SUPPRESSION OF *BELONOLAIMUS LONGICAUDATUS* WITH *IN VITRO*-PRODUCED *PASTEURIA* SP. ENDOSPORES

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ABSTRACT

Luc, J. E., W. T. Crow, R. McSorley, and R. M. Giblin-Davis. 2010. Suppression of *Belonolaimus Longicaudatus* with *in vitro*-produced *Pasteuria* sp. endospores. Nematropica 40:217-225.

A rate study was conducted in a growth room to determine the effects of increasing levels of *in vitro*-produced *Pasteuria* sp. endospores in soil on populations of *Belonolaimus longicaudatus*. Another study was conducted to determine if the size of *in vitro*-produced *Pasteuria* sp. sporangia influence the ability of the bacterium to infect *B. longicaudatus*. The rate study consisted of five endospore levels (0; 28,000; 56,000; 140,000; or 280,000 endospores/cm^3 of soil). Effects of treatments on *B. longicaudatus* populations, endospore attachment, and turf root length were compared. All inoculum levels suppressed *B. longicaudatus* populations compared to the non-inoculated control at all sampling dates. Population levels of *B. longicaudatus* declined linearly at inoculum levels ≥28,000 endospores/cm^3 soil. The endospore size study consisted of three endospore treatments: i) non-treated, ii) 25% large (≥4 µm-diam) endospores, and iii) 45% large endospores. Endospore treatments were incorporated at the rate of 280,000 endospores/cm^3 of soil. Treatments consisting of 25% and 45% large endospores suppressed nematode populations by as much as 59% and 69%, respectively at 84 days. Total root length differences were observed between the 45% large endospore treatment and non-treated control. Results suggest high levels of *in vitro*-produced *Pasteuria* sp. endospores can suppress *B. longicaudatus* populations, while sporangium size did not affect the level of nematode suppression.

Key words: biological control, management, sting nematode, suppression, turfgrass.

RESUMEN

Luc, J. E., W. T. Crow, R. McSorley, and R. M. Giblin-Davis. 2010. Supresión de *Belonolaimus longicaudatus* con endosporas de *Pasteuria* sp. producidas *in vitro*. Nematropica 40:217-225.

Se condujo un estudio en condiciones controladas para determinar el efecto que diferentes tasas de endosporas de *Pasteuria* sp. producidas *in vitro* causan en las poblaciones de *Belonolaimus longicaudatus*. Se condujo otro estudio para determinar si el tamaño de los esporangios de *Pasteuria* sp. producidos *in vitro* influye sobre la habilidad de la bacteria para infectar a *B. longicaudatus*. El primer estudio incluyó cinco tasas de endosporas (0; 28,000; 56,000; 140,000; ó 280,000 endosporas/cm^3 de suelo). Se compararon los efectos de los tratamientos sobre las poblaciones de *B. longicaudatus*, la adhesión de endosporas y el crecimiento de las raíces del pasto. Todos los niveles de inóculo suprimieron las poblaciones de *B. longicaudatus* comparados con el control no inoculado. Las poblaciones de *B. longicaudatus* declinaron linearmente con inóculo mayor o igual a 28,000 endosporas/cm^3 suelo. En el segundo estudio se incluyeron tres tratamientos: i) control, ii) 25% endosporas grandes (≥4 µm-diam), y iii) 45% endosporas grandes. Los tratmien-

tos de endosporas se aplicaron a razón de 280,000 endosporas/cm^3 de suelo. Los tratamientos con 25% y 45% de endosporas grandes suprimieron las poblaciones del nematodo en un 59% y 69%, respectivamente, a los 84 días. Se observaron diferencias en la longitud total de las raíces entre el tratamiento con 45% de endosporas grandes y el control no tratado. Los resultados sugieren que se pueden suprimir las poblaciones de *B. longicaudatus* con niveles altos de endosporas de *Pasteuria* sp. producidas *in vitro*, y que el tamaño del esporangio no afecta el grado de supresión.

