SPECIFICITY OF *IN VITRO*-PRODUCED *PASTEURIA* SP. ENDOSPORES TO SUPPRESS *BELONOLAIMUS LONGICAUDATUS*

J. E. Luc*[1], W. Pang[1], W. T. Crow[1], R. McSorley[1], and R. M. Giblin-Davis[2]

[1]Post Doctoral Research Associate, Graduate Student, Associate Professor, and Professor, respectively, Entomology and Nematology Department, Building 970 Natural Area Drive, University of Florida, Gainesville, FL 32611. [2]Professor, University of Florida-IFAS, Fort Lauderdale Research and Education Center, 3205 College Ave., Davie, FL 33314. *Corresponding author, e-mail: barzona71@yahoo.com

ABSTRACT

Luc, J. E., W. Pang, W. T. Crow, R. McSorley, and R. M. Giblin-Davis. 2011. Versatility of *in vitro*-produced *Pasteuria* sp. endospores to suppress *Belonolaimus longicaudatus*. Nematropica 41:248-253.

The objective of this research was to evaluate the isolate specificity among *in vitro*-produced *Pasteuria* sp. endospores and its host *Belonolaimus longicaudatus*. Five geographically diverse isolates of *Pasteuria* sp. were collected from infected *B. longicaudatus*, cultured *in vitro*, and tested for their ability to attach to and control two genetically and geographically diverse isolates of *B. longicaudatus* in greenhouse trials. There were no differences ($P \geq 0.05$) among the *in vitro*-produced *Pasteuria* sp. isolates or between the two *B. longicaudatus* populations in the ability of the spores to attach to and reduce numbers of *B. longicaudatus*. All isolates of *in vitro*-produced *Pasteuria* sp. endospores reduced numbers of *B. longicaudatus* nearly 70%, compared to pots without endospores, and the geographical origins of *in vitro*-produced *Pasteuria* sp. endospores would not appear to affect efficacy. However, these results differ from previous research conducted with *in vivo* produced endospores, suggesting that a closer look at the effects of *in vitro* fermentation process on the resulting progeny may be warranted.

Keywords: biological control, management, sting nematode, suppression, turfgrass.

RESUMEN

Luc, J. E., W. Pang, W. T. Crow, R. McSorley, and R. M. Giblin-Davis. 2011. Versatilidad de las endosporas de *Pasteuria* sp. producidas *in vitro* para el control de *Belonolaimus longicaudatus*. Nematropica 41:248-253.

El objetivo de este trabajo fue evaluar la especificidad de aislamientos en endosporas de *Pasteuria* sp. producidas *in vitro* para el control de *Belonolaimus longicaudatus*. Se colectaron cinco aislamientos de *Pasteuria* sp. a partir de *B. longicaudatus* infectados, se cultivaron los aislamientos *in vitro*, y se probó su habilidad de adhesión y de control en dos poblaciones de *B. longicaudatus* provenientes de dos localidades diferentes, en pruebas de invernadero. No se encontraron diferencias ($P \geq 0.05$) entre los aislamientos o entre las dos poblaciones de *B. longicaudatus* tanto en la adhesión de las esporas como en el control de *B. longicaudatus*. Todos los aislamientos de *Pasteuria* sp. producidos *in vitro* redujeron las poblaciones de *B. longicaudatus* en casi un 70%, comparados con macetas sin endosporas. El origen geográfico de los aislamientos de *Pasteuria* sp. no tuvo efecto sobre su eficacia. Sin embargo, estos resultados difieren de los obtenidos en otros trabajos con endosporas producidas *in vivo*, lo cual sugiere la necesidad de estudiar mejor los efectos del proceso de fermentación *in vitro*.

Palabras clave: *Belonolaimus longicaudatus*, céspedes, control biológico, manejo de nematodos.

