatments were each incorporated at 280,000 endospores/cm\(^3\) of soil. Subsequent steps of establishment, nematode inoculation, and turf maintenance were conducted as described for the rate study; except nematode counts and root lengths were assessed only once at 84 d after nematode inoculation. All data sets were tested for normality and homoscedasticity without issue. Analysis of variance (ANOVA) and Fisher’s LSD were performed to compare counts of \( B. \ longicaudatus \), percent endospore attachment, and root lengths among treatments using SAS (SAS Institute, Cary, NC).

**Results**

**Rate Study**

All inoculum levels suppressed \((P \leq 0.05)\) \( B. \ longicaudatus \) populations compared to the noninoculated control at all sampling dates. Endospore rate was negatively correlated with \( B. \ longicaudatus \) at all dates and the slopes increased negatively at each date. In trial one, at 28,000 endospores/cm\(^3\) of soil, nematode populations were suppressed by 20%, 26%, and 46% and at 280,000 endospores/cm\(^3\) of soil, nematode populations were suppressed by 46%, 58%, and 74% at 28, 56, and 84 d, respectively compared to the noninoculated control (Figure 3-1 A-C). Results of trial two were similar to those in trial one, with 28,000 endospores/cm\(^3\) of soil, nematode populations were suppressed by 5%, 22%, and 44% and with 280,000 endospores/cm\(^3\) of soil, nematode populations were suppressed by 45%, 56%, and 74% at 28, 56, and 84 d, respectively compared to the noninoculated control (Figure 3-2 A-C). In both trials, there were no differences
in endospore attachment among inoculation levels \( (P \geq 0.05) \) (Figure 3-3 A-C, Figure 3-4 A-C) and inoculation with IVP did not improve root length compared to the noninoculated treatment (Figure 3-5 A-B, Figure 3-6 A-B).

**Sporangium Size Study**

Both endospore treatments suppressed \( (P \leq 0.05) \) *B. longicaudatus* populations compared to the nontreated control but there were no differences among the size treatments. The 25% and 45% large endospore treatments suppressed nematode populations by 67% and 75% during trial one and 61% and 51% during trial two, respectively (Table 3-1). Differences \( (P \leq 0.05) \) in total root length were observed in the 45% large endospore compared to nontreated control. In both trials, the 45% large endospore treatments suppressed nematode populations allowing for increased root retention by 54% and 30% during trials one and two, respectively (Table 3-1). No treatment differences in endospore attachment were observed among the spore-size treatment \( (P \geq 0.05) \) (Table 3-1).

**Discussion**

At all dates, increasing endospore levels of IVP suppressed *B. longicaudatus* populations. The highest level of endospores suppressed *B. longicaudatus* populations to near the initial inoculum level of 31.5 nematodes/100 cm\(^3\) at all dates. The negative slope of the regression line between nematodes and endospores levels decreased as the study progressed due to increases in nematode populations at the lower endospore levels.

Studies conducted previously indicated that 5,000 CPu endospores/cm\(^3\) of soil could reduce *B. longicaudatus* populations to very low numbers over an 18-month period (Giblin-Davis, 2000). These differences may result from several factors. First, treatment differences in previous studies were not observed until 13 months after endospore inoculation. These experiments have shown that higher levels of IVP endospores were required to suppress *B.*
Belonolaimus longicaudatus population within a shorter 12-wk period. Second, while the Pastueria sp. isolate used in these experiments was collected and cultured from B. longicaudatus, we were unable to confirm its identity as CPu (Giblin-Davis, 2000). Isolate differences in pathogenicity, virulence, or percent endospore attachment may have contributed to higher levels of IVP endospores being required to suppress B. longicaudatus populations (Davies et al., 1994; Bekal et al., 2001).

Lastly, bacterial virulence is multifaceted and can only be completely manifested in vivo (Smith, 1998). Up or down regulation of genes in vitro have been shown to affect the virulence of Salmonella typhimurium, Aeromonas salmonicida, and many other bacteria (Buchmeiner and Heffron, 1990; Thornton et al., 1993). In vitro production of Pasteuria spp. may reduce virulence and effectiveness of IVP to control B. longicaudatus.

The finding that increased IVP endospore density did not increase percent attachment differs from studies with P. penetrans (Chen et. al., 1996b). However, the P. penetrans studies investigated endoparasitic nematodes (i.e. Meloidogyne spp.) and involved endospore attachment of second-stage juveniles before root penetration. Belonolaimus longicaudatus is an ectoparasitic nematode remaining in the soil, with all life stages observed. Belonolaimus longicaudatus is exposed to the endospores throughout its life and the loss of endospores during molts may explain the lack of treatment differences for percent endospore attachment. Further study is required to determine if endospore attachment is a reliable indicator of endospore levels in the soil for B. longicaudatus and other ectoparasitic nematodes.

The lack of improvement in root lengths in the rate studies can be attributed to three factors: (i) nematode inoculation levels, (ii) duration of the experiment, and (iii) host plant. Experiments conducted to determine the tolerance of zoysia (Zoysia japonica Steud.) and bermudagrasses (Cynodon dactylon [L.] Pers. var. dactylon × C. transvaalensis Burtt-Davy) to B.
*longicaudatus* have shown that an inoculation level of 100 nematodes/100 cm³ of soil is needed to show root differences for trials lasting 90 d. Lower inoculum levels may be effective during experiments with a longer duration (Schwartz et al., 2008). Moreover, bentgrass produces a finer and more fibrous root system that bermudagrass, and under similar nematode and environmental conditions, may require more time for root length differences to be observed.

Both endospore size treatments suppressed nematode populations compared to the nontreated control indicating both endospore treatments were effective. The lack of differences between the 25% and 45% large endospore treatments indicates that at least for these levels, endospore size was not relevant to the level of suppression provided. In vitro sporangium diam. did not affect efficacy of IVP endospores. Regardless, Pasteuria Bioscience LLC has continued to improve the in vitro method of producing endospores and this issue of inconsistent endospore size seems to be resolved. While nematode and IVP inoculum levels were consistent with the rate study, turfgrass in these studies experienced some drought stress in addition to nematode feeding, which may explain increased root lengths in the treatment with 45% large endospores, when compared to the nontreated control.

In conclusion, these experiments indicate that high levels of IVP endospores can suppress *B. longicaudatus* populations. Similarly, endospore size related to batch differences does not appear to greatly affect efficacy. In addition, a few nematode cadavers were observed to have vegetative cells or endospores throughout the pseudocoelom, suggesting that recycling of the *Pasteuria* sp. was occurring on *B. longicaudatus*. However, the identity of the *Pasteuria* isolate from Sebring, FL remains unconfirmed.
Table 3-1. Effects of the endospore size of in vitro produced *Pasteuria* sp. on *Belonolaimus longicaudatus* populations, percent endospore attachment, and total root length in pots planted with 'Penncross' creeping bentgrass and grown in a growth room for 84 days after nematode inoculation.

<table>
<thead>
<tr>
<th>Treatments&lt;sup&gt;a&lt;/sup&gt;</th>
<th>*B. longicaudatus/pot&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Percent Endospore Attachment&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Total Root Length&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trial 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>141.1 ± 21.1 a&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>0.0 ± 0.0 a</td>
<td>865.2 ± 117.6 a</td>
</tr>
<tr>
<td>25%</td>
<td>58.0 ± 20.4 b</td>
<td>8.0 ± 7.8 b</td>
<td>1165.0 ± 275.2 ab</td>
</tr>
<tr>
<td>45%</td>
<td>44.0 ± 22.8 b</td>
<td>10.0 ± 5.7 b</td>
<td>1333.5 ± 332.3 b</td>
</tr>
<tr>
<td></td>
<td>Trial 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>131.0 ± 17.4 a</td>
<td>0.0 ± 0.0 a</td>
<td>642.9 ± 84.3 a</td>
</tr>
<tr>
<td>25%</td>
<td>65.5 ± 23.7 b</td>
<td>9.0 ± 8.2 b</td>
<td>790.9 ± 137.7 ab</td>
</tr>
<tr>
<td>45%</td>
<td>51.3 ± 24.1 b</td>
<td>11.0 ± 10.3 b</td>
<td>835.6 ± 176.6 b</td>
</tr>
</tbody>
</table>

<sup>a</sup>One batch of endospores had 25% with sporangium diameters ≥ 4µm (25% large spores) the other batch had 45% with sporangium diameters ≥ 4µm (45% large spores). <sup>b</sup>All plants were inoculated with 31.5 ± 2.25 *B. longicaudatus* per 100 cm<sup>3</sup> of soil. <sup>c</sup>Percent nematodes out of twenty that had at least one endospore attached. <sup>d</sup>Total root length measured in centimeters. <sup>e</sup>Means and standard deviation for five replications. <sup>f</sup>Treatments within a columns with common letters are not different (*P* ≤ 0.05), according to Fisher’s Least Significant Difference procedure.
Figure 3-1. Effects of increasing levels of in vitro produced Pasteuria sp. endospores on Belonolaimus longicaudatus on creeping bentgrass in a growth room, A) 28, B) 56, and C) 84 days after nematode inoculation during trial one. All plants were inoculated with $31.5 \pm 2.5$ B. longicaudatus per 100 cm$^3$ of soil. Each treatment level consists of four replications.
Figure 3-1. Continued.

Figure 3-2. Effects of increasing levels of in vitro produced Pasteuria sp. endospores on Belonolaimus longicaudatus on creeping bentgrass in a growth room, A) 28, B) 56, and C) 84 days after nematode inoculation during trial two. All plants were inoculated with $31.5 \pm 2.5$ B. longicaudatus per 100 cm$^3$ of soil. Each treatment level consists of four replications.
Figure 3-2. Continued
Figure 3-3. Effects of increasing levels of in vitro produced *Pasteuria* sp. endospores on percent endospore attachment to *Belonolaimus longicaudatus* inoculated onto creeping bentgrass in a growth room, A) 28, B) 56, and C) 84 days after nematode inoculation during trial one. All plants were inoculated with 31.5 ± 2.5 *B. longicaudatus* per 100 cm³ of soil. Each treatment level consists of four replications.
Figure 3-3. Continued

Figure 3-4. Effects of increasing levels of in vitro produced *Pasteuria* sp. endospores on percent endospore attachment to *Belonolaimus longicaudatus* inoculated onto creeping bentgrass in a growth room, A) 28, B) 56, and C) 84 days after nematode inoculation during trial two. All plants were inoculated with $31.5 \pm 2.5$ *B. longicaudatus* per 100 cm$^3$ of soil. Each treatment level consists of four replications.
B

\[ y = -0.00001x + 15.128 \]

\[ r^2 = 0.049 \quad CV = 26.3 \]

\[ P \leq 0.2319 \]

C

\[ y = 0.00001x + 15.765 \]

\[ r^2 = 0.0011 \quad CV = 21.5 \]

\[ P \leq 0.9334 \]

Figure 3-4. Continued
Figure 3-5. Effects of increasing levels of in vitro produced *Pasteuria* sp. endospores on total root length of creeping bentgrass inoculated with *Belonolaimus longicaudatus* in a growth room, A) 28, and B) 84 days after nematode inoculation during trial one. All plants were inoculated with $31.5 \pm 2.5$ *B. longicaudatus* per 100 cm$^3$ of soil. Each treatment level consists of four replications.
Figure 3-6. Effects of increasing levels of in vitro produced Pasteuria sp. endospores on total root length of creeping bentgrass inoculated with Belonolaimus longicaudatus in a growth room, A) 28, and B) 84 days after nematode inoculation during trial two. All plants were inoculated with $31.5 \pm 2.5$ $B. longicaudatus$ per $100 \text{ cm}^3$ of soil. Each treatment level consists of four replications.
CHAPTER 4
EFFECTS OF IRRIGATION, THATCH, AND WETTING AGENT ON MOVEMENT OF IN VITRO PRODUCED PASTEURIA ENDOSPORES IN TURF

Introduction

Management of plant-parasitic nematodes in turf has become increasingly difficult over the last decade due to the limited number of effective post-plant nematicides. In particular, *Belonolaimus longicaudatus* (sting nematode) is considered the most destructive nematode on turf in Florida (Crow et al., 2003; Crow, 2005). Biological control of *B. longicaudatus* may offer another management tactic to turfgrass managers in addition to chemical and cultural control methods. ‘*Candidatus Pasteuria usgae*’ (CPu) has been recognized as a naturally occurring biological agent that can suppress *B. longicaudatus* in turf (Giblin-Davis, 2000). Use of in vitro produced endospores (IVP) as a bionematicide for sting nematode is being explored. The movement of IVP endospores in the soil profile may greatly affect its efficacy and success as a biopesticide on turf. In previous experiments, in vivo produced *Pasteuria* spp. endospores have been inoculated into pots, micro-plots, and field plots using various sources of inoculum laden with endospores: ground root material, soil, second stage juvenile nematodes encumbered with endospores, or endospores in water suspension (Stirling and Wachtel, 1980; Dube and Smart, 1987; Chen and Dickson, 1996b; Weibelzahl-Fulton et al., 1996; Giblin-Davis, 2000; Kariuki and Dickson, 2007). However, these methods used disturbance of the soil profile to incorporate the endospore for testing. Unlike seasonal crops that are cultivated regularly allowing for incorporation of endospores throughout the soil profile, established turfgrass is relatively undisturbed. Movement of topically applied endospores into the turfgrass soil profile might be hindered by dense turf growth, thatch, or localized hydrophobic soil conditions (Murray and Juska, 1977; Tan, 1998). Thatch is an intermingled layer of living and dead plant tissues, and decaying organic matter between the turf canopy and soil surface. Excessive thatch layers ($\geq 2.5$...
cm-depth) can affect the movement of air, water, and pesticides (Dunn and Diesburg, 2004). Pesticides applied to control soil pests can be adsorbed and held in the thatch above. Likewise, localized hydrophobic soil conditions can be created when fulvic acid coats soil particles and then dries. The dry fulvic acid then repels water hindering pesticide movement (Tan, 1998). Wetting agents can be used to decrease hydrophobic soil conditions and aid pesticide penetration. Golf course greens are constructed predominately of sand to have high percolation rates and as a result have high leaching potential. Once in the mineral soil, increased percolation due to rainfall or irrigation following treatment may affect the distribution of endospores or cause endospores to be moved below the turf root zone (upper 10 cm of soil), reducing effectiveness (Cetintas and Dickson, 2005; Dabiré et. al., 2005). The objective of this research was to determine the effect of thatch and wetting agent on movement of IVP endospores into the soil profile and to determine if IVP endospores were subject to leaching from large irrigation or rainfall events.

**Materials and Methods**

A greenhouse experiment was conducted and replicated simultaneously in the Turfgrass Envirotoron at the University of Florida in Gainesville, FL, from August 2007 to August 2008. The ten treatments evaluated were five watering levels: i) 0.6 cm of water with a wetting agent (Lesco Wet®) at 2.54 ml/m², ii) 0.6 cm of water without a wetting agent, iii) 2.5 cm of water without a wetting agent, iv) 7.5 cm of water without a wetting agent, and v) 15.2 cm of water without a wetting agent. Each watering level was tested at two thatch levels: with thatch (2.5-cm depth) or without thatch.

Fifty lysimeters (5.08-cm-diam., 45.5-cm-deep, 927-cm³-volume) were used to simulate a golf course putting green soil profile. In the bottom of the lysimeters was placed 15 cm of gravel...
(2-mm-diam.) covered with an additional 30 cm of nematode-free U.S. Golf Association specification root-zone sand (Anonymous, 1993). Plugs of recently established 'Tifdwarf' bermudagrass (Cynodon dactylon [L.] Pers. var. dactylon × C. transvaalensis Burtt-Davy) were harvested from pots using a soil probe (5.08-cm-diam.) and planted into 25 lysimeters to simulate turf with minimal to no thatch. Conversely, plugs of ‘Tifdwarf’ bermudagrass from a putting green with an extensive thatch layer were harvested and soil removed to simulate turf with a heavy thatch layer. Prior to turf being harvested, nematode and CPu bioassay tests were performed and no B. longicaudatus or CPu were detected. Following planting, approximately 0.25 cm of sand was added to the surface of each lysimeter as top-dressing. During grow-in the turf was watered 3 times/d with 10 ml of water for 3 wk.

Following establishment, the turf was watered daily with 25 ml of water. Turf was fertilized every 2 wk with Peters® 24-4-16 (N-P₂O₅-K₂O) fertilizer (United Industries Corp., St. Louis, MO). Nutrient inputs were 49 kg/ha N, 3.6 kg/ha P, 27.1 kg/ha K/month, along with trace amounts of essential micronutrients. Turf was mowed to 3-cm-height once/wk. The turf was allowed to establish a root system for 7 months before applying endospores.

In-vitro endospores were produced from an isolate of Pasteuria spp. that was collected and cultured from B. longicaudatus on turf from Sebring, Florida. The endospores were obtained from Pasteuria Bioscience LLC (Alachua, FL) and refrigerated at 4 °C for 3 d to allow time to quantify endospores/ml and obtain morphometrics measurements of the IVP endospores. In-vitro endospore measurements indicate that mean core diam. is consistent with previously published measurements for CPu, however mean sporangium diam. is variable (Giblin-Davis et al., 2001). Furthermore, molecular identification of the Pasteuria sp. was unsuccessful. In vitro produced Pasteuria sp. endospores were applied topically at 1,990,000 endospores/cm² of soil. Endospores
were applied to all lysimeters as a drench using 0.6 cm (12 ml) of water, spore media, and wetting agent if assigned. Following treatment the lysimeters remained undisturbed for 2 d, and then water treatments were applied once.

One week after the water treatments were applied, destructive sampling occurred. The entire soil profile (5.08-cm-diam., 30-cm-deep) was removed from each lysimeter. These soil profiles were cut into sections to determine endospore presence at four soil depths (0 to 2.5 cm, 2.5 to 10 cm, 10 to 20 cm, and 20 to 30 cm-deep) providing four depth samples per soil profile. Each sample was placed in a paper bag and allowed to air dry.

After drying, each sample was gently mixed, and a subsample (100 g) was placed onto a coffee filter within a sterile polyethylene container (6.5-cm-wide, 6.5-cm-length, 10-cm-deep). Twenty-five ml of water were added to each sample to rehydrate the soil allowing for increased nematode movement (Brown and Smart, 1984). Two hundred mixed life stages of *B. longicaudatus* in 5 ml of water were added and the containers left uncovered at room temperature. Three days later the nematodes were extracted from the soil using the centrifugal-flotation method (Jenkins, 1964). Subsequently, twenty nematodes were randomly selected from each sample population and number of endospores attached per nematode was made by observation with an inverted microscope at ×400 magnification (Chen and Dickson, 1997).

Data for thatch and wetting agent were subjected to analysis of variance (ANOVA) and orthogonal contrast with SAS software (SAS Institute, Cary, NC). Microsoft Excel (Microsoft, Redmond, WA) was used for regression analysis. Data for percent endospore attachment were transformed using natural log (\( \ln \)) before statistical analysis. Transformed numbers are presented in the figures.
Results

During trial one, increasing the amount of irrigation reduced ($P \leq 0.01$) percent endospore attachment in soil depths 0 to 2.5 and 2.5 to 10 cm and increased ($P \leq 0.01$) percent endospore attachment at soil depths 10 to 20 and 20 to 30 cm, compared to 0.6 cm of irrigation. Application of 2.5, 7.6, and 15.2 cm of irrigation reduced percent endospore attachment by 16, 47, and 73%, respectively compared to 0.6 cm of irrigation at soil depth 0 to 2.5 cm (Figure 4-1 A). Similarly, irrigation treatments reduced percent endospore attachment by 8, 26, and 46%, respectively compared to 0.6 cm of irrigation at soil depth 2.5 to 10 cm (Figure 4-1 B). However, at soil depth 10 to 20 cm, irrigation treatments increased percent endospore attachment by 12, 51, and 136%, respectively compared to 0.6 cm of irrigation (Figure 4-1 C). At the deepest soil depth (20 to 30 cm) irrigation treatments increased percent endospore attachment by 54, 359, and 2512%, respectively compared to 0.6 cm of irrigation (Figure 4-1 D). The placement of endospores within the root zone (0 to 10 cm soil depth) with one application of a 0.6 cm of irrigation should make endospore applications, relatively easy. Thatch and wetting agent treatments showed no effect ($P \leq 0.05$) on placement of endospores into the soil profile (Table 4-1).

Results of trial two were similar to those of trial one, application of 2.5, 7.6, and 15.2 cm of irrigation reduced percent endospore attachment by 12, 37, and 62%, respectively compared to 0.6 cm of irrigation at soil depth 0 to 2.5 cm (Figure 4-2 A). Similarly, irrigation treatments reduced percent endospore attachment by 6, 21, and 39%, respectively compared to 0.6 cm of irrigation at soil depth 2.5 to 10 cm (Figure 4-2 B). However, at soil depth 10 to 20 cm, irrigation treatments increased percent endospore attachment by 9, 38, and 95%, respectively compared to 0.6 cm of irrigation (Figure 4-2 C). At the deepest soil depth (20 to 30 cm) irrigation treatments increased percent endospore attachment by 52, 358, and 2297%, respectively compared to 0.6 cm of irrigation (Figure 4-2 D).
cm of irrigation (Figure 4-2 D). Thatch and wetting agent treatments showed no effect ($P \leq 0.05$) on placement of endospores into the soil profile (Table 4-1).

**Discussion**

Water is the basic method of bacteria dispersal throughout the soil profile and is a major reason for *Pasteuria* spp. endospore losses from the cultivated soil horizon (Gammack et al., 1992). One application of 0.6 cm of irrigation was sufficient to place IVP endospores within the turfgrass root zone (0 to 10 cm soil depths), with some endospores moving into the 10 to 20 cm depth. A slight reduction in irrigation to 0.5 cm might prevent the movement of endospores below the root zone, concentrating the endospores were most nematodes are actively feeding and causing damage. Previous research has shown that repeated applications of water could leach *Pasteuria* spp. endospores, reducing endospore levels and effectiveness (Zyman and Sorber, 1988; Cetintas and Dickson, 2005; Dabiré et. al, 2005). In our studies, a single application of water simulating a large rainfall event was sufficient to move many of the IVP endospores below the turf root zone. Timing of application may need to be scheduled to times of the year when rainfall is minimal. In Florida, this might be best accomplished during early fall and spring. Reapplication of IVP following rainfall events might be required to maintain endospores at sufficiently high levels to cause nematode reductions.

Thatch did not hinder the movement of IVP endospores into the soil profile and is unlikely to impact its utility as a bionematicide. Wetting agents are used to improve the movement of water and other liquids into the soil profile, especially when the soil is hydrophobic. In this experiment, turf was watered daily, preventing the soil from becoming dry or hydrophobic. However in the field, wetting agents might help to provide a more even distribution of IVP endospores by creating conditions more favorable to water movement. Because the wetting agent
did not increase leaching of IVP endospores, it should not have a detrimental effect on spore movement in turf.

In conclusion, greenhouse studies indicate irrigation has a great impact on the placement of IVP endospores in turf and thereby can affect its utility as a bionematicide. Movement of topically applied IVP endospores into the turfgrass root zone is likely achieved by routine irrigation practices. Conversely, too much irrigation or rainfall might leach IVP spores out of the turf root zone and thereby reduce efficacy. Clearly water management is critical for placement and retention of IVP and other biopesticides in the typically sandy soils of turf.
Table 4-1. Effect of thatch or wetting agent on movement of topically applied in vitro produced *Pasteuria* sp. in greenhouse lysimeters planted with “Tifdwarf” bermudagrass at four soil depths determined by bioassay of endospore attachment to *Belonolaimus longicaudatus*. Thatch data pooled across irrigation levels because thatch and irrigation level interaction was not significant (\(P \geq 0.05\)).

<table>
<thead>
<tr>
<th>Soil Depth (cm)</th>
<th>Thatch(^a)</th>
<th>No Thatch</th>
<th>(P)</th>
<th>Wetting Agent(^b)</th>
<th>No Wetting Agent</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trial 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 to 2.5</td>
<td>11.18 ± 10.22(^c)</td>
<td>11.40 ± 12.54</td>
<td>NS(^d)</td>
<td>25.78 ± 2.91(^e)</td>
<td>25.94 ± 3.08</td>
<td>NS</td>
</tr>
<tr>
<td>2.5 to 10.0</td>
<td>20.62 ± 5.58</td>
<td>18.11 ± 8.28</td>
<td>NS</td>
<td>22.21 ± 3.48</td>
<td>22.36 ± 4.63</td>
<td>NS</td>
</tr>
<tr>
<td>10.0 to 20.0</td>
<td>17.19 ± 7.67</td>
<td>16.50 ± 8.06</td>
<td>NS</td>
<td>1.50 ± 3.34</td>
<td>1.50 ± 3.37</td>
<td>NS</td>
</tr>
<tr>
<td>20.0 to 30.0</td>
<td>9.97 ± 9.33</td>
<td>11.87 ± 8.43</td>
<td>NS</td>
<td>1.03 ± 2.16</td>
<td>0.00 ± 0.00</td>
<td>NS</td>
</tr>
<tr>
<td>All depths</td>
<td>14.74 ± 8.97</td>
<td>14.47 ± 9.41</td>
<td>NS</td>
<td>12.63 ± 11.94</td>
<td>12.45 ± 12.33</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Trial 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 to 2.5</td>
<td>20.28 ± 8.79</td>
<td>12.79 ± 7.93</td>
<td>NS</td>
<td>28.05 ± 8.39</td>
<td>24.09 ± 4.64</td>
<td>NS</td>
</tr>
<tr>
<td>2.5 to 10.0</td>
<td>15.55 ± 4.96</td>
<td>17.28 ± 7.98</td>
<td>NS</td>
<td>18.08 ± 4.16</td>
<td>19.41 ± 2.96</td>
<td>NS</td>
</tr>
<tr>
<td>10.0 to 20.0</td>
<td>9.19 ± 8.16</td>
<td>13.71 ± 4.12</td>
<td>NS</td>
<td>1.00 ± 2.11</td>
<td>1.50 ± 3.37</td>
<td>NS</td>
</tr>
<tr>
<td>20.0 to 30.0</td>
<td>11.06 ± 10.53</td>
<td>10.23 ± 7.45</td>
<td>NS</td>
<td>0.00 ± 0.00</td>
<td>1.00 ± 2.11</td>
<td>NS</td>
</tr>
<tr>
<td>All depths</td>
<td>14.02 ± 9.29</td>
<td>12.79 ± 7.93</td>
<td>NS</td>
<td>11.78 ± 12.83</td>
<td>11.50 ± 11.01</td>
<td>NS</td>
</tr>
</tbody>
</table>

\(^a\) Thatch layer was 2.54 cm in depth.  
\(^b\) Wetting agent was Lesco Wet \textsuperscript{®} at 2.54 ml/m\(^2\)  
\(^c\) Means and standard deviation for twenty-five replications.  
\(^d\) NS = No statistical difference \(P \leq 0.05\).  
\(^e\) Means and standard deviation for five replications.
Figure 4-1. Effect of increasing irrigation on depth placement of in vitro produced *Pasteuria* sp. endospores in soil columns as determined by bioassay with *Belonolaimus longicaudatus* during trial 1. Soil depth ranges evaluated were: A) 0 to 2.5 cm, B) 2.5-10 cm, C) 10-20 cm and D) 20-30 cm. Data are means of five replications.
Figure 4-1. Continued

\[ y = 0.0588x + 2.2827 \]
\[ r^2 = 0.4742 \quad CV = 20.4 \]
\[ P \leq 0.0008 \]

\[ y = 0.2227x + 0.2611 \]
\[ r^2 = 0.7594 \quad CV = 38.8 \]
\[ P \leq 0.0001 \]
Figure 4-2. Effect of increasing irrigation on depth placement of in vitro produced *Pasteuria* sp. endospores in soil columns as determined by bioassay with *Belonolaimus longicaudatus* during trial 2. Soil depth ranges evaluated were: A) 0 to 2.5 cm, B) 2.5-10 cm, C) 10-20 cm and D) 20-30 cm. Data are means of five replications.
Figure 4-2. Continued
CHAPTER 5
VERSATILITY OF IN VITRO PRODUCED PASTEURIA ENDOSPORES TO SUPPRESS BELONOLAIMUS LONGICAUDATUS

Introduction

Pasteuria spp. is a group of Gram-positive endospore-forming bacteria having great potential as biological control agents of plant-parasitic nematodes (Dickson et al., 1994). A major obstacle to the commercial development of these bacteria as biological control agents is their limited host range. Isolates of Pasteuria penetrans were found to not only exclusively attach to a particular Meloidogyne species, but also had different levels of attachment among isolates within a Meloidogyne species (Stirling 1985; Davies et al. 1988). The S-1 strain of ‘Candidatus Pasteuria usgae’ (CPu) was shown to have differing levels of attachment to different populations of Belonolaimus longicaudatus (Bekal et al., 2001). Narrow host-isolate specificity could limit the practicality of in vitro produced endospores (IVP) as a commercial biopesticide (Davies et al. 1988).

Belonolaimus longicaudatus has been shown to have great genetic diversity among populations within Florida and even greater diversity among populations from differing states (Gozel et al., 2006). The genetic diversity observed among populations of B. longicaudatus is most likely due to amphimictic reproduction and geographical population isolation. Similarly, research has shown that there is a high level of diversity within what was regarded as a relatively homogeneous endospore population of P. penetrans (Davies et al., 1994). However, in vivo culturing of the bacterium on a particular nematode host can reduce the diversity present within the original Pasteuria population (Davies et al., 1994). If Pasteuria spp. is to be used successfully for control of nematodes, it is important to know the effect of diversity of both the target nematode and the hyperparasite (Davies et al., 1994). The objective of this research was to
determine if IVP exhibits host-parasite isolate specificity in its ability to manage *B. longicaudatus*.

Initially, *Pasteuria* spp. identification was accomplished using morphology, ultrastructure, host range, and life cycle (Chen and Dickson, 1998). However, the reliability of these methods for accurate identification has been challenged (Cianco, 1995). More than 300 nematode species have been reported with *Pasteuria* spores attached to their cuticle, but only four *Pasteuria* species have been described in detail. Many of the undescribed *Pasteuria* spp. may have overlapping morphometrics and host ranges, making accurate species identification increasingly difficult (Chen and Dickson, 1998). Furthermore, among the nematode genus *Belonolaimus*, considerable overlap of morphometrics measurements can be observed among species (Rau, 1958, 1961, 1963). Therefore, for these studies molecular analysis of internal transcribed region (ITS) and 16S were preformed to confirm the respective identification of the nematode host and hyperparasite, as well as to determine if genetic diversity exists between the isolates to be used in these experiments.

**Materials and Methods**

**Isolate Maintenance**

Two clay pots (15-cm-diam.; 20-cm-high; 1500-cm³-volume) were cleaned, autoclaved, and then filled with 1430 cm³ of nematode free USGA sand. ‘Penncross’ creeping bentgrass (*Agrostis palustris* Huds.) was seeded at 98 kg/ha (0.14 g/pot) and allowed to germinate and establish a root system for 13 d before being inoculated with nematodes. Isolates of *B. longicaudatus* collected from Jefferson, SC and Sun City, FL were extracted using the decant and sieve method (Cobb, 1918). Nematodes were hand-picked and then inoculated into the soil at 500 mixed-life stages nematodes/pot; every 3 months the pure cultures of *B. longicaudatus* were
extracted, counted, and inoculated onto new pots; increasing the number of pots each time to increase the nematode population available for the experiment.

Five isolates of *Pasteuria* spp. were collected and cultured from *B. longicaudatus* on turf from Sebring, FL; Gainesville, FL; The Villages, FL; Columbia, SC; and Conway, SC. Pasteuria Bioscience LLC (Alachua, FL) maintained the isolate collection until in vitro endospores were produced for this experiment. The methods for maintenance and production of in vitro endospore are proprietary to Pasteuria Bioscience LLC. Following in-vitro production, the endospores were obtained from Pasteuria Bioscience LLC and refrigerated at 4 °C for 3 d to allow time to quantify endospores/ml and determine endospore core and sporangia size. In-vitro endospore measurements indicate that mean core diam. is consistent with previously published measurements for ‘*Candidatus Pasteuria usgae*’, however mean sporangium diam. is variable (Giblin-Davis et al., 2001).

**Belonolaimus rDNA Analysis**

For molecular analysis, DNA was extracted from individual female nematodes for each population using Isohair extraction kit (Nippon Gene Co. LTD., Toyama, Japan). Ribosomal DNA of the ITS was PCR amplified using the 18S (5’-TTG ATT ACG TCC CTG CCC TTT-3’) (forward) and 26S (5’-TTT CAC TCG CCG TTA CTA AGG -3’) (reverse) primers designed by Vrain (1993) which bind to the posterior 3’ portion of the 18S small ribosomal subunit (forward), and the 5’ end of the 28S subunit region (reverse). Polymerase chain reactions were carried out in 30 μl-volumes. The following PCR components were added to each tube: 15.0 μl of GoTaq® Green Master Mix (Promega Corp., Madison, WI), 1.5 μl of 10 pM forward primer, 1.5 μl of 10 pM reverse primer (Intergrated DNA Technologies, Coralville, IA), 11.0 μl of distilled water and 1 μl of DNA. All PCR reactions were run in a icycler (BioRad Laboratories, Inc., Hercules, CA) with the cycling sequence: 1 cycle of 94 °C for 7 min followed by 35 cycles of 94 °C for 1 min,
55 °C for 1 min, 72 °C for 1 min. The final step was 72 °C for 10 min. PCR products were visualized by ethidium bromide staining (0.3 μg/ml) in 1.5% agarose gel. The resultant PCR products were purified using the Montage® PCR centrifugal filter kit (Millipore Corp., Billerica, MA). All products were sequenced at the University of Florida ICBR sequencing core facility on Perkin Elmer/Applied Biosystems automated DNA sequencers. The primers used for sequencing were the same used for PCR amplification. Sequences were edited using Sequencher (4.1.2 Gene Codes Corp., Ann Arbor, MI). The sequences obtained in this study were aligned to each other and the outgroup taxon *Pratylenchus coffeae* (GenBank accession #AF170443) using the default parameters of Clustal X 1.83 (Thompson *et al.*, 1997). The alignments were adjusted manually in MacClade 4.0 (Maddison and Maddison, 2000). Data was transferred to PAUP 4.0b8 to determine phylogenetic relationships using maximum parsimony, maximum likelihood, and evolutionary distance (Swofford, 2001). Bootstrapping was conducted with 1000 replications per analysis (Figure 5-1).

**Pasteuria rDNA Analysis**

In vitro produced endospores (1 x 10⁷) were pipetted into a sterile 1.5 ml eppendorf tube and centrifuged at 14,000 g for 10 minutes to produce a pellet, the supernatant (in vitro media) was removed. The endospores were re-suspended in 1 ml of 10 mM Tris, 1.0 mM EDTA, pH 8.0, containing 20 mg/ml lysozyme, and incubated at 37 °C for 30 min. The endospores were transferred to a 2.0-ml impact-resistant tube containing approximately 1 g of glass beads (100 μm-diam.) (Biospec Products Inc., Bartlesville, OK). The endospores were pulverized using a Mini-beadbeater (Biospec Products, Inc., Bartlesville, OK). The mixture was then transferred to a DNeasy blood and tissue kit (Qiagen Inc., Valencia, CA) for further purification. Ribosomal DNA of the 16S region was PCR amplified using the 440f (5’-CAT CGG GAA GAA ATG-3’) (forward) and 1492r (5’-TAC GGT TAC CTT GTT ACG ACT T-3’) (reverse) primers
designed by Lane (1991). Polymerase chain reactions were carried out in 30 μl volumes. The following PCR components were added to each tube: 15.0 μl of GoTaq® Green Master Mix (Promega Corp., Madison, WI), 1.5 μl of 10 pM forward primer, 1.5 μl of 10 pM reverse primer (Integrated DNA Technologies, Coralville, IA), 11.0 μl of distilled water and 1 μl of DNA. All PCR reactions were run in a iCycler (BioRad Laboratories, Inc., Hercules, CA) with the cycling sequence: 1 cycle of 94 °C for 10 min followed by 45 cycles of 94 °C for 1 min, 52 °C for 1 min, 72 °C for 2 min. The final step was 72 °C for 10 min. PCR products were visualized by ethidium bromide staining (0.3 μg/ml) in 1.5% agarose gel. Following these trials, 100 B. longicaudatus from each of the Pasteuria treatments were hand-picked. Nematodes were digested using an Isohair extraction kit (Nippon Gene Co. LTD., Toyama, Japan), incubated at 55 °C for one hour. In vivo produced endospores were then subjected to the same extraction methods, PCR reactions, and gel electrophoresis as above. No PCR products were obtained using the above extraction method or combination of the above steps for in vitro or in vivo produced endospores.

**Experiment Establishment and Sampling**

The experiments were conducted and replicated simultaneously in a growth room on the campus of the University of Florida in Gainesville, FL from May to August 2009. This trial used a factorial design consisting of two B. longicaudatus populations: Jefferson, SC (WP) and Sun City, FL (SUN) and six treatments of IVP endospores collected from: i) Sebring, FL (SBRG), ii) Gainesville, FL (AGRO), iii) The Villages, FL (POLO), iv) Columbia, SC (USC), v) Conway, SC (BR) and vi) nontreated with five replications. Sixty pots (10.16-cm-diam.; 10.16-cm-high; 500-cm³-volume) were cleaned and autoclaved. The respective endospore treatments were prepared as a liquid suspension (50 ml) of water, growth media, and endospores at 280,000 endospores/cm³ of sand. Each endospore treatment was added to a plastic bag containing 400-cm³ nematode-free United States Golf Association specification sand (Anonymous, 1993), gently
hand mixed for two minutes, and then potted. ‘Penncross’ creeping bentgrass was seeded at 98 kg/ha (0.08 g/pot) and allowed to germinate and establish a root system for 13 d before being inoculated with nematodes. Experimental units were kept in a growth room with a light period of 14 hr/d and soil temperature maintained at 24 °C ± 0.5 °C.

Following turf establishment, isolates of *B. longicaudatus* originally from Jefferson, SC and Sun City, FL were extracted using the decant and sieve method (Cobb, 1918) from pure nematode populations maintained on ‘Penncross’ creeping bentgrass. Nematode population density was determined by counting the *B. longicaudatus* in 1-ml aliquots on a counting slide (Hawksley and Sons Limited, Lancing, Sussex, UK.) Nematode counts were replicated five times. Nematode inoculum was pipetted into two holes (1-cm-diam. x 2.5-cm-deep) in the soil at 120 ± 6 mixed-life stages nematodes/pot (30.0 ± 1.5 nematodes/100 cm³ of soil).

Turf was watered twice/d with 10 ml of water. Turf was fertilized every 2-wk with Peters® 20-20-20 (N-P₂O₅-K₂O) fertilizer (United Industries Corp., St. Louis, MO). Nutrient inputs were 12.3 kg/ha N, 5.4 kg/ha P, 10.2 kg/ha K (0.010 g/pot N, 0.004 g/pot P, and 0.008 g/pot K), and trace amounts of essential micronutrients. Turf was trimmed to 3-cm height once per wk.

Nematode populations and root lengths were assessed with destructive sampling 84 d after nematode inoculation. The entire soil profile of each pot was used to obtain nematode and root samples. Each sample was placed onto a 135-µm sieve. The roots were rinsed with water and the sand and nematodes collected. Rinsates were agitated and nematodes were extracted by centrifugal-flotation (Jenkins, 1964) using a 25-µm sieve to catch any *B. longicaudatus* present. Nematodes were collected and counted using an inverted light microscope x40 magnification. Subsequently, 20 nematodes were randomly selected from each sample and numbers of attached endospores were counted (Chen et al., 1996a). Roots were collected and then placed into a clear
plastic tray and scanned with Epson Perfection 4990 Photo Desktop Scanner (Epson, America Inc., Long Beach, CA) to obtain bitmap images of the root system (Pan and Bolton, 1991). The bitmap images were imported into the WinRhizo (Regent Instruments, Chemin Sainre-Foy, Quebec) software program for analysis. This program is designed to determine root length in millimeters. All data sets were tested for normality and homoscedasticity without issue. Analysis of variance (ANOVA) and Fisher’s LSD were performed to compare counts of *B. longicaudatus*, percent endospore attachment and total root lengths for main effects and interactions using SAS (SAS Institute, Cary, NC).

**Results**

No interaction between *B. longicaudatus* populations and isolates of IVP endospores were observed for counts of *B. longicaudatus*, percent endospore attachment, or total root lengths. Therefore, the data for IVP isolates were pooled among *B. longicaudatus* populations. No differences (P ≥ 0.05) were observed among isolates of IVP endospores for number of *B. longicaudatus* and percent endospore attachment (Table 5-1). However, all isolates of IVP endospores reduced numbers of *B. longicaudatus* compared to the nontreated control (P ≤ 0.05). Isolates of IVP endospores reduced *B. longicaudatus* populations by 69% to 71%, during trial 1, and 71% to 72% during trial 2, compared to nontreated control. No difference (P ≥ 0.05) was observed among IVP treatments for total root length (Table 5-1).

**Discussion**

Phylogenetic analysis of the ITS region of *B. longicaudatus* indicated genetic divergence between the WP and SUN populations (Figure 5-1). However, these IVP trials revealed no difference between *B. longicaudatus* populations for any data collected. While the ITS regions are well suited for distinguishing between species or populations within a species (Cherry et al.,
1997) it appears that differences in the ITS regions of *B. longicaudatus* do not address the ability of IVP endospores to attach or control nematode populations.

All isolates of IVP endospores suppressed *B. longicaudatus* populations equally. The lack of differences between geographically distinct isolates of IVP endospores was unexpected. Nevertheless, without phylogenetic data available for the isolates of IVP endospores, we cannot address their apparent lack of diversity. Previous research has revealed that in vivo culturing of *Pasteuria* on a particular nematode host can reduce the diversity of the bacterium (Davies et al., 1994). Furthermore, in vitro culturing of bacteria has been shown to affect the virulence of many bacteria (Buchmeiner and Heffron, 1990; Thornton et al., 1993). In vitro production may affect genetic diversity and virulence of IVP endospores explaining the relatively uniform suppression of *B. longicaudatus*.

The lack of improvement in root lengths in these trials can be attributed to three factors: i) nematode inoculation levels, ii) duration of the experiment, and iii) host plant. Experiments conducted to determine the tolerance of zoysia- (*Zoysia japonica* Steud.) and bermuda-grasses (*Cynodon dactylon* [L.] Pers. var. *dactylon × C. transvaalensis* Burtt-Davy) to *B. longicaudatus* have shown that an inoculation level of 100 nematodes/100 cm$^3$ of soil is needed to demonstrate root differences for trials lasting 90 d (Schwartz et al., 2008). Lower inoculum levels may be effective during experiments with a longer duration (Schwartz et al., 2008). Moreover, bentgrass produces a finer and more fibrous root system than bermudagrass, and under similar nematode and environmental conditions may require more time for root length differences to be observed.

In conclusion, these experiments indicate that geographical differences between the *B. longicaudatus* tested will likely not affect the efficacy of a bionematicide based on IVP endospores. Similarly, the use of IVP endospores from the geographically distinct isolates
collected here does not appear to affect efficacy. The versatility of a biopesticide is paramount. These studies indicate that a bionematicide using IVP may be broadly effective against *B. longicaudatus*. The observation of a few nematode cadavers with vegetative cells or endospores throughout the pseudocoelom suggest that recycling of the *Pasteuria* sp. was occurring on *B. longicaudatus*. However, the identity of the *Pasteuria* isolates used in these experiments remains unconfirmed.
Table 5-1. Effect of isolates of *Belonolaimus longicaudatus* and in vitro produced *Pasteuria* spp. on *Belonolaimus longicaudatus* populations, percent endospore attachment, and total root length in pots planted with ‘Penncross’ creeping bentgrass and grown in a growth room for 84 days after nematode inoculation. Data pooled across main effects because *B. longicaudatus* and *Pasteuria* spp. isolate interaction was not significant (*P* ≥ 0.05).

<table>
<thead>
<tr>
<th>Belonolaimus&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Pasteuria&lt;sup&gt;b&lt;/sup&gt;</th>
<th>B. longicaudatus/pot</th>
<th>Percent Endospore Attachment&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Total Root Length&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Trial 1</td>
<td></td>
</tr>
<tr>
<td>WP</td>
<td>-</td>
<td>149.2 ± 99.4&lt;sup&gt;e&lt;/sup&gt; a&lt;sup&gt;f&lt;/sup&gt;</td>
<td>18.2 ± 4.0 a</td>
<td>1719.7 ± 473.5 a</td>
</tr>
<tr>
<td>SUN</td>
<td>-</td>
<td>144.3 ± 95.1 a</td>
<td>17.8 ± 3.9 a</td>
<td>1510.4 ± 356.8 a</td>
</tr>
<tr>
<td>- Nontreated</td>
<td></td>
<td>355.1 ± 39.6 A&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.0 ± 0.0 A</td>
<td>1466.9 ± 413.2 A</td>
</tr>
<tr>
<td>- SBRG</td>
<td>99.3 ± 13.5 B</td>
<td>21.5 ± 4.7 B</td>
<td>1647.4 ± 292.6 A</td>
<td></td>
</tr>
<tr>
<td>- AGRO</td>
<td>106.3 ± 13.8 B</td>
<td>22.5 ± 2.6 B</td>
<td>1629.7 ± 363.9 A</td>
<td></td>
</tr>
<tr>
<td>- POLO</td>
<td>107.7 ± 13.1 B</td>
<td>21.0 ± 3.9 B</td>
<td>1604.2 ± 206.5 A</td>
<td></td>
</tr>
<tr>
<td>- USC</td>
<td>110.2 ± 25.8 B</td>
<td>22.0 ± 3.5 B</td>
<td>1726.9 ± 589.3 A</td>
<td></td>
</tr>
<tr>
<td>- BR</td>
<td>102.1 ± 17.4 B</td>
<td>21.0 ± 4.0 B</td>
<td>1615.2 ± 622.2 A</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Trial 2</td>
<td></td>
</tr>
<tr>
<td>WP</td>
<td>-</td>
<td>158.9 ± 106.4 a</td>
<td>15.0 ± 5.0 a</td>
<td>1835.5 ± 555.5 a</td>
</tr>
<tr>
<td>SUN</td>
<td>-</td>
<td>162.4 ± 119.0 a</td>
<td>14.8 ± 4.5 a</td>
<td>2009.4 ± 419.3 a</td>
</tr>
<tr>
<td>- Nontreated</td>
<td></td>
<td>398.3 ± 75.0 A</td>
<td>0.0 ± 0.0 A</td>
<td>1791.4 ± 462.6 A</td>
</tr>
<tr>
<td>- SBRG</td>
<td>110.8 ± 16.3 B</td>
<td>17.5 ± 4.9 B</td>
<td>1894.5 ± 441.1 A</td>
<td></td>
</tr>
<tr>
<td>- AGRO</td>
<td>110.1 ± 12.5 B</td>
<td>18.5 ± 4.1 B</td>
<td>1876.5 ± 618.5 A</td>
<td></td>
</tr>
<tr>
<td>- POLO</td>
<td>113.8 ± 17.3 B</td>
<td>18.0 ± 5.4 B</td>
<td>1900.9 ± 637.2 A</td>
<td></td>
</tr>
<tr>
<td>- USC</td>
<td>115.8 ± 15.9 B</td>
<td>17.0 ± 4.8 B</td>
<td>1993.8 ± 556.4 A</td>
<td></td>
</tr>
<tr>
<td>- BR</td>
<td>115.3 ± 16.4 B</td>
<td>18.5 ± 5.3 B</td>
<td>2077.4 ± 457.2 A</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Nematode populations (WP) and (SUN) were collected from golf courses planted with ‘Tifway 419’ bermudagrass in Jefferson, SC and Sun City, FL, respectively. <sup>b</sup>*Pasteuria* isolates were harvested for in vitro culturing from *B. longicaudatus* that were collected from Sebring, FL (SBRG), Gainesville, FL (AGRO), The Villages, FL (POLO), Columbia, SC (USC), and Conway, SC (BR). <sup>c</sup>Percent nematodes out of twenty that had at least one endospore attached. <sup>d</sup>Total root length measured in centimeters. <sup>e</sup>Main effect means and standard deviation for thirty replications. <sup>f</sup>Main effect differences *P* ≤ 0.05 are indicated with different letters within each column for trials 1 and 2. <sup>g</sup>Main effect means and standard deviation for ten replications.
Figure 5-1. Relationships among the two populations of Belonolaimus longicaudatus used in this study, five previously studied B. longicaudatus populations and a representative outgroup taxa based on parsimony analysis of the ITS subunit gene. Branch support indices appear to the left of each estimated node. Bootstrap values for maximum parsimony (1,000 replicates) are italicized. Bootstrap values for maximum likelihood (1000 replicates) are in bold. Bootstrap values for evolutionary distance (1000 replicates) are in parenthesis. Belonolaimus longicaudatus used in our studies were obtained from Jefferson, SC (WP) and Sun City, FL (SUN). The previously studied B. longicaudatus populations originated from: Hartsville, SC (HART); Tifton, GA (GA); Sanford, FL (MF); Sanford, FL (UNI); and Palatka, FL (PAL). The outgroup taxon Pratylenchus coffeae (accession #AF170443) was obtained from National Center for Biotechnology Information Genbank database.
CHAPTER 6
EFFECTS OF FORMULATION AND NEMATODE POPULATION ON THE ABILITY OF IN VITRO PASTEURIA ENDOスポRES TO CONTROL ITS HOST BELONOLAIMUS LONGICAUDATUS

Introduction

In recent years, researchers have continued to investigate the usefulness of biocontrol agents for management of plant-parasitic nematodes. ‘Candidatus Pasteuria usgae’ (CPu) has been recognized as a biological agent that can suppress Belonolaimus longicaudatus in turf (Giblin-Davis, 2000). Previously, CPu was cultivated on B. longicaudatus grown in aseptic root culture, greenhouse cultures, or collected from suppressive sites in the field (Giblin-Davis et al., 1990; Bekal et al., 2001). Recently, Pasteuria Bioscience LLC developed an in vitro method of culturing Pasteuria spp. that may allow it to be commercialized as a biopesticide.

In vivo produced Pasteuria spp. endospores have been applied into soil using various sources of inoculum laden with endospores: ground root material, soil, or second stage juvenile nematodes encumbered with endospores (Stirling and Wachtel, 1980; Dube and Smart, 1987; Chen and Dickson, 1996b; Weibelzahl-Fulton et al., 1996; Giblin-Davis, 2000; Kariuki and Dickson, 2007). Recent research suggests that a liquid spore suspension of in vitro produced endospores (IVP) readily enters the turfgrass soil profile, but also can be leached below the turfgrass root zone with heavy rainfall or irrigation. This leaching may affect the efficacy and success as a biopesticide on turf (Luc et al., unpublished). To counteract IVP leaching from the turf rootzone, Pasteuria Bioscience LLC has developed a clay granular formulation of IVP endospores. This formulation should release IVP endospores slowly so that endospores are in the turfgrass rootzone longer, but also would reduce the quantity of IVP endospores present in the root zone at a given time.
Belonolaimus longicaudatus and CPu form a density-dependent relationship (Giblin-Davis, 2000). Similarly, studies with in vivo P. penetrans and Meloidogyne arenaria found that populations of both organisms fluctuated in the field in a density-dependent manner as explained by the Lotka-Volterra model (Orajay, 2009). In previous growth room studies, Luc et al. (unpublished) inoculated approximately 30 B. longicaudatus/100 cm\(^3\) of soil containing 280,000 IVP endospores/cm\(^3\) of soil and suppressed B. longicaudatus by about 75% after 12 wk. However, the application of IVP endospores as an inundative control method raised concern that the effectiveness of IVP as a bionematicide might be affected by B. longicaudatus population density at time of application. The objectives of this research were to determine if the efficacy of IVP as a biopesticide is affected by B. longicaudatus population density at the time of application, and to compare the efficacy of the clay granule formulation with the liquid spore suspension.

**Materials and Methods**

The experiment consisted of two trials conducted in a growth room on the campus of the University of Florida in Gainesville, FL from May to August 2009. This experiment utilized a factorial design consisting of two B. longicaudatus inoculum levels: 30 or 300 nematodes/100 cm\(^3\) of soil and three formulations of IVP endospores (nontreated, granular, or liquid) with five replications. Thirty clay pots (10.16-cm-diam.; 10.16-cm-high; 500-cm\(^3\)-volume) were cleaned, autoclaved, and then filled with 400-cm\(^3\) of nematode-free United States Golf Association specification sand (Anonymous, 1993). In-vitro endospores were produced from an isolate of Pasteuria sp. that was collected and cultured from B. longicaudatus on turf from Sebring, Florida. The endospores were obtained from Pasteuria Bioscience LLC (Alachua, FL) and refrigerated at 4 °C for 3 day to allow time to quantify endospores/ml and determine endospore core and sporangia size. In-vitro endospore measurements indicate that mean core diam. is
consistent with previously published measurements for CPu, however mean sporangium diam. is variable (Giblin-Davis et al., 2001). Furthermore, molecular identification of the Pasteuria sp. was unsuccessful. Prior to applying the formulation treatments, the liquid formulation was prepared as a liquid suspension (50 ml) of water, growth media, and endospores. The granular formulation was prepared by pipetting 1120 µl of growth media and endospores onto 2 g of a clay blank provided by Pasteuria Bioscience LLC; this process was repeated 10 times to provide the granular formulation. The mixture of clay, media, and endospores was stirred periodically and allowed to dry at 35 °C for 24 hours. The respective endospore treatments were applied topically to the pots at 1,380,000 endospores/cm². Following topical application of the nontreated and granular formulations, 50 ml of water was applied to maintain equal soil moisture between formulations and facilitate endospore release from the granular material. The application of 50 ml of water is the equivalent of 0.6 cm of irrigation previously shown to move endospores into the top 10 cm of the soil profile. Subsequently, ‘Penncross’ creeping bentgrass (Agrostis palustris Huds.) was seeded at 98 kg/ha (0.08 g/pot) and allowed to germinate and establish a root system for 13 d before being inoculated with nematodes. Experimental units were keep in a growth room with a light period of 14 hr/d and soil temperature maintained at 24 °C ± 0.5 °C.

Following turf establishment, B. longicaudatus originally obtained from Sun City, FL were extracted from pure nematode populations maintained on ‘FX313’ St. Augustinegrass (Stenotaphrum secundatum [Walt.] Kuntze) (Giblin-Davis et al., 1992; Busey et el., 1993) using the decant and sieve method (Cobb, 1918). Nematode population density was determined by counting the B. longicaudatus in 1-ml aliquots on a counting slide (Hawksley and Sons Limited, Lancing, Sussex, UK) Nematode counts were replicated five times. Nematode inoculum was
pipetted into two holes (1-cm-diam. x 2.5-cm-deep) in the soil at two rates: 120 ± 6 mixed-life stages nematodes/pot (30.0 ± 1.5 nematodes/100 cm³ of soil) or 1200 ± 60 mixed-life stages nematodes/pot (300.0 ± 15 nematodes/100 cm³ of soil).

Turf was watered twice/d with 10 ml of water. Turf was fertilized every 2-wk with Peters® 20-20-20 (N-P₂O₅-K₂O) fertilizer (United Industries Corp., St. Louis, MO). Nutrient inputs were 12.3 kg/ha N, 5.4 kg/ha P, 10.2 kg/ha K (0.010 g/pot N, 0.004 g/pot P, and 0.008 g/pot K), and trace amounts of essential micronutrients. Turf was trimmed to a 3-cm height once/wk.

Nematode populations and root lengths were assessed with destructive sampling 84 d after nematode inoculation. The entire soil profile of each pot was used to obtain nematode and root samples. Each sample was placed onto a 135-µm sieve. The roots were rinsed with water and the sand and nematodes collected. Rinsates were agitated and nematodes extracted by centrifugal-flotation (Jenkins, 1964) using a 25-µm sieve to catch *B. longicaudatus*. Nematodes were collected and counted using an inverted light microscope x40 magnification. Subsequently, 20 nematodes were randomly selected from each sample and the number of attached endospores counted (Chen et al., 1996a). Roots were collected and then placed into a clear plastic tray and scanned with Epson Perfection 4990 Photo Desktop Scanner (Epson, America Inc., Long Beach, CA) to obtain bitmap images of the root system (Pan and Bolton, 1991). The bitmap images were imported into the WinRhizo (Regent Instruments, Chemin Sainre-Foy, Quebec) software program for analysis. This program is designed to determine root length in millimeters. All data sets were tested for normality and homoscedasticity without issue. Factorial analysis of variance (ANOVA) and Fisher’s LSD were performed to compare counts of *B. longicaudatus*, percent
endospore attachment, and total root lengths for main effects and interactions using SAS (SAS Institute, Cary, NC).

Results

No interaction between *B. longicaudatus* inoculum levels and formulations of IVP endospores were observed for counts of *B. longicaudatus* and percent endospore attachment. Therefore, the data for the formulation comparisons were pooled across inoculum levels (Table 6-1). A 10-fold increase in nematode inoculum increased (*P* ≤ 0.05) Pf counts of *B. longicaudatus* per pot by 59% and 26%, respectively for trials 1 and 2 (Table 6-1). Granular and liquid formulations of IVP endospores suppressed nematode populations by 22% and 59% respectively, during trial one, and by 20% and 63% during trial two at 84 d after nematode inoculation, respectively compared to the nontreated controls (Table 6-1). No differences were observed in percent endospore attachment between nematode inoculum levels during either trial. The liquid formulation was more effective than the granular formulation, reducing *B. longicaudatus* populations by an additional 48% and 53%, during trial 1 and 2, respectively (Table 6-1). Similarly, the liquid formulation was more effective than the granular formulation increasing percent endospore attachment by an additional 147% and 158%, during trial 1 and 2, respectively (Table 6-1).

An interaction between *B. longicaudatus* inoculum levels and formulations of IVP endospores was observed for total root lengths. The greatest root retention was observed with the combination of a low *B. longicaudatus* inoculum level and the application of a liquid formulation of IVP endospores. Conversely, the greatest root losses were observed when a high number of *B. longicaudatus* was applied without application of IVP endospores (Table 6-2).
Discussion

Increasing the *B. longicaudatus* inoculum levels increased counts of *B. longicaudatus* per pot as would be expected. However, increased *B. longicaudatus* populations did not increase the efficacy of IVP endospores. While *Belonolaimus longicaudatus* and CPu form a density-dependent relationship in natural soil environments, the inundative application of IVP endospores as a biopesticide reduces *B. longicaudatus* populations equally at high or low levels. However, these experiments were not run sufficiently long to observe whether density dependent recycling (classical biological control) was indeed possible. In fact, Koch’s postulates have not been accomplished for the IVP formulation of CPu, so it is unclear what the mode of action is for the formulation under these conditions.

Both formulations of IVP endospores suppressed nematode populations compared to the nontreated control, indicating both formulations were effective. However, the liquid formulation was more effective at suppressing *B. longicaudatus* populations and exhibited increased percent endospore attachment compared to the granular formulation. Previous research has shown that 0.6 cm of irrigation (50 ml/pot) was sufficient to move a liquid formulation of IVP endospores to a soil depth of 10 cm (Luc et al., unpublished). However, 0.6 cm of irrigation may not have been enough water to release a majority of the IVP endospores from the clay substrate and then move them into the soil profile, resulting in reduced nematode suppression and decreased endospore attachment. Furthermore, the liquid formulation provided increased root abundance relative to the nontreated formulation in both trials.

In conclusion, these experiments indicate that *B. longicaudatus* levels in the soil at time of application does not affect efficacy of IVP endospores to suppress *B. longicaudatus* in short-term experiments. Furthermore, while the granular formulation reduced *B. longicaudatus* populations, it was not as effective as the liquid formulation. Further research studying spore release rate from
clay and quantifying the number of IVP endospores/g soil in the turfgrass root zone over time and with increasing irrigation rates would be very helpful in predicting efficacy in the field. Biopesticides using IVP may be an important component of integrated pest management for *B. longicaudatus* in the future.
Table 6-1. Effect of inoculum level of *Belonolaimus longicaudatus* originally obtained from Sun City, FL and formulation of in vitro produced *Pasteuria* sp. collected from Sebring, FL, on *Belonolaimus longicaudatus* populations and percent endospore attachment in pots planted with ‘Penncross’ creeping bentgrass and grown in a growth room for 84 days after nematode inoculation. Data pooled across main effects because inoculum level and formulation interaction was not significant (*P*≥0.05).

<table>
<thead>
<tr>
<th>Inoculum Level</th>
<th>Formulation</th>
<th>B. longicaudatus/pot Trial 1</th>
<th>B. longicaudatus/pot Trial 2</th>
<th>Percent Endospore Attachment Trial 1</th>
<th>Percent Endospore Attachment Trial 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>-</td>
<td>336.5 ± 133.3 c a d</td>
<td>238.7 ± 94.8 a</td>
<td>12.7 ± 7.8 a</td>
<td>12.3 ± 9.4 a</td>
</tr>
<tr>
<td>Low</td>
<td>-</td>
<td>211.1 ± 87.9 b</td>
<td>188.9 ± 71.4 b</td>
<td>11.7 ± 9.5 a</td>
<td>10.3 ± 9.1 a</td>
</tr>
<tr>
<td>-</td>
<td>Nontreated</td>
<td>376.6 ± 108.6 A c</td>
<td>295.2 ± 55.4 A</td>
<td>0.0 ± 0.0 A</td>
<td>0.0 ± 0.0 A</td>
</tr>
<tr>
<td>-</td>
<td>Granular</td>
<td>292.7 ± 96.3 B</td>
<td>236.6 ± 26.6 B</td>
<td>10.5 ± 3.7 B</td>
<td>9.5 ± 5.0 B</td>
</tr>
<tr>
<td>-</td>
<td>Liquid</td>
<td>152.2 ± 55.7 C</td>
<td>109.7 ± 15.4 C</td>
<td>26.0 ± 2.1 C</td>
<td>24.5 ± 3.7 C</td>
</tr>
</tbody>
</table>

a High inoculum was 1200 ± 60 B. longicaudatus/pot and low inoculum was 120 ± 6 B. longicaudatus/pot. b Percent nematodes out of twenty that had at least one endospore attached. c Inoculum level effect means and standard deviation for fifteen replications. d Main effect differences *P* ≤ 0.05 are indicated with different letters within each column; lower case letters for inoculum level and capital letters for formulation. e Formulation effect means and standard deviation for ten replications.
Table 6-2. Interaction effect of inoculum level of *Belonolaimus longicaudatus* and formulation of in vitro produced *Pasteuria* sp. endospores on total root length of ‘Penncross’ creeping bentgrass at 84 days after nematode inoculation.

<table>
<thead>
<tr>
<th>Inoculum Level&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Formulation&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Total Root Length (cm)</th>
<th>Trial 1</th>
<th>Trial 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>Liquid</td>
<td>1102.8 ± 430.7&lt;sup&gt;c&lt;/sup&gt;&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;d&lt;/sup&gt;</td>
<td>578.6 ± 155.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>Granular</td>
<td>818.9 ± 230.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>369.5 ± 174.5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>Nontreated</td>
<td>443.8 ± 281.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>316.3 ± 150.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>Liquid</td>
<td>900.9 ± 247.0&lt;sup&gt;A&lt;/sup&gt;</td>
<td>556.6 ± 252.7&lt;sup&gt;A&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>Granular</td>
<td>712.8 ± 203.6&lt;sup&gt;A&lt;/sup&gt;</td>
<td>402.2 ± 146.4&lt;sup&gt;AB&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>Nontreated</td>
<td>312.1 ± 160.1&lt;sup&gt;B&lt;/sup&gt;</td>
<td>300.1 ± 128.9&lt;sup&gt;B&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> High inoculum was 1200 ± 60 *B. longicaudatus*/pot and low inoculum was 120 ± 6 *B. longicaudatus*/pot.  
<sup>b</sup> Liquid and granular formulations were applied topically at 1,380,000 endospores/cm<sup>2</sup> of soil.  
<sup>c</sup> Means and standard deviation for five replications.  
<sup>d</sup> Formulation differences within inoculum levels *P* ≤ 0.05 are indicated with different letters within each column for trials 1 and 2; lower case letters for low inoculum level and capital letters for high inoculum level.
CHAPTER 7
SUMMARY

During the last decade, management of plant-parasitic nematodes in turf has become a daunting task, due to the limited number of effective post-plant nematicides. The use of in vitro produced *Pasteuria* sp. (IVP) as a biological control of *Belonolaimus longicaudatus* (sting nematode) offers an additional management tool to existing chemical and cultural controls. *Pasteuria* sp. and *B. longicaudatus* form a complex and dynamic parasite-host relationship, complicated further by the soil environment in which they live. Several factors were studied to determine their effect on the efficacy of IVP endospores to suppress *B. longicaudatus*: endospore level in soil, sporangia size, amount of irrigation after endospore application, thatch and wetting agent on movement of endospores, nematode levels at time of endospore application, bionematicide formulation, and different isolates of *Pasteuria* sp. and *B. longicaudatus*. While these experiments focused on the use of IVP to suppress *B. longicaudatus* in turf, this information may be transferable to other agronomic, horticultural, and ornamental crops.

Growth room experiments revealed that all endospore levels suppressed *B. longicaudatus* populations compared to the noninoculated control. At 280,000 endospores/cm$^3$ of soil, nematode populations were suppressed by 75% compared to the noninoculated control. The IVP endospore level required to achieve suppression of *B. longicaudatus* populations in 12 weeks was considerably higher than in previous studies. However, previous studies used in vivo endospores and the experiments ran over a period of 1 to 2 years. For IVP to be a viable nematode strategy, higher endospore levels may be required to achieve nematode suppression quickly.

Greenhouse experiments indicate that IVP endospore movement into the turf profile was not hindered by thatch or aided by a wetting agent. However, large inputs of water (which could
occur from large irrigation or rainfall events) can move endospores below the turfgrass root zone, possibly reducing the effectiveness. Water is the basic method of bacteria dispersal throughout the soil profile and is a major reason for *Pasteuria* spp. endospore losses from the cultivated soil horizon (Gammack et al., 1992). One application of 0.6 cm of irrigation was sufficient to place a liquid suspension of IVP endospores within the turfgrass root zone (0 to 10 cm soil depths). However, increasing the amount of irrigation caused IVP endospores to be leached.

In an attempt to address the issue of IVP endospore leaching a spore-impregnated clay granule formulation was produced by Pasteuria Bioscience LLC. Furthermore the density dependent relationship between *Pasteuria* spp. and their nematode host suggested that nematode density at the time of endospore application might affect efficacy of the IVP endospores. Subsequently, research was conducted to determine if the efficacy of IVP as a biopesticide is affected by *B. longicaudatus* population density at the time of application, and to compare the efficacy of a spore-impregnated clay granule formulation with the liquid spore suspension. The level of control provided by IVP endospores was independent of the nematode population density at time of application, reducing *B. longicaudatus* populations equally at high or low levels. Furthermore, both formulations of IVP endospores suppressed nematode populations compared to the nontreated control, indicating both formulations were effective. However, the liquid formulation was more effective than the granular formulation reducing *B. longicaudatus* populations by an additional 50% and increased percent endospore attachment by an additional 150%, during both trials. All endospore treatments received 0.6 cm of irrigation that had been shown to be sufficient to move the liquid formulation of IVP endospores throughout the soil profile of the experimental units without leaching endospores. However, 0.6 cm of irrigation may
not have been enough water to release a majority of the IVP endospores from the clay substrate and then move them into the soil profile, which resulted in reduced nematode control and decreased endospore attachment. This suggests that a granular formulation can mitigate endospore loses due to leaching by acting as a slow release mechanism. However, the initial and subsequent release rates of IVP endospores from the granular material must be identified, so a suppressive level of endospore can be achieved initially then maintain with additional applications of endospores.

Further research was conducted to determine if IVP endospores exhibit host-parasite isolate specificity in their ability to manage *B. longicaudatus*. These experiments revealed no difference between *B. longicaudatus* populations for nematode population density, percent endospore attachment, or total root length. This supports previous research that indicated endospore attachment and nematode control were not linked to nematode phylogeny. Additionally, all isolates of IVP endospores suppressed *B. longicaudatus* populations by approximately 70%. The lack of differences between geographically distinct isolates of IVP endospores was unexpected. Nevertheless, without phylogenetic data available for the isolates of IVP endospores, we cannot address their lack of diversity. This research suggests that a bionematicide utilizing IVP endospores is likely to be effective against populations of *B. longicaudatus* and the original geographical source of IVP endospores does not appear to affect efficacy, at least among the isolates evaluated.

In conclusion, these experiments indicate that high levels of IVP endospores can suppress *B. longicaudatus* populations, irrigation has a great impact on the placement of IVP endospores, a granular formulation may reduce leaching of endospores, and IVP endospores may be broadly effective against *B. longicaudatus*. These trials have provided invaluable information, which is
assisting to explain this complex ecosystem. Biopesticides using IVP may be an important component of integrated pest management for *B. longicaudatus* in the future.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

John Eric Luc was born in Hialeah, Florida to John Theodore and Lynda Alden Luc. They moved to Citrus County, Florida in 1972 where he held residency for 37 years. He graduated from Citrus High School in 1990. After a couple of unsuccessful years in college, he decided to leave college in 1992. He took a position at Citrus County Parks and Recreation in 1994, where a love of turfgrass was born. However, his thirst for knowledge and desire to improve himself could not be satisfied there. Through the advice of Jeff Hayden, he sought out Dr. Grady L. Miller, assistant professor of turfgrass science and later mentor. He attended Central Florida Community College and graduated with an Associate of Arts degree in 1999. Upon entrance to the University of Florida, he relocated to Gainesville and he sought out employment in an agricultural field, to assist with learning and to obtain experience in research. At that time, Dr. William T. Crow, Landscape Nematologist, University of Florida, Gainesville, Florida was searching for student workers to assist with his budding research program. He became a graduate student and a research assistant in 2002, conducting master’s research on ‘Effects of Plant-Parasitic Nematodes and Nitrogen Fertility Management on Hybrid Bermudagrass’, until he graduated in 2004. He was a graduate student and research assistant conducting doctor of philosophy research on ‘Utilization of ‘Candidatus Pasteuria usgae’ as a Biological Control for Belonolaimus longicaudatus’, until he received his PhD. from the University of Florida in the fall of 2009.