Palabras clave: Belonolaimus longicaudatus, control biológico, manejo, supresión, pastos

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 those agents studied, *Pasteuria* spp. has been recognized as having great potential for the biological control of nematodes (Dickson *et al.*, 1994). Field plots inoculated with '*Candidatus* Pasteuria usgae' demonstrated a significant reduction of *B. longicaudatus* populations after 13 months (Giblin-Davis, 2000). Research conducted on peanut showed 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 non-treated control plots (Chen *et. al.*, 1996b). The following year peanut was replanted into the same micro-plots without additional endospore inoculum and, root and pod galls were reduced by 61% and 82% and 81% and 90%, respectively in 10,000 and 100,000 endospores/g treatments compared to non-treated control plots.

Previously, '*Candidatus* Pasteuria usgae' was cultivated on *B. longicaudatus* grown in aseptic root culture, greenhouse cultures, or collected from suppressive sites in the field (Bekal *et al.*, 2001; Giblin-Davis *et al.*, 1990; 2003). 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 occur *in vivo*. Giblin-Davis *et al.* (2001) described mature '*Candidatus* Pasteuria usgae' endospores produced *in vivo* as having a mean core diameter of 1.54 ± 0.24 µm and a mean sporangium diameter of 6.05 ± 0.36 µm. *In vitro*-produced *Pasteuria* sp. endospore measurements indicate that mean core diameter is consistent with these published measurements, while mean sporangium diameter is variable. *In vitro* sporangium diameter variability may be due to nutrient availability and duration of the fermentation process (Smith, 1998). *In vitro* sporangium diameter 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 *in vitro*-produced *Pasteuria* sp. 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 *in vitro*-produced *Pasteuria* sp. endospores that suppress *B. longicaudatus* populations below damaging numbers and to determine if sporangia size affects efficacy of *in vitro*-produced *Pasteuria* sp. 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* sp. that was collected and cultured from *B. longicaudatus* on turf from Sebring, Florida. The endospores were grown *in vitro* by Pasteuria Bioscience LLC (Alachua, FL) and refrigerated at 4°C for 3 days to allow time to quantify endospores/ml and determine endospore core and sporangia size. *In vitro* endospore measurements indicate that mean core diameter is consistent with previously published measurements for '*Candidatus* Pasteuria usgae'; however mean sporangium diameter is variable (Giblin-Davis *et al.*, 2001). Furthermore, molecular identification of the *Pasteuria* sp. isolate was unsuccessful. Data for these experiments were collected over an 84-day period for each trial.

Rate Study

This experiment utilized a randomized complete block design (RCBD) consisting of five 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 days) 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*) 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. Experimental units were kept in a growth room and maintained at 27°C ± 3°C with a light period of 14 hours/day.

Following turf establishment, *B. longicaudatus* were extracted using Cobb's decant and sieve method from pure nematode populations maintained on 'FX313' St. Augustine grass (*Stenotaphrum secundatum*), an excellent host for this nematode (Giblin-Davis *et al.*, 1992; 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 × 2.5-cm-deep) in the soil at 450 ± 35 mixed-life stages nematodes/pot (32 ± 2 nematodes/100 cm^3 of soil).

Turf was watered twice/day with 25 ml of water, and fertilized every 2-week with Peters® 20-20-20 (N-P_2O_5-K_2O) 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/week.

Nematode populations and root lengths were assessed with destructive sampling 28, 56, and 84 days after nematode inoculation. Nematode and root samples were obtained from a single core sample (5.08-cm-diam) taken from the middle of each pot. These cores 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 recover *B. longicaudatus* that were present. Nematodes were collected and counted using an inverted light microscope 40X magnification. Subsequently, 20 nematodes were randomly selected from each sample and attached endospores were counted (Chen *et al.*, 1996a). Washed roots were 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. 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 centimeters. All data was tested for trial × parameter interact without issue, so trial one and two data were combined. Initially, all treatments (0 to 280,000 endospores cm^3 of soil) were subjected to regression analysis using various transformations (log, natural log, x^2+1, etc.) to fulfill regression assumptions without success. However, removing the non-inoculated control treatment from the regression analysis revealed a linear relationship between endospore treatments and *B. longicaudatus* populations at all sampling dates. Data were subjected to regression analysis using SAS (SAS Institute, Cary, NC).