INTRODUCTION

Pasteuria spp. are a group of Gram-positive endospore-forming bacteria with great potential as biological control agents of plant-parasitic nematodes (Dickson *et al.*, 1994; Chen and Dickson, 1998). A major obstacle to the commercial development is their limited host range. Isolates of *Pasteuria penetrans* were found to not only exclusively attach to a particular *Meloidogyne* sp., but also had different levels of attachment among isolates within a *Meloidogyne* sp. (Stirling 1985; Davies *et al.*, 1988). The S-1 strain of '*Candidatus* Pasteuria usgae' 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 *Pasteuria* sp. endospores 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; Timper, 2009). 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* 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 parasite (Davies *et al.*, 1994; Timper, 2009). The objective of this research was to determine if *in vitro*-produced *Pasteuria* sp. exhibit host specificity in their ability to manage *B. longicaudatus*.

MATERIALS AND METHODS

Isolate Maintenance: Seven geographically distinct populations of *Belonolaimus* were collected and reared for genetic comparison. These populations originated from sites planted with bermudagrass for at least 5 years in several U.S. States: South Carolina, Jefferson, SC (WP) and Hartsville, SC (HART); Georgia, Tifton, GA (GA); and Florida, Sun City, FL (SUN), Sanford, FL (MF), Sanford, FL (UNI), and Palatka, FL (PAL). Clay pots were cleaned, autoclaved, and then filled with 1430 cm^3 of nematode-free sand meeting the specification of the United States Golf Association (USGA) (Anonymous, 1993). 'Penncross' creeping bentgrass (*Agrostis palustris*) was seeded at 98 kg/ha (0.14 g/pot) and allowed to germinate and establish a root system for 13 days before being inoculated with nematodes. Isolates were extracted using the decanting and sieving method (Cobb, 1918). Nematodes were hand-picked and then inoculated into the soil at 500 mixed life-stages/pot. Every 3 month, the nematode cultures were extracted, counted, and inoculated into new pots to increase the nematode population available for the experiment.

Five different geographical isolates of *Pasteuria* sp. attached to *B. longicaudatus* on turf were collected and cultured. These isolates originated from: Sebring, FL (SBGR); Gainesville, FL (AGRO); The Villages, FL (POLO); Columbia, SC (USC); and Conway, SC (BR). *Pasteuria* Bioscience LLC (Alachua, FL) was responsible for surface sterilization of the *Pasteuria* infected nematodes, *in vitro* production of a small quantity of endospores for the isolate collection, and storing the *Pasteuria* isolate collection at 4°C until larger quantities of *in vitro* endospores were produced for this experiment. The specific methods for maintenance and production of *in vitro* endospores are proprietary to *Pasteuria* Bioscience LLC and are therefore not reported herein (Hewlett *et al.*, 2002). Following *in vitro* production, the suspensions of

endospores were refrigerated at 4°C for 3-d to allow time to quantify endospores/ml and determine endospore core and sporangia size. *In vitro*-produced endospore measurements indicated that mean core diameter was consistent with previously published measurements for '*Candidatus Pasteuria* usgae' (Giblin-Davis *et al.*, 2001, 2003); however mean sporangium diameter was variable (Luc *et al.*, 2010).

Belonolaimus isolate selection: Due to time and space constraints it was not practical to compare attachment rates of all the *Pasteuria* sp. isolates on all of the *B. longicaudatus* isolates collected. Therefore, attachment rates among *Pasteuria* sp. isolates was compared on only two of the *B. longicaudatus* isolates. It was assumed that use of nematode isolates that maximized geographical origin and also genetic diversity would increase the likelihood of identifying attachment rate differences among the *Pasteuria* sp. isolates.

Molecular analysis was conducted to select the two most genetically diverse *B. longicaudatus* populations from among the seven populations available to conduct this research. For molecular analysis of *Belonolaimus* isolates, DNA was extracted from individual female nematodes for each population using Isohair extraction kit (Nippon Gene Co. LTD., Toyama, Japan). The ITS1, 5.8S, and ITS2 regions of rDNA 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 an 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 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 (Life Technologies Corp., Carlsbad, CA). The primers used for sequencing were the same used for PCR amplification. Sequences were edited using Sequencher (version 4.1.2, Gene Codes Corporation, Ann Arbor, MI). The sequences obtained in this study were aligned to each other and the outgroup taxon *Pratylenchus coffeae* (Sher and Allen) (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 were transferred to PAUP 4.0b8 to determine phylogenic relationships using maximum parsimony, maximum likelihood, and evolutionary distance (Swofford, 2001). Bootstrapping was conducted with 1000 replications per analysis.

Experiment Establishment and Sampling: The experiments were conducted twice in a growth room at the University of Florida in Gainesville, FL from March to August 2009. These trials used a factorial design consisting of two *B. longicaudatus* populations: WP and SUN and six treatments of *in vitro*-produced *Pasteuria* sp.: SBRG, AGRO, POLO, USC, BR, and non-treated with five replicates each. Sixty pots (10.16-cm-diam.; 10.16-cm-high; 500-cm^3-volume) were cleaned and autoclaved. The respective endospore treatments were prepared as a liquid suspension (50 ml) of distilled water, growth media, and endospores at 280,000 endospores/cm^3 of sand (Luc *et al.*, 2010). Each endospore treatment was added to a plastic bag containing 400-cm^3 nematode-free USGA specification sand, 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, the isolates of *B. longicaudatus* were extracted using the decanting and sieving method (Cobb, 1918) from nematode populations maintained on 'Penncross' creeping bentgrass. Nematodes were quantified by counting the *B. longicaudatus* in five random 1-ml aliquots on a counting slide (Hawksley & Sons Limited, Lancing, Sussex, UK). Nematode inoculum was pipetted into two holes (1-cm-diam. x 2.5-cm-deep) in the soil at 120 ± 6 mixed-life stages/pot (30.0 ± 1.5 nematodes/100 cm^3 of soil).

Turf was watered twice/d with 10 ml of water and fertilized every 2-wk with Peters® 20-20-20 (N-P2O5-K2O) fertilizer (United Industries Corp., St. Louis, MO). Bi-weekly 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 weekly.

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 at x40 magnification. Subsequently, 20 nematodes were randomly selected from each sample

and numbers of endospores attached were counted (Chen *et al.*, 1996). Washed roots were placed into a 50-ml conical polypropylene tube, submerged in water, and refrigerated at 4°C for 1 to 2-d until the samples could be scanned with an Epson Perfection 4990 Photo Desktop Scanner (Epson, America Inc., Long Beach, CA) to obtain bitmap images of the root system. 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 centimeters (Bauhus and Messier, 1999). 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

Based on morphological characteristics and phylogenetic analysis of the ITS1, 5.8S, and ITS2 regions, the PAL population was identified as *B. euthychilus*, and the other six populations were *B. longicaudatus*. Among the six *B. longicaudatus* populations, the greatest genetic divergence was between the WP and SUN populations (Fig. 1). These also were geographically separate isolates (South Carolina and western Florida). Therefore, these two populations were chosen to compare *Pasteuria* sp. attachment rates.

In the growth room pot trials, no differences between nematode populations were observed for counts of *B. longicaudatus*, percent endospore attachment, or total root lengths (Table 1). Furthermore, no interaction between nematode populations and endospore isolates were observed. Therefore, the data for *Pasteuria* sp. isolates are pooled among the two *B. longicaudatus* populations. No differences ($P \geq 0.05$) were observed among isolates of *in vitro*-produced *Pasteuria* sp. for number of *B. longicaudatus*, percent endospore attachment, or total root lengths (Table 1). However, all endospore isolates suppressed numbers of *B. longicaudatus* by 69% to 71%, during trial 1, and 71% to 72% during trial 2, compared with the nontreated control.

