# Biological Control Studies on the Reniform nematode (*Rotylenchulus reniformis*) on Cotton in Alabama.

by

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#### Abstract

Cotton production in the Southeastern United States is limited by the reniform nematode (Rotylenchulus reniformis) causing an estimated loss of \$98,000.000 per year. Currently, management is based on crop rotation and use of nematicides because there are no resistant cotton cultivars to R. reniformis. Therefore, biological control of R. reniformis is an option that needs to be explored. The objective of this work was to isolate, identify, and evaluate fungi as biological control agent associated with R. reniformis in cotton plants. Soil samples were collected in cotton fields naturally infested with R. reniformis and from cotton stock plants cultured in the greenhouse. Nematodes were extracted from the soil by gravity screening and centrifugation-flotation method. Nematode samples were observed under the stereoscope and vermiforms colonized with mycelia and discolored eggs were tacitly collected, placed in a syracuse dish, and rinsed with sterile water. These nematodes were cultured on 1.5% water agar supplemented with antibiotics, and incubated at 27°C. Fungal growth from the nematodes was transferred to Potato Dextrose Agar (PDASA) plates supplemented with streptomycin sulfate to establish pure cultures. Identification of the nematophagous fungi was based on morphological characters and the ITS regions and 5.8S rDNA amplified by PCR using the primers ITS1 and ITS4. A total of 16 fungi were identified from R. reniformis with four of the fungal isolates previously reported as nematophagous fungi: three isolates of Drechslerella dactyloides and one isolate each of Drechslerella brochopaga, Paecilomyces lilacinus, and Fusarium oxysporum. In vitro pathogenicity tests for Drechslerella dactyloides and Dr. brochopaga were conducted and

revealed the start of conidial germination after 14 hours. Ring formation began after 16 hours, and its completed between 36 and 42 hours. Nematodes were attracted to the ring after 60 hours, and 12 hours later the nematodes were trapped. The pathogenicity of these fungi was tested in the greenhouse in a factorial arrangement of a RCBD with two soil types (autoclaved and natural) and three fungal carriers (wheat, oat, and corn meal) replicated 6 times. Cotton plants ST5599 BGRR were cultivated in 500 cm<sup>3</sup> pots and 1% (v/v) of fungal inoculum was added per pot with 3000 R. reniformis nematodes at planting. After 60 days plants were harvested, and plant height, shoot, and root mass, number of R. reniformis in soil, and eggs in root were recorded. In autoclaved soil, Dr. dactyloides, Dr. brochopaga, and P. lilacinus reduced ( $P \le 0.05$ ) the number of vermiform R. reniformis nematodes in soil. Paecilomyces lilacinus and F. oxysporum reduced (P < 0.05) the number of juveniles and eggs in the roots. In natural soil, A. dactyloides and F. oxysporum reduced R. reniformis numbers in soil, but none of the fungal isolates affected R. reniformis numbers of eggs in roots. The fungal isolates did not exhibited phytoxicity and did not reduce plant shoot or root mass or reduce plant height. Interestingly, oat when used as a fungal carrier, increased plant root mass. All six fungal strains of Dr. dactyloides, Dr. brochopaga, P. lilacinus, and F.oxysporum reduced R. reniformis numbers in autoclaved soil. The reduction in numbers of nematodes was consistently observed in autoclaved soil.