Endospore Size Study

Pasteuria sp. endospores produced from two *in vitro* batches obtained from Pasteuria Bioscience LLC were the experimental treatments. Each treatment consisting of 50 ml of water, media, and endospores was vigorously hand shaken for 20 seconds to resuspend the endospores, a 5 µl aliquot was pipetted to each slide, and five permanent slide mounts were made. Subsequently, 20 endospores from each slide were randomly selected to measure core and sporangium diameter at 1000X magnification. Individual endospores were designated large or small based on sporangium diameter. Endospores with sporangium diameters ≥4 µm-diam were considered large and <4 µm-diam small. One batch was determined to have 25% large endospores; the second had 45% large endospores.

Experimental design was a RCBD consisting of three endospore treatments (non-treated 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, 84 days after nematode inoculation. All data sets were tested for normality and homoscedasticity without issue. Analysis of variance (ANOVA) and Fisher's protected least significant difference test were performed to compare counts of *B. longicaudatus*, percent endospore attachment and root lengths among treatments. Analysis of variance and mean separation tests were performed using SAS (SAS Institute, Cary, NC).

RESULTS

Rate Study

All inoculum levels suppressed ($P \leq 0.05$) *B. longicaudatus* populations compared to the

non-inoculated control at all sampling dates. Endospore rate was negatively correlated with *B. longicaudatus* at all dates and the slopes increased negatively at each date (Fig. 1A-C). At 28,000 endospores/cm^3 of soil, nematode populations were suppressed relative to the non-inoculated control by 8%, 30%, and 48% at 28, 56, and 84 days, respectively; and at 280,000 endospores/cm^3 of soil, nematode populations were suppressed by 48%, 60%, and 74% at 28, 56, and 84 days, respectively (Fig. 1A-C). In both trials, there were no differences in endospore attachment among inoculation levels ($P \geq 0.05$) (Fig. 2A-C) and inoculation with *in vitro*-produced *Pasteuria* sp. endospores did not

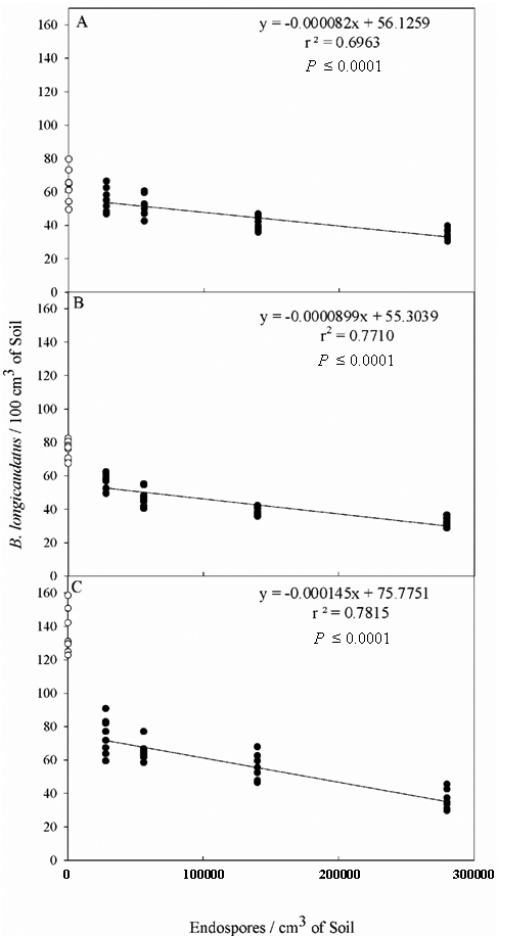

Fig. 1. Effects of increasing levels of *in vitro*-produced *Pasteuria* sp. endospores on *Belonolaimus longicaudatus* on creeping bentgrass in a growth room, A) 28 days, B) 56 days, and C) 84 days after nematode inoculation during trial one and two. All plants were inoculated with 32 ± 2 *B. longicaudatus* per 100 cm^3 of soil. Combined data for both trials, each treatment level consists of eight replications.

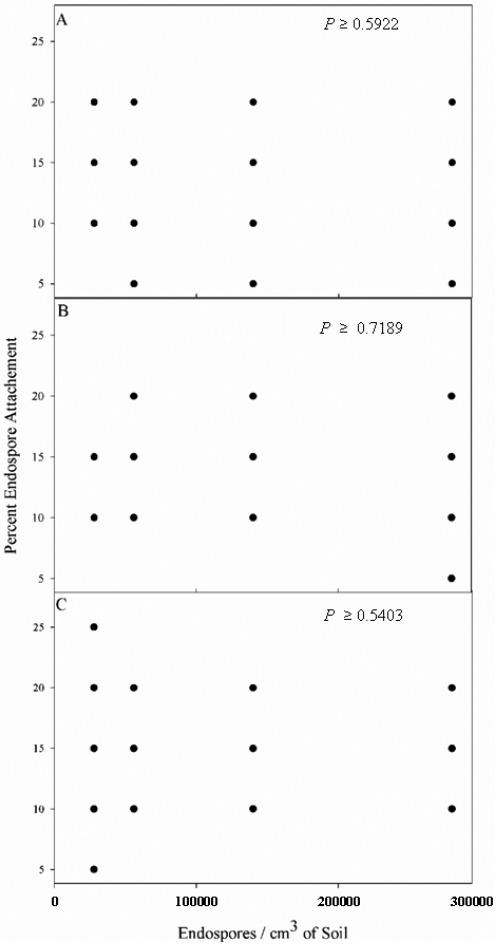

Fig. 2. Effects of increasing levels of *in vitro*-produced *Pasteuria* sp. endospores on percent endospore attachment to *Belonolaimus longicaudatus* on creeping bentgrass in a growth room, A) 28 days, B) 56 days, and C) 84 days after nematode inoculation during trial one and two. All plants were inoculated with 32 ± 2 *B. longicaudatus* per 100 cm^3 of soil. Combined data for both trials, each treatment level consists of eight replications.

improve root length compared to the non-inoculated treatment (Fig. 3A-B).

Endospore Size Study

Both endospore treatments suppressed ($P \leq 0.05$) *B. longicaudatus* population densities compared to the non-treated control but there were no differences among the size treatments. The 25% and 45% large endospore treatments suppressed nematode populations by 59% and 69%, respectively, during trial one and by 50% and 61% during trial two (Table 1). Differences ($P \leq 0.05$) in total root length were observed in the 45% large endospore compared to non-treated 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 1). No differences in endospore attachment were observed among the spore-size treatments ($P \geq 0.05$) (Table 1).

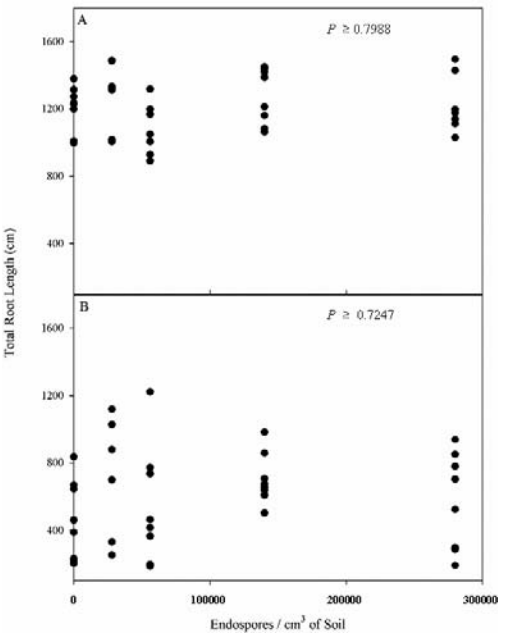

Fig. 3. Effects of increasing levels of in vitro produced *Pasteuria* sp. endospores on total root length of creeping bentgrass inoculated with *Belonolaimus longicaudatus* in a growth room, A) 28 days and B) 84 days after nematode inoculation during trial one and two. All plants were inoculated with 32 ± 2 *B. longicaudatus* per 100 cm^3 of soil. Combined data for both trials, each treatment level consists of eight replications.

DISCUSSION

Rate Study

At all dates, increasing endospore levels suppressed *B. longicaudatus* population density. The highest level of endospores suppressed *B. longicaudatus* population density to near the initial inoculum level of 32 nematodes/100 cm^3 at all dates. The negative slope of the regression line between nematode and endospore levels increased as the study progressed due to increases in nematode populations at the lower endospore levels.

Studies conducted previously indicated that 5,000 '*Candidatus* Pasteuria usgae' endospores/cm^3 of soil could reduce *B. longicaudatus* populations to very low numbers over an 18-mo 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 *in vitro Pasteuria* sp. endospores were required to suppress *B. longicaudatus* population densities within a shorter 12-week period. Second, while the *Pasteuria* sp. isolate in these experiments was collected and cultured from *B. longicaudatus,* we were unable to confirm its identity as '*Candidatus* Pasteuria usgae' (Giblin-Davis, 2000). Isolate differences in pathogenicity, virulence, or percent endospore attachment may have contributed to higher levels of *in vitro*-produced *Pasteuria* sp. endospores being required to suppress *B. longicaudatus* population densities (Davies

Table 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 grown in a growth room for 84 days after nematode inoculation.

Treatments[u]	*B. longicaudatus*/pot[v]	Percent Endospore Attachment[w]	Total Root Length[x]
	Trial 1		
Non-treated	141.1 ± 21.1 a[yz]	0.0 ± 0.0 a	865.2 ± 117.6 a
25%	58.0 ± 20.4 b	8.0 ± 7.8 b	1165.0 ± 275.2 ab
45%	44.0 ± 22.8 b	10.0 ± 5.7 b	333.5 ± 332.3 b
	Trial 2		
Non-treated	131.0 ± 17.4 a	0.0 ± 0.0 a	642.9 ± 84.3 a
25%	65.5 ± 23.7 b	9.0 ± 8.2 b	790.9 ± 137.7 ab
45%	51.3 ± 24.1 b	11.0 ± 10.3 b	835.6 ± 176.6 b

[u]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); non-treated treatments did not receive endospores.
[v]All plants were inoculated with 32 ± 2 *B. longicaudatus* per 100 cm^3 of soil.
[w]Percent nematodes out of 20 that had at least one endospore attached.
[x]Total root length measured in centimeters.
[y]Data are means and standard deviations for five replications.
For each trial, treatments within a column with common letters are not different ($P \leq 0.05$), according to Fisher's Least Significant Difference procedure.

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 (Buchmeier and Heffron, 1990; Thornton *et al.*, 1993). *In vitro* production of *Pasteuria* sp. may reduce virulence and effectiveness of *Pasteuria* sp. to control *B. longicaudatus*.

The fact that increased *in vitro* produced *Pasteuria* sp. 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 with all life stages occurring in the soil. Consequently, *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 growth, reflected in root lengths, in the rate studies may 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 bermuda grasses (*Cynodon dactylon* Var. *dactylon* × *C. transvaalensis*) to *B. longicaudatus* have shown that an inoculation level of 100 nematodes/100 cm^3 of soil is needed to show root differences for trials

lasting 90 days. 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.

Sporangium Size Study

Both endospore size treatments suppressed nematode populations similarly when compared to the non-treated 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 diameter did not affect efficacy of *Pasteuria* sp. endospores. Regardless, Pasteuria Bioscience LLC has continued to improve the *in vitro* method of producing endospores and this issue of inconsistent endospore size has been reduced. While nematode and *Pasteuria* sp. inoculum levels were consistent with the rate study, differences in total root length were observed between 45% large endospore and non-treated control. Differences in total root length were not observed in the rate study or previously conducted experiments. However, during the endospore trials, the turfgrass experienced some drought stress in addition to nematode feeding; the combination of these stress factors may explain the observed differences in total root lengths.

In conclusion, these experiments indicate that high levels of *in vitro*-produced *Pasteuria* sp. 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* sp. isolate from Sebring, FL remains unconfirmed.

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