DISCUSSION

Phylogenetic analysis of the ITS1, 5.8S, and ITS2 regions of *B. longicaudatus* indicated the greatest genetic divergence between the WP and SUN populations (Fig. 1). Coincidentally, these two nematode populations were the most geographically divergent among the six *B. longicaudatus* populations collected. However, these trials revealed no differences between
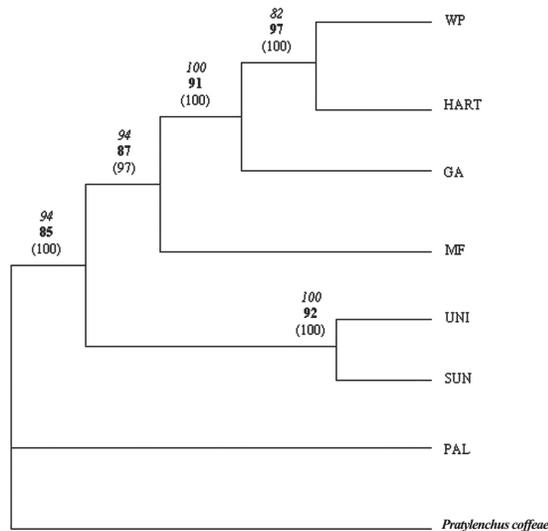
Fig.1. Relationships among the two populations of *Belonolaimus longicaudatus* used in this study, four previously studied *B. longicaudatus* populations, a population of *B. euthychilus* and a representative outgroup taxon based on maximum parsimony (MP) analysis of the ITS subunit gene. Branch support indices appear to the left of each estimated node. Bootstrap values for MP (1,000 replicates) are italicized. Bootstrap values for maximum likelihood (ML) (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); and Sanford, FL (UNI). While the Palatka, FL (PAL) isolate was determined to be *B. euthychilus* (Gozel *et al.*, 2006). The outgroup taxon *Pratylenchus coffeae* (Sher and Allen) (accession #AF170443) was obtained from the National Center for Biotechnology Information Genbank database.

the two *B. longicaudatus* populations for any *Pasteuria* data collected. Davies *et al.* (1994) demonstrated cuticle heterogeneity in respect to *in vivo* endospore attachment between populations of *Meloidogyne*. Furthermore, populations of root-knot nematodes that appeared to be comparatively homogeneous from studies using DNA, exhibited considerable level of variation in the binding of *in vivo Pasteuria* spores. This would suggest the ability of nematodes to develop levels of cuticlar heterogeneity faster than phylogenetic analysis can observe. However, phylogenetic analysis in these experiments indicated diversity between the WP and SUN populations, but no difference in spore attachment were observed. The ITS1, 5.8S, and ITS2 regions may be better suited for distinguishing between species or populations within a species (Cherry *et al.*, 1997), than previously investigated DNA regions, but these differences do not address the ability of *in vitro*-

produced *Pasteuria* sp. to attach to the nematode cuticle. Bekal *et al.* (2001) showed that soil naturally infested with spores of *Pasteuria* strain S-1 had different levels of percent endospore attachment (51% to 96%) for geographically different populations of *B. longicaudatus*. Differences in percent endospore attachment also were observed among three Florida populations (Bekal *et al.*, 2001). The lack of differences in percent endospore attachment for these two genetically and geographically distinct nematode populations in our study was not expected.

All endospore isolates suppressed *B. longicaudatus* populations equally and exhibited similar levels of percent endospore attachment. Davies *et al.* (2001) using 25 populations of *Pasteuria* endospores demonstrated varying levels of *in vivo* endospore attachment to *Meloidogyne arenaria* and *M. incognita*. Field research data suggests that heterogeneity within nematode and *Pasteuria* populations within a field occurs and that subpopulations of *Pasteuria* resistant nematodes and resistance breaking *Pasteuria* develop in response to selection pressure from each other continuously (Timper, 2009). The lack of differences among geographically distinct isolates of *in vitro*-produced *Pasteuria* sp. is of interest. Previous research has revealed *in vivo* culturing of *Pasteuria* spp. 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). The lack of host-parasite interaction during *in vitro* production may affect genetic diversity and virulence of *Pasteuria* sp. explaining the relatively uniform nematode suppression and endospore attachment.

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*) and bermudagrasses (*Cynodon dactylon* var. *dactylon* × *C. transvaalensis*) to *B. longicaudatus* have shown that an inoculation level of 100 nematodes/100 cm^3 of soil was needed to observe root differences for trials lasting 90-d (Schwartz *et al.*, 2008). Lower inoculum levels were used in these experiments to minimize plant biomass effect on the nematode population density and spore attachment. With lower inoculum levels the duration of the experiments may need to be extended to observe root differences (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 measurable damage to the root system.

In conclusion, these experiments may indicate that geographical differences between *B. longicaudatus*

Table 1. Effect of isolates of *Belonolaimus longicaudatus* and *in vitro*-produced *Pasteuria* sp. on the density of *B. longicaudatus* populations, percent endospore attachment, and total root length in pots planted with 'Penncross' creeping bentgrass and grown in a growth room for 84-d after nematode inoculation. Data pooled across main effects because *B. longicaudatus* and *Pasteuria* sp. isolate interaction was not significant ($P \geq 0.05$).

	Trial 1		
Belonolaimus Population[t]	*B. longicaudatus*/pot	Percent Endospore Attachment[u]	Total Root Length[v]
WP	149.2 ± 99.4[w] a[x]	18.2 ± 4.0 a	1719.7 ± 473.5 a
SUN	44.3 ± 95.1 a	17.8 ± 3.9 a	1510.4 ± 356.8 a
Pasteuria treatments[y]	355.1 ± 39.6 a[z]	0.0 ± 0.0 a	1466.9 ± 413.2 a
Nontreated	99. 3 ± 13.5 b	21.5 ± 4.7 b	1647.4 ± 292.6 a
SBRG	106.3 ± 13.8 b	22.5 ± 2.6 b	1629.7 ± 363.9 a
AGRO	107.7 ± 13.1 b	21.0 ± 3.9 b	1604.2 ± 206.5 a
POLO	110.2 ± 25.8 b	22.0 ± 3.5 b	1726.9 ± 589.3 a
USC	102.1 ± 17.4 b	21.0 ± 4.0 b	1615.2 ± 622.2 a
BR			
	Trial 2		
Belonolaimus Population	*B. longicaudatus*/pot	Percent Endospore Attachment	Total Root Length
WP	158.9 ± 106.4 a	15.0 ± 5.0 a	1835.5 ± 555.5 a
SUN	162.4 ± 119.0 a	14.8 ± 4.5 a	2009.4 ± 419.3 a
Pasteuria treatments			
Nontreated	398.3 ± 75.0 a	0.0 ± 0.0 a	1791.4 ± 462.6 a
SBRG	110.8 ± 16.3 b	17.5 ± 4.9 b	1894.5 ± 441.1 a
AGRO	110.1 ± 12.5 b	18.5 ± 4.1 b	1876.5 ± 618.5 a
POLO	113.8 ± 17.3 b	18.0 ± 5.4 b	1900.9 ± 637.2 a
USC	115.8 ± 15.9 b	17.0 ± 4.8 b	1993.8 ± 556.4 a
BR	115.3 ± 16.4 b	18.5 ± 5.3 b	2077.4 ± 457.2 a

[t] Nematode populations (WP) and (SUN) were collected from golf courses planted with Tifway 419 in Jefferson, SC and Sun City, FL, respectively.
[u] Percent nematodes out of 20 that had at least one endospore attached.
[v] Total root length measured in centimeters.
[w] Data are means and standard deviations for thirty replications.
[x] Means in columns followed by the same letters were not different according to least significant difference (LSD) at $P \leq 0.05$.
[y] *Pasteuria* endospores were provided by Pasteuria Biosciences LLC from *B. longicaudatus* that were collected for Sebring, FL (SBRG), Gainesville, FL (AGRO), The Villages, FL (POLO), Columbia, SC (USC), Conway, SC (BR).
[z] Data are means and standard deviations for ten replications.

tested will likely not affect the efficacy of a bionematicide based on *in vitro*-produced *Pasteuria* sp. endospores. Similarly, the use of *in vitro*-produced *Pasteuria* sp. endospores from geographically distinct isolates does not appear to affect efficacy. However, the differences observed between these experiments with in-vitro produced endospores and previous work conducted with *in vivo* endospores should not be ignored. Further testing should be conducted to determine if the in-vitro fermentation process is affecting the genetic diversity and virulence of the *Pasteuria* isolates.

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