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#### I. LITERATURE REVIEW

Cotton (*Gossypium hirsutum* L.) is susceptible to multiple species of plant parasitic nematodes. Currently, the most economically damaging plant parasitic nematodes affecting cotton in the United States are the root-knot nematode (*Meloidogyne incognita*), the reniform nematode (*Rotylenchulus reniformis*), the Columbia lance nematode (*Hoplolaimus columbus*), and the sting nematode (*Belonolaimus longicaudatus*) (Robinson, 2007; Overstreet and McGawley, 2001). *Meloidogyne incognita* and *R. reniformis* are the widest spread parasitic nematodes in cotton crops in the U.S. (Robinson, 2008). In the past, *M. incognita* was considered the most economically damaging plant parasitic nematode in the cotton production region of the United States which covers the entire southern region of the country from the east to west coast. Recent data indicate *R. reniformis* nematode populations have increased in zones where the predominant nematode was *M. incognita* and as a result have displaced it (Robinson, 2007). *Rotylenchulus reniformis* causes cotton yield losses in the United States, especially in the states of Mississippi, Alabama, and Louisiana, where 9% (142,000 bales), 8.5% (41,463 bales), and 4% (32,558 bales) of the total cotton production is lost (Blasingame *et al.*, 2008).

Rotylenchulus reniformis was first reported on pineapple in Hawaii. It was identified by Lindford and Oliveira in 1940. The initial report of *R. reniformis* in cotton in the continental United States was in Georgia in 1940 (Smith, 1941). Later it was found in the states of Louisiana and Florida in 1942 (Steiner, 1942), Alabama and Texas in 1959 (Motsinger *et al.*, 1976), and Mississippi in 1968 (Heald and Robinson, 1990). It has spread as far north as Tennessee (Robinson, 2007; Heald and Robinson, 1990). This nematode has not moved through the western region of the U.S. and has not been reported in New Mexico, Arizona, or California. Currently, the economic loss from *R. reniformis* in cotton in the southeastern United States is estimated to be \$98 million per year (Blasingame *et al.*, 2008). Cotton pest surveys have indicated that the acreage infested with *R. reniformis* has continually increased in the southeastern and midsouth regions of the U.S. The increase in estimated cotton losses due to *R. reniformis* over time is attributed to the lack of resistant varieties (Weaver *et al.*, 2007, Usery *et al.*, 2005), limited use of crop rotation, ability of *R. reniformis* to colonize a wide range of soils types at depths over 122 cm (Moore *et al.*, 2008; Robinson *et al.*, 2005; Koenning *et al.*, 1996), and the cost and efficacy of nematicides (Starr *et al.*, 2007; Robinson, 2007; Lawrence *et al.*, 2005a; McLean and Lawrence, 2003).

*Rotylenchulus reniformis* has some biological advantages that make it successful in attacking cotton plants and difficult to control. The nematode can survive severe soil desiccation in the vermiform stage (Robinson *et al.*, 2005). Moore *et al.*, (2008) found *R. reniformis* in extreme drought soil conditions in non-irrigated plots in a depth up to 90 cm. Also, *R. reniformis* has been recovered from soil stored at 4°C for 1,080 days (Lawrence *et al.*, 2005b). In cotton plants, the life cycle of R. *reniformis* is shorter than other nematodes that attack cotton (Diez *et al.*, 2003; Robinson *et al.*, 1997), requiring 17-23 days at 27°C to 32°C from egg to egg (Sivakumar and Seshadri, 1971; Birchfield, 1962), compared to *Meloidogyne incognita* that requires between 21 - 28 days at 28°C (Veech and Starr, 1986). Also, the retention of the cuticles during the three juvenile molts provides protection from soil antagonists (Gaur and Perry, 1991, Robinson *et al.*, 1997). *Rotylenchulus reniformis* establishes its feeding sites in all root tissues and colonized the pericycle. This is an advantage over *M. incognita* which enters the root only in the apical meristem region of the developing roots. *Rotylenchulus reniformis* also has a high

reproduction potential. A single female produces 20 to 300 eggs per egg matrix and can build up high population densities in different types of soil (Davis *et al.*, 2003, Koenning *et al.*, 2007).

#### Rotylenchulus reniformis life cycle.

It is thought that the most common reproduction of R. reniformis populations is amphimictic where there are numerous males (40-60% males) (Gaur and Perry, 1991, Nakasono, 1977). However, there are some populations with parthenogenic reproduction where there are few or no males (Nakasono, 2004). 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 2/3 of the female body remains outside the root (Robinson *et al.*, 1997). 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 (Gaur and Perry, 1991). The cytoplasm is dense and contains various nuclei, plastids, and mitochondria (Agudelo et al., 2005). Six to 15 days after the initial infection, the female matures and develops the characteristic kidney shaped stage. 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 (Nakasono 1977; Starr et al., 2007). Mature females start laying eggs in a gelatinous matrix composed of glycoproteins, which are secreted by six specialized cells around the vagina and protect the eggs from desiccation when they are exposed completely outside the roots (Agudelo *et al.*, 2004).

Depending on the host and environmental conditions, between 20 and 300 eggs are laid 7-20 days after invasion of the plant roots (Robinson *et al.*, 1997; Gaur and Perry, 1991; Sivakumar and Seshadri, 1971). The eggs go through embryonic stages, where they divide continuously until they reach a multi-cell stage that gives rise to a tadpole stage (Sivakumar and Seshadri, 1971). Once hatching occurs, R. reniformis goes through three different juvenile stages Nakasono (2004) describes the twenty-one (vermiform stages) in soil without feeding. developmental processes from the J<sub>2</sub> to pre-adult stage in great detail. Basically the J<sub>1</sub> is formed within the egg in 3 days, and a first moult occurs in about 24 hours into the J<sub>2</sub> stage. Then, after 1-3 days of J<sub>2</sub> formation, hatching starts (Sivakumar and Seshadri, 1971). At this stage, the stylet of the nematode shows movement, and 6-7 days after the egg-laying a second molt occurs. The J<sub>2</sub> cuticule remains enclosing the J<sub>3</sub>, that remains vermiform, but sexes start to differentiate (Gaur and Perry, 1991). After two to three days, the J<sub>4</sub> stage develops, and the nematode has a new stylet and a well-developed cephalic region (Nakasono, 2004; Gaur and Perry, 1991). Females develop ovaries and a vulva, and males have spicula and testis. 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 develop a kidney-shape. The complete life cycle of the reniform nematode in a cotton root requires 17 - 22 days at 27 - 32°C (Robinson et al., 1997; Sivakumar and Seshadri, 1971).

#### Current management of R. reniformis.

Management of this nematode in cotton production in the United States is based on the use of nematicides, such as aldicarb, oxamyl, thiodicarb, metam sodium, 1,3 dichloropropene, and abamectin. These nematicides are generally recommended for *R. reniformis* management in cotton production with variations in the beltwide cotton production area of the United States. Aldicarb, oxamyl and thiodicarb are all in the carbamate chemical class, suggesting that they have similar modes of action (interfering with the functioning of the enzyme acetylcholinesterase) and with a high mammalian toxicity (Cox, 1992a). Metham sodium and 1,

3 dichloropropene are soil fumigants that binds to oxygen carrying molecules preventing tissues from using oxygen, and are also highly poisonous to human by inhalation, oral and dermal exposures (Cox, 1992b). Abamectin is a fermentation product of the bacteria *Streptomyces avermictilus*, and mode of action consists on binding to glutamate-gated chloride channels expressed on the nematode neurons and muscle cells (Wolstenholme and Rogers, 2005). 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 usually reduced the first 30 to 60 days after nematicide application, but numbers increase during the season reaching populations similar or higher than the untreated plots at harvest time (Robinson, 2007; Kinloch and Rich, 2001).

Aldicarb has been recommended for nematode management in cotton crop production since the early 1970's, and it is applied as a granule at 0.8-1.2 kg/ha in furrow (Koenning *et al.*, 2004; Koenning *et al.*, 2007). Unfortunately, biodegradation is now occurring causing a reduction in efficacy of aldicarb (McLean and Lawrence, 2003). The degradation of aldicarb is accelerated by soil microflora, and increasing the application rate does not maintain the efficacy of aldicarb (McLean and Lawrence, 2003). Studies based on HPLC techniques reveal that the presence of aldicarb or its metabolites was reduced from 42 days to less than 6 days, thereby severely reducing the chemical effect on nematodes (Lawrence *et al.*, 2005). The nematicides such as 1,3-dichloropropene and metam sodium are applied in the top 25 to 45 cm of soil and provide good control in that area, but *R. reniformis* can be found up to 90 cm deep in the soil profile (Moore *et al.*, 2008; Robinson *et al.*, 2005), The *R. reniformis* nematode populations found at the deep in the soil have been show to increase to threshold levels during the following season in cotton. Koenning *et al.*, (2007) found that the fumigant nematicides 1,3-

dichloropropene and metam sodium can enhance cotton yield in the presence of *R. reniformis*. Other studies where the nematicides: 1,3-dichloropropene, fenamiphos, phorate, terbufos, and aldicarb where evaluated, show that the application of 1,3-dichloropropene with aldicarb reduce *R. reniformis* nematode numbers and increased cotton height and yield under field conditions (Lawrence *et al.*, 1990).

Recent studies on seed treatments with abamectin reported suppression on penetration and infection by *Meloidogyne incognita* and *R. reniformis* on cotton taproots at a length of 5 cm *in vitro*. This suppression decreased when the taproot length increased (Faske and Starr, 2007). Field tests done by Lawrence and Lawrence, (2007) show that seed treatments with Abamectin (Avicta®) and Thiodicarb (Aeris®) have shown increase in cotton yields by an average and 7.6%, and 5.9% respectively, compared to the non-treated control. These seed treatments are offering a new management option.

A common cultural practice to lower *R. reniformis* reniform nematode populations is crop rotation. In the beltwide region of the United States, crop rotation with corn, peanut, rice, sorghum, and resistant cultivars of soybean can effectively reduce soil populations of *R. reniformis* nematodes (Gazaway et al., 2000, 2007). Corn is a non-host of *R. reniformis* and does not support reproduction of this nematode. An increase in cotton yield and a decrease in *R. reniformis* populations are often observed with a rotation including corn and resistant soybean, compared to continual cotton crops. A one-year rotation to corn and resistant soybean increases cotton yield and reduces *R. reniformis* numbers, but nematode populations rebuild within one growing season when cotton is produced (Davis et al., 2003). Thus the benefit of reduced *R. reniformis* numbers was demonstrated to last for one subsequent cotton crop (Davis et al., 2003, Gazaway et al., 2007). In the case of corn rotation, economic estimates are required to compare the income corn yield and price plus the increase in yield of the cotton following the rotation crop, with not growing cotton during a year of rotation.

A commonly used technique to deter pests in crops is to develop a resistant variety to the pest. For cotton, cultivars resistant to *R. reniformis* are not available (Robinson, 2008; Starr *et al.*, 2007; Davis *et al.*, 2003). Under greenhouse conditions, Weaver *et al.*, (2007), evaluated 1973 genotypes or accessions of cotton based on the *R. reniform* egg production per gram of root and vermiform numbers per plant and none of the genotypes showed high levels of resistance to *R. reniformis*. Usery *et al.*, (2005), evaluated 53 cultivars of transgenic and conventional cotton based on the ratio of the final to the initial nematode population, known as the reproductive factor (Rf). An Rf value of less than 1 indicated the nematode population density decreased and the host is considered resistant. The Rf values of all the cultivars in the study ranged between 4.1 and 59, indicating that none of the cultivars common to southern cotton production were resistant to the reniform nematode. Two cotton breeding lines resistant to *R. reniformis*, LONREN-1 and LONREN-2 where released by the USDA in 2007. These lines are reported to suppress *R. reniformis* numbers by 85% to 98% in different field trials (Starr *et al.*, 2007).

#### Biological control as an alternative management option.

Considering the lack of resistant varieties and cost of chemical nematicides, biocontrol or *R. reniformis* should be assessed. There is a misconception that nematodes are almost impossible to control biologically. Although, soil interactions are very complex, making nematode management difficult, nematode are sensitive to a general or specialized antagonists (Sikora, 1992). Biological control of *R. reniformis* nematodes has not yet been implemented in cotton crops in the United States, but it is a possible option for management in the future (Robinson, 2007). Previous studies have reported fungi and bacteria as pathogenic to different stages of the

*R. reniformis* life cycle. For the egg stage, the fungi *Pochonia chlamydosporia, Paecilomyces lilacinus*, and an unidentified fungus named Arkansas Fungus (ARF) have been documented as parasites of the *R. reniformis* nematode (Wang et al., 2004).

Wang et al. (2005) identified 117 isolates of *Pochonia chlamydospora* obtained from eggs of *R. reniformis* nematodes. Only 12 of the isolates parasitized eggs, colonizing up to 35% of the eggs under *in vitro* conditions and 77% under greenhouse conditions. Three of 12 isolates reduced the numbers of *R. reniformis* nematodes by 35% on cotton roots and in soils at a concentration of 5,000 chlamydospores per gram of soil. Walters and Barker (1994) found *Paecilomyces lilacinus* reduced *R. reniformis* nematodes from 36 to 59% under greenhouse and microplot trials on tomato. The fungal isolate used for this experiment was not isolated from the *R. reniformis* nematode. Instead, it was obtained from *Meloidogyne* spp. in the International Potato Center in Lima, Peru, where the isolate had been grown on rice grains and was added with the rice grains to the tomato plots.

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*. The strains of ARF are closely related as determined by mitochondrial DNA Restriction Fragment Length Polymorphism (mtDNA RFLP) patterns, but the morphology of the colony growth differs. ARF strains isolated from *R. reniformis* nematodes are also pathogenic to the cyst nematode. Greenhouse experiments reveal that ARF parasitized eggs, sedentary females and juveniles. The parasitism of this fungus ranges from 48% to 79% *in vitro*, and reduces *R. reniformis* population from 87 to 98% under greenhouse conditions (Wang *et al.*, 2004).

For the vermiform stages of *R. reniformis*, the fungi *Arthrographis* sp., *Pseudorobillarda* sp., and *Fusarium equiseti* have been isolated from *R. reniformis* vermiform life stages, and all

were found to significantly reduce the nematode population on cotton in the greenhouse (McLean *et al.*, 2000). Strains from the bacterium *Pseudomonas fluorescens* were isolated from the rhizosphere of cotton plants from different locations in India (Jayakumar *et al.*, 2003). Isolates reduced the soil and root *R. reniformis* populations as much as 70.4 and 44.8%, respectively. Furthermore, 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.

Robinson *et al.* (2008) report suppressive soils from five fields in the Lower Rio Grande Valley of Texas and five fields in Louisiana. The greatest suppression of *R. reniformis* populations was from the Lower Rio Grande Valley by up to 95%, however, the organisms involved in this suppression were not identified. Recently, isolates of *Pasteuria* spp. were isolated from *R. reniformis* from cotton fields in Alabama, Florida, and Mississippi (Hewlett *et al.*, 2009).

*Rotylenchulus reniformis* is a semi-endoparasite where the posterior portion of the females body remains outside the root exposed to the soil microflora. This exposure of the nematode and eggs makes it sensitive to wide or specialized antagonist microorganisms in the soil. Thus, biological control is an option that has to be explored in more detail in further years and can be integrated with current management to improve yields and reduce impact of this nematode in the cotton crop.

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# II. ISOLATION, AND MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF FUNGI ASSOCIATED WITH *ROTYLENCHULUS RENIFORMIS*

#### ABSTRACT

The objective of this work was to isolate, and identify, fungi associated with R. reniformis in cotton roots. Soil samples were collected in cotton fields naturally infested with R. reniformis and from cotton stock plants cultured in the greenhouse. Nematodes extracted from the soil were observed under the stereoscope. Vermiforms colonized with mycelia and discolored eggs were tacitly collected and cultured on 1.5% water agar supplemented with antibiotics, and incubated at 27°C. Identification of the nematophagous fungi was based on the morphological characters, and the ITS regions and 5.8S rDNA amplified by PCR using the primers ITS1 and ITS4. The parasitism percentage on vermiform nematodes from greenhouse samples was 21.2%, and the percentages from Limestone, Henry, and Baldwin counties were 3%, 23.2%, and 5.6%, respectively. A total of 12 fungi were identified from *R*. reniformis. The most frequently isolated were Arthrobotrys dactyloides (49%) and Paecilomyces lilacinus (15%). Penicillium waksmanii and Phoma exigua were isolated from 3.8% of the nematodes, followed by Aspergillus glaucus group (2.5%), and Cladosporium cladiosporoides, Cladosporium herbarum, Fusarium oxysporum, Torula herbarum, Dactylaria brachophaga, Aspergillus fumigatus, and unidentified basidiomycete were less frequent (1.25%). A high percentage (17.5%) of colonized nematodes did not produce a fungal culture on the media. Out of those 12 fungi, only four have been previously reported as nematophagous fungi: three isolates of Arthrobotrys dactyloides, and one isolate of *Dactylaria brochopaga*, *Paecilomyces lilacinus*, and *Fusarium oxysporum*. Molecular identification of *Arthrobotrys dactyloides* and *Dactylaria brochopaga* confirm that these two fungi belong to the new genus *Drechslerella* proposed by Scholler et al., 1998.

#### **INTRODUCTION**

*Rotylenchulus reniformis* is a serious problem in cotton production in the southeastern United States, causing an economic loss of \$98 million per year (Blasingame *et al.*, 2007). There are no commercial cotton varieties resistant to *R. reniformis* (Weaver *et al.*, 2007; Starr *et al.*, 2007; Usery *et al.*, 2005), consequently management is based on the application of nematicides, such as aldicarb, oxamyl, 1,3-dichloropropene, thiodicarb, and metam sodium (Koenning *et al.*, 2007; Lawrence *et al.*, 2005; Kinloch and Rich, 2001; Lawrence *et al.*, 1990), and crop rotation with corn, peanut, sorghum, and resistant cultivars of soybean (Davis *et al.*, 2003; Gazaway *et al.*, 2000). The economic cost of managing this nematode pest indicates the need to explore new management options that can be integrated in sustainable systems. Biological control options for *R. reniformis* nematode management are not available in the United States (Robinson, 2007).

Numerous studies of fungi colonizing plant parasitic nematodes have been reported on soybean cyst (*Heterodera glycines*) and root-knot nematodes (*Meloidogyne* spp.) (Kim and Riggs, 1991; Carries and Glawe, 1989; Jatala, 1986). In the case of *R. reniformis*, suppressive soils from five fields in the Lower Rio Grande Valley (LRGV) of Texas and five fields in Louisiana have been reported. In the LRGV the suppression of *R. reniformis* populations was as great as 95%, but the organisms involved in this suppression were not identified (Robinson *et al.,* 2008). In other studies, nematophagous fungi have been isolated and evaluated on different life stages of *R. reniformis*. *Paecilomyces lilacinus* significantly reduced numbers of *R. reniformis* 

eggs under greenhouse conditions (Walters and Barker, 1994). *Pochonia chlamydospora* reduced numbers of *R. reniformis* on cotton roots or in soil after a single application of 5,000 chlamydospores per gram of soil (Wang *et al.*, 2005). Also, a fungus known as Arkansas Fungus (ARF) parasitized 17 -21% of eggs (Wang *et al.*, 2004). For the juvenile stages, *Arthrographis* sp., *Pseudorobillarda* sp., and *Fusarium equiseti* significantly reduced *R. reniformis* population development on cotton (McLean *et al.*, 2000).

Taxonomy of nematophagous fungi is being redefined with the development of new molecular techniques. In the past, identification was based solely on the morphology of the conidia and conidiophores, but Scholler *et al.* (1999) reclassified the *Orbiliaceae* genera based on the ITS1, ITS2, and 5.8S rDNA sequences, demonstrating that trapping devices are more informative than other morphological structures.

Based on the life cycle of *R. reniformis*, we hypothesize that the nematodes are constantly exposed to many fungal antagonists and other microorganisms in the rhizosphere. The objective of this study was to isolate and identify nematophagous fungi which parasitize *R. reniformis* vermiform life stages and eggs. Fungal isolates were identified by morphology and molecular characters by use of ITS regions and 5.8S rDNA.

#### **MATERIALS AND METHODS**

#### Collection of nematode samples.

Rotylenchulus reniformis nematodes were collected from the roots of cotton plants grown to maintain stock cultures of nematodes at the Plant Science Research Center of the Alabama Agricultural Experiment Station on the campus of Auburn University. We have observed that the continuous culture of cotton has increased populations of antagonistic fungi against this nematode in our greenhouse cultures (Lawrence, personal communication). These nematodes were cultured in 15-cm-diameter polystyrene pots, inoculated with 2,000 eggs/500 cm<sup>3</sup> of soil per pot. The cotton was grown in the greenhouse for 6 -18 months. *Rotylenchulus reniformis* nematode samples were also collected in naturally infested cotton fields across the state to determine what fungal parasites are naturally colonizing the nematodes. Nematodes from samples of four different cotton production counties of Alabama (Escambia, Henry, Limestone, and Baldwin) were collected. In the field sites, approximately 1,000 cm<sup>3</sup> of soil were collected to a depth of 15 cm in the root zone of the cotton plants during the growing season from June through August. Soils were placed in plastic bags, labeled, and stored in a cool ice chest for transport to the greenhouse. Vermiform adults and nematode juveniles were extracted from the soil by a modified gravity screening and centrifugation-flotation method. Eggs were extracted from cotton roots by shaking the roots in 0.6% NaOCI and sieving the solution.

Nematode samples were observed at 6.3X magnification under the stereoscope (Nikon SMZ800). Vermiform life stages of *R. reniformis* nematodes colonized with fungal mycelia and discolored eggs were carefully removed from the counting dish, placed in a syracuse dish, and rinsed with sterile water. Colonized nematodes were aseptically cultured on 1.5% water agar supplemented with 12.5 mg of chlortetracycline HCl and 300 mg of streptomycin sulfate per liter (Kim and Riggs, 1994). Cultures were incubated at 27°C and identified after 5 days. Hyphal tips from the fungal growth of the eggs and vermiform life stages of the nematodes were aseptically transferred to a Potato Dextrose Agar (PDA/SA) (Sigma Chemical Co.) culture plates supplemented with 300 mg/liter of streptomycin sulfate (Sigma Chemical Co.) to establish pure cultures.

Identification of the nematophagous fungi was based on the morphological characteristics of conidiophores and conidia. Fusarium isolates were identified based on Nelson *et al.*, 1983, and Gerlach and Nirenberg, 1982, *Paecilomyces lilacinus* based on Domsch *et al.*, 1980, and *Arthrobotrys dactyloides* and *Dactylaria brochopaga* on Barron, 1977 and Drechsler, 1937. For some fungi it was necessary to induce sporulation on water agar media (WA) adding 20 *R. reniformis* vermiform stages to each culture. Sporulation was also induced in some cultures by exposing fungal mycelium to a black light lamp (Model X-15B 115 volts 60Hz).

#### Molecular identification of fungi.

The fungi isolated from *R. reniformis* and previously reported as nematophagous fungi were identified by nucleic acid extraction and PCR amplification. The nematophagous fungal isolates were grown on PDA/SA at 27°C for 7 days. Fungal mycelia were harvested, placed in a mortar, frozen with liquid nitrogen and transferred to an eppendorf tube without letting the sample thaw. DNA was extracted using the Dneasy Plant Mini Kit® (Quiagen Inc.). The internal transcribed spacer (ITS) regions 1 and 2, including the 5.8 rRNA, were amplified in 50 µL reactions on a Multigene Labnