Biocontrol Studies of *Rotylenchulus reniformis* in Cotton Crops in Alabama.

by

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Abstract

Rotylenchulus reniformis is the most important pathogen of cotton in the southeastern United States. Aldicarb, which is the most used nematicide in cotton production, will be retired from the market in 2018 by the EPA. Thereby, this is the time to introduce biocontrol agents against *R. reniformis* that can reduce nematode damage on cotton plants. Several reports of fungi and bacteria antagonistic to *R. reniformis* have been published. The general objective of this research was to evaluate different commercial biocontrol agents, new formulations for biocontrol agents, and new antagonists that can be implemented to control *R. reniformis* in cotton crops. Specific objectives were: *i)* to evaluate the biocontrol potential of *Bacillus firmus* GB-126 and *Paecilomyces lilacinus* 251 under greenhouse, microplot, and field conditions against *R. reniformis*; *ii)* to understand the mechanisms of acton of *B. firmus* GB-126 under *in vitro* conditions; and *iii)* to identify morphologically and molecularly a new strain of *Catenaria auxiliaris* found paratizing *R. reniformis.* Reductions of all life stages of *R. reniformis* was observed with the combination of *B. firmus* ($1.4x10^7$ spores/seed) and *P. lilacinus* (0.3% v/v) under greenhouse cultivation 30 days after planting (DAP). The two biologicals reduce *R. reniformis* vermiform life stages at 60 DAP, and increased plant height and stem diameter in microplot and field trials. Cotton yields were similar between the biological *B. firmus* and *P. lilacinus* combination treatment and aldicarb. *In vitro* studies indicate that the mechanism of action of *B. firmus* works as a biosurfactant produced by the bacterium at concentrations of 1 and 2 ppm which paralyses the juvenile stages within 30 minutes *in vitro*. Further studies found *Catenaria auxiliaris* parasitized 42% of *R. reniformis* populations. This obligate-parasite fungus

was identified based on the 18S and 28S rDNA. *Catenaria auxiliaris* has the potential of become a successful biocontrol agent because it colonizes all life stages of the nematode, zoospores give mobility to this biocontrol agent, and the resistant spore stage can extend the survival of the fungus in the commercial formulation process. At this time there are commercial biocontrol agents and new antagonists of *R. reniformis* that can be implemented in a nematode management program when aldicarb is removed from the market.

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List of Abbreviations

PGPR	Plant-Growth Promoting Rhizobacteria
PCR	Polimerase Chain Reaction
PDA	Potato Dextrose Agar
TSA	Triptic Soy Agar

Chapter I. Biological control of the reniform nematode (*Rotylenchulus reniformis*): an exploration of a new management alternative.

Abstract

Rotylenchulus reniformis is the most important pathogen of cotton in the southeastern United States, and it is a continuous threat to cotton production with annual losses of $30 million dollars. Currently, management of this nematode is based on crop rotation and chemical nematicides. However, aldicarb, which is the most used nematicide in cotton production, will be retired from the market in 2018 by the EPA. Therefore, biocontrol of *R. reniformis* is an option that needs to be explored. During the last decade, several reports of *R. reniformis* antagonists have been published including fungi and bacteria. Additionally, suppressive soils have already been reported in Louisiana and Texas, although the agent responsible for the suppression was not identified. Private companies have developed commercial biocontrol agents using different formulations (seed treatment, thixotropic, wettable powder), and it is necessary to evaluate these commercial agents under different field conditions to understand their performance. Furthermore, biocontrol field studies have shown inconsistent results over the last years, thus it is important to understand the biology of the antagonists when they are applied in the soil. The perfect antagonist has yet to be discovered. Therefore, evaluation of possible interactions among existing antagonists, and combinations of antagonists that attack at different stages of the life cycle of the nematode, can reduce *R. reniformis* damage. The objective of this review is to discuss the different antagonists (fungi and bacteria) of *R. reniformis,* the commercial biocontrol agents currently available and the production systems in which they can be used against this nematode.

I. Introduction.

Plant-parasitic nematodes are the most important pathogens in cotton production in the United States, resulting in the highest percentage of bales lost at 4.16% (7). The most economically damaging plant parasitic nematodes affecting cotton are the root-knot nematode (*Meloidogyne incognita* Kofoid and White), the reniform nematode (*Rotylenchulus reniformis* Linford and Oliveira), the Columbia lance nematode (*Hoplolaimus columbus* Sher), and the sting nematode (*Belonolaimus longicaudatus* Rau) (63, 66). Among them, *M. incognita* and *R. reniformis* are the widest-spread parasitic nematodes in cotton crops in the United States (67). *Meloidogyne incognita* primarily affects production in Texas, Georgia, and Arkansas, while *R. reniformis* most often reduces the production in Alabama, Mississippi, and Louisiana. Furthermore, *R. reniformis* is an emerging problem in states where it was not present before, such as Tennessee, Texas, Arkansas, and South Carolina (7).

The first report of *R. reniformis* in the continental United States was in 1940 in Georgia (82), and subsequently it spread to the above mentioned states. Currently, cotton losses due to *R. reniformis* damage in Alabama, Mississippi, and Louisiana are 25,670, 46,450, and 20,340 bales, respectively (7). Based on the price of $480 per bale, this represents losses of $44 million dollars per year. The increase in estimated cotton losses due to *R. reniformis* is attributed to the lack of resistant varieties (90, 99), limited use of crop rotation, the ability of *R. reniformis* to colonize a wide range of soil types at depths over 122 cm (44, 57, 68, 69), and the cost and efficacy of nematicides (46, 53, 67, 83).

In Alabama, *R. reniformis* was found in 46% of the fields sampled, and within these infested fields, 47% had nematode population above the damage threshold (24). Historical data in Mississippi show a shift from *M. incognita* to *R. reniformis*, with all the crop producing counties highly infested with *R. reniformis* (74). The northeast and central parts of Louisiana have a high incidence of *R. reniformis*, and have been increase its spreading during lasts years (63).

Frequent reports suggest that *R. reniformis* has displaced *M. incognita* in specific fields over the years (66). Several biological attributes of *R. reniformis* can explain this displacement. Cotton is an excellent host for *R. reniformis*. *Rotylenchulus reniformis* can survive under dry conditions and has a shorter life cycle, completing it (egg to egg) in 17 days at 27 ºC to 32ºC, compared to *Meloidogyne incognita* that requires between 21 to 28 days at 28°C (47, 69, 80, 91). In concomitant infections *R. reniformis* affected the hatching and number of eggs and second stage juveniles of *M. incognita* 60 days after inoculation (21). Additionally, *R. reniformis* establishes feeding sites in primary, secondary, and tertiary roots, and present more mature females per gram of root than *M. incognita* (56, 67, 68). This plant-parasitic nematode retaines it's cuticle from the three juvenile stages in the soil, which may provided protection to antagonists. *Rotylenchulus reniformis* can build up high populations in a wide range of soils (high silt and clay content) compared to other cotton nematodes that are limited to sandy soils (44, 66). Further, this nematode not only represents a threat to cotton production, but also has a wide host range, reproducing in approximately 330 different plant species in tropical and subtropical regions (69).

II. *Rotylenchulus reniformis* life cycle.

Infection starts when *R. reniformis* females enter the root in a non-selective manner and establish a feeding site. Root penetration is intracellular through the cortex, and two-thirds of the female's body remains outside the root (Figure 1A) (69). The feeding nematode inserts the stylet into an endodermal cell, inducing the formation of a syncytium, which is the feeding cell to the nematode. The syncytium is formed when the cytoplasm of root cells mix following the destruction of the cell wall (25). The cytoplasm is dense and contains various nuclei, plastids, and mitochondria (1). Six to 15 days after the initial infection, the female matures and develops the characteristic kidney shaped stage (Figure 1B-D). There is no evidence of copulation before the young female establishes in the root. The female starts feeding on the root and attracts males by a chemical stimulus (60, 82). Mature females start laying eggs in a gelatinous matrix composed of glycoproteins (Figure 1E-F), which are secreted by six specialized cells around the vagina, and protect the eggs from desiccation when they are exposed outside the roots (2).

Depending on the host and environmental conditions, between 20 and 300 eggs are laid and can be seen between 7-20 days after invasion of the plant roots (25, 69, 80). The eggs go through embryonic stages, where they divide continuously until they reach a multi-cell stage that gives rise to a tadpole stage (80). Once hatching occurs, *R. reniformis* goes through three different juvenile stages (vermiform stages) in soil without feeding. Nakasono (2004) describes the twenty-one developmental processes from the J_2 to pre-adult stage in great detail. The J_1 is formed within the egg in 3 days, and a first molt occurs about 24 hours into the J_2 stage. Then, after 1-3 days of J_2 formation, hatching begins (80). At this stage, the stylet of the nematode shows movement, and six to seven days after the egg-laying a second molt occurs. The J_2

cuticule remains, enclosing the J_3, which continues in the vermiform state, but sexes start to differentiate (25). After two to three days, the J_4 stage develops, and the nematode has a new stylet and a well-developed cephalic region (25, 59).

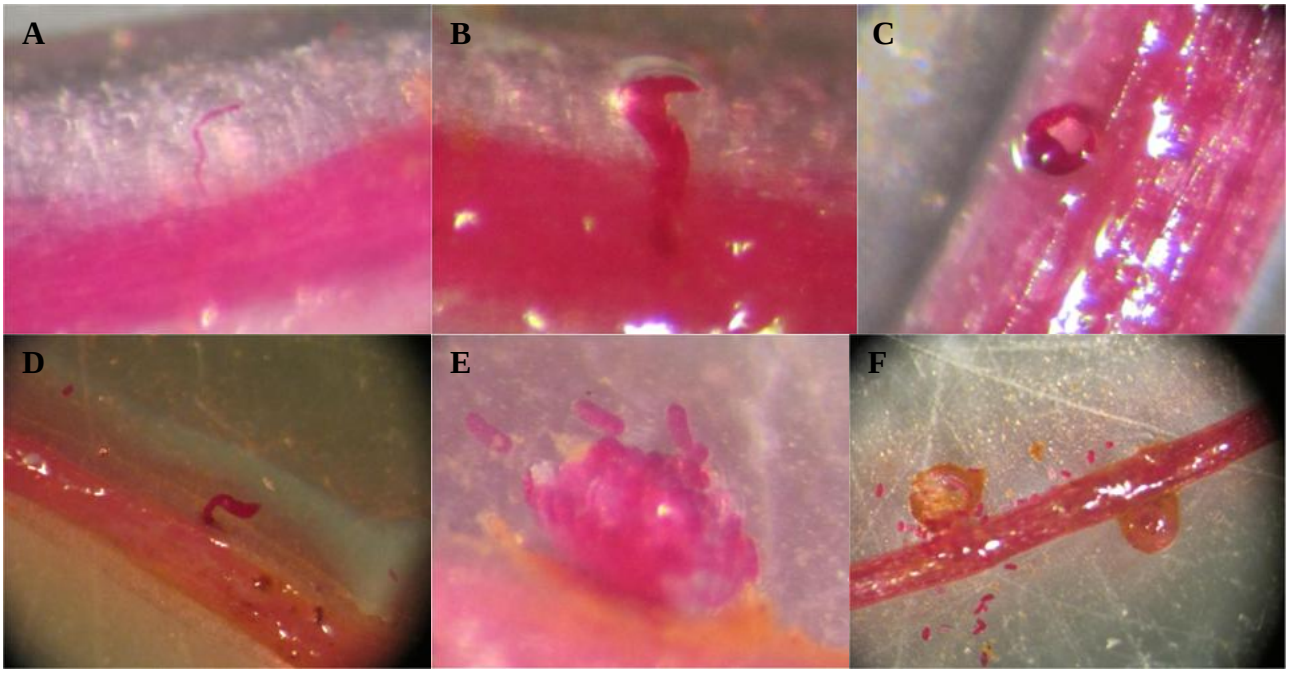

Figure 1. Life cycle of *R. reniformis* in a cotton root under greenhouse conditions: (A) juvenile infecting the root 5 days after planting (DAP); (B-C) female taking the reniform shape 10-15 DAP; (D) ~~M~~mature female beginning to lay eggs 20 DAP; (E-F) eggs being laid in a gelatinous matrix 25-30 DAP.

Females develop ovaries and a vulva, and males have spicula and testes. The adult male and female are vermiform in shape and are often surrounded by the cuticular sheaths from previous juvenile stages. Males are not parasitic and have not been observed to feed. In plant roots, adult vermiform females start feeding from the root until they obtain a kidney-shape. The complete life cycle of the reniform nematode in a cotton root has been shown to require from 17 to 22 days

at 27 - 32°C (69, 80). The most common type of reproduction of *R. reniformis* populations is amphimictic where there are numerous males (40-60% males) (25, 60). However, there are ~~few~~ populations with parthenogenic reproduction where there are few or no males present (59).

III. Current *R. reniformis* management.

Current management is based on crop rotation with *R. reniformis*-resistant soybean cultivars, corn, and rice; and use of chemical nematicides, such as aldicarb, oxamyl, thiodicarb, metam sodium, 1,3-dichloropropene, and abamectin. Aldicarb, oxamyl, and thiodicarb are all in the carbamate chemical class with possible similar modes of action (interfering with the functioning of the enzyme acetylcholinesterase), and a high mammalian toxicity (17). Metham sodium and 1, 3 dichloropropene are soil fumigants that bind to oxygen-carrying molecules, preventing tissues from using oxygen, and are also highly poisonous by inhalation and oral and dermal exposures (18). Abamectin is a fermentation product of the bacteria *Streptomyces avermictilus*, Kim and Goodfellow, which binds to glutamate-gated chloride channels expressed on the nematode neurons and muscle cells (96).

Nematicides are economically sustainable for some areas; however, they require annual applications and can significantly increase the cost of crop production. Typically, *R. reniformis* numbers are reduced the first 30 to 60 days after nematicide application, but numbers increase during the season, reaching populations similar to or higher than the untreated plots at harvest time (42, 48, 52, 66).

During recent years, the Environmental Protection Agency (EPA) has restricted the use of the nematicide aldicarb in cotton production and will remove it from the market by 2018. Therefore, there is a need to explore nematode biocontrol as a management alternative that can be integrated in a cropping system (crop rotation, cover crops, tolerant varieties, etc.) to reduce losses. This review is to discuss the importance of biological control as a management alternative that can be incorporated with other management practices to reduce the losses of nematodes in cotton crops and also provide information on commercial products available in the market that can be used in the future for *R. reniformis* management.

IV. Biocontrol of nematodes.

Plant pathologists define biocontrol as the reduction of inoculum density or disease-producing activities of a pathogen or parasite in its active or dormant state by one or more organisms. This reduction is accomplished naturally or through manipulation of the environment, host, or by mass introduction of one or more antagonists (5). For the specific case of nematodes, the host is not considered part of biocontrol. Therefore, biocontrol is defined as the reduction of nematode populations through the action of living organisms other than nematode-resistant host plants, which occur naturally or through the manipulation of the environment or the introduction of antagonists (84). The antagonists can be parasites, pathogens, competitors, or other organisms that repel, inhibit, or kill plant parasitic nematodes (36, 77).

The main nematode-antagonists primarily used in biocontrol are fungi (endoparasites, egg-parasites, nematode-trapping, and mycorrhiza), bacteria (PGPR, obligate parasites, nematode-toxin and enzyme producers), and predatory nematodes. Although there are other antagonists

(i.e., viruses, mites, collembola, turbellarians, oligochaetes, and protozoans) that can reduce nematode numbers, there is limited information about their efficacy (16, 66, 77, 84).

There is a common misconception that nematodes are almost impossible to control biologically. Soil interactions are very complex, making nematode management difficult, but nematodes, being obligate parasites, are sensitive to a general or specialized antagonist (77). Biological control of *R. reniformis* nematodes has not yet been implemented in cotton crops in the United States, but it is an option for management in the future (66).

Rotylenchulus reniformis is a sedentary semi-endoparasite, which is sensitive to antagonists in all life stages. Mature females feed from the root and the posterior portions of their bodies remain outside the root exposed to the soil microflora (Figure 2 A-B). Eggs are also laid outside the root in a gelatinous matrix. Juvenile stages molt in the soil and need to overcome the rhizosphere microorganisms to colonize roots and establish a feeding channel. The rhizosphere is a dynamic and complex environment where more bacteria, fungi, and free-living and plant-parasitic nematodes can be found compared to a bulk of soil (29). Hence, the antagonist has to be able to compete in the rhizosphere, colonize the root, and offer protection against nematodes (36).

Recent studies have reported *R. reniformis* suppressive soils in Louisiana and Texas (65) and different fungi and bacteria as pathogenic to different stages of its life cycle. Thus, biological control is an option that has to be explored in more detail in future years, and integrated with current management to improve yields and reduce impact of this nematode in the cotton crop.

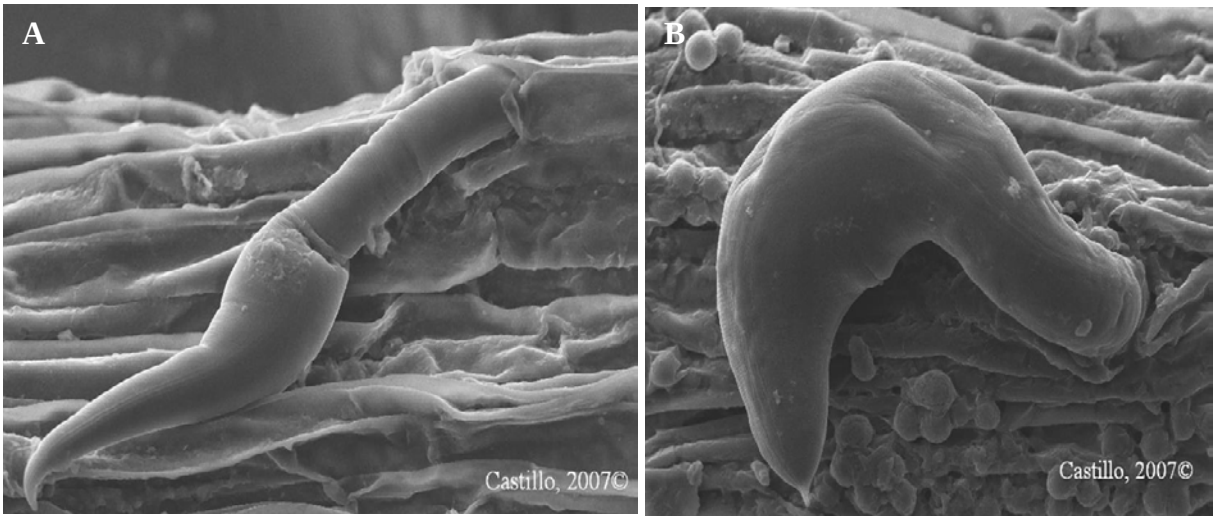

Figure 2. *Rotylenchulus reniformis* (A) young female, and (B) mature female feeding from the cotton root.

V. Antagonists for *R. reniformis*.

Several studies report fungi and bacteria as pathogens of *R. reniformis* (Table 1). Some of these antagonists have been widely studied, reported in other plant-parsitic nematodes, and available in the market under different formulations (i.e., seed treatments and wettable powders). In contrast, the fungi *Arthrographis* sp., *Pseudobillarda* sp., and *Fusarium equiseti* have been identified parasitizing *R. reniformis* (53), but no reports on other nematodes or modes of action have been reported.

Table 1. Antagonistic fungi and bacteria reported for *R. reniformis*.

Organism	Name	*R. reniformis* life stage	Mode of action	Reference
Fungi	*Paecilomyces lilacinus*	Egg	Appresoria and haustoria in eggs	12, 13, 92
	Pochonia chlamydosporia	Egg		93
	ARF (Arkansas fungus)	Egg		94
	Arthrographis sp.	Vermiform	Unknown	53
	Pseudobillarda sp.			
	Fusarium equiseti			
	Drecheslerella dactyloides	Vermiform	Trapping-rings	12, 13
	Drecheslerella brochopaga			
	Catenaria auxiliaris	All	Swimming zoospores encyst	11
Bacteria	*Pasteuria* spp.	Vermiform	Endospores	30, 31, 77
	Bacillus firmus	Vermiform	Metabolite	10
	Pseudomonas fluorescens	Vermiform	Unknown	34

a) Egg-parasite-fungi.

These types of fungi have great potential in biocontrol programs due to their ability to survive in the soil as saprophytes (57, 85). The three egg-parasite fungi that attack *R. reniformis* are *Pochonia chlamydosporia* (Goddard) Zare&Gams, *Paecilomyces lilacinus* (Thom) Samson, and an unidentified isolate named Arkansas fungus (ARF).

Pochonia chlamydosporia initiates infection when it comes in contact with the eggs. The fungus develops germ tubes that will differentiate into aspresoria (32), that is covered by a muscilaginous substance that acts as an adhesive (50). In addition to this substance, proteinases are also important during the infection. *Pochonia chlamydosporia* produce the protease VCP1, which allows infection of *M. incognita* eggs (75).

In Arkansas soils, 117 isolates of *Pochonia chlamydosporia* obtained from eggs of *R. reniformis* nematodes were identified (93). Only 12 of the isolates parasitized eggs, colonizing up to 35% of the eggs *in vitro* and 77% under greenhouse conditions. However, only three of the 12 isolates reduced the numbers of *R. reniformis* nematodes up to 35% on cotton roots and in soils at a concentration of 5,000 chlamydospores per gram of soil.

Paecilomyces lilacinus was initially isolated from eggs of *M. incognita* in Peru (33), and since then has been reported in *Meloidogyne* spp. and *Heterodera* spp. in different parts of the world (19, 84). Its pathogenicity varies depending on the isolate. The mechanism of infection is similar to *Pochonia* spp., where *P. lilacinus* penetrates the egg shell by producing penetrating hyphae and appressoria (49), but the protease used by the fungus is called PL (8). Production of chitinases, proteases, and acetic acid has also been reported to be involved in the mode of action (39). Australian isolates of *P. lilacinus* produce leucinotoxins, a group of toxins responsible for biocontrol activity (64).

Walters and Barker (1994) found that *P. lilacinus* reduced populations of *R. reniformis* nematodes 36% under greenhouse conditions and 59% in microplot trials on tomato

(*Lycopersicon esculentum*). The fungus was grown on rice (*Oryza sativa*) grains and was added to the tomato plots. Further, an isolate of *P. lilacinus* from eggs was reported in cotton plants. The isolate reduced the number of eggs under greenhouse conditions when the fungus was applied at planting using wheat and oats as carriers (13). *Paecilomyces lilacinus* has been shown to grow around and in the epidermis on tomato roots (9), and under *in vitro* conditions parasitizes eggs within 48 hours (Fig. 3A) (13).

Arkansas Fungus (ARF) strains were originally isolated from eggs of the soybean cyst nematode (*Heterodera glycines*), and later five strains were isolated from *R. reniformis*. All the strains of ARF are closely related based on mitochondrial DNA Restriction Fragment Length Polymorphism (mtDNA RFLP) patterns, but the morphology of the colony growth differs. ARF infects and parasitizes eggs of *R. reniformis*, and is belived to produce a natural substance that inhibits the embryonic development and hatching of the eggs. Greenhouse experiments reveal that ARF parasitized eggs, sedentary females, and juveniles. These strains are also pathogenic to the cyst nematode. The parasitism of this fungus ranges from 48% to 79% under *in vitro* conditions, and reduces *R. reniformis* population from 87 to 98% at different rates under greenhouse conditions (94).

b) Ring-trapping fungi.

Drechslerella dactyloides (Drechsler) M. Scholler, Hagedorn & A. Rubner, and *D. brochopaga* (Drechsler) M. Scholler, Hagedorn & A. Rubner were previously reported to reduce populations of *Meloidogyne graminicola* in rice (45, 79) and were also found parasitizing juveniles of *R.*

reniformis nematodes. These fungi produce constricting rings that trap the vermiform stage of the nematodes (Figure 3B).

In Alabama cotton crops, *D. dactyloides* and *D. brochopaga* were isolated from crops and evaluated under *in vitro* and greenhouse conditions (13). Under *in vitro* conditions, conidia of *D. dactyloides* and *D. brochopaga* require at least 72 hours (3 days) to germinate, produce trapping-rings, and start ensnaring *R. reniformis* vermiforms. The conidia of *D. dactyloides* and *D. brochopaga* have a lag phase to adapt to changing environments and begin forming trapping rings to ensnare *R. reniformis* vermiforms.

Under greenhouse conditions, *R. reniformis* population was reduced with the application of these fungi in autoclaved soil; however, in non-autoclaved soil there were no differences with the untreated control. Ring-trapping fungi are weak competitors in soil and grow at a slower rate compared to the egg-parasite fungi. They only produce rings under certain conditions (e.g., when food source is scarce) (12, 13, 35). Nevertheless, they are very efficient and specific in trapping vermiform life stages. Therefore, mixing them with egg-parasitic fungi can improve nematode reduction. Granuled formulations of *D. dactyloides* reduced the number of *Meloidogyne javanica* more than 90% and reduced the galling in tomato roots 57-98% (83).

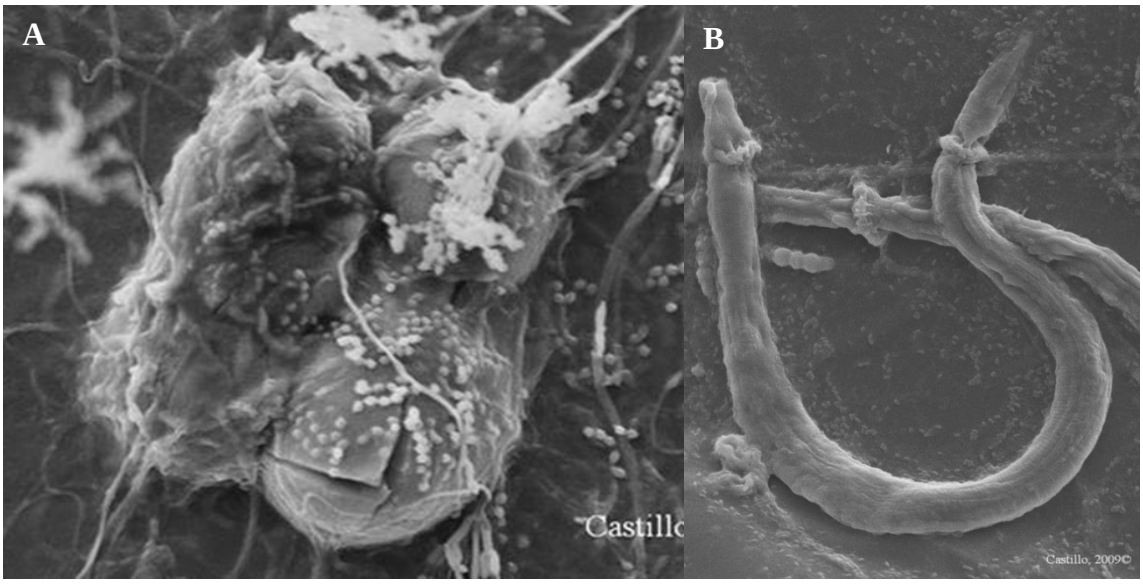

Figure 3. *Paecilomyces lilacinus* parasitizing *R. reniformis* eggs (A); *Drechslerella dactyloides* trapping a *R. reniformis* vermiform (B).

c) Zoosporic fungi.

Catenaria auxuliaris (Kuhn) Tribe has been reported to parasitize *R. reniformis* populations on cotton plants under greenhouse conditions, where 46% of the stock nematode population was colonized (11). Egg, females, and vermiform life stages were microscopically observed under light microscopy and SEM. All life stages were sensitive to this obligate-parasite fungus (Fig.4). Eggs become internally colonized with zoosporangia, and in advanced stages of infection, they darken in color. Subsequently, zoosporangia erupt through the cuticle. In vermiform life stages, *C. auxiliaris* forms a rhizomycelium in the initial phase of infection, and adult females exhibit zoospores encysted in the metacorpus region. The zoospores swim short distances, maneuvering in the direction of the flagellum.

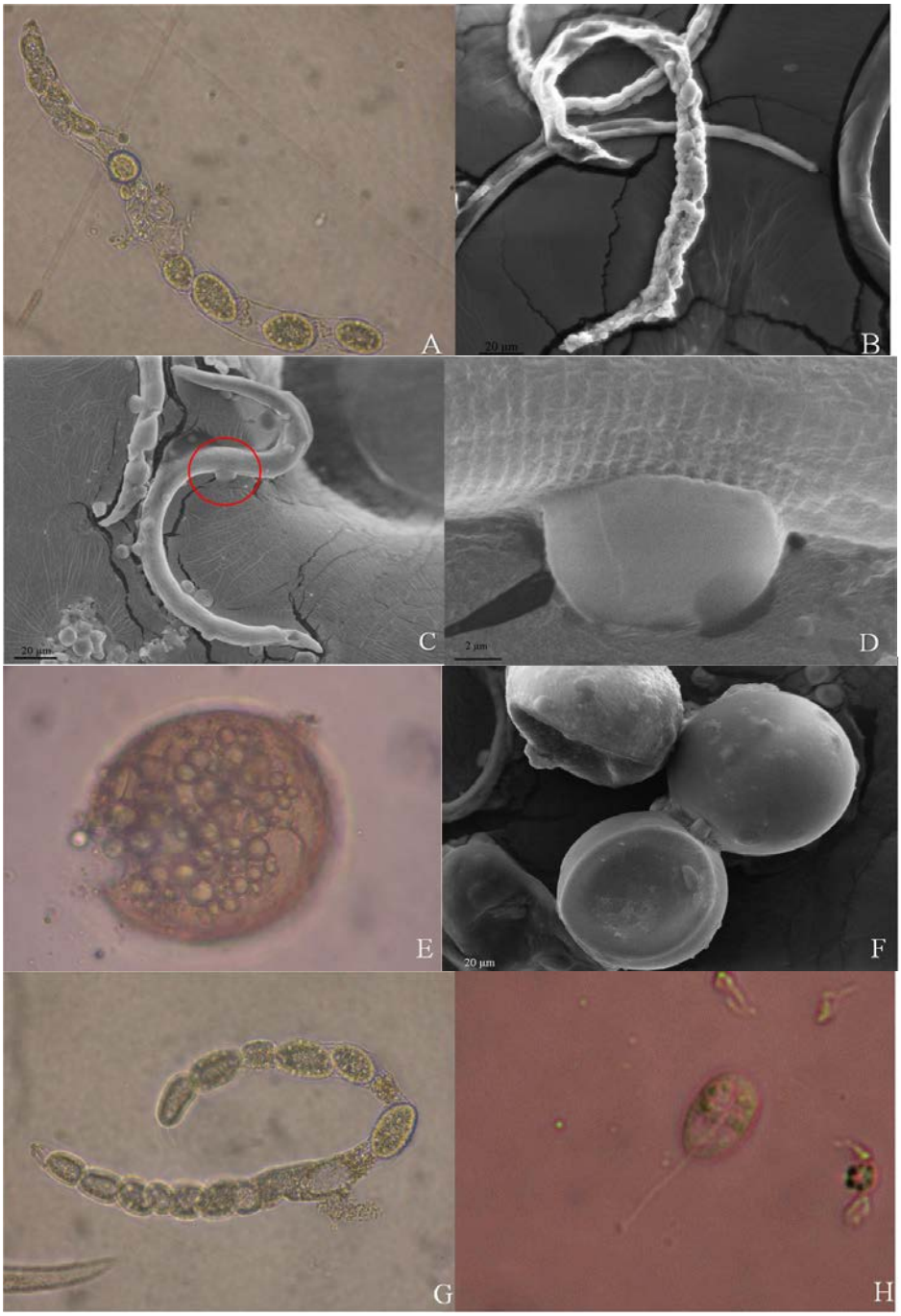

Figure 4. Rhizomycelium of *C. auxiliaris* inside the body of *R. reniformis* under light microscope (40X) (A) and under Scanning Electron Microscopy (SEM) (B); Vermiform stage with swellings caused by the sporangia formation (C) and zoom of the sporangia in the red circle (D); Resting spores under light microscope (E) and under SEM (F); Zoosporangia inside the nematode corpse releasing zoospores (40X) (G); uniflagellated zoospore (100X) (H).

This nematophagous fungus has been previously reported on the beet cyst nematode (*Heterodera schachtii*) in Europe (38, 87). Biocontrol potential of this fungus depends on the moisture of the soil, thus zoospores can swim and search for their host. Scarce research exists on this fungus. The fact that it is an obligate parasite makes it difficult to study, however molecular research based on rDNA has been completed (Castillo et al. unpublished). In contrast, the infection process of the facultative endoparasite of nematodes, *Catenaria anguillulae* Sorokin, has been studied in detail by Deacon and Saxena (1997).

d) *Pasteuria* spp.

Pasteuria spp. are gram-positive, dichotomously-branched bacteria, with septate mycelium. They form endospores that are non-motile structures that reside in the soil and attach to the nematode (70). *Pasteuria* spp. are aggressive parasites of plant-parasitic nematodes, converting each adult nematode into a mass of bacterial endospores through its life. Endospores are released and attach to other nematodes (70).

To date, six species of *Pausteria* sp. have been described based on their host preference (27). Five of those have been described on plant-parasitic nematodes: *P. penetrans* (on *Meloidogyne* spp.), *P. thornei* (on *Pratylenchus penetrans*), *P. nishizawae* (on *Heterodera* spp. and *Globodera* spp.), Candidatus *P. usage* (on *Belonolaimus longicaudatus*), and *P. hartismeri* (on *M. ardenensis*). *Pasteuria ramosa* is a parasite of the water flea *Daphnia magna* Straus, and is the type specie of the genus (22). Identification and characterization of *Pasteuria* spp. are based on morphology, life cycle and development, host range, and DNA sequences (6, 26, 70, 71).

Pasteuria penetrans is mistakenly used to refer to other *Pasteuria* members that parasitize other nematodes than *Meloidogyne* spp. Studies with bacterial DNA obtained from disrupting endospores within the host nematodes, followed by PCR with universal primers 27F and 39F that recognize the 16S rRNA, reveal that *Pasteuria* is closely related to Bacillus-Clostridium clade, and the species designation is related to the host from which they were isolated (3, 14). At present there are 58 sequences of the *Pasteuria* 16S rRNA on the GenBank that can be used to characterize the different isolates (27).

In cotton field soils in Alabama, *Pasteuria* spp. strain Pr3 was isolated from *R. reniformis.* This strain was able to infect and complete the life cycle in juveniles, males, and females (72). Cotton seeds treated with *Pasteuria* spp. Pr3 at a rate of $1x10^8$ endospores/seed suppressed *R. reniformis* similar to the seed treatment Aeris® (imidacloprid + thiodicarb) (72).

e. Plant Growth Promoting Rhizobacteria (PGPR)

This group of bacteria has beneficial effects on plants. They enhance emergence, colonize the roots, stimulate plant growth, and can also suppress diseases (43, 76). For nematode biocontrol, they have great potential because they become intimately associated with their host plant at the root surface. Therefore they can be in direct contact with the target sites of entrance and feeding sites of nematodes (51). Antagonists that colonize the rhizosphere can strongly influence root exudates and thereby potentially affect the development of the nematode (77).

Rhizosphere bacteria have been reported to reduce nematode invasion of roots by modifying rood exudates or by producing toxins that kill the nematodes before they invade or feed on host

tissue (36, 37). Rhizobacteria use several mechanisms for nematode suppression such as production of ammonia compounds, production of toxins and chemicals, production of lytic enzymes (chitinases and collagenases), and induction of resistance in the host plant (4, 51).

Induced systemic resistance (ISR) is the process of active resistance dependent on the host plant's physical barriers, activated by biotic or abiotic agents (inducing agents) (43). Induced resistance is systemic because the stimulated defensive capacity is increased not only in the site where the inducer was applied but also in spatially separated tissues (90). Early studies in Germany report induction of resistance and reduction of potato cyst nematode (*Globodera pallida*) by *Bacillus sphaericus* and *Agrobacterium radiobacter* in split-root trials under greenhouse conditions (28).

In the case of *R. reniformis*, root penetration in tomato was reduced by 44.5% in a split-root trial and 51% in unsplit root trial with *Bacillus subtilis* isolate Bs_t at a cell suspension of 10^{10} cells/ml (61). No study has been published on systemic resistance inductors for nematodes in cotton. However, the rhizobacterial strain *Bacillus subtilis* A-13 was tested against *M. incognita* on cotton and sugarbeet and *R. reniformis* on cotton. A reduction of 38% to 68% was observed in all plants in autoclaved soil. In non-autoclaved soil a reduction was observed in *M. incognita* in sugarbeet and *R. reniformis* in cotton, but not in *M. incognita* in cotton (78).

Strains from the bacterium *Pseudomonas fluorescens* were isolated from the rhizosphere of cotton plants from different locations in India (34). Isolates reduced the soil and root *R. reniformis* populations by up to 70.4 and 44.8%, respectively. Further, root and shoot weights were significantly higher in plants treated with isolates of *P. fluorescens*. The reduction in

nematode population was due to the ability of the bacterium to develop or bind on the root surface lectins, thereby interfering with normal host recognition by nematodes (34).

Bacillus firmus strain GB-126 was originally isolated in Israel and has been reported to reduce *M. incognita* under field conditions and *Radopholus similis*, *Ditylenchus dipsaci*, and *Heterodera glycines* under *in vitro* conditions showing that the mode of action is a metabolite that inhibits the egg development and root infection (54, 73, 86). Currently, a seed treatment formulation is being studied for *R. reniformis* in cotton roots under *in vitro*, greenhouse, and field conditions (Castillo unpublished data).

In cotton trials under greenhouse conditions, the rate of 1.4 $x10^7$spores per seed is able to reduce *R. reniformis* damage during the first 30 days after planting (DAP). A reduction in numbers of vermiform life stages of *R. reniformis* was observed at 15 DAP. As a result, there were fewer females per gram of root at 20 to 30 DAP and fewer eggs produced at 30 DAP (10). The mode of action of this bacterium against *R. reniformis* remains unclear; however, studies on ISR and production of metabolites are on going.

VI. Antagonists as commercial products

Since the EPA has restricted the use of the nematicide aldicarb (Temik®) and will be removing it from the market in 2018, other nematode management alternatives have become a matter of interest. Biological control of *R. reniformis* nematodes has not yet been implemented in cotton crops in the United States, but can be an option for nematode management (66).

The ideal antagonist should produce inoculum in excess, be able to resist, escape, or tolerate other antagonists, germinate and grow rapidly, and survive and grow in the rhizosphere (5). However, the ideal antagonist does not exist, and the main limiting factor in the commercialization of these antagonists is the inconsistent performance in the field. Currently, many private companies are searching for antagonists or by-products of antagonists that can be used in nematode control programs (Table 2).

Table 2. Commercial formulations of nematode-antagonists (Table 2).

Product	Microorganism	Host Nematodes	Company and country	Reference
BioAct ® WG (MeloCon)	*Paecilomyces lilacinus*[1] strain 251	*Meloidogyne* spp. *Radopholus similis* *Heterodera schachtii*	Prophyta (Germany)	39, 40, 41
DiTera ®	*Myrothecium verrucaria*[2]	*Globodera* spp. *Heterodera* spp. *Meloidogyne* spp. *Radopholus similis*	Valent Biosciences Corp. (U.S.A.)	23, 88
Xianchongbike	*Pochonia chlamydosporium*[1]	*Meloidogyne* spp.	Yunnan University (China)	58
Econem ®	*Pasteuria penetrans*[4,5]	*Belonolaimus longicaudatus* *Meloidogyne* spp. *Rotylenchulus reniformis*	Pasteuria Bioscience (U.S.A.)	29, 30,72
Votivo ® WP	*Bacillus firmus* strain *GB-126*[4]	*Meloidogyne incognita* *Ditylenchus dipsaci* *Rotylenchulus reniformis*	Bayer CropScience[6] (Germany)	86
Pathway Consortia ®	*Bacillus subtilis* *B. licheniformis* *B. megaterium* *B. coagulans* *Pseudomonas fluorescens* *Streptomyces* spp. *Trichoderma* spp.	*Meloidogyne* spp. *Rotylenchulus reniformis*	Pathway Holdings (U.S.A.)	Castillo, unpublished data

[1] Egg-parasitizing fungi.
[2] Fungi that produce toxins against nematodes.
[4] Bacteria.
[5] EPA (Environmental Protection Agency) Registration.

Paecilomyces lilacinus strain 251 is a commercial strain that is currently available in several countries under the names BioAct®, MeloCon®, and MycoNema®. It is formulated by Prophyta

as a wettable powder ($1x10^9$ spores/gram) and is registered by the EPA. This strain has been demonstrated to reduce populations of *Meloidogyne* spp., *R. similis*, and *H. schachtii* in different crops (39, 40, 41, 54). Different studies on this strain show that there are no adverse effects on mutualistic fungal endophytes, mychorhizas, entomopathogenic nematodes, or other fungal antagonists (39). Further, nematode control improves when the strain has been applied before planting and with other antagonists. When applied six days before tomato planting, it is able to reduce *M. incognita* galls between 58 and 74% (40). Additionally, in bananas the strain was able to reduce *R. similis* with three applications: before transplant, 24 hours before transplant, and at transplant (41). The combination of *P. lilacinus* strain 251 with *Fusarium oxysporum* strain 162 caused a 68.5% reduction of *R. similis* in banana roots, compared to the individual application of *P. lilacinus* that reduced *R. similis* by 54% compared to the control (54).

DiTera® is the product of the fermentation of the fungi *Myrothecium verrucaria*, where the main compounds are sugars and proteins. This product has adverse effects on the hatching of juveniles of *Globodera rostochiensis* and *G. pallida*. Even though the mode of action remains unclear, it seems to be related to a direct interference in the egg hatching process of the nematode (88). The egg hatching of *M. incognita* is not affected directly by the product; however, the cuticle permeability seems to be affected. Under field conditions, the inhibition in the egg hatching seems to indirectly influence changes in the soil microflora. Fernandez *et al.* (2001) reported significant changes in the bacterial population in the rhizosphere after the product was applied, with increases in the enzymatic activity of the soil and the population of proteolytic bacteria.

Pochonia chlamydosporium (Xianchongbike®) is a product developed at Yunnan University in China. Its formulation is based on conidia and mycelia in a liquid media. It was found to reduce populations of *Meloidogyne* spp. in tobacco crops (58). The mode of action is as described above in the egg-parasite fungi.

Pasteuria penetrans is the most studied bacteria with a high biocontrol potential (15). However, being an obligate parasite, it was unculturable. Therefore it was considered impossible to commercialize it as a biological nematicide. In 2003 the company Pasteuria Bioscience Inc. was able to mass produce this bacterium in fermenters. The main objective has been to reduce populations of sting nematode (*Belonolaimus longicaudatus*) in golf fields, *Meloidigyne* spp. in different crops, and currently *R. reniformis* in cotton (29, 30, 72).

Bacillus firmus strain GB-126 is the antagonistic bacterium and biocontrol commercial product most recently developed. It was originally isolated and commercially formulated by Agrogreen in Israel, and due to its biocontrol potential it was purchased by Bayer Crop Science. It is currently commercialized with the insecticide imidacloprid as a seed treatment on soybean and cotton against cyst and reniform nematode, respectively. It is also formulated as a wettable powder for turf grass against sting nematode. Its mode of action is uncertain, although *in vitro* experiments show inhibition of egg eclosion and immobilization of juveniles of *M. incognita* by the production of nematostatic compounds (86). Field applications of 200 and 400 Kg/ha reduce the galls (75-84%) and increase the height (29-31%) and weight of the plants (20-24%) after 45 days. When *B. firmus* is applied with other antagonists, nematode control is enhanced. Combined

application with *F. oxysporum* was effective in controlling *R. similis* on banana (86.2%), compared to *B. firmus* alone (63.7%) (54).

Pathway Consortia® is a recent biocontrol product under development that is formulated in liquid, granular, and thixotropic (properties of gels becoming fluid when disturbed) forms. These formulations mix multiple PGPR strains of *Bacillus subtilis, B. licheniformis, B. megaterium, B. coagulans, P. fluorescens, Streptomyces* spp., and *Trichoderma* spp. Preliminary results on cotton under greenhouse conditions show growth promotion and *R. reniformis* reduction in autoclaved and non-autoclaved soils (Castillo, unpublished data). This product is recommended to be applied at least three times: 7 days before planting, once at planting, and 7 days after planting.

There have been nematode biocontrol products that were retired from the market because they were reported to be hazardous to human health. Deny® was a product based on the bacterium *Burkholderia cepacia*, which reduced egg hatch and juvenile motility of *M. incognita* in pepper and increased the shoot weight (55). However, this bacterium has been reported as a nosocomial bacterium on patients with cystic fibrosis. It was withdrawn from the market in the United States in 2005.

VII. Conclusion.

Biocontrol of *R. reniformis* can be a viable practice that reduces cotton losses. More research on understanding the colonization of nematode antagonists and their interactions in the cotton rhizosphere is necessary. *Rotylenchulus reniformis* is a sedentary semi-endo parasitic nematode,

which feed from the cotton root inserting one-quarter of its body to establish a feeding channel, and three-quarters of are outside the root. Eggs are laid in a gelatinous matrix outside the root. They hatch to juvenile stages (J_2, J_3 and J_4) that will be outside the root searching for new hosts. Therefore, *R. reniformis* is exposed to different antagonists in the soil during its life cycle. Juveniles and female stages are vulnerable to parasitism or exposure to compounds that repel or affect the feeding channel establishment in the root.

There is no ideal biocontrol agent; hence it is necessary to understand the life cycle of *R. reniformis* under different environmental conditions (e.g., soil type, temperatures, pH, moisture), the biology of the antagonists, and the plant response during this three-way interaction. Based on that understanding, there can be several possible applications of antagonists that act at different nematode life stages, such as applying a juvenile and egg antagonists before planting, using a juvenile antagonist with PGPR strains at planting, and applying an egg antagonist plus a PGPR strain after planting. However, commercial companies need to continue improving the formulations (seed treatments, thixotropic formulations, wettable powder carriers) of the biocontrol products to make them safe, reliable, practical, and economically feasible to the growers. Biocontrol of *R. reniformis* has to be part of an integrated management program where other control practices such as crop rotation, cover crops, and use of less nematode-susceptible cotton varieties are applied.

The impact of losing aldicarb from the market in future years will be reduced if more research is conducted exploring biocontrol antagonists. *Rotylenchulus reniformis* suppressive soils in Texas and Louisiana (65) have been reported, but the organisms involved in this suppression were not

identified. Study of the interactions among *R. reniformis*, antagonist (commercial biocontrol agent), and cotton rhizosphere in different types of soils is needed. Field studies with biocontrol agents seem to be inconsistent. Therefore it is necessary to understand the variations in the performance of the biocontrol agent under different field management systems. Further, some antagonists can adapt better to certain environmental and soil types than others. Hence, it is important to isolate more antagonists, evaluate their activity, and determine the soil and environmental conditions in which they perform best. *Rotylenchulus reniformis* biocontrol is possible, but more research is needed to understand rhizosphere interactions under field conditions.

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Chapter II. Biocontrol potential of *Bacillus firmus* strain GB-126 and *Paecilomyces lilacinus* strain 251 to the reniform nematode (*Rotylenchulus reniformis*) on cotton.

Abstract

Biological control options are becoming more prevalent and economically feasible in sustainable agriculture production systems. Due to increased EPA restrictions of chemical nematicides, the search for nematode management alternatives such as biocontrol is needed. The objectives of this study were: *i)* to evaluate the biocontrol potential of *Bacillus firmus* GB-126 and *Paecilomyces lilacinus* 251 in commercial formulations to manage *Rotylenchulus reniformis* in cotton; and *ii)* to evaluate the response on the nematode population when these products are applied separately or concomitantly on cotton grown under greenhouse, microplot, and field conditions. In the greenhouse, cotton seeds treated with *B. firmus* (1.4 x 10^7 cfu/seed), or an application of *P. lilacinus* (0.3% v/v of water), and the combination of both reduced the number of females, eggs, and vermiform life stages of *R. reniformis* ($P \leq 0.02$) and increased free-living nematode population 30 DAP ($P \leq 0.01$). In microplots, populations of *R. reniformis* vermiform life stages decreased through the growing season when exposed to both biocontrol agents ($P \leq 0.04$). Cotton plant stem diameter also increased. Free-living nematode populations increased at mid-season ($P \leq 0.01$). Consistently in the field, the populations of *R. reniformis* vermiform life stages were reduced. These populations decreased at mid-season when cotton began blooming until bloom peak ($P \leq 0.01$). During this same time period, stem diameter and free-living nematode numbers also increased ($P \leq 0.01$). There was a reduction of females, eggs, and vermiform life stages at the end of the growing season. Cotton yields from the application of *B. firmus* GB-126 and *P. lilacinus* 251 were similar to those from the chemical standard aldicarb.

I. Introduction

Rotylenchulus reniformis (Linford and Oliveira), the reniform nematode is the primary economic pathogen in cotton production in the southeastern region of the U.S (41, 42). This plant-parasitic nematode causes an annual average of 5% loss of the total cotton production in Louisiana, Mississippi, and Alabama (5). Current management is based on crop rotation and use of chemical nematicides. The Environmental Protection Agency (EPA) has restricted the use of aldicarb, which is the most common nematicide used in cotton production. The aldicarb application rate for cotton is restricted to 7.8 kg/ha (7 lb/acre) and will be retired from the market on August 31, 2018 (2). Therefore, there is a need to explore nematode management alternatives such as biocontrol options.

Over the past 20 years, studies on biological control of *R. reniformis* have reported antagonistic activity by nematophagous fungi and strains of bacteria at different stages of the nematode life cycle (22, 48, 49, 50). *Paecilomyces lilacinus*, *Pochonia chlamydospora*, and an unidentified fungus named Arkansas Fungus (ARF) have been documented as parasites of the egg stage of *R. reniformis* (50). The vermiform life stages have been colonized by the fungi *Arthrographis* sp., *Pseudorobillarda* sp., and *Fusarium equiseti*. All were found to reduce nematode populations on cotton (33). The zoosporic fungi *Catenaria auxiliaris* has also been reported colonizing *R. reniformis* vermiform life stages (8). Furthermore, the bacteria *Pasteuria* spp. and isolates of *Pseudomonas fluorescens* have been reported to reduce the number of *R. reniformis* in soil (17, 22, 43). Recently, soils suppressive to *R. reniformis* have been reported in Louisiana and Texas; however, the agents responsible for this suppression have not been identified (40).

Currently there are two biocontrol commercial products available on cotton for nematode control. The biocontrol agents are *Paecilomyces lilacinus* strain 251 and *Bacillus firmus* strain GB-126. *Paecilomyces lilacinus* strain 251 is an egg-parasite fungus that has been reported to reduce eggs of *Meloidogyne javanica* (14) and *R. reniformis* (47). It is currently commercialized in a wettable powder formulation under the name of NemOut®. *Bacillus firmus* strain GB-126 is a bacterium originally isolated in Israel, and currently formulated as a seed treatment under the name of Votivo® or wettable powder under the name of Nortica 5%WP®. It has been reported to produce metabolites responsible for the reduction of *Radopholus similis*, *Meloidogyne incognita*, and *Ditylenchus dipsaci* under *in vitro* conditions (34).

Paecilomyces lilacinus was initially isolated from eggs of *M. incognita* in Peru (21), and later reported colonizing *Meloidogyne* spp. and *Heterodera* spp. in other parts of the world (12, 26, 45). Its pathogenicity varies depending on the isolates. The mechanism of infection is by penetration of the nematode egg shell with an appressoria that develops from hyphae and secretes the PL protease (6, 32). Additionally, the production of the chitinase enzymes and acetic acid has also been reported to be involved in the mode of action (25). In the Australian isolates of *P. lilacinus*, a group of toxins called the leucinotoxins, are the responsible for biocontrol activity (38).

Walters and Barker (1994) found that *P. lilacinus* reduced populations of *R. reniformis* nematodes 36% under greenhouse conditions and 59% in microplot trials on tomato, when the fungus was applied via infested rice grains. Further, an isolate of *P. lilacinus* from eggs was

reported in cotton plants (10). The isolate reduced the number of *R. reniformis* eggs under greenhouse conditions when the fungus was applied at planting using wheat and oat as carriers (9). *Paecilomyces lilacinus* has been observed growing intercellularly in the epidermis of tomato roots (7), and under *in vitro* conditions, it parasitizes eggs within 48 hours (9).

Bacillus firmus strain GB-126 under field conditions is able to reduce *M. incognita* in tomato roots with a single application. Furthermore, it has been reported reducing *Radopholus similis*, *Ditylenchus dipsaci*, and *Heterodera glycines* under *in vitro* conditions (34, 44, 47). *In vitro* studies suggest that the mode of action is a metabolite that inhibits egg development and root infection of *M. incognita* (34, 47). The objective of this study was to evaluate the commercial biocontrol products *Bacillus firmus* GB-126 and *Paecilomyces lilacinus* 251 to determine their potential to reduce *R. reniformis* populations and enhance cotton plant growth under greenhouse, microplot, and field production systems. Additionally, each biocontrol agent was evaluated separately and in combination to determine their individual and combined pathogenicity to *R. reniformis* life stages.

II. Materials and Methods

To evaluate the response of *R. reniformis* to applications of *B. firmus* and *P. lilacinus* on cotton, three trials were conducted under greenhouse conditions in the Plant Science Research Center (PSRC) of Auburn University. In the first trial, each biocontrol agent was evaluated independently in autoclaved field soil at three different rates. The soil was autoclaved using two 90 minute cycles at 130ºC at 1.0 kg/cm^3 presure with a 24 hour cool down between cycles to remove any natural competition for the microflora. In the second trial, rates that reduced

nematode numbers were evaluated in non-autoclaved field soil with microflora competition. In the third trial, each biocontrol agent, *B. firmus* and *P. lilacinus,* were evaluated separately and in combination. This third trial was replicated under microplot and field conditions. In all the experiments the cotton cultivar used was 'Stoneville 5458B2RF', and the soil was a Decatur silty clay loam (sand-silt-clay: 17.5-51.3-31.2%; nitrogen: 0.16%; organic matter: 2.2; pH 7.24) from the Tennessee Valley Research and Extension Center (TVREC) near Belle Mina, AL.

In all the trials the vermiform stages were extracted from the soil by the modified gravity screening and sucrose centrifugation-flotation (23). Eggs stages were extracted from cotton roots by shaking the root system in a 1% NaOCl solution for four minutes at 120 rpm. The nematode suspension was collected and rinsed with water through a 25 µm sieve (20). Females in roots were stained with acid fuschin to facilitate enumeration of the females invading the root (18). Vermiform life stages and eggs were counted under an inverted TS100 Nikon microscope at 40x magnification. Females embedded in the root systems were quantified at 5x magnification utilizing the Nikon SMZ800 compound microscope. Cotton seeds were treated with *Bacillus firmus* by the manufacturer in a liquid seed dresser Hege11(Hege Maschinen GmbH, Germany), and the presence of the bacterium in the seed was confirmed by culturing the treated seed on Triptic Soy Agar (TSA) adjusted to pH 8.0 and recording the bacterial growth after 16 hours. The formulation of *P. lilacinus* is a wettable powder applied at $1x10^{10}$ spores of the fungus per gram. Percent of spore germination was determined by counting one hundred spores 24 hours after culturing on Potato Dextrose Agar (PDA) at 27ºC.

Data collected in all the trials were analyzed in SAS 9.1 (SAS Institute Inc.). The distributional assumption was evaluated with the student panel graphs of the GLIMMIX procedure. Dunnett's option was used to assess the differences between treatments, the untreated control, and aldicarb. Adjusted *P*-values rather than significance classes are presented in the tables.

Greenhouse trials

The first greenhouse trials were conducted in the Decatur silt clay loam autoclaved field soil described previously. In the *P. lilacinus* initial trial, treatments were applied at planting and consisted of the following: *i)* control without nematodes; *ii)* control with nematodes; *iii) P. lilacinus* (0.02% v/v); *iv) P. lilacinus* (0.1% v/v); and *v) P. lilacinus* (0.2% v/v). In the *B. firmus* trial, seed treatments were as follows: *i)* untreated seed with nematodes; *ii)* imidacloprid (500 g ai/100kg) a standard insecticide; *iii) B. firmus* ($7x10^4$ cfu/seed) plus imidacloprid (500 g ai/100kg); *iv) B. firmus* ($7x10^5$ cfu/seed) plus imidacloprid (500 g ai/100kg); and *v) B. firmus* ($7 x10^6$ cfu/seed) plus imidacloprid (500 g ai/100kg). The standard insecticide seed treatment imidacloprid was included because this insecticide is commonly applied as a combination seed treatment and was tested to determine its effect on *B. firmus* and *R. reniformis*.

Variables measured included plant height, shoot and root weights, females and eggs per gram of root, and the number of vermiform life stages in 500 cm^3 of soil. Data were recorded every five days until day thirty for a total of six harvest timings. Roots were stained with acid fuchsin and numbers of females and eggs per gram of root determined. The number of vermiform life stages in 500 cm^3 of soil was also recorded. Greenhouse average temperatures where plants were grown

were 29˚C. Soil moisture was kept in the ideal moisture range between 40-60% of the maximum water holding capacity, and all the experimental units received the same amount of water.

A second set of trials was conducted in the same soil type; however, it was not autoclaved to determine the competitive effect of the biological control agents with the natural soil microflora. Treatments with *P. lilacinus* were applied at planting and consisted of: *i)* untreated control; *ii)* aldicarb (5.6 kg/ha); *iii) P. lilacinus* (0.1% v/v); *iv) P. lilacinus* (0.2% v/v); and *v) P. lilacinus* (0.3% v/v). In the *B. firmus* trial, treatments included the following: *i*) untreated control; *ii*) aldicarb (5.6 kg/ha); *iii*) *B. firmus* (1 x10^6 cfu/seed); *iv*) *B. firmus* (7 x10^6 cfu/seed); and *v) B. firmus* (1.4 x10^7 cfu/seed). In both trials the *B. firmus* and *P. lilacinus* rates were compared with an untreated seed control and the industry standard chemical nematicide aldicarb. Plant variables and nematode populations were monitored and recorded as in the first trial. For the second greenhouse test, root architecture was measured to determine root length, surface area, and number of tips using WinRHIZO Pro (Regent Instruments Inc.)

In the final greenhouse experiment, the most effective rates of each biological were selected from the previous trials and were evaluated separately and in combination. Treatments included: *i)* untreated control; *ii)* aldicarb (5.6 kg/ha); *iii) B. firmus* (1.4 x10^7 cfu/seed); *iv*) *P. lilacinus* (0.3% v/v) (1.5 gr/100 ml); and *v) B. firmus* (1.4 x10^7 cfu/seed) plus *P. lilacinus* (0.3% v/v). Parameters measured were the same as in the previous trials.

All six greenhouse trials were arranged in a RCBD experimental design with five replications and each was repeated twice over time for a total of 12 trials. Plant height, shoot weight, and root

architecture (root length, surface area, and number of tips) were recorded in each. Roots were stained with fuchsin acid and numbers of females and eggs per gram of root determined. In the first and second trials, numbers of vermiform life stages found in 500 cm^3 of soil were also enumerated. Data were recorded every five days for thirty days. In the third trial, data were recorded 15 DAP and 30 DAP.

Microplot trial

This trial was conducted in Plant Science Research Center located on the Auburn University main campus. The treatments included the following: *i)* untreated control; *ii)* aldicarb (5.6 kg/ha); *iii) B. firmus* (1.4 $x10^7$ cfu/seed); *iv*) *P. lilacinus* (0.3% v/v); and *v) B. firmus* (1.4 $x10^7$ cfu/seed) and *P. lilacinus* (0.3% v/v). In this trial, the biological treatments were applied at three timings (7 days before planting, at planting, and 7 days after planting). Two formulations of *B. firmus* were utilized in the microplot trials. First one as a seeds treatment of *B. firmus* at planting, and the second one as a 5% wettable powder formulation (Nortica) at 7 days pre-plant and 7 days post-planting. This wettable powder formulation contained 3 x 10^9 spores per gram of product. The microplots are 4500 cm^3 and were filled with 12.5 Kg of the same Decatur silty clay loam field soil described previously. Each microplot was planted with four Stoneville 5458 B2RF cotton seed. Plant parameters recorded included plant height, stem diameter, number of nodes, and yield. Nematode populations were monitored by determining numbers of females and eggs per gram of root, and number of vermiform life stages and free-living nematodes per 150 cm^3 of soil. All parameters were recorded 30, 60, 90, and 150 DAP. As previously, an RCBD design was utilized with five replications and the entire test was repeated twice. An extra microplot was connected to a temperature and moisture data logger to record these environmental variables

during the growing season. Average soil temperature during the whole trial was 26.8°C and volume water content of 0.2% v/v (field capacity).

Field trial

A field trial was conducted at the Tennessee Valley Research and Extension Center near Belle Mina, AL in a field which was infested with *R. reniformis* in 2005. The soil in this field is a Decatur silty clay loam. The treatments were exactly the same as the microplot trial with the biological treatments being applied 7 days pre-plant, at plant, and 7 days post-planting. Each experimental unit consisted of a two-row plot (7.6 x 2 m) planted with 100 cotton seeds each. The experimental design was a RCB with five replications and the entire test was repeated twice. For each plot, the biocontrol treatment was suspended in 4 liters of water and applied to each row using a garden sprinkler can. The same amount of water was applied to the control and aldicarb treatments. A data logger was installed within the field to record moisture and temperature during the growing season. Soil moisture was at field capacity with average of volume water content of 0.27 m^3/m^3. The field was irrigated as needed with a linear overhead sprinkler irrigation system.

Three plants in each plot were marked with a fluorescent tape to allow plant variables to be determined from the same plants during the entire growing season. Variables recorded were plant height, stem diameter, and number of nodes. Nematode variables included numbers of females and eggs per gram of root and number of vermiform life stages and free-living nematodes in soil. Data were collected at 0, 30, 60, 90, and 150 DAP. At the end of the growing season cotton yield was collected with a Case IH plot picker.

III. Results

Greenhouse trials

In the autoclaved soil trial, all rates of *P. lilacinus* reduced the number of *R. reniformis* females per gram of root from 15 to 25 DAP. The higher rate (0.3% v/v) resulted in lower numbers from 5 to 30 DAP ($P \leq 0.001$). This rate also reduced the number of eggs per gram of root during the 5 to 30 DAP time period ($P \leq 0.001$) and reduced numbers of vermiform life stages in the soil 10 to 30 DAP. Root fresh weight of the cotton plants was increased at this rate when compared to the untreated control ($P \leq 0.01$). The highest rate of the *B. firmus* seed treatment reduced the number of *R. reniformis* females and eggs per gram of root, and vermiform life stages in the soil from 20 to 30 DAP compared to the control ($P \leq 0.01$). The insecticide imidaclorprid seed treatment control did not influence any plant growth parameters or reduce populations of *R. reniformis* ($P \leq 0.77$).

In the non-autoclaved field soil trial which allowed for natural soil competition, all three rates of *P. lilacinus* reduced the number of females and eggs in the root, and vermiform life stages in the soil ($P \leq 0.01$). The higher (0.3% v/v) rate consistently reduced the number of females and eggs per gram of root ($P \leq 0.001$), from 10 to 30 DAP compared to the control (Figure1). Vermiform life stages in the soil were reduced by the higher (0.3% v/v) and lower (0.1% v/v) rates of *P. lilacinus* at 20 to 30 DAP ($P \leq 0.003$). The higher rate increased the number of free-living nematodes ($P \leq 0.001$) (Table 1). Plant height and shoot dry weight ($P \leq 0.01$) were also greater in the higher rate of *P. lilacinus* as well as root fresh weight, and root surface area at 30 DAP ($P \leq 0.026$). The medium (0.1% v/v) and lower rates (0.01% v/v) of *P. lilacinus* and aldicarb did not influence root variables as compared to the untreated control ($P \leq 0.273$) (Table 2).

In the non-autoclaved field soil trial the number of *R. reniformis* females and eggs per gram of root were lower in the highest rate (1.4 $x10^7$ cfu/seed) of *B. firmus* when compared to the untreated control at 30 DAP ($P \leq 0.036$) (Fig.2). At the medium and high rates of *B. firmus*, vermiform life stages in the soil were reduced compared to the untreated control ($P \leq 0.009$), and similar to the aldicarb treatment ($P \leq 0.160$) at 15 to 30 DAP (Table 3). There were no differences between the number of free-living nematodes with the *B. firmus* rates and the untreated control 30 DAP ($P \leq 0.410$) (Table 3). Additionally, the higher rate increased the number of cotton root tips at 30 DAP ($P \leq 0.001$). However, there were no differences on plant height, shoot dry weight or root fresh weight among the treatments (Table 4).

In the final greenhouse trial, the combination of the two biologicals at the rates evaluated resulted in lower number of females and eggs per gram of root, and vermiform life stages in the soil 30 DAP ($P \leq 0.022$). Additionally, *B. firmus* and *P. lilacinus* reduced the number of females and eggs when applied individually ($P \leq 0.049$). *Paecilomyces lilacinus* increased free-living nematodes compared to the control, aldicarb, and *B. firmus* ($P \leq 0.001$) (Table 5).

Microplot trial

In this trial, the number of vermiform life stages in the soil was reduced by all the biological treatments during the first 60 DAP compared to the control ($P \leq 0.001$). The reduction of *R. reniformis* populations was similar to that of the aldicarb standard ($P \leq 0.399$) (Table 6). Number of females per gram of root were reduced at 30 DAP with *B. firmus* ($P \leq 0.01$) and *P. lilacinus* ($P \leq 0.05$) individually, but not when they were applied together ($P \leq 0.22$). At the end of the growing season, there were no differences in the number of females in the root among the

biological and aldicarb ($P \leq 0.99$) treatments. There was an increase in free-living nematodes at 30 DAP with the combination of the biologicals compared to aldicarb and untreated control ($P \leq 0.04$). Plant parameters were also affected. *Bacillus firmus* ($P \leq 0.005$) and *B. firmus* + *P. lilacinus* ($P \leq 0.053$) influenced plant architecture by supporting larger stem diameters at 60 DAP. Also, plants from the combination of *B. firmus* + *P. lilacinus* were taller ($P \leq 0.001$) when compared to the control (Figure 3). All the treatments, including aldicarb, provided higher cotton seed yields (Figure 4).

Field trial

The biologicals *B. firmis* and *P. lilacinus* reduced the number of vermiform life stages of *R. reniformis* in the soil at 90 and 150 DAP ($P \leq 0.01$). The control provided by the biologicals and aldicarb were similar ($P \leq 0.97$). *Paecilomyces lilacinus* and aldicarb began reducing vermiform life stages earlier at 60 DAP ($P \leq 0.021$) (Table 7). The number of females per gram of root were reduced at the end of the season at 150 DAP by the two biological combined ($P \leq 0.02$), and by aldicarb ($P \leq 0.03$). As observed in the microplots, the *P. lilacinus* treatment increased the number of free-living nematodes 60 DAP ($P \leq 0.01$) in the field as well. The combination of the biologicals increased free-living nematodes (Table 8), and the plants were taller, had increased stem diameter, and higher plant height 90 DAP ($P \leq 0.01$). At harvest, cotton seed yields were similar between the *B. firmis* and *P. lilacinus* and aldicarb.

IV. Discussion

Initial evaluations of *B. firmus* without microflora competition (autoclaved soil) found the higher rate of 7 x10^6 cfu/seed reduced *R. reniformis* females, eggs, and vermiform life stages with in the

first 30 days of planting. In this test, the insecticide imidacloprid did not show any nematicidal activity. This insecticide is formulated as a seed treatment with *B. firmus*. The effect of this insecticide has been previously evaluated against *Meloidogyne ethiopica* were no deleterious effect was observed in the reproduction of this nematode (46). Moreover, this insecticide has synergistic activity with the entomopathogenic nematodes *Sterneinema glaseri* and *Heterorhabditis bacteriophora* against white grubs (30).

In the natural soil microflora competition (non-autoclaved field soil), the higher seed treatment rate (1.4 $x10^7$ cfu/seed) of *B. firmus* showed 37% fewer vermiform life stages, and 21% fewer eggs of *R. reniformis* compared to the untreated seed control. Similar results of this strain have been reported under greenhouse conditions, when 8gr/1200cm^3 of sterilized soil of a wettable powder formulation of *B. firmus* was applied to tomato seedlings a week after transplanting; reducing the final nematode population by 76%, and the number of eggs of *M. incognita* by 45% (47). Furthermore, the application of the wettable powder formulation of *B. firmus* at rates of 1.8 and 3.6 gr/kg of soil to cucumber plants resulted in a lower number of eggs hatched of *Meloidogyne* spp. (15). In these trials a wettable powder formulation was used, and the main action against the nematodes was unclear for the authors. They suggest a major contribution of the additives of the formulation, and that *B. firmus* plays an additional role in nematode control. However, *in vitro* studies demonstrate that the production of bioactive secondary metabolites by this bacterium were toxic to *Radopholus similis, Meloidogyne incognita,* and *Ditylenchus dipsaci,* and inhibited hatching of *M. incognita* eggs (34). Our results indicated that the seed treatment formulation of *B. firmus* allows direct contact of the bacterium with the emerging cotton roots which is advantegeous against other root colonizers within the soil; therefore there is

an improvement in nematode control. Studies on seed treatments with abamectin report suppression penetration and infection of *M. incognita* and *R. reniformis* on cotton at a root length of 5 cm *in vitro*, but protection decreases as the taproot length increases (12). In the case of *B. firmus*, it is possible that the protection lasts longer due to the multiplication of the live bacteria with the cotton root as it grows in length. Cotton plants treated with higher seed treatment rates of *B. firmus* produced more root tips than the untreated control, and had a longer root length than the aldicarb treatment. Increases of root tips can represent higher uptake of nutrients and water from the soil. Studies in tomato also showed an increase of 50% in plant biomass, and a 91% reduction of the gall formations in the root (47).

In autoclaved soil, *P. lilacinus* at a rate of 0.2% v/v delayed the infection of *R. reniformis* and reduced vermiform life stages and eggs during the first 30 days. As a result of this protection, root fresh weight was higher than the untreated control and aldicarb treatment. Previous trials using a different strain of *P. lilacinus* on cotton plants under greenhouse conditions showed a reduction of *R. reniformis* eggs in autoclaved soil but not in non-autoclaved soil at a rate of 0.1% v/v (9). On the contrary, *P. lilacinus* strain 251 reduced females, eggs, and vermiform life stages at 0.2 and 0.3% v/v in nematode-infested field soil. This coincides with earlier greenhouse studies where *R. reniformis* eggs and vermiform life stages were reduced in tomato plants by 41% when *P. lilacinus* was applied at a rate of 5 gr of colonized rice (48), and 51% at a rate of 2 gr per plant after 60 days (39). In castor plants (*Ricinus communis* L.) and basil (*Ocimum basilicum* L.) reduction of this nematode was observed after 100 days using 8 gr of *P. lilacinus* inoculum on a rice seed carrier (1). Moreover, this fungus has been reported to reduce root galling caused by *Meloidogyne incognita* on tomato plants by 36%, with the application of 20 gr

fungus-infested wheat seed 10 days after transplanting (7). *Paecilomyces lilacinus* is an aggressive egg-parasite fungus that can be implemented in *R. reniformis* management programs. Eggs of *R. reniformis* are laid outside the cotton root, and second stage juveniles hatch usually within 7 days. These characteristics of *R. reniformis* reproduction probably enhance their susceptibility to paratisism by *P. lilacinus*. This fungus invades the eggs within 48 hours under *in vitro* conditions (9). Biocontrol provided by *P. lilacinus* was different than the control achieved with aldicarb. Aldicarb drastically reduces all the nematodes in the soil including the free-living nematodes. In contrast, these free-living nematodes were higher in all *P. lilacinus* rates evaluated.Comparable outcomes were observed with free-living nematodes responses to fenamifos and *P. lilacinus,* where the chemical nematicide decline all nematodes while *P. lilacinus* increase free-living populations (31). This fungus colonizes around and in the epidermis of the roots (7), which may explain the protection and increase in root fresh weight, root surface area, and number of tips at the rate of 0.3% v/v.

In vitro observations culturing *B. firmus* and *P. lilacinus* in petri dishes did not show any antagonism between this bacteria and fungi. Previous studies indicated no incompatibility between *P. lilacinus* and *B. firmus* under *in vitro* conditions (35). The combination of *B. firmus* and *P. lilacinus* under greenhouse conditions improved the biocontrol of *R. reniformis* in the root compared to their separated applications. Similar results were reported when biocontrol of *Radopholus similis* was increased with the application of *Fusarium oxysporum* with *B. firmus*, and *P. lilacinus* in banana plants (35). Furthermore, combination of *P. lilacinus* with *Monacrosporium lysipagum* reduced populations of *M. javanica, R.similis,* and *Heterodera avenae* in tomato, banana, and barley plants, respectively (25). Results from these trials conclude

that the combination of biocontrol agents with different mechanisms of action provide enhanced control over a single agent.

Under microplot and field conditions, reduction of *R. reniformis* was consistent to greenhouse trials when the two biocontrol agents were applied together. Reductions of nematode population occur when cotton plants start blooming (60 DAP) until full blooming (90 DAP). At this time stem diameter, plant height, and free-living nematodes populations also increased. Combinations of the two biocontrol agents resulted in a seed cotton yield comparable with chemical nematicide. Previous studies have reported reductions of plant-parasitic nematodes and increased yields under field conditions. *Bacillus firmus* reduced *Meloidogyne* spp. on *Hypericum* plants with a single pre-plant application through irrigation system, and protection lasted for three-months (4). Additionally, *B. firmus* suppressed *Meloidogyne* spp. on cucumber plants from 60 DAP until the end of the season (16). Moreover, when *B. firmus* was combined with soil solarization, reduction of nematodes improves similarly to the soil fumigant dazomet (15). Furthermore, *Paecilomyces lilacinus* reduced *Meloidogyne incognita* on *Piper betle* L.with three field applications (24).

In summary, the two commercial biocontrol products consistently reduced vermiform life stages and number of females in the roots in all the environments tested. There were no differences in the control provided by aldicarb and the combination of the biologicals. This suggests that combining antagonists that attack different life stages of *R. reniformis* provide a more effective control with multiple modes of action than using only one biocontrol agent. *Paecilomyces lilacinus* reduced females and eggs, while *B. firmus* affected the vermiform life stages. Similar

results were obtained in the reduction of *Radopholus similis* on banana plants were the application of these two biologicals with the endophytic *Fusarium oxysporum* strain 162 reduced nematode damage in the roots (35). Additionally, the combination of these biologicals increased free-living nematodes, which play an important role in nutrient cycling in soil ecosystems and can be used as biological indicators of soil health (13, 37). The orders of free-living nematodes that increased in the combined biological treatment were Rhabditida, Acrobeles, and Mononchida. The first two are bacterial feeders and the third one is reported as an omnivore. Mononchida, in their initial stages, could be bacterial feeders and in later stages predators of protozoa or nematodes (51). Furthermore, these nematodes, depending on their biology or feeding habit, can help to spread fungi or bacteria in the soil in their cuticles or by the bacteria/fungi passage through their intestines. Studies on wheat seeds treated with *Pseudomonas fluorescens* SBW25 show an increase in root colonization when free-living nematodes were present (29). Hirouchi et al. (2005) reported that *Caenorhabditis elegans* spreads *Sinorhizobium meliloti* to legume roots in response to plant-released volatiles that attract the nematode. Recent publications show interest in the potential of these nematodes as antagonists of plant-parasitic nematodes (3).

Understanding the interaction of free-living nematodes with *B. firmus* and *P. lilacinus* can enhance the reduction of *R. reniformis*. Research is needed to determine if the free-living nematode can decrease *R. reniformis* by predation or by enhancing biocontrol agents' performance through helping them spread through the soil. This finding will be an advantage because this nematode colonizes roots up to 90 cm deep in silty clay loam soils (36). Future experiments need to evaluate results from several growing seasons to determine if there is an

accumulative biocontrol effect in the soil after continuous applications and confirm if yield data remain consistent. Therefore, there are new alternatives arising to manage nematode problems in cotton crops once aldicarb is retired from the market.

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VI. Appendix

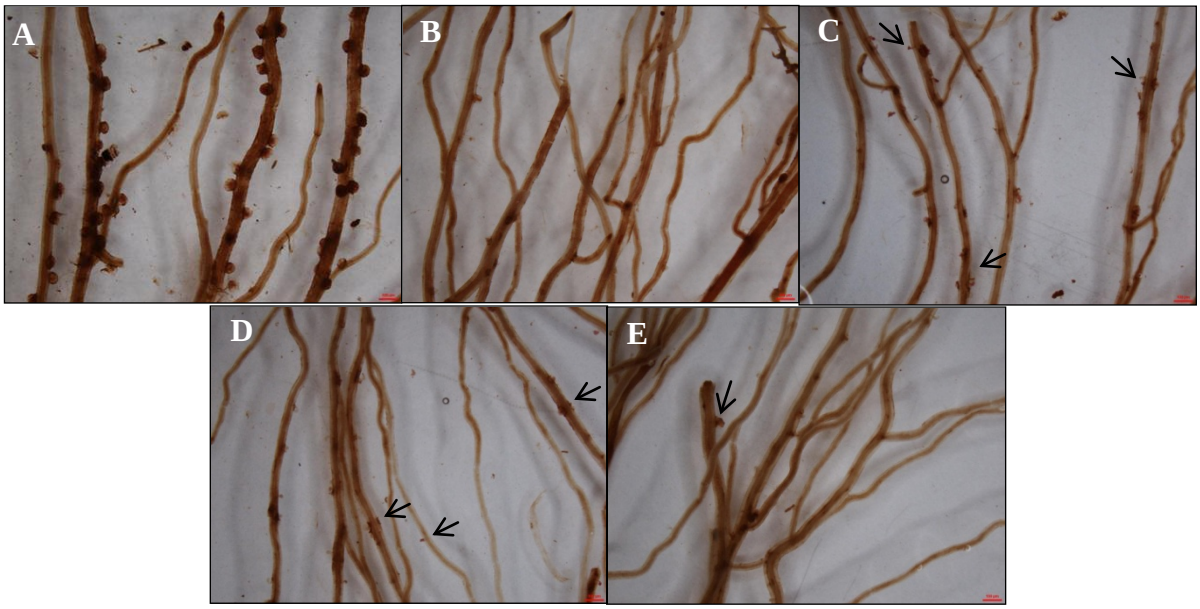

Figure 1. *Rotylenchulus reniformis* females feeding from the cotton roots treated with *P. lilacinus* strain 251 at 30 DAP. **A.** Untreated control, **B.** Aldicarb (5.6 kg/ha), **C.** *P. lilacinus firmus* (0.1% v/v), **D.** *P. lilacinus* (0.2% v/v), **E.** *P. lilacinus* (0.3% v/v).

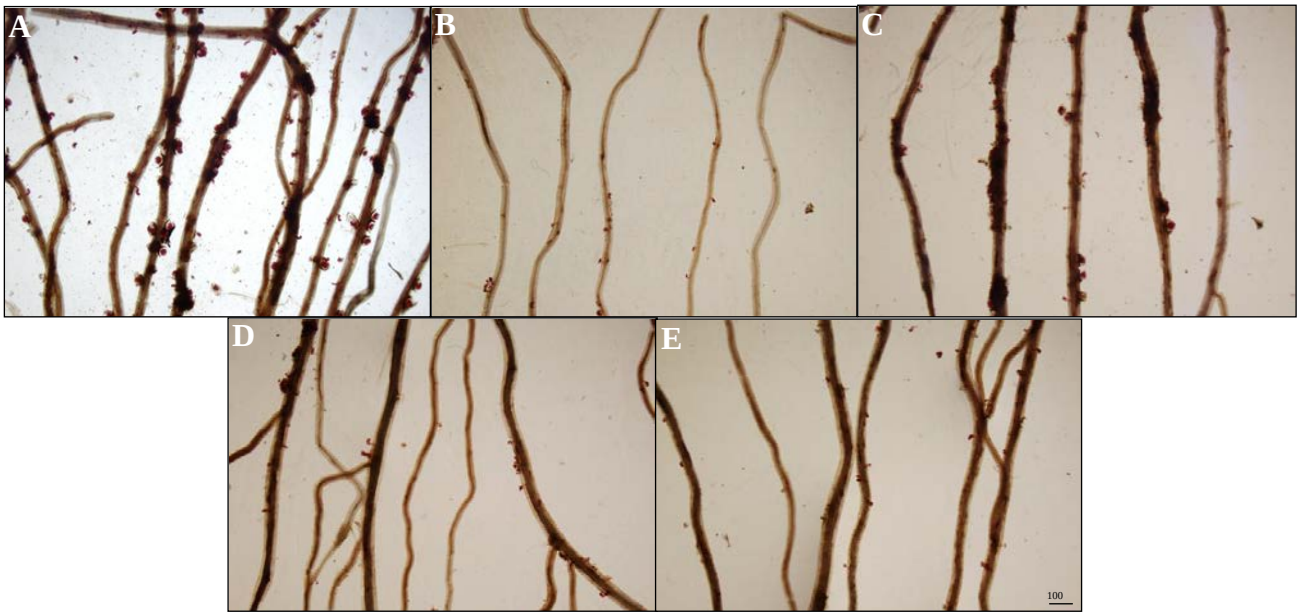

Figure 2. *Rotylenchulus reniformis* females feeding from the cotton roots treated with *B. firmus* strain GB-126 at 30 DAP. **A.** Untreated control, **B.** Aldicarb (5.6 kg/ha), **C.** *B. firmus* (1 x10^6 cfu/seed), **D.** *B. firmus* (7 x10^6 cfu/seed), **E.** *B. firmus* (1.4 x10^7 cfu/seed).

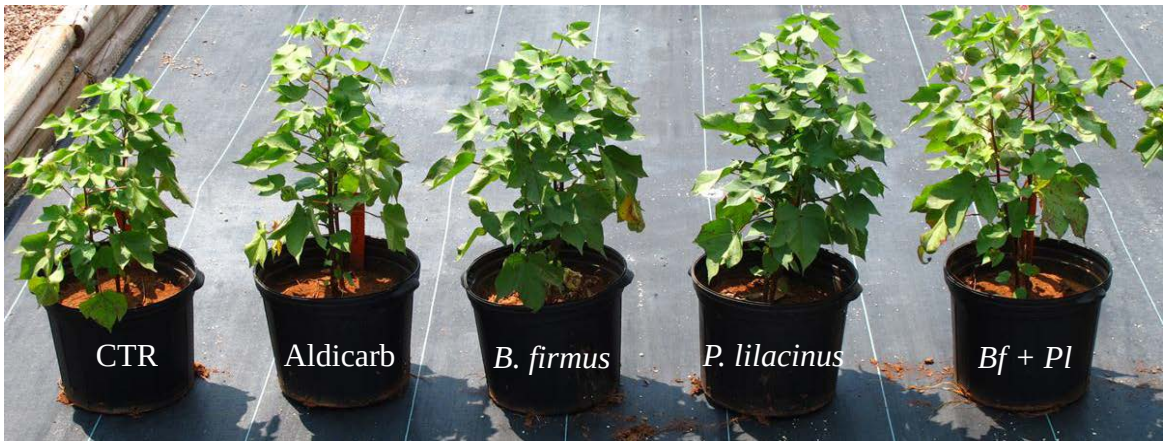

Figure 3. Cotton microplots at 60 DAP.

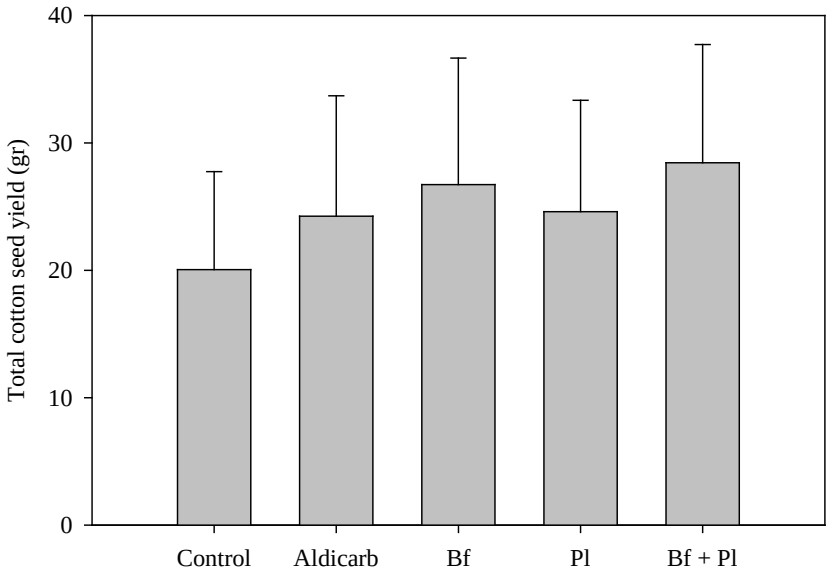

Figure 4. Cotton microplot yield in gr/microplot for *B. firmus*, *P. lilacinus*, aldicarb and the untreated control ($P \leq 0.05$)

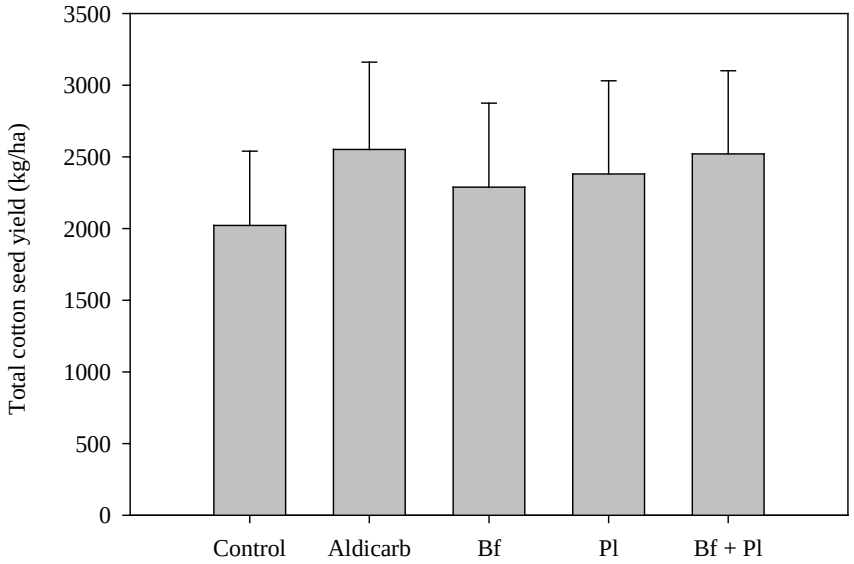

Figure 5. Cotton yield under field conditions in kg/ha for *B. firmus*, *P. lilacinus*, aldicarb, and the untreated control ($P \leq 0.05$).

Table 1. Effect of *Paecilomyces lilacinus* strain 251 on nematodes in roots and soil at 30 DAP under greenhouse conditions ($P \leq 0.05$).

***Rotylenchulus reniformis* per gm of root**						
Treatments	Females	Dunnett's *P* vs.		Eggs	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb
Control	484			3668		
Aldicarb	105	0.001		547	0.001	
Pl (0.1% v/v)	248	0.001	0.001	1841	0.066	0.003
Pl (0.2% v/v)	266	0.001	0.001	2264	0.637	0.361
Pl (0.3% v/v)	255	0.001	0.001	1767	0.030	0.002
Nematodes per 500 cm^3 of soil						
Treatments	Vermiforms	Dunnett's *P* vs.		Free-living	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb
Control	1771			459		
Aldicarb	302	0.001		70	0.001	
Pl (0.1% v/v)	945	0.019	0.001	518	0.891	0.001
Pl (0.2% v/v)	1177	0.171	0.001	751	0.593	0.001
Pl (0.3% v/v)	993	0.005	0.001	1685	0.003	0.001

Table 2. Effect of *Paecilomyces lilacinus* strain 251 on plant variables and root architecture at 30 DAP under greenhouse conditions ($P \leq 0.05$).

Plant height, shoot dry weight, and root fresh weight									
Treatments	Height (cm)	Dunnett's *P* vs.		ShDW[a] (g)	Dunnett's *P* vs.		RFW[b] (g)	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb
Control	13.6			0.8			3.1		
Aldicarb	13.9	0.764		0.8	0.976		3.6	0.226	
Pl (0.1% v/v)	14.3	0.605	0.704	0.8	0.805	0.711	3.6	0.343	0.772
Pl (0.2% v/v)	16.0	0.121	0.103	0.9	0.397	0.324	3.8	0.273	0.909
Pl (0.3% v/v)	17.0	0.012	0.003	1.1	0.003	0.001	4.7	0.026	0.021

Root Architecture									
Treatments	Lenght (cm)	Dunnett's *P* vs.		SA[c] (cm^3)	Dunnett's *P* vs.		Number of tips	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb
Control	354			171			469		
Aldicarb	380	0.657		167	0.764		474	0.872	
Pl (0.1% v/v)	391	0.419	0.694	179	0.605	0.704	432	0.745	0.530
Pl (0.2% v/v)	411	0.309	0.497	178	0.121	0.103	542	0.295	0.272
Pl (0.3% v/v)	437	0.226	0.348	225	0.012	0.003	550	0.304	0.294

[a]Shoot dry weight [b]Root fresh weight [c]Superficial area

Table 3. Effect of *Bacillus firmus* strain GB-126 on nematodes in roots and soil at 30 DAP under greenhouse conditions ($P \leq 0.05$).

***Rotylenchulus reniformis* per gm of root**						
Treatments	Females	Dunnett's *P* vs.		Eggs	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb
Control	650			2121		
Aldicarb	260	0.001		1326	0.021	
Bf ($1x10^6$ cfu/seed)	497	0.131	0.008	1910	0.507	0.060
Bf ($7x10^6$ cfu/seed)	477	0.071	0.001	1636	0.116	0.237
Bf ($1.4x10^7$ cfu/seed)	448	0.036	0.022	1684	0.001	0.512
Nematodes per 500 cm^3 of soil						
Treatments	Vermiforms	Dunnett's *P* vs.		Free-living	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb
Control	1731			316		
Aldicarb	725	0.001		262	0.548	
Bf ($1x10^6$ cfu/seed)	1416	0.218	0.194	474	0.519	0.301
Bf ($7x10^6$ cfu/seed)	1005	0.002	0.160	324	0.981	0.539
Bf ($1.4x10^7$ cfu/seed)	1099	0.009	0.105	402	0.410	0.207

Table 4. Effect of *Bacillus firmus* strain GB-126 on plant variables and root architecture 15 and 30 DAP under greenhouse conditions at three different seed treatment rates ($P \leq 0.05$).

Plant height, shoot dry weight, and root fresh weight									
Treatments	Height (cm)	Dunnett's *P* vs.		ShDW[a] (g)	Dunnett's *P* vs.		RFW[b] (g)	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb
Control	10.4			0.6			3.1		
Aldicarb	10.4	1.000		0.56	0.625		2.3	0.002	
Bf ($1x10^6$ cfu/seed)	9.8	0.849	0.843	0.56	0.673	0.965	3.3	0.629	0.009
Bf ($7x10^6$ cfu/seed)	10.4	1.000	1.000	0.53	0.319	0.569	3	0.704	0.022
Bf ($1.4x10^7$ cfu/seed)	10.6	0.987	0.989	0.6	0.968	0.472	3.6	0.148	0.000

Root Architecture									
Treatments	Lenght (cm)	Dunnett's *P* vs.		SA[c] (cm^3)	Dunnett's *P* vs.		Number of tips	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb
Control	354			180			424		
Aldicarb	382	0.091		145	0.420		500	0.021	
Bf ($1x10^6$ cfu/seed)	363	0.999	0.123	181	1.000	0.517	450	0.507	0.0595
Bf ($7x10^6$ cfu/seed)	350	0.127	0.926	146	0.527	1.000	424	0.116	0.2371
Bf ($1.4x10^7$ cfu/seed)	403	0.823	0.018	185	1.000	0.114	621	0.000	0.5117

[a] Shoot dry weight [b] Root fresh weight [c] Superficial area

Table 5. Effect of *P. lilacinus* strain 251 and *B. firmus* strain GB-126 alone and in combination on plant variables, root architecture, and nematodes in root and soil 30 DAP under greenhouse conditions ($P \leq 0.05$).

Plant height, shoot dry weight, and root fresh weight									
Treatments	Height (cm)	Dunnett's *P* vs.		ShDW[a] (g)	Dunnett's *P* vs.		RFW[b] (g)	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb
Control	18			0.9			2.9		
Aldicarb	18.5	0.990		0.85	0.992		2.5	0.347	
Bf ($1.4x10^7$ cfu/seed)	17.5	0.989	0.597	0.81	0.906	0.993	3	1.000	0.104
Pl (0.3% v/v)	17.4	0.984	0.570	0.79	0.811	0.961	3	1.000	0.139
Bf + Pl	17	0.822	0.231	0.85	0.991	1.000	3	1.000	0.358

Root Architecture									
Treatments	Lenght (cm)	Dunnett's *P* vs.		SA[c] (g)	Dunnett's *P* vs.		Number of tips	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb
Control	440			179			415		
Aldicarb	381	0.891		166	0.982		417	0.982	
Bf ($1.4x10^7$ cfu/seed)	500	0.897	0.269	196	0.918	0.397	457	0.918	0.397
Pl (0.3% v/v)	407	0.989	0.996	167	0.991	1.000	433	0.991	1.000
Bf + Pl	413	0.995	0.985	196	0.942	0.534	460	0.942	0.534

***Rotylenchulus reniformis* females, eggs, and vermiform life stages ~~Nematode variables~~**									
Treatments	Females[d]	Dunnett's *P* vs.		Eggs[d]	Dunnett's *P* vs.		Vermiforms[e]	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb
Control	86			3294			1561		
Aldicarb	32	0.001		1069	0.001		328	0.001	
Bf ($1.4x10^7$ cfu/seed)	63	0.004	0.001	1808	0.007	0.037	702	0.001	0.004
Pl (0.3% v/v)	55	0.001	0.008	1615	0.049	0.778	966	0.063	0.015
Bf + Pl	40	0.022	0.001	1523	0.006	0.641	882	0.013	0.004

[a]Shoot dry weight [b]Root fresh weight [c]Superficial area [d]Per gram of root [e]Per 500 cm^3 of soil

Table 6. Effect of *P. lilacinus* strain 251 and *B. firmus* strain GB-126 alone and in combination on plant height, stem diameter, and *R. reniformis* vermiform stage in soil under microplot conditions ($P \leq 0.05$).

Plant Height									
Treatments	30 DAP			60 DAP			90 DAP		
	Height (cm)	Dunnett's *P* vs.		Height (cm)	Dunnett's *P* vs.		Height (cm)	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb
Control	19.0			36.8			44.8		
Aldicarb	19.4	0.791		38.4	0.297		45.9	0.422	
Bf($1.4x10^7$ cfu/seed)	18.1	0.633	0.466	38.9	0.260	0.795	43.4	0.422	0.218
Pl (0.3% v/v)	20.1	0.550	0.707	39.5	0.046	0.444	45.8	0.474	0.920
Bf + Pl	19.4	0.805	0.989	42.2	0.001	0.013	47.7	0.085	0.366

Stem Diameter									
Treatments	30 DAP			60 DAP			90 DAP		
	SD (cm)	Dunnett's *P* vs.		SD (cm)	Dunnett's *P* vs.		SD (cm)	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb
Control	3.6			6.6			6.9		
Aldicarb	3.7	0.998		7.1	0.793		7	1.000	
Bf($1.4x10^7$ cfu/seed)	3.6	1.000	0.996	8.2	0.005	0.02	8.2	0.021	0.008
Pl (0.3% v/v)	3.8	0.984	0.999	7.2	0.764	1.00	7.6	0.702	0.698
Bf + Pl	3.7	0.993	1.000	7.8	0.053	0.24	7.6	0.603	0.567

***Rotylenchulus reniformis* vermiform life stages per 150 cm^3 of soil**									
Treatments	30 DAP			60 DAP			90 DAP		
	R. reniformis	Dunnett's *P* vs.		*R. reniformis*	Dunnett's *P* vs.		*R. reniformis*	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb
Control	122			597			1037		
Aldicarb	60	0.001		197	0.001		502	0.020	
Bf($1.4x10^7$ cfu/seed)	51	0.001	0.561	259	0.001	0.386	964	0.455	0.187
Pl (0.3% v/v)	84	0.039	0.030	232	0.001	0.280	621	0.059	0.442
Bf + Pl	78	0.025	0.512	211	0.001	0.399	578	0.071	0.277

Table 7. Effect of *P. lilacinus* strain 251 and *B. firmus* strain GB-126 alone and in combination on plant height, stem diameter, and *R. reniformis* vermiform stage in soil under field conditions ($P \leq 0.05$).

Plant Height									
Treatments	30 DAP			60 DAP			90 DAP		
	Height (cm)	Dunnett's *P* vs.		Height (cm)	Dunnett's *P* vs.		Height (cm)	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb
Control	14.9			52.5			80.8		
Aldicarb	16.8	0.022		60.2	0.113		91.2	0.013	
Bf ($1.4x10^7$ cfu/seed)	15.4	0.571	0.041	54.5	0.642	0.241	86.1	0.236	0.265
Pl (0.3% v/v)	15.7	0.282	0.109	58.5	0.155	0.719	90.8	0.027	0.925
Bf + Pl	15.8	0.291	0.251	58.7	0.162	0.761	91.3	0.007	0.980

Stem Diameter									
Treatments	30 DAP			60 DAP			90 DAP		
	SD (cm)	Dunnett's *P* vs.		SD (cm)	Dunnett's *P* vs.		SD (cm)	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb
Control	3.7			8.0			10.7		
Aldicarb	4.2	0.021		9.2	0.030		12.7	0.001	
Bf ($1.4x10^7$ cfu/seed)	3.9	0.396	0.082	8.4	0.388	0.181	12.1	0.016	0.342
Pl (0.3% v/v)	4.1	0.048	0.496	8.8	0.102	0.519	12.1	0.014	0.323
Bf + Pl	3.9	0.633	0.155	9.3	0.013	0.896	12.4	0.006	0.371

***Rotylenchulus reniformis* vermiform life stages per 150 cm^3 of soil**									
Treatments	30 DAP			60 DAP			90 DAP		
	R. reniformis	Dunnett's *P* vs.		*R. reniformis*	Dunnett's *P* vs.		*R. reniformis*	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb
Control	1458			586			2651		
Aldicarb	1102	0.214		289	0.002		2066	0.087	
Bf ($1.4x10^7$ cfu/seed)	1094	0.398	0.540	424	0.151	0.122	1906	0.001	0.948
Pl (0.3% v/v)	1269	0.737	0.308	348	0.021	0.496	1882	0.009	0.826
Bf + Pl	1202	0.804	0.238	443	0.221	0.082	1933	0.011	0.975

Table 8. Effect of *P. lilacinus* strain 251 and *B. firmus* strain GB-126 alone and in combination on free-living nematodes in soil under microplot and field conditions ($P \leq 0.05$).

Microplot free-living nematodes per 150 cm^3 of soil									
Treatments	30 DAP			60 DAP			90 DAP		
	FL	Dunnett's *P* vs.		FL	Dunnett's *P* vs.		FL	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb
Control	22			30			27		
Aldicarb	6	0.116		14	0.319		11	0.002	
Bf ($1.4x10^7$ cfu/seed)	22	0.640	0.119	19	0.590	0.669	73	0.250	0.002
Pl (0.3% v/v)	32	0.251	0.091	16	0.840	0.514	16	1.000	0.319
Bf + *Pl*	41	0.039	0.006	32	0.416	0.114	43	0.327	0.001

Field free-living nematodes per 150 cm^3 of soil									
Treatments	30 DAP			60 DAP			90 DAP		
	FL	Dunnett's *P* vs.		FL	Dunnett's *P* vs.		FL	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb
Control	59			27			32		
Aldicarb	119	0.033		35	0.366		57	0.63	
Bf ($1.4x10^7$ cfu/seed)	81	0.343	0.011	62	0.049	0.278	100	0.011	0.072
Pl (0.3% v/v)	111	0.759	0.260	70	0.007	0.066	86	0.008	0.054
Bf + *Pl*	138	0.105	0.789	51	0.080	0.352	130	0.001	0.013

Chapter III. Determination of biocontrol activitiy of *Bacillus firmus* strain GB-126 against the reniform nematode (*R. reniformis*).

Abstract

Previous studies have demonstrated the antagonistic effect of bioactive secondary metabolites of *Bacillus firmus* GB-126 against plant-parasitic nematodes. However, the type of metabolite and enzymes produced by *B. firmus* GB-126, as well as other possible modes of action against nematodes remain unknown. In our present study, we evaluated the effect of *B. firmus* GB-126 against *R. reniformis* under *in vitro* and greenhouse conditions. The objectives were: *i*) to determine pathogenicity or toxicity of *B. firmus* GB-126 living cells and secondary metabolites against *R. reniformis*, *ii*) to identify the type of enzymes produced by *B. firmus* GB-126 responsible for nematode suppression, and *iii*) to evaluate systemic induced resistance by *B. firmus* GB-126 on cotton as possible mode of action. Cell concentration of $15x10^7$ cfu/ml, and secondary metabolites of *B. firmus*, inhibited the hatching of *R. reniformis* eggs within 48-72 hours, and paralyzed juveniles after 1 hour. From the metabolites of *B. firmus* GB-126, a biosurfactant was purified and evaluated at different concentrations. The biosurfactant applied at 2ppm and 1ppm paralyzed 100% and 45.9% of the *R. reniformis* juveniles within 30 minutes, respectively. Enzymes produced by *B. firmus* GB-126 were proteases, amylases, lipases, and cellulases. Scanning electronic microscopy indicated that there was no mechanical damage on the cuticle of *R. reniformis*, suggesting that the paralysis was due to a toxic effect of the biosurfactant. Systemic induced resistants to *R. reniformis* by *B. firmus* GB-126 on cotton was not demonstrated by reduced females and eggs per gram of root.

I. Introduction

Bacteria are an important group of natural antagonists of plant-parasitic nematodes. Bacteria are distributed broadly, have diverse modes of action, and have broad host ranges (27). They exhibit diverse modes of action against nematodes that include parasitism, production of toxins, antibiotics, or lytic enzymes; induce systemic resistance, and promote plant health (1, 10, 11, 13, 21, 26, 27, 30). Furthermore, bacteria can be in direct contact with the entrance sites of the nematodes and influence root exudates that can affect the nematode development (13, 23). The genera *Pasteuria*, *Pseudomonas*, and *Bacillus* have shown promising potential for nematode biocontrol (16, 21, 26, 27).

Bacillus firmus strain GB-126 is a nematode biocontrol agent registered initially in a bionematicide in Israel under the trade name of Bionem® WP (3, 9). This formulation was shown to reduce galling index caused by *Meloidogyne* spp. on cucumber and tomato plots (9). Also, under field conditions, suppression of *Meloidogyne* spp. was observed within 2 months of transplanting cucumbers and continued through the end of the experiments (5). Control provided by *B. firmus* GB-126 was less effective than the soil fumigant dazomet. However, its combination with soil solarization improves nematode control giving results similar to dazomet use (4). Furthermore, when *B. firmus* GB-126 was evaluated in tomato seedlings in the greenhouse, it reduced gall formation by 91%, final nematode population by 76%, and the number of *M. incognita* eggs by 45% (29).

In other study, a formulation of *B. firmus* that contains seaweed extract (BioNem® L) was able to reduce *Helicotylenchus* spp. and *Tylenchorhynchus* spp. in golf greens (32). Furthermore,

synergism of *B. firmus* with other nematode biocontrol agents has been reported to improve nematode reduction (15). In banana, *B. firmus* was evaluated against *R. similis* and applied in combination with *F. oxysporum* and *P. lilacinus*, which reduced the infection of this migratory endoparasitic nematode (15). Under *in vitro* conditions *B. firmus* was evaluated against the plant-parasitic nematodes *Radopholus similis*, *Meloidogyne incognita*, and *Ditylenchus dipsaci*. *Bacillus firmus* produced bioactive secondary metabolites that were toxic to these nematode juveniles and reduced egg hatching (14).

Nevertheless, the types of secondary metabolites and enzymatic properties involved and the role of possible induction of plant resistance have not been evaluated. The objectives were: *i*) to determine pathogenicity or toxicity of *B. firmus* GB-126 living cells and secondary metabolites against *R. reniformis*, *ii*) to identify the type of enzymes produced by *B. firmus* GB-126 responsible for nematode suppression, and *iii*) to evaluate systemic induced resistance by *B. firmus* GB-126 on cotton as possible mode of action.

II. Materials and Methods

In vitro tests against *R. reniformis.*

An initial test was conducted to evaluate the effect of the metabolites produced by *B. firmus* against second stage juveniles and egg hatching of *R. reniformis*. Eggs of *R. reniformis* were extracted from cotton roots by shaking the root system in a 1% NaOCl solution for four minutes at 120 rpm. The suspension of eggs was collected and rinsed with water through a 25 µm sieve (8). Eggs were rinsed with streptomycin sulfate (300 mg/L) and chlortetracycline (12.5 mg/L) for bacterial disinfection, then with metalaxyl (25mL/L) and iprodine (20mL/L) for fungal

disinfection, then finally with distilled water. For the second stage juvenile trial, the eggs were placed in a modified Baerman dish on a slide warming tray at 27ºC. Second stage juveniles were hatched after three days. To obtain the bacterial metabolite, *Bacillus firmus* GB-126 was grown in 50 mL of Tryptic Soy Broth (TSB) (Bacto^{TM}) for four days and then placed in 50 mL plastic tubes and centrifuged for 20 minutes at 4000 x*g*. The supernatant was collected and filtered through a Millipore filter 0.45-0.22 ųm to obtain the final bacterial metabolite product. *In vitro* trials were conducted on 96-well plates, where volumes of 100µL of the treatments were transferred to each well, which contained approximately 16 juveniles or 20 eggs of *R. reniformis*. Treatments were *i*) water control, *ii*) TSB media control, *iii*) metabolite 100%, and *iv*) metabolite 50%. Each treatment had 6 replications, and the entire trial was repeated twice. The number of eggs hatched releasing juveniles was recorded at 0, 24, 48, and 72 hours after inoculation. For the second stage juveniles, number of moving and paralyzed nematodes was recorded at 0, 1, 2, 4, 6, and 12 hours after inoculation. Data were analyzed on SAS 9.1 (SAS Institute Inc.) using the GLIMMIX procedure where the distributional assumption was evaluated with the student panel graphs. Dunnett's option was used to assess the differences with the water and TSB controls.

Enzyme characterization of *B. firmus*.

Different enzymatic properties of *B. firmus* GB-126 were evaluated to test their capacity to degrade different media. Production of enzymes was evaluated as positive when a transparent halo was formed around the bacterium culture. *Bacillus firmus* GB-126 was grown for 24 hours on milk agar to test the production of proteases, starch agar for the production of amylases, carboximetil cellulose (CMC) agar for the production of cellulases, chitinase agar for the

production of chitinases, and trybutirin agar for the production of lipases. The CMC agar and chitinase agar required the application of 5 ml of congo red to stain the media and a transparent halo after 24 hours of culturing the bacteria. In the case of the starch media, culture was stained with lugol.

Determination of production of biosurfactant by *B. firmus*.

Three initial tests were conducted to determine the production of biosurfactant from *B. firmus*. In the first test, the bacterium was grown under blood agar, where a positive production of biosurfactant is indicated by a transparent halo around the bacterial colony. For the second and third tests, *B. firmus* was cultured on nutrient broth at 30°C for 24 hours. Subsequently, the living cells were recovered by centrifugation at 5,181 x*g* for 15 minutes, and cells were washed twice with NaCl 0.85% (w/v) and later suspended in 5 ml of NaCl 0.85% (w/v). They were used to inoculate 45 ml of saline Davis minimal broth with an inoculum ratio of 1% (v/v). The composition was K_2HPO_4 5.23 g/l, KH_2PO_4 1.91 g/l, $MgSO_4$ 0.09 g/l, $(NH_4)2SO_4$ 1 g/l, as well as 1 ml/l of trace elements solution ($CoCl_3$ 20 mg/l, H_3BO_3 30 mg/l, $ZnSO_4$ 10 mg/l, Cu_2SO_4 1 mg/l, Na_2MoO_4 3 mg/l, $FeSO_4$ 10 mg/l and $MgSO_4$ 2.6 mg/ l). Cultures were incubated at 30 °C ± 2 at 150 rpm for 3 days. Again *B. firmus* living cells were separated from the supernatant by centrifugation (20 minutes at 4000 x*g*). The supernatant was filtered through a Millipore filter 0.45-0.22 ɥm to obtain the final bacterial biosurfactant product. This final product was autoclaved twice for 30 minutes at 120°C at 1 kg/cm^3 pressure to kill all the bacterium's living cells and inactivate its enzymes.

In the second test, emulsifying activity of the cell-free supernatant was evaluated by mixing 0.5 ml with 0.5 ml of kerosene and 4 ml of distilled water to a disposable culture tube (borosilicate glass 16x150 mm). The negative control consisted of distilled water and kerosene, and the positive control consisted of distilled water, kerosene, and Triton X-100 (100 mg/ml). Each tube was agitated in a vortex for 1 min and was left to stand for 24 hours. The height of the emulsification ring was then measured in millimeters and compared to that of the chemical emulsifier. If there was positive production of surfactant, the kerosene emulsified it and produced foam. The third test consisted of an oil drop collapse in which one drop of the supernatant was placed on parafilm paper and a drop of oil was placed on top of it. If the drop of oil increased its diameter compared to the media control, the bacteria was considered to have produced a biosurfactant.

Finally, the biosurfactant product was again tested under *in vitro* conditions in 96 well-plate against second stage juveniles of *R. reniformis*. Volumes of 100µL of the treatments were transferred to each well, which contained approximately 16 juveniles of *R. reniformis*. Treatments for this trial were *i*) water control, *ii*) BPM control, *iii*) biosurfactant 100%, and *iv*) *B. firmus* $15x10^7$ cfu/ml. Each treatment was replicated 8 times, and the entire trial was repeated twice. The number of juveniles paralyzed or dead was recorded 30 minutes after inoculation. Data were analyzed on SAS 9.1 (SAS Institute Inc.) as described previously.

Purification and production of biosurfactant and *in vitro* evaluations at different concentrations against *R. reniformis*.

For biosurfactant production, *B. firmus* GB-126 was grown aerobically on minimal salt medium containing (per liter) KH_2PO_4 (2.0 g), K_2HPO_4 (5.0 g), $(NH_4)_2SO_4$ (3.0 g) , $NaNO_3$ (2.0 g), NaCl

(0.1 g), $MgSO_4$ H_2O (0.2 g), 0 $FeSO_4$ $7H_2O$ (0.01g), CaCl2 (0.01g), and 1 ml of a trace element solution. The stock solution of trace elements contained (per liter) $ZnSO_4$ $7H_2O$ (2.32 g), $MnSO_4$ $4H_2O$ (1.78 g), H_3BO_3 (0.56 g), $CuSO_4$ $5H_2O$ (1 g), Na_2MoO_4 $7H_2O$ (0.39 g), $CoCl_2$ $6H_2O$ (0.42 g), EDTA (1 g), NiCl2 $6H_2O$ (0.004g), and KI (0.66 g). The medium was supplemented with 0.05% yeast extract (31). Glucose was added as a carbon source at a concentration of 2% (wt/vol). The medium pH was 7.1 to 7.2. The organism was grown at 37°C for 48 h in 2-liter Erlenmeyer flasks containing 800 ml of medium and shaken at 200 rpm in a shaker incubator.

For biosurfactant isolation, bacterial cells were removed from the surfactant-containing medium by centrifugation (13000 x*g* for 15 min at 4°C). The biosurfactant was precipitated from the supernatant by adding 6 N HCl to obtain a final pH of 2.0. The acid precipitates were recovered by centrifugation (13000 x*g* for 15 min at 4°C) and were extracted with dichloromethane or methanol (lipopeptide fraction). When methanol was used as the solvent, the extract was neutralized immediately to avoid formation of methyl esters. After precipitation with HCl, the crude fraction dissolved in methanol or dichloromethane was evaporated in a rotary evaporator (Model Buchi R) under a vacuum pump (Model Gem 8890) (31). The final purified biosurfactant product was diluted in distilled water at concentrations of 2 ppm, 1 ppm, 0.5 ppm, 0.2 ppm, 0.1 ppm, and 0.02 ppm. These concentrations were evaluated, compared to distilled water control under *in vitro* conditions on second stage juveniles as described above, with 8 replications per treatment, and repeated twice.

Greenhouse trials.

To evaluate if *B. firmus* GB-126 induces systemic resistance the following treatments were in tested in a split root system. Treatments consisted of *i)* water control without nematodes, *ii)*

water control with nematodes, *iii) B. firmus* $1x10^7$ cfu/mL, *iv) B. firmus* $1x10^6$ cfu/mL, or *v) Serratia marcescens* $1x10^7$ cfu/mL. Stoneville 5458 B2RF cotton seeds were germinated in potting mixing soil under greenhouse conditions. Emerging root radicals approximately 2.5 cm in length were split with a razer blade. At 5 days after planting (DAP), soil was removed from the roots and divided into two equal halves. Plants were planted in 960 cm^3 pots with each root half in a different cup. At 7 days after splitting the roots, a suspension of 50 mL of treatments was applied on the left side of the root. Five days later, the right side of the root was inoculated with 500 second stage juveniles of *R. reniformis*. The trial was harvested 45 DAP, and plant height, root fresh weight, and number of females and eggs per gram of root were measured. Each treatment had 6 replications and the entire trial was repeated twice.

To evaluate the response of *R. reniformis* to cotton seeds treated with *B. firmus* GB-126, a trial in autoclaved soil was conducted in the Plant Science Research Center (PSRC) of Auburn University. Cotton seeds from cultivar Stoneville 5458 B2RF were treated with *B. firmus* GB-126 by the manufacturer in a liquid seed dresser Hege11 (Hege Maschinen GmbH, Germany). Presence of the bacterium in the seed was confirmed by culturing the treated seed on Triptic Soy Agar (TSA) adjusted to pH 8.0 and recording the growth after 16 hours. The soil was a Decatur silty clay loam (sand-silt-clay: 17.5-51.3-31.2%; nitrogen: 0.16%; organic matter: 2.2; pH 7.24) from the Tennessee Valley Research and Extension Center (TVREC) near Belle Mina, AL. The soil was autoclaved using two 90-minute cycles at 130ºC at 15 psi with a 24 hour cool down between cycles. Seed treatments were as follows: *i)* untreated seed with nematodes; *ii)* imidacloprid (500 g ai/100kg) a standard insecticide; *iii) B. firmus* ($7x10^4$ cfu/seed) plus imidacloprid (500 g ai/100kg); *iv) B. firmus* ($7x10^5$ cfu/seed) plus imidacloprid (500 g ai/100kg);

and *v) B. firmus* (7 x10^6 cfu/seed) plus imidacloprid (500 g ai/100kg). The standard insecticide seed treatment imidacloprid was included because this insecticide is commonly applied as a seed treatment and was tested to determine if it has any effect on *B. firmus* or *R. reniformis* life stages.

Rotlyenchulus reniformis vermiform life stages were extracted from the soil by modified gravity screening and sucrose centrifugation-flotation. Eggs were extracted from cotton roots by shaking the root system in a 1% NaOCl solution for four minutes at 120 rpm. The nematode suspension was and rinsed with water and collected on a 25 µm sieve. Females in roots were stained with acid fuschin to facilitate enumeration of the females invading the root. Vermiform life stages and eggs were counted under an inverted TS100 Nikon microscope at 40x magnification. Females embedded in the root systems were quantified at 5x magnification utilizing the Nikon SMZ800 compound microscope. Variables measured were plant height, shoot and root weight, females and eggs per gram of root, and the number of vermiform life stages in 500 cm^3 of soil. Greenhouse average temperature where plants were grown was 29˚C. Soil moisture was maintained between 40-60% of the maximum water holding capacity. Data were analyzed in SAS 9.1 (SAS Institute Inc.). The distributional assumption was evaluated with the student panel graphs of the GLIMMIX procedure. Dunnett's option was used to assess the differences with the untreated control.

III. Results

In the first *in vitro* trial, *R. reniformis* egg hatch was reduced at 48 and 72 hours, when eggs were exposed to *B. firmus* metabolites at 100% and 50%, when compared to the water and media control ($P \leq 0.01$) (Figure1). Furthermore, paralysis of second stage juveniles of *R. reniformis*

observed within one hour of inoculation in 100% and 50% metabolite through 12 hours when all the second stage juveniles were paralyzed ($P \leq 0.01$) (Figure 2). No differences were observed between the water and the media controls in these trials ($P \leq 0.99$).

Biosurfactant production was confirmed by the emulsification of kerosene, oil drop collapse, and halo formation in blood agar (Figure 3). Biosurfactant and living cells of *B. firmus* at a concentration of $15x10^7$ cfu/mL paralyzed all the second stage juveniles within 30 minutes after inoculation when compared to the BP media and water controls ($P \leq 0.01$) (Fig.3). There were no differences between the two controls ($P \leq 0.99$). Finally, in the last *in vitro* trial where the pure *B. firmus* biosurfactant was evaluated at different concentrations, the biosurfactant at 2 ppm and 1 ppm paralyzed 100% and 45.9%, of the second stage juveniles of *R. reniformis*, respectively, within 30 minutes (Figure 4).

These two concentrations produced an increase in paralysis of the second stage juveniles compared to the water control ($P \leq 0.001$). Biosurfactant concentrations of 0.5ppm, 0.2 ppm, 0.1 ppm, and 0.02 ppm did not paralyze second stage juveniles and were not different from the water control ($P \leq 0.932$). When second stage juveniles from the water control and 2 ppm treatments were observed under SEM, no mechanical damage to the cuticle was observed (Fig. 5A, B). The enzymatic profile of *Bacillus firmus* GB-126 indicated a high enzymatic activity for proteases, amylases, and cellulases forming a transparent halo in milk agar, starch agar, and CMC agar, respectively, within 24 hours. In contrast, no production of chitinases was observed under chitinase agar (Figure 7).

The induction of systemic resistance trial indicated cotton plants treated with *B. firmus* GB-126 ($1x10^6$ cfu/ml) was taller than control with nematodes ($P \leq 0.05$) and *S. marcecens* ($1x10^9$ cfu/ml) treatment ($P \leq 0.01$). There were no differences in left or right root fresh weights or the number of *R. reniformis* females and eggs among the treatments ($P \leq 0.99$) (Fig. 6). In contrast, *B. firmus* GB-126 at a rate of 7 $x10^6$ cfu/seed reduced the number of females per gram of root ($P \leq 0.001$) and juveniles per 500 cm^3 of soil 30 days after planting ($P \leq 0.01$) (Fig. 8). The insecticide imidacloprid did not have any effect on cotton plant growth or *R. reniformis* life stages.

IV. Discussion

Bacillus firmus GB-126 inhibits the hatch of *R. reniformis* eggs and paralyzes second stage juveniles under *in vitro* conditions using secondary metabolites from this bacterium and also living cells at a concentration of $15x10^7$cfu/ml. Similar results were obtained in previous studies under *in vitro* conditions, where the bioactive secondary metabolites of *B. firmus* reduced egg hatching of *M. incognita* and paralyzed *R. similis*, *M. incognita*, and *D. dipsaci* (14, 15). In our greenhouse trial, *B. firmus* GB-126 applied as a seed treatment at a rate of 7 $x10^6$ cfu/seed reduced number of *R. reniformis* females in the root and juveniles in soil within the first 30 days of planting. The effect of the insecticide imidacloprid, which is used as a seed treatment and formulated with this bacterium, did not show any nematicidal activity. The effect of this insecticide has been previously evaluated against *Meloidogyne ethiopica* were no deleterious effect was observed in the reproduction of this nematode (24). Moreover, this insecticide has synergistic activity with the entomopathogenic nematodes *Sterneinema glaseri* and *Heterorhabditis bacteriophora* against white grubs (*Cyclocephala* spp.) (12). Previous studies of *B. firmus* formulated as a wettable powder in tomato plants was reduced *Meloidogyne* spp. with a

higher efficiency than *Pasteuria penetrans* in the field. However the biocontrol effect in this study was partially attributed to the stimulating effect that the additives in this formulation have on the rhizosphere (4).

The present study demonstrates that the mode of action of *B. firmus* GB-126 against nematodes is a secondary metabolite toxic to nematodes. This secondary metabolite is a biosurfactant which is responsible for the paralysis of *R. reniformis* juveniles and inhibition of egg hatch under *in vitro* conditions. *Bacillus firmus* GB-126 biosurfactant needs a minimum concentration of 1 ppm to paralyze second stage juveniles within 30 minutes. Production of metabolites against nematodes has been previously reported on *Bacillus* species. The secondary metabolites produced by the endophytic bacteria *Bacillus megaterium* reduced egg hatching of *Meloidogyne graminicola* by 60% (20). Furthermore, metabolites from several strains of *Bacillus* spp. and *B. megaterium* obtained from sugar beet (*Beta vulgaris*) were able to reduce hatching of *Heterodera schachtii* (17). Deleterious effects on nematodes by a biosurfactant occurred when rhamnolipid was found at a concentration of 250 ppm and caused collapse and disintegration of root-knot eggs after 7 days (2).

The presence of enzymes (amylases, cellulases, and proteases) suggests the possibility that *B. firmus* GB-126 can have other modes of action against *R. reniformis* and other nematode species during different stages of the life cycle. The production proteases by this bacterium can affect on egg hatching and cause nematode paralysis. Proteases from *Bacillus* spp. deleterious to nematodes have been reported (7,19,28). *Brevibacillus laterosporus* G4 contains the extracellular alkaline protease BLG4, which kills (cuticle degradation) between 43-71% of the free-living

nematode *Panagrellus redivius* and the plant-parasitic nematode *Bursaphelenchus xylophilus* (7, 28). An alkaline serine protease from *B. nematocida* can kill about 90% of the free-living nematode *P. redivius* within 24 hours (19).

Induction of systemic resistance of *B. firmus* GB-126 in cotton plants was not observed at the concentrations evaluated. *Bacillus subtilis* strain Bs_t at a cell concentration of 10^{10} cells/ml was able to reduce root penetration in tomato by *R. reniformis* by 44.5% in a split-root trial and 51% in whole root trial (18). Additionally, studies in Germany report induction of resistance and reduction of potato cyst nematode (*Globodera pallida*) by *Bacillus sphaericus* and *Agrobacterium radiobacter* in split-root trials under greenhouse conditions (6). However, the rhizobacterial strain *Bacillus subtilis* A-13 was tested against *M. incognita* on cotton and sugar beet and against *R. reniformis* on cotton. A reduction of 38% to 68% was observed in all plants in autoclaved soil. In non-autoclaved soil a reduction was observed in *M. incognita* in sugar beet, and *R. reniformis* in cotton, but not in *M. incognita* in cotton (24).

In summary, the biocontrol activity of *B. firmus* GB-126 observed in previous trials under greenhouse and field conditions where eggs and juvenile stages were reduced can be explained because the bacterium is producing a biosurfactant that is toxic to the plant-parasitic nematode. No ISR was observed at rates tested. However, *B. firmus* GB-126 possibly has other mechanisms of action against *R. reniformis* due to the presence of proteases that can be deleterious to the nematode. It is necessary to evaluate the root colonization of this bacterium, the effect it has in non-autoclaved soil against *R. reniformis*, and other type interactions that can be beneficial to cotton plants.

V. Literature cited.

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VI. Appendix

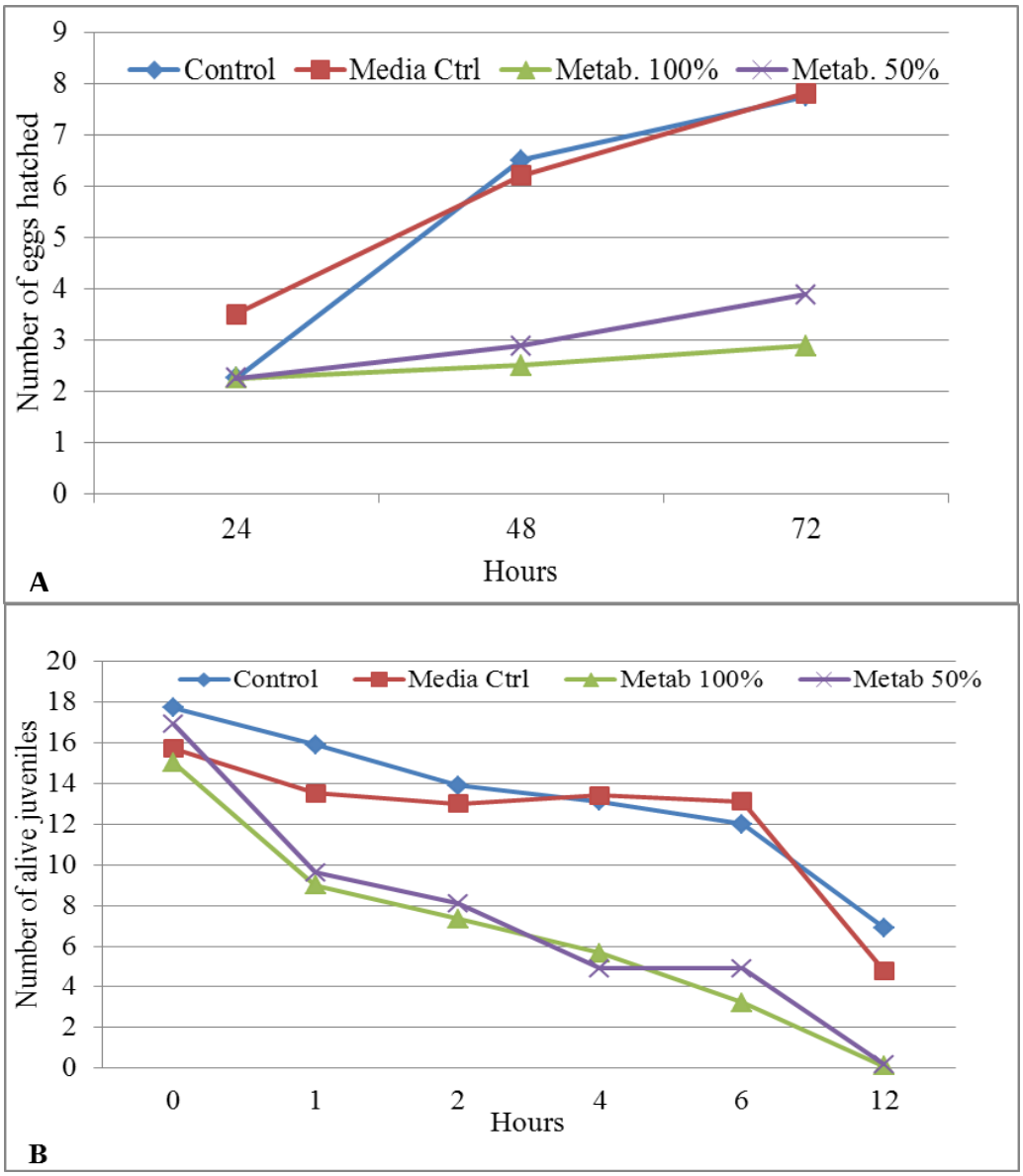

Figure 1. Effect of *B. firmus* metabolite at 100% and 50% concentration on eggs (A), and second stage juveniles (B).

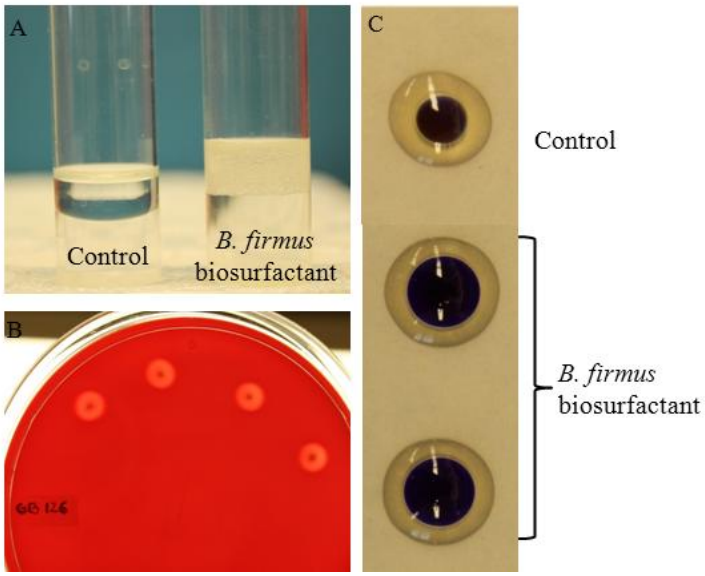

Figure 2. Tests of biosurfactant production of *B. firmus* GB-126. (A) Positive emulsification of kerosene, (B) halo formation due cell lysis in blood agar, and (C) Positive for drop collapse test.

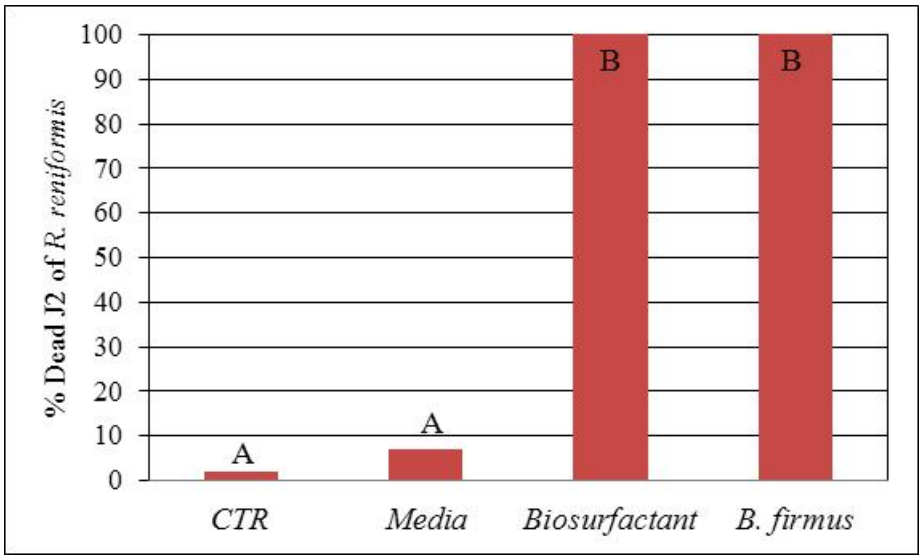

Figure 3. Effect of biosurfactant produced by *B. firmus* on *R. reniformis* second stage juveniles after 30 minutes after inoculation ($P \leq 0.05$)

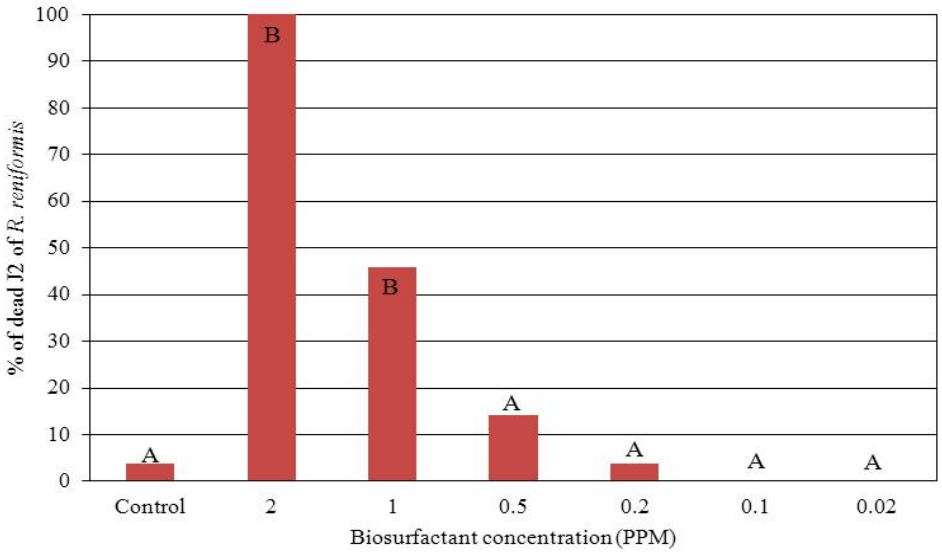

Figure 4. Effect of purified biosurfactant at different concentrations (ppm) on *R. reniformis* second stage juveniles after 30 minutes of inoculation under *in vitro* conditions ($P \leq 0.05$).

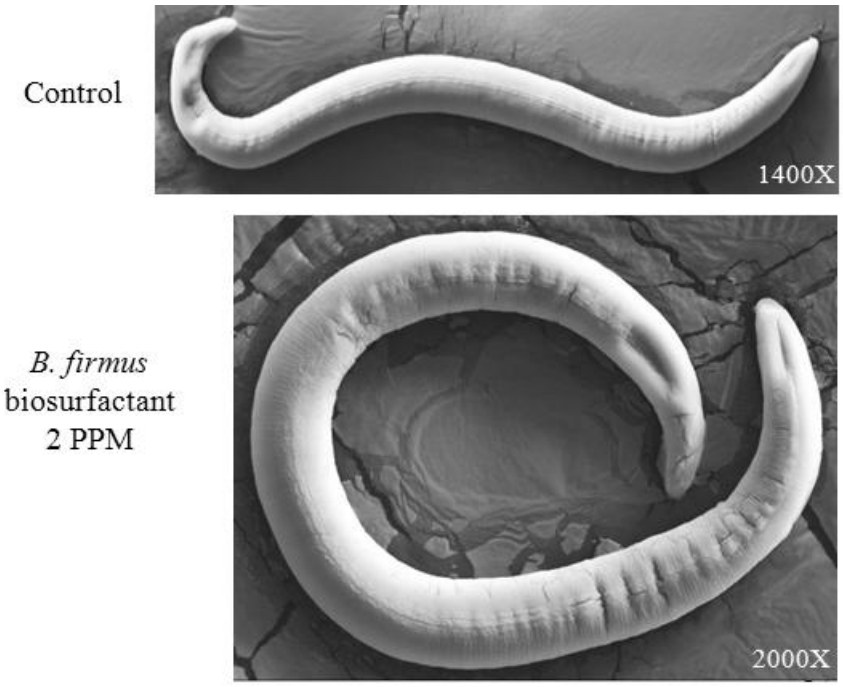

Figure 5. Second stage juveniles of *R. reniformis* form *in vitro* trial. No differences or mechanical damage is observed in the cuticle of juveniles treated with 2 ppm of pure *B. firmus* biosurfactant.

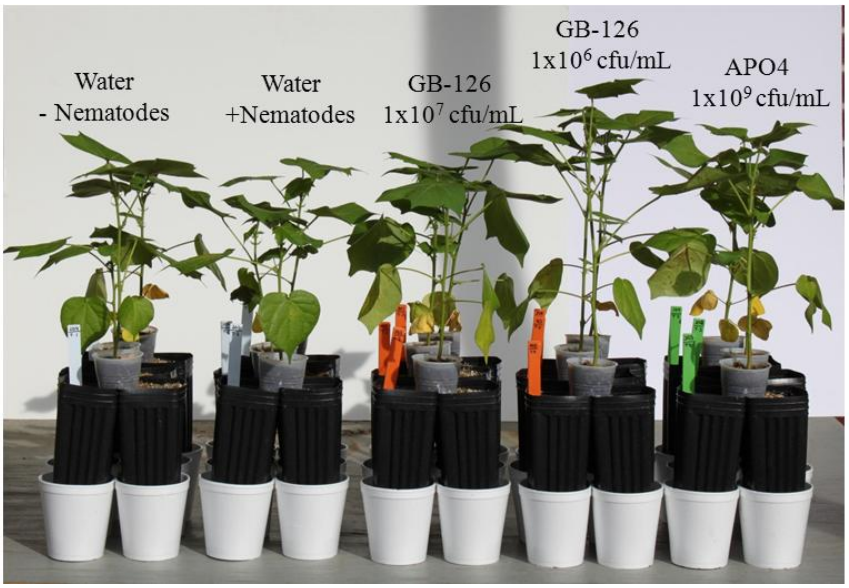

Figure 6. There were no induced systemic resistance trial of *B. firmus* GB-126 and *S. marcescens* against *R. reniformis* in cotton plants.

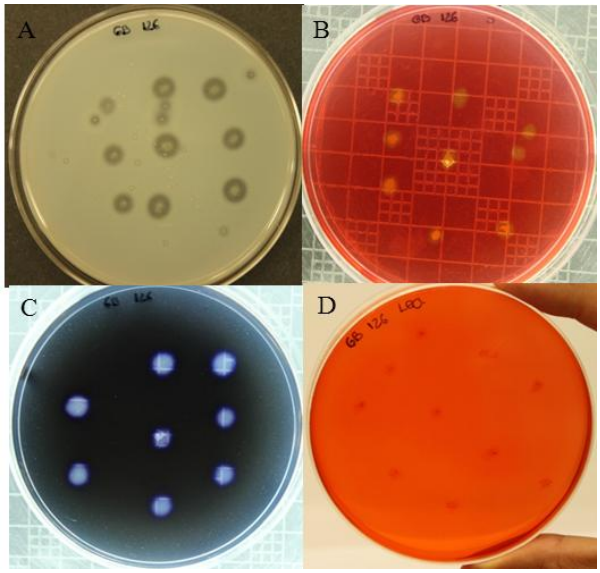

Figure 7. Enzyme reaction test of *B. firmus* GB126: production of proteases (A), amylases (B), celulases (C), and lack of chitinases (D).

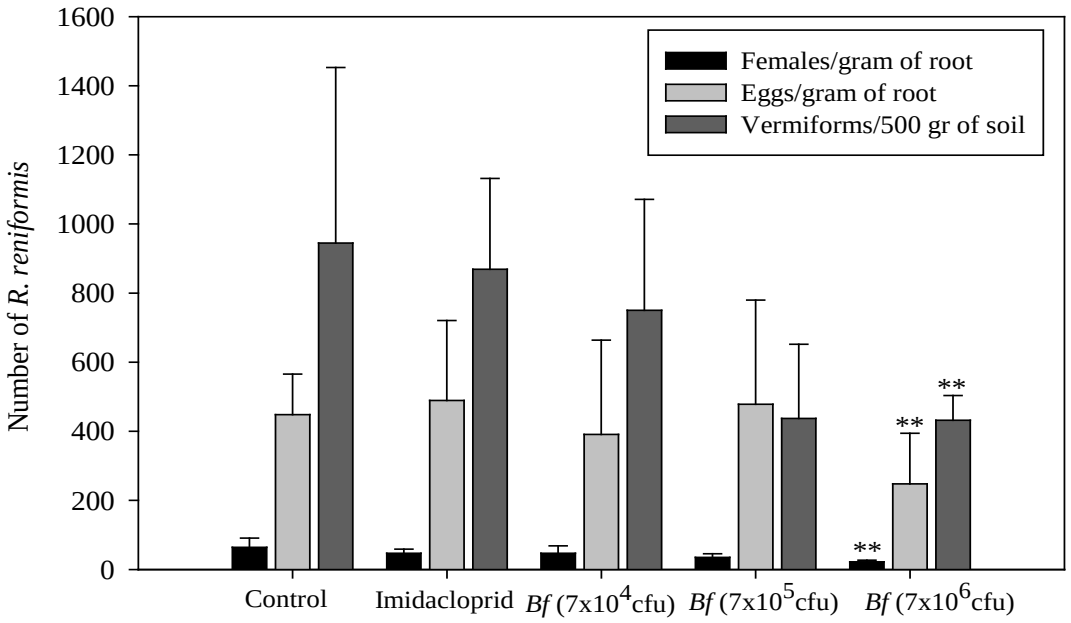

Figure 8. Reduction of *R. reniformis* life stages with cotton seeds treated with *B. firmus* GB-126 at $7x10^6$ cfu/seed under greenhouse conditions in autoclaved soil ($P \leq 0.05$).

Chapter IV. *Catenaria auxiliaris*: a new parasite of different stages of *Rotylenchulus reniformis.*

Abstract

A new pathogen to *Rotylenchulus reniformis* has been observed in greenhouse stock cultures of this nematode. Microscope observations reveal the presence of a zoosporic fungus infecting vermiform and mature female life stages of *R. reniformis.* The objective of this study was to identify morphologically and molecularly this new fungus infecting *R. reniformis* on cotton plants. Observations and measurements under light and electronic microscopy reveal the formation of a rhizomycelium composed by ovoid sporangia inside the nematodes body. In advanced stages of infection sporangia forms swellings in the cuticle of the nematode, which break and release posteriorly-uniflagellated zoospores. Zoospores are maneuvered by its flagellum. Zoospore encysts often near the excretory pore of the female nematode. Morphological characters and molecular identification based on rDNA (SSU+5.8S+LSU) confirm that *Catenaria auxiliaris* is a parasite of *R. reniformis*. This fungus has features that make it a promising biocontrol agent for *R. reniformis*: *i)* it is an obligate parasite that infects vermiform and female life stages of *R. reniformis*; *ii)* it releases zoospores that can search for its host within the soil profile; and *iii)* the fungus produces resistant spores that make formulation possible. Developing a media to cultivate this fungus will facilitate research studies, and can be the first step to develop it as future commercial biocontrol agent.

I. Introduction.

The reniform nematode (*Rotylenchulus reniformis* Linford and Oliveira) is an economic problem on cotton and soybean in tropical and temperate regions of North and South America (1). This nematode has been reported to parasitize 314 host plants, and colonizes a wide range of soils types at depths over 122 cm (10,11,14). Over the growing season *R. reniformis* may survive at these depths waiting till the host crop is planted again. Thus, there is a potential to improve nematode control with biological antagonists.

Several fungal and bacterial species have been reported as antagonists of *R. reniformis* (2,3,7,15). Among those found in the literature, zoosporic fungi have been reported as nematode antagonists. *Catenaria auxiliaris*, *Nematophtora gynophila*, and 'Langenidiaceous fungus' have been identified as parasites of cyst nematode (8, 9). The zoosporic fungus has a resting spore stage, and the moisture level is a key factor for zoospores infection to host nematodes. The stock cultures of *R. reniformis* we increase for our research projects have become colonized by a new and unknown biocontrol agent. Nematode numbers were reduced and vermiform stages were visible colonized by a zoosporic fungus. Therefore, the objective of this study was to identify, describe and document pathogenicity of this *R. reniformis* parasite.

II. Materials and methods.

Morphological identification.

Cotton plants infected with *R. reniformis* were grown in 500 cm^3 pots with sandy loam field soil (nitrogen: 0.08; organic matter: 1.4%) in the Plant Science Research Center of Auburn

University. Vermiform nematodes were extracted from the soil by gravity screening followed by sucrose centrifugation. Eggs were extracted from the roots by shaking in 10% NaOCl for 4 minutes and multiple rinses with water and collected on a 25 µm sieve. Structures of *C. auxiliaris* infecting female, juvenile and egg stages were observed, counted, and measured under the inverted microscope Nikon Eclipse E400, and electron microscope SEM EVO40. Average measurements of sporangia, resting spores, zoospores, and flagella were obtained by measuring each structure from approximately 100 random nematode life stages.

The life stages of infected *R. reniformis* nematodes were removed from the petri dish and placed on 12mm diameter aluminum stubs. Fixation of the tissues included vapor exposure to a 2% aqueous solution of osmium tetroxide (OsO_4 2%) in the dark for 2 hours. Samples were air dried for 2 more hours and after the stubs were transferred to the sputter coater (EMS 550x) for gold layering. SEM observations were made with a Carl Zeiss EVO 50 microscope.

Molecular identification

Vermiform life stages of *R. reniformis* with signs of *C. auxiliaris* of were hand-picked and placed in an Eppendorf tube with distilled water. DNA from infected vermiform nematodes were extracted by using a modified cetyl methyl ammonium bromide (CTAB) (6). To amplify the SSU rDNA region we used the primers 18S-Cs-1F [5'-GAGGCCTACCRTGGTGAT-3'] and NS4 [5'-CTTCCGTCAATTCCTTTAAG-3'] (13, 21). Thermal cycles for SSU were: 95°C for 2 min, followed by 37 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 3.5 min, and a final 72°C for 10 min. To amplify the ITS and LSU regions, we used the designed set of primers LF01F [5'-GATGAAGAACGCAGCGAAATGCGA-3'] with LF01R [5'-

ATGCTTAAGTTCAGCGGGTAGCCT-3'], and LR0R (14) with LR5 (20). The thermal cycles were: 95°C for 2 min, followed by 35 cycles of 94°C for 1 min, 58.5°C for 1 min, 72°C for 1 min, and a final 72°C for 10 min. Amplicons were purified using the Qiaquick PCR purification kit (Quiagen). The resulting amplified products were sequenced at the Auburn University Genomics facility. Sequence analyses were edited using Chromas Lite 2.01 software (www.technelsyum.com.au). Alignments of the sequences were done in Mega 4.1 software (17), and then were subjected to blast analysis in National Center for Biotechnology Information **(**NCBI**).**

III. Results and Discussion

Observations under the compound and electronic microscope display the presence of *C. auxiliaris* colonizing various life stages of *R. reniformis.* In the greenhouse stock cultures, 39.5% of the *R. reniformis* were parasitized by this fungus. In vermiform life stages, the rhizomycelium is composed by forming ovoid sporangia (40 X 16 ųm) inside the nematode body. Sporangia can be connected with short fragments of hyphae 1-1.5 ųm long (Figure 1). The rhizomycelium matured forming ovoid sporangia swellings on the cuticle of the nematode (Figure 2). Later the sporangia break through the swellings and release zoospores (Figure 3A). The zoospores are ovoid (2.94 x 4.90 µm), posteriorly uniflagellated, (9-11 µm long), and have globules visible in the anterior region (Figure 3B). Zoospores swim short distances maneuvered by the flagellum. Mature sporangia form yellow circular resting spores with a reticulate appearance (20-25µm diameter) (Figure 4). In the nematode mature female stage, zoospores encyst in the metacorpus region of the body (Figure 5). Nematode eggs were observed colonized by the rhizomycelium with precursor sporangia inside the egg (Figure 6A). The mature resting sporangia are visible on the outer cuticle of the egg (Figure 6B), that later burst releasing the zoosporangia (Figure 6C).

This fungus belong to the order of Blastocladiales, and was initially described in the beet cyst-nematode (*Heterodera schachtii*) as mycelia fungus with resting spores, named *Tarichium auxiliare* by Kühn in 1877. A century later, Tribe did a complete description of this fungus, including its biology and distribution, and renamed it *Catenaria auxiliaris* (18). Furthermore, he reported *C. auxiliaris* not only in populations of *H. schachtii*, also in cereal cyst-nematode (*H. avenae*), clover cyst-nematode (*H. trifoli*), and soybean cyst-nematode (*H. glycines*) (19). Among the fungal antagonists, there is a first time report of the zoosporic fungus *Catenaria auxiliaris* infecting different life stages of *R. reniformis* (2).

Blast analysis on GenBank results in 97% match with *Catenaria* sp., and 95% *C. anguillulae* BR105 (Table 1). *Catenaria anguillulae* BR105 is the type specie of the genus. Studies of *C. anguillulae* BR105 zoospore attachment and encystment have been conducted (5). There are differences and similarities between our strain of *C. auxiliaris* and *C. anguillulae*. Both species are posteriorly uniflagellated compared to other nematode-parasite zoosporic fungi (7). Additionally, both species encyst in nematode natural openings; zoospores in *C. anguillulae* accumulate and encyst near the mouth, excretory pore, and anus of *Panagrellus reduvivus* (5). In *C. auxiliaris* encyst in the excretory pore, but zoospores were not observed to accumulate. One zoospore encysted in the female excretory pore was able to kill the nematode. The surface-recognition for encystment by *C. anguillulae* is mediated by the zoospore soma, not by the flagellum. In contrast, *C. auxiliaris* zoospores swim straight following its flagella. Therefore, flagella in *C. auxiliaris* can play an important role in surface-recognition during the encystment to *R. reniformis*. *Catenaria anguillulae* is a facultative endoparasite of nematodes, while *C. auxiliaris* is an obligate parasite of cyst nematodes and *R. reniformis* (2,9,10,18,19).

Morphological characters and molecular identification based on rDNA (SSU+5.8S+LSU) confirm that this fungal parasite of *R. reniformis* is *Catenaria auxiliaris.* Molecular phylogeny of the Blastocladiomycota has been recently described based on nuclear ribosomal DNA, using sequences from strains identified by traditional morphological and ultrastructural characters (13).

This fungus has three characteristics that are advantageous for nematode biocontrol agents: *i)* it releases zoospores that are mobile and can search for its host within the soil profile; and *iii)* it produce resistant spores which are an advantage in the formulation. More research needs to be conducted evaluating *C. auxiliaris* population levels for nematode pathogenicity. Additionally, developing a media to cultivate this fungus will facilitate research studies, and can be a future commercial biocontrol agent.

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V. Appendix

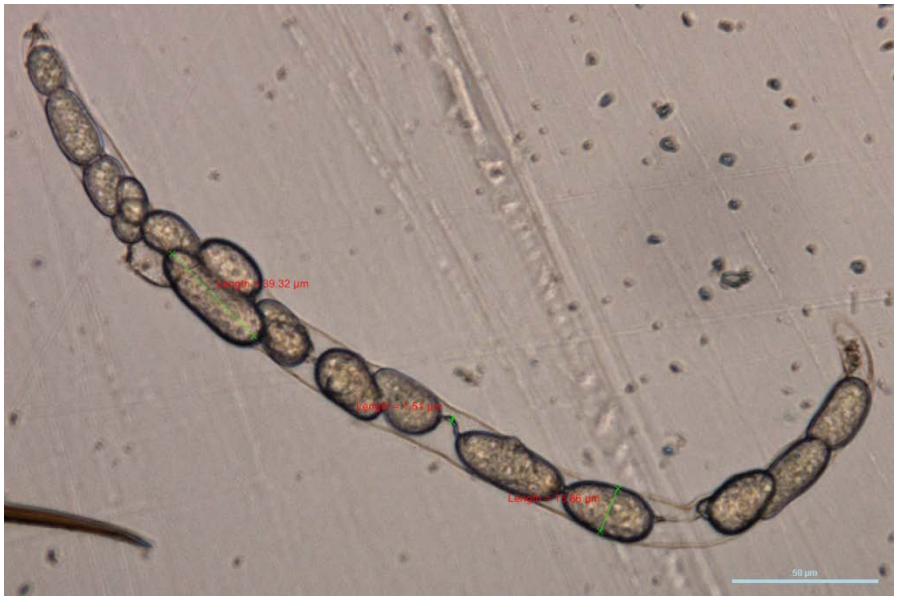

Figure 1. Rhizomycelium of *C. auxiliaris* growing inside a *R. reniformis* vermiform nematode.

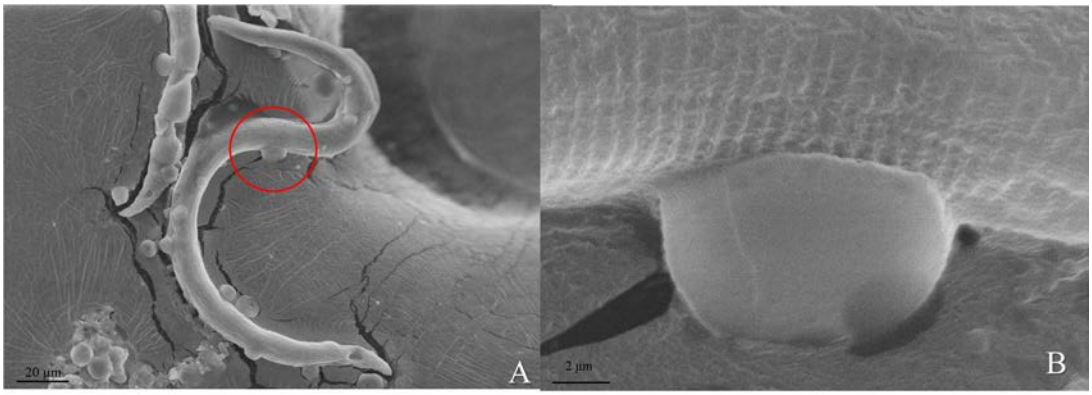

Figure 2. (A) Nematode with swellings caused by the sporangia formation; (B) Higher magnification of the sporangia in red circle.

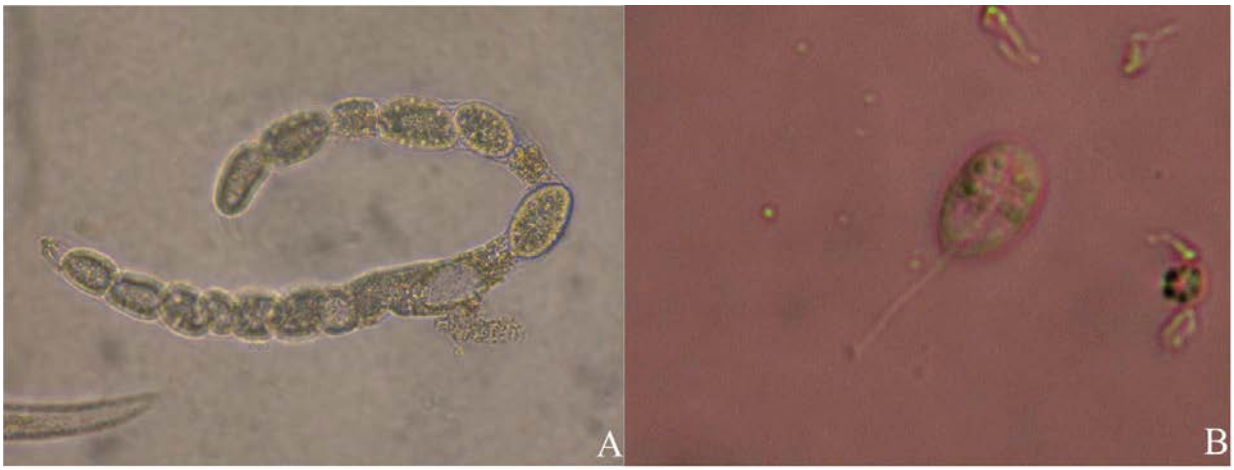

Figure 3. (A) Zoosporangia inside the nematode corpse releasing zoospores (40x); **(B)** Uniflagellated zoospore (100x)

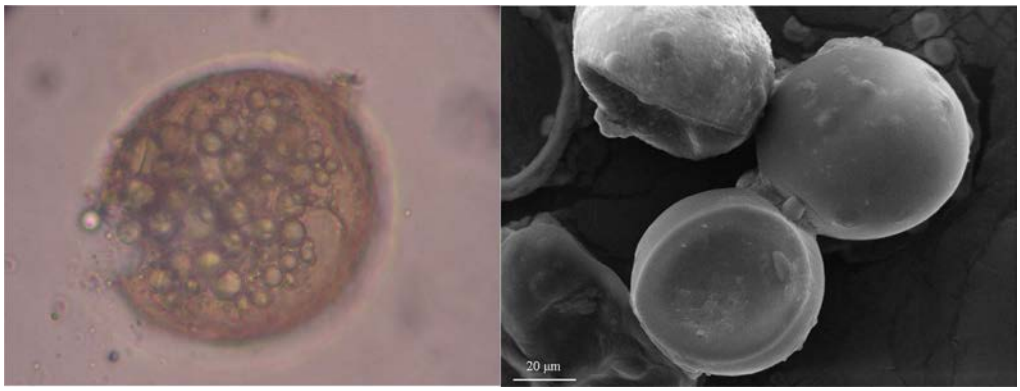

Figure 4. Resting spores (A) under light microscope (40x); and (B) under SEM.

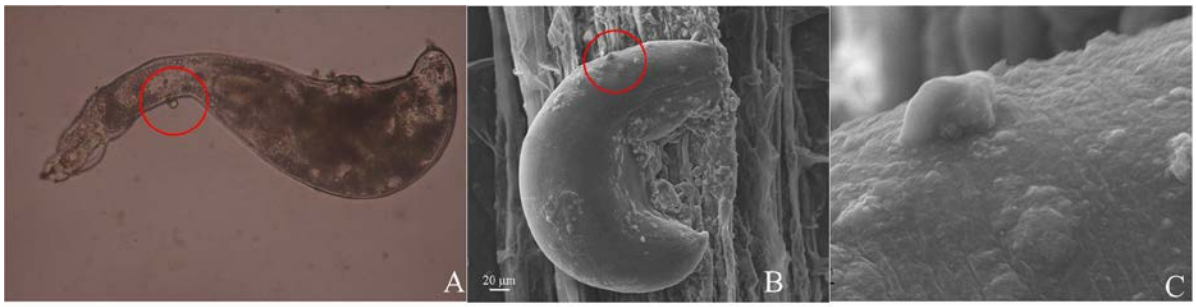

Figure 5. A) Female with a encysted zoospore (40x); (B) SEM view of a female with an encysted zoospore; (C) Zoom of the encysted zoospore on the female cuticle.

Table 1. Results from blastn analysis in GenBank

rDNA region	Amplicon Fragment (bp)	Highest Match in GenBank
SSU	831	97% *Catenaria* sp. Poly Ad 2-0 (HQ888758.1) 96% *Catenaria* sp. APO4 (HQ888760.1) 95% *Catenaria anguillulae* BR105 (HQ888755.1)
ITS-5.8S	455	
LSU	907	

Chapter V. Evaluation of thixotropic formulation for PGPR strains against soil-borne diseases on cucumber plants and nematodes in cotton crops.

Abstract

Biological control agents are an alternative for managing soil pathogens in a system that demands environmentally friendly practices. Currently, commercially available Plant-Growth promoting-rhizobacteria-based products for managing soil pathogens contain a single strain or a combination of two. Application is recommended as seed treatment or at transplanting time. In this study we report the effect of the thixotropic formulation, which is a novel formulation that represents a new advance in the PGPR application, because a complex mixture of PGPR can be delivered periodically during the growing season. The objective of this study was to test the effect of this formulation (Restore®) against the soil-borne pathogens *Pythium ultimum*, *P. aphanidermatum* and *Rhizoctonia solani* on cucumber plants under greenhouse conditions, and against the reniform nematode (*Rotylenchulus reniformis*) on cotton plants under greenhouse, microplot, and field conditions. Greenhouse results show that three applications of Restore to cucumber and cotton plants significantly increase shoot and root weight, and root architecture variables ($P \leq 0.001$), and reduce the disease incidence and nematode numbers, respectively ($P \leq 0.001$). Microplot and field results show an increase of stem diameter during the growing season and a reduction of females per gram of root 30 days after planting. Microplot cotton harvest was statistically higher than the untreated control ($P \leq 0.001$). However, under field conditions yield obtained was not statistically different from the control ($P \leq 0.461$). Thereby, it is necessary to evaluate a second and third growing season to test if there is any accumulative effect of Restore that can increase yield obtained. Results indicate that thixotropic formulation

represents a promising advance in the development of PGPR as microbial inoculants for biocontrol purposes.

I. Introduction

Various groups of microorganisms have been investigated for their capacity to improve plant growth or reduce plant disease. Plant growth-promoting rhizobacteria (PGPR) are beneficial root-colonizing bacteria that elicit increased plant growth, enhance emergence, induce resistance or reduced damage due to plant diseases (Siddiqui and Shaukat, 2003; Kloepper et al., 1992). These benefits that result from inoculation of seeds or plant parts with PGPR are due both to production of bacterial metabolites such as plant growth regulators and antibiotics that alter the plant or the native rhizosphere community. In addition, some PGPR alter the host plant's physiology in a way that induces resistance to diseases (Kloepper et al. 2004a) and stress (Yang et al. 2009). Several reviews summarize the theoretical basis of PGPR, their mechanisms of action, and efficacy in greenhouse and field trials (Bashan 2010; Kloepper et al. 2004b; Persello-Cartieaux et al. 2003; Vissey 2003).

Interest on PGPR has focused on their use as growth promoters and biological control agents. With an increasing global concern about reducing fertilizer run-off and contamination of surface and ground waters, searching for PGPR that increase root system will enable the plant to enhance the uptake of applied fertilizers while reducing application rates (Adesemoye et al., 2009). As biocontrol agents for soil borne pathogens and plant-parasitic nematodes they have great potential, because they become intimately associated with their host plant at the root surface. This direct contact with the target sites of entrance and feeding sites of nematodes can strongly

influence root exudates, and thereby affect the development of the nematode (Martinez-Ochoa, 2000; Sikora, 1992). Furthermore, rhizobacteria can also produce ammonia compounds, toxins, and lytic enzymes (chitinases and collagenases) that kill the nematodes before they invade or feed on host tissue (Kerry, 2000, 1987; Martinez-Ochoa, 2000; Aalten et al., 1998).

Over the past decade, there has been a proliferation in PGPR-based products in the United States. The majority of these products contain a single purified strain of PGPR applied as a seed treatment. Seed treatments have been the preferred delivery system in the U.S. partly due to integration of agricultural inputs into the seed. Growers tend to prefer purchasing inputs from a single source, such as the seed supplier, rather than purchasing multiple inputs from various sources. Hence, companies developing microbial inoculants have focused on seed treatment. A decision to limit the delivery system to a seed treatment has profound implications and limitations for which kinds of PGPR can be developed. For example, many strains of *Pseudomonas* are active as PGPR, however, they cannot be formulated as seed treatments. This is because the sales and distribution network of seeds requires that a bacterial seed treatment remain viable for 12-18 months and that it be compatible with seed treatment chemicals such as fungicides and insecticides which are also added into the seeds. This requirement means that for all practical purposes, only spore-forming PGPR, such as *Bacillus* spp. or *Streptomyces* spp. can be formulated as seed treatments. Examples of such PGPR that have been registered by the U.S. Environmental Protection Agency and that are sold as seed treatment products include *Bacillus pumilus* strain INR-7 (Hu et al., 2003; Kloepper et al., 2004a), *Bacillus subtilis* strain GB03 (Brannen and Backman, 1993; 1994; Hammon and Berrada, 2001; Koepper et al., 2004b; Turner,

Backman 1991), and *Bacillus subtilis* strain MBI 600 (Bennett et al., 2003; Kumar et al., 2009; Rossall and McKnight, 1991).

Applying PGPR as seed treatments has been a successful approach for the products that are currently registered. However, as stated above, the PGPR which can be used as seed treatments are limited. In addition, seed treatments are obviously only applied once per year, thereby eliminating the potential to apply additional dosages, commonly referred to as "boosters", of PGPR during the growing season. Also, many research studies demonstrate that a combination of the living cells of PGPR and their metabolic products produced by fermentation can have more consistent or stronger beneficial effects on plants than application of only spores of the PGPR. Hence, there have been many attempts to produce PGPR products that are not limited to seed treatments.

The objective of this work is to evaluate a common core consortium of PGPR strains (*Bacillus subtilis, B. licheniformis, B. megaterium, B. coagulans, P. fluorescens, Streptomyces* spp., and *Trichoderma* spp.) which have recently been blended into a thixotropic (properties of gels becoming fluid when disturbed) formulation commercialized under the name of Restore®. We test this formulation in cucumber against the soil-borne pathogens: *Pythium ultimum, P. aphanirdermatum*, and *Rhizoctonia solani* under greenhouse conditions, and in cotton against the reniform nematode *Rotylenchulus reniformis* under greenhouse, microplot, and field conditions.

II. Materials and Methods

2.1. *Cucumber greenhouse trials*

Five tests were conducted in the greenhouse with Restore on cucumber plants in the Plant Science Research Center (PSRC) of Auburn University. Restore test 1 was designed to calculate the populations of PGPR that are delivered to plants when the product is applied through irrigation. Two application methods were used, a tank mix and a process column, which represent the two common ways in which farmers are now using the product in irrigated agriculture. For the test, small scale models were designed of the actual tank mix and process columns used by farmers.

The model for the tank mix application consisted of a 20 L container with a circulating pump and a section of pipe (PVC - polyvinylchloride) 8 cm diameter X 20 cm long, capped at the bottom end with slits in the cap (Figure 1A). Using products and rates provided by the manufacturer, a thixotropic formulation of nutrients for the microorganisms was placed into the pipe and a powdered formulation of the Restore microorganisms was placed into container. The mixture was incubated at room temperature with the circulating pump for 10 hrs. The resulting suspension was diluted 1:20 with water to recreate the concentration applied through irrigation systems, and the population was determined using most probable number (MPN) technique in tryptic soy broth with two replications and three tubes of each dilution per replication.

The model for the process column was a section of PVC pipe 20 cm diameter X 75 cm long with caps on both ends and an output line that delivered the contents in the same concentration used in field irrigation systems (Figure 1B). Populations were determined in the process column by

inserting into the column a thixotropic formulation that contained both the microorganisms and the microbial nutrients, pressurizing the system by connecting it to a residential water hose, and collecting the suspension that flowed from the output line for a 15 min. period. Aliquots were removed and used to determine the population using MPN. Each test with the tank mix and process column was conducted three times, and populations were calculated as means of the three tests.

Restore tests 2-5 evaluated the effects of the PGPR consortia applied in a manner designed to replicate the commonly used system in vegetable production, by alternating applications of PGPR from the tank mix and the process column. Each experiment was a randomized block design with 3 treatments, and 10 replications per treatment. Treatments were 1) tank mix alone, 2) tank mix + process column and 3) water control. Each experimental unit consisted of a 25-cm-diameter pot containing a soilless potting mix. Applications began one week prior to planting 10 cucumber seeds in each pot, by adding 100 ml of formulation per pot. Treatment 1 was applied three times during each test: 7 days before planting, day of planting, and 7 days after planting (DAP). With treatment 2, the application from the process column was applied at the same times as treatment 1, and the application from the tank mix was applied 4 days after each of these. Treatment 3 was treated with water on the same schedule.

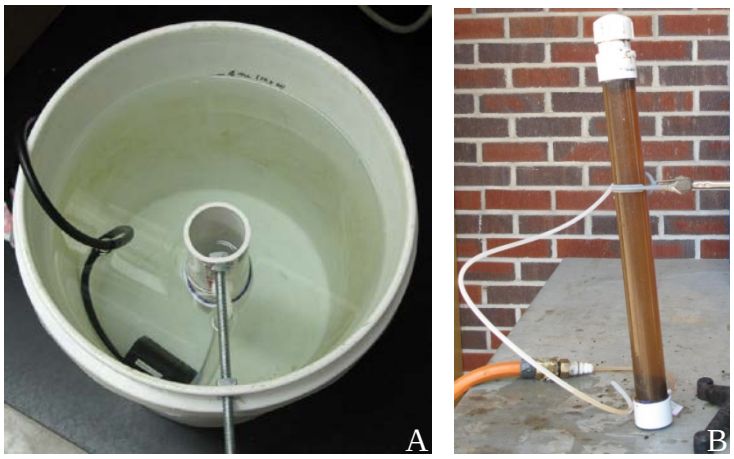

Figure 1. Tank mix model (A); Process column model (B).

Restore test 2 evaluated seedling growth promotion using the PGPR applications. The experimental design was doubled so that the test could be destructively sampled at 7 and 14 days after planting. At these times, seedlings were washed and weighed to determine whole seedling and root weights. Then, using WinRhizo analysis, the mean total root length, surface area, and number of root tips were determined as measurements of root architecture.

Restore tests 3-5 were biological control assays of the PGPR applications against three soilborne pathogens: *Pythium ultimum* (test 3), *Pythium aphanirdermatum* (test 4), and *Rhizoctonia solani* (test 5). Pathogens were applied at the time of planting by pipetting 5 ml of inoculum over the cucumber seeds before covering. Inoculum was prepared by growing cultures on corn meal agar (*Pythium* sp.) or potato dextrose agar (*R. solani* for 5 days and then homogenizing agar from 4 plates in 400 ml sterile water. All of the pathogens can cause both pre- and post-emergence damping off and stunting of plant growth. Hence, data were collected on emergence at 5 DAP, final stand of healthy seedlings at 14 DAP, and disease incidence calculated as the number of missing, dead, or wilted seedlings at 20 DAP.

2.2. *Cotton greenhouse, microplot, and field trials.*

To test the effect of Restore on cotton plants infested with *R. reniformis,* five trials were conducted under greenhouse, microplot, and field conditions. In all the trials the cotton cultivar used was ST5458B2RF, and the soil was a silty clay loam collected from a naturally-infested *R. reniformis* cotton field from Tennessee Valley Research and Extension Center (TVREC) in Belle Mina, AL. Initial trial was conducted in the greenhouse using autoclaved-field soil, and then in non-autoclaved field soil. Later the same treatments were evaluated under microplot and field conditions to test if there was any effect in yield.

Vermiform stages were extracted from the soil by the modified gravity screening and centrifugation-flotation method (Jenkins, 1964). Egg stages were extracted from cotton roots by shaking them under 1% NaOCl for four minutes at 120 rpm and sieving the solution (Hussey and Baker, 1973). Females in roots were stained with fuschin acid to count the females invading the root (Hooper, 1986). Vermiform life stages and eggs were counted under an inverted TS100 Nikon microscope, and females in the root under compound microscope Nikon SMZ800. Data collected in all the trials were analyzed in SAS 9.1 (SAS Institute Inc.) using GLIMMIX procedure at the ($P \leq 0.05$) level of significance.

2.2.1 *Greenhouse trials.*

Three trials were conducted under greenhouse conditions in the PSRC of Auburn University. In the first trial Restore was evaluated in a silty clay loam autoclaved field soil (sand-silt-clay: 17.5-51.3-31.2%; nitrogen: 0.16%; organic matter: 2.2%; pH 7.24). Treatments were 1) untreated control, 2) untreated control with 1000 *R. reniformis* vermiforms, and 3) tank mix + process

column with 1000 *R. reniformis.* The experiment was a RCBD with six replications per treatment, and the trial was repeated twice. Application frequency for treatment 3 was the same as described above in cucumber trials, adding 100 ml of formulation per pot. Treatments 1 and 2 were treated with water on the same schedule. Experimental unit consisted of 500 cc styrofoam pots with one cotton plant. Variables recorded were plant height, shoot and root weight, females and eggs per gram of root, and vermiforms in 500 cc of soil. Data were recorded 15 and 30 DAP. Average temperatures were plants were grown was 29˚C.

On the second trial, PGPR consortia were evaluated in a non-autoclaved silty clay loam field soil (nitrogen: 0.16%, organic matter: 2.1%, pH 6.8). The purpose on this trial was to compare Restore with an untreated control and aldicarb (nematicide used for commercial cotton production). Treatments were 1) untreated control, 2) aldicarb (5.6 kg/ha), 3) tank mix + process column. The trial was conducted using a RCB experimental design with five replications per treatment, and was repeated twice. Data were recorded 15 and 30 DAP. Variables recorded were the same as in the autoclaved-soil trial including root architecture on WinRhizo analysis software (Regent Instruments Inc.).

In the final greenhouse trial, the effect of Restore was evaluated only with only three applications (7 days prior planting, at planting, and 7 DAP), and compared to an untreated control and aldicarb. Treatments were: 1) untreated control, 2) aldicarb (5.6 kg/ha), and 3) tank mix + process column. Experimental design was a RCBD with five replications per treatment and repeated twice. Variables recorded were the same as in the second greenhouse trial.

2.2.2 *Microplot trial.*

Pots of 4500 cm^3 were filled with 12.5 kg of silty clay loam field soil (sand-silt-clay: 21.2-46.2-32.5%; nitrogen: 0.22 %; organic matter: 3.7%; pH 4.9). Each pot contained four cotton plants, and data were recorded 30, 60, 90 and 150 DAP. The trial had an RCBD experimental design with five replications per treatment, and was repeated twice. An extra pot was connected to a temperature and moisture data logger to record these variables during the growing season.

2.2.3 *Field trial*

This trial was conducted in the TVREC of Auburn University, in a naturally infested silty clay loam field soil (sand-silt-clay: 21.2-46.2-32.5%; nitrogen: 0.17%; organic matter: 2.3%; pH 6.3). The experimental unit was two-row plots 2 x 7.6m with 200 cotton seeds each. The experimental design was a RCBD with five replications per treatment, and repeated twice. The treatments were the same as the microplot trial, however the formulation was changed, but the group of PGPR bacteria used in previous trials was applied. In this trial a granular formulation of Restore was applied 7 days prior planting. At planting and 7 DAP, an emulsification formulation of Restore was applied. Each two-row plot received 8 L of the product on each application, and in the untreated control and aldicarb received the same volume of water.

Within each two-row plot, three plants were marked with a fluorescent tape and plant variables recorded were: plant height, stem diameter, and number of nodes. At the end of the growing season yield, was recorded. A data logger was installed within the field to record moisture and temperature during the growing season. Nematode variables were number of females and eggs

per gram of root, and number of vermiforms and free-living nematodes in soil. Data were collected 0, 30, 60, 90 and 150 DAP.

III. Results

3.1. *Cucumber greenhouse trials*

In Restore test 1, which simulated two delivery systems for the thixotropic formulations of the PGPR consortia, the mean populations determined from three tests were log 6.06/ml with the process column and log 9.82/ml for the tank mix. In Restore test 2 (Table 1), both application systems of PGPR (tank mix alone and tank mix + process column) promoted early cucumber seedling growth. This growth promotion was expressed as significantly increased overall seedling weight and root weight as well as changes in the root architecture, including increased root length, surface area, and numbers of root tips.

Results from the three biological control assays (Table 2) showed that the disease pressure in the gfrom 35% with *Pythium ultimum* to 45% with *P. aphanidermatum*. Under these conditions, Restore, applied with both applications systems significantly reduced damage from all three soilborne pathogens. Protection was evident as reductions in both pre-emergence damping-off (emergence at 5 DAP) and post-emergence damping off (final stand). Restore also resulted in healthier root systems (Figure 2).

Table 1. Effect of Restore applications on cucumber seedling growth and root architecture (Restore test 2)

7 days after planting					
Treatment	Seedling Fresh Weight (g)	Root Fresh Weight (g)	Root length (cm)	Root Surface Area (cm^2)	Number of root tips
Control	0.67	0.07	17	3.01	158
Tank mix alone	0.98*	0.22*	77*	17.2*	487*
Tank mix + Process column	1.1*	0.16*	43.7*	13.7*	405*
LSD ($P \leq 0.05$)	0.18	0.04	20.7	10.7	163

14 days after planting					
Treatment	Seedling Fresh Weight (g)	Root Fresh Weight (g)	Root length (cm)	Root Surface Area (cm^2)	Number of root tips
Control	3.14	0.63	97	37.8	426
Tank mix alone	4.03*	0.86*	155*	64.2*	671*
Tank mix + Process column	3.96*	0.85*	177*	62.2*	838*
LSD ($P \leq 0.05$)	0.67	0.21	35	13.9	197

*Indicates significant difference from the control value on the same column at $P \leq 0.05$

Table 2. Biological control activity of Restore against soilborne pathogens on cucumber (Restore test 3-5).

Restore Test 3: *Pythium ultimum*			
	5 DAP	14 DAP	20 DAP
Treatment	Seedling Emergence	Number of healthy plants	Disease Incidence[1]
Control	8.1	6.1	3.9*
Tank mix alone	9.5*	8.8*	1.2*
Tank mix + Process column	9.4*	9.0*	1.0*
LSD ($P \leq 0.05$)	0.82	0.84	0.84

Restore Test 4: *Pythium aphanidermatum*			
	5 DAP	14 DAP	20 DAP
Treatment	Seedling Emergence	Number of healthy plants	Disease Incidence[1]
Control	6.3	5.5	4.5*
Tank mix alone	9.2*	8.1*	1.9*
Tank mix + Process column	8.8*	8.4*	1.6*
LSD ($P \leq 0.05$)	0.78	0.93	0.9

Restore Test 4: *Rhizoctonia solani*			
	5 DAP	14 DAP	20 DAP
Treatment	Seedling Emergence	Number of healthy plants	Disease Incidence[1]
Control	8.1	6.1	3.9
Tank mix alone	9.5*	8.8*	1.2*
Tank mix + Process column	9.4*	9.0*	1.0*
LSD ($P \leq 0.05$)	0.82	0.84	0.84

*Indicates significant difference from the control value of the same column at $P \leq 0.05$

[1] Number of missing, dead or wilted plants.

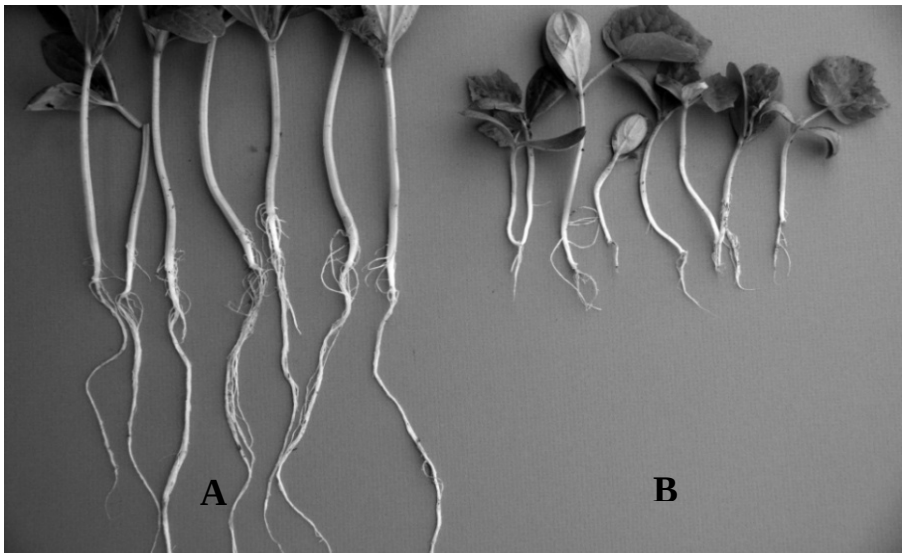

Figure 2. Biological control of *Pythium ultimum* damping-off by Restore. From Restore test 3. Right = water control; left = Restore; both were inoculated with the pathogen.

3.2. *Cotton greenhouse, microplot, and field trials.*

3.2.2. *Cotton greenhouse trials*

In the first trial (in autoclaved soil), plant height, root and shoot fresh weight were higher in the plants treated with Restore when compared to the control without nematodes ($P \leq 0.001$), and untreated control with nematodes 30 DAP ($P \leq 0.001$). All *R. reniformis* nematodes were reduced when Restore was applied. Vermiforms per 500 cc of soil were reduced since 15 DAP ($P \leq 0.003$) until 30 DAP ($P \leq 0.026$). Furthermore, eggs and females per gram of root were lower when compared to the untreated control ($P \leq 0.001$).

For the second trial, plant height, shoot dry weight, root fresh weight, and root architecture (Root length, surface and number of tips) were higher in the Restore treatment when compared to the untreated control and aldicarb ($P \leq 0.001$) (Table 3). Females and eggs per gram of root, and vermiforms in soil were reduced by the thixotropic formulation delivered by tank mix and process column ($P \leq 0.001$), when compared to the untreated control. However, the reduction in

nematode life stages was not different from the control provided by aldicarb ($P \leq 0.816$) (Table 4). Furthermore, free-living nematodes were increased with the Restore treatment when compared to the untreated and aldicarb control ($P \leq 0.001$) (Table 5).

In the final greenhouse trial, the effect of three Restore applications were consistent with the trial described above, increasing shoot dry weight, root fresh weight, root surface and number of tips when compared to untreated, and aldicarb control 30 DAP ($P \leq 0.001$) (Figure 3). Females and eggs per gram of root were lower in the Restore treatment when compared to the untreated control ($P \leq 0.021$) but not different from the control provided by aldicarb ($P \leq 0.830$) (Figure 4). Vermiforms in soil were lower in Consortia when compared to the untreated control ($P \leq 0.010$); however, it did not reduce vermiforms as aldicarb did ($P \leq 0.001$). Free-living were increased when compared to the untreated and aldicarb control ($P \leq 0.001$).

Table 3. Effect of Restore on cotton plant variables under greenhouse conditions 30 DAP ($P \leq 0.05$).

Plant Variables									
Treatments	Height (cm)	Dunnett's *P* vs.		ShDW[1] (g)	Dunnett's *P* vs.		RFW[2] (g)	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb
Control	16.3			0.95			3.54		
Aldicarb	17.2	0.999		0.96	0.999		2.84	0.971	
Restore	23.4	0.001	0.001	2.21	0.001	0.001	6.8	0.001	0.001
Root Architecture									
Treatments	Lenght (cm)	Dunnett's *P* vs.		SA[3] (cm^2)	Dunnett's *P* vs.		# Tips	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb
Control	16.3			218.9			218.9		
Aldicarb	17.1	0.999		162.3	0.999		162.3	0.999	
Restore	23.4	0.001	0.001	355.2	0.001	0.001	355.2	0.001	0.001

[1]Shoot dry weight [2]Root fresh weight [3]Surface area

Table 4. Reduction of *R. reniformis* life stages under greenhouse conditions 30 DAP with Restore ($P \leq 0.05$).

	***R. reniformis* life stages**								
Treatment	Females[1]	Dunnett's *P* vs.		Eggs[1]	Dunnett's *P* vs.		Vermiforms[2]	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb
Control	254			43			1507		
Aldicarb	53	0.001		12	0.001		245	0.001	
Restore	44	0.001	0.816	8	0.001	0.811	414	0.001	0.816

[1]Per gram of root [2]Per 500cc of soil

Figure 3. Increase in plant variables under greenhouse conditions 30 DAP. (A) Increase in height and shoot dry weight (left: untreated control, center: aldicarb, and right: Restore). (B) Increase in root length, surface area, and number of tips (left: untreated control, center: aldicarb, and right: Restore).

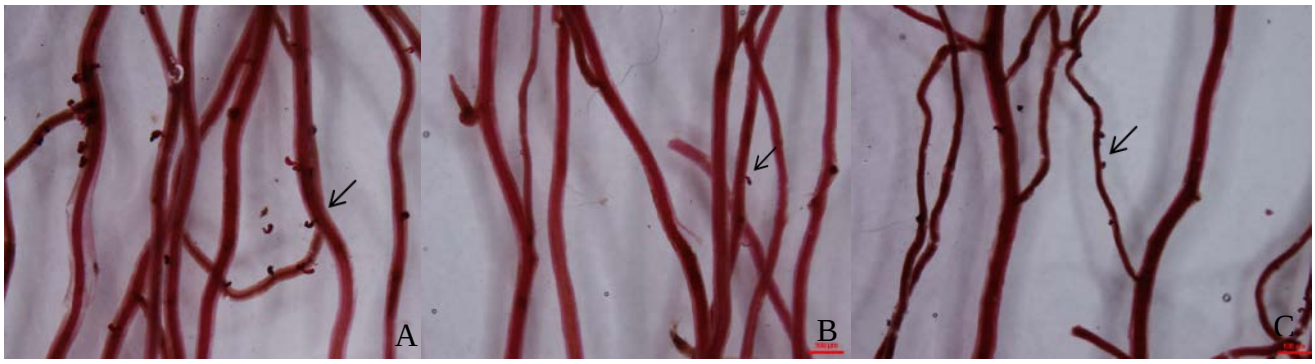

Figure 4. Reduction of females per gram of cotton root 30 DAP. (A) untreated control; (B) aldicarb at 5.6 kg/ha; (C) Restore.

Table 5. Increase on free-living nematodes population 30 DAP under greenhouse, microplot, and field conditions ($P \leq 0.05$).

Treatment	Greenhouse			Microplot			Field		
	Free-living[1]	Dunnett's *P* vs.		Free-living[2]	Dunnett's *P* vs.		Free-living[2]	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb
Control	600			21			59		
Aldicarb	184	0.002		6	0.56		118	0.251	
Restore	1674	0.001	0.001	165	0.001	0.001	221	0.001	0.031

[1] Nematodes per 500cc [2] Nematodes per 150cc

3.2.3. *Cotton microplot trials*

Shoot dry weight, and root fresh weight were higher than the untreated control ($P \leq 0.018$) and aldicarb 30 DAP ($P \leq 0.019$) (Table 6). Furthermore, plants treated with Restore were taller and had higher stem diameter than the two controls during the trial ($P \leq 0.006$) (Figure 5). Eggs and vermiforms were not different from the untreated control ($P \leq 0.291$) and aldicarb ($P \leq 0.891$) 30 DAP. However, 60, 90 and 150 DAP there was a reduction of vermiforms compared to the untreated control ($P \leq 0.001$). The vermiform reduction was not different than the one provided by aldicarb ($P \leq 0.98$) (Table 7). Additionally, females per gram of root were reduced 30 DAP ($P \leq 0.038$) (Table 8). Free-living nematodes were increased during the whole trial with the Restore treatment compared to the two controls ($P \leq 0.001$) (Table 5). The harvest obtained 150 DAP from was higher in the Restore treatment compared to the untreated control and aldicarb ($P \leq 0.001$) (Figure 6).

Table 6. Effect of Restore on cotton plant variables under microplot conditions 30 DAP, and stem diameter 30, 60, and 90 DAP ($P \leq 0.05$).

Plant Variables									
Treatment	Height (cm)	Dunnett's *P* vs.		ShDW [1] (g)	Dunnett's *P* vs.		RFW [2] (g)	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb
Control	18.9			1.25			1.23		
Aldicarb	19.4	0.998		1.24	0.909		1.17	0.995	
Restore	29.3	0.006	0.008	3.8	0.018	0.019	2.9	0.032	0.025

Stem Diameter									
Treatment	30 DAP			60 DAP			90 DAP		
	SD [3] (mm)	Dunnett's *P* vs.		SD [3] (mm)	Dunnett's *P* vs.		SD [3] (mm)	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb
Control	3.6			6.7			6.9		
Aldicarb	3.7	0.997		7.1	0.792		7.2	1	
Restore	5.4	0.001	0.004	9.6	0.001	0.001	9.6	0.001	0.001

[1] Shoot dry weight [2] Root fresh weight [3] Stem diameter

Figure 5. Increase in plant height and stem diameter boosted by Restore under microplot conditions. (A) 30 DAP; (B) 60 DAP; (C) 90 DAP. (Left: untreated control, center: aldicarb, right: Restore).

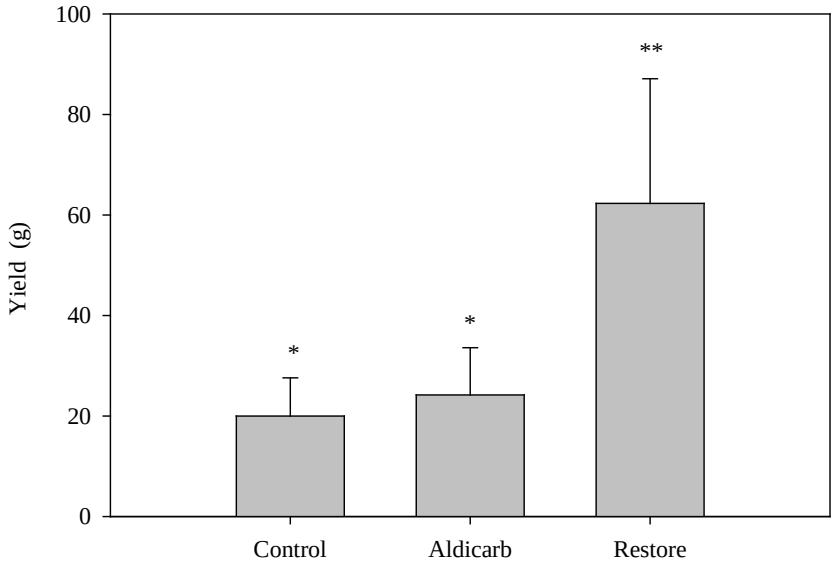

Figure 6. Cotton harvest obtained under microplot conditions 150 DAP.

3.2.4. *Cotton field trial*

During this trial plants treated with Restore were taller than the untreated control ($P \leq 0.011$), and had heavier shoot and root weight compared the two controls 30 DAP ($P \leq 0.01$). Furthermore, stem diameter was higher than the untreated control ($P \leq 0.001$), and similar to aldicarb ($P \leq 0.13$) 30, 60 and 90 DAP (Table 9; Figure 7). Reduction of females per gram of root using Restore was similar that the one provided by aldicarb when compared to the untreated control ($P \leq 0.014$) (Table 8). Number of eggs per gram of root and vermiforms were not different to the untreated control and aldicarb ($P \leq 0.99$) (Table 7). Free-living nematodes were increased during the trial when treated with Restore ($P \leq 0.001$) (Table 5). Harvest obtained was not different from the untreated control ($P \leq 0.461$) and aldicarb ($P \leq 0.433$) (Figure 8).

Table 7. Number of vermiforms per 150cc of soil under microplot, and field conditions 30, 60, 90, and 150 DAP ($P \leq 0.05$).

Microplot

Treatment	30 DAP			60 DAP			90 DAP			150 DAP		
	Vermiforms	Dunnett's *P* vs.		Vermiforms	Dunnett's *P* vs.		Vermiforms	Dunnett's *P* vs.		Vermiforms	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb
Control	121			596			1036			2616		
Aldicarb	60	0.001		197	0.001		502	0.021		1703	0.091	
Restore	92	0.989	0.169	226	0.003	0.989	456	0.007	1	1247	0.001	0.914

Field

Treatment	30 DAP			60 DAP			90 DAP			150 DAP		
	Vermiforms	Dunnett's *P* vs.		Vermiforms	Dunnett's *P* vs.		Vermiforms	Dunnett's *P* vs.		Vermiforms	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb
Control	1458			585			2651			2956		
Aldicarb	1101	0.81		288	0.023		2065	0.497		2097	0.007	
Restore	1341	0.994	0.977	464	0.927	0.179	2443	0.948	0.965	2116	0.007	0.998

Table 8. Number of females per gram of cotton root under microplot and field conditions 30 DAP ($P \leq 0.05$).

Treatment	Microplot			Field		
	Females	Dunnett's *P* vs.		Females	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb
Control	25			80		
Aldicarb	13	0.032		58	0.693	
Restore	15	0.038	0.891	40	0.014	0.249

Table 9. Effect of Restore on cotton plant variables under field conditions 30 DAP, and stem diameter 30, 60, and 90 DAP ($P \leq 0.05$).

Plant Variables									
Treatment	Height (cm)	Dunnett's *P* vs.		ShDW [1] (g)	Dunnett's *P* vs.		RFW[2] (g)	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb
Control	14.9			2.78			2.16		
Aldicarb	16.7	0.599		2.75	1		2.06	0.998	
Restore	17.8	0.011	0.817	3.9	0.006	0.006	2.92	0.01	0.019

Stem Diameter									
Treatment	30 DAP			60 DAP			90 DAP		
	SD[3] (mm)	Dunnett's *P* vs.		SD[3] (mm)	Dunnett's *P* vs.		SD[3] (mm)	Dunnett's *P* vs.	
		Control	Aldicarb		Control	Aldicarb		Control	Aldicarb
Control	3.7			7.9			10.6		
Aldicarb	4.2	0.184		9.1	0.249		12.5	0.064	
Restore	4.7	0.001	0.13	9.8	0.001	0.812	12.7	0.043	1

[1] Shoot dry weight [2] Root fresh weight [3]Stem diameter

Figure 7. Field trial 90 DAP. Untreated control: two rows on the left; Restore treatment: two rows on the right.

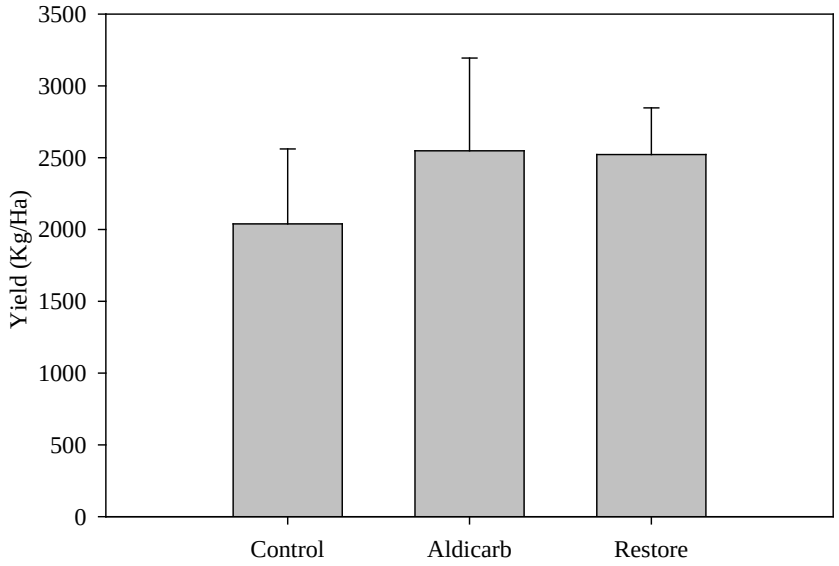

Figure 8. Cotton harvest obtained under field conditions 150 DAP.

IV. Discussion

Results obtained with the application of Restore on cucumber plants show an increase in seedling fresh weight, root weight and architecture (length, surface area, and number of tips), and a consistent reduction of disease incidence of the soil-borne pathogens *Pythium ultimum*, *P. aphanirdermatum*, and *Rhizoctonia solani* under greenhouse conditions. Furthermore, there was a similar trend in cotton plants, were height, and shoot and root weight, and root architecture were boosted, with lower number of females in the root and vermiforms in the soil.

Under microplot conditions there was also an increase in plant variables, including stem diameter and harvest at the end of the season. Reduction in vermiform and female stage was consistent with greenhouse results obtained. However, under field conditions even if there was in increase in plant variables and a reduction in females after a month of planting, the reduction of vermiform stage through the season and final harvest was not statistically significant compared to the untreated control. It is important to evaluate a second and third field growing season to test if there is any accumulative effect that shows statistically differences on vermiform stage and harvest obtained. Contrary to aldicarb, this PGPR product under thixotropic formulation has a beneficial effect in the soil that is evidenced in the increase on free-living nematodes in soil under all conditions tested. This type of nematodes plays an important role in soil as key actors in the food web and nutrient cycling.

Plant growth promotion and biological control with Restore (the thixotropic formulation of PGPR from Pathway Holdings) and earlier product formulations of the same core bacterial consortium (Equity, Naturize Potted Plant Food, and Plant Growth Activators (PGA)) are in agreement with results from several published studies with two of the earlier products. For

example, two publications have reported effects of Equity. In the first study, Equity was reported to increase plant growth and to reduce salt uptake of squash plants growing under conditions of salinity stress (Yildirim et al., 2006). In another study with root-knot nematode (*Meloidogyne incognita*) on tomato, treatment with Equity induced significant reductions in nematode eggs per gram root, vermiforms in soil, and root galls (Burkett-Cadena et al. 2008).

Three publications have reported benefits of PGA. Kalogridis *et al*. (2006) presented results of two field trials in Florida using PGA against Fusarium wilt disease, caused by *Fusarium oxysporum*, on the ornamental plant Lisianthus (*Eustoma grandiflorum*) demonstrating that the PGPR consortium provided protection against the pathogen and that this protection with the consortium "was superior to products with limited or single microbial species." The same publication also presented results of a field trial showing that PGA reduced disease incidence on two varieties of basil (*Ocimum basilicum*) transplanted into a field infested with the Fusarium wilt pathogen, *F. oxysporum.* As summarized by the authors, basil treated with the bacterial consortium "showed a drastic decrease in the incidence of Fusarium wilt with both susceptible and resistant varieties benefiting from applications." In the second report with PGA (Adesemoye et al. 2008), PGA alone and with mycorrhizae increased yield and nutrient uptake in a two-year field study on corn under conventional tillage and no-till with inorganic fertilizer and poultry litter. The third report (Adesemoye et al. 2009) was a greenhouse study with tomato under different fertilization levels. Inoculation with PGA resulted in levels of plant growth, yield and nitrogen uptake per gram of tomato shoot at 70% and 80% of recommended fertilizer that were statistically equivalent to the values for non-inoculated plants with 100% fertilizer.

The thixotropic formulation, as embodied in the product Restore, represents a promising advancement in the development of PGPR as microbial inoculants by allowing formulations of

mixed communities together with microbial food bases that can aid bacterial population development. Considering that a typical drip, furrow, or overhead irrigation system can deliver 100 ml per plant, the finding in Restore test 1 that the populations of PGPR delivered were log 6.06 per ml with the process column and 9.82 per ml with the tank mix suggests that Restore can be applied at populations of log 7 – 11 per plant with each irrigation. In some agricultural settings, such as vegetable production in Florida, plants are irrigated 4 – 6 times per week. Adding thixotropic formulations such as Restore into each irrigation has the potential to dramatically modify the rhizosphere microflora and to maintain the introduced PGPR in a metabolically active stage during the entire growing season. In addition, because the components of the thixotropic formulation can be changed relatively easily and inexpensively, one can envision a future with customized PGPR consortia being applied periodically during a season to address a particular site-specific need such as biocontrol of a disease outbreak or alleviation of an abiotic stress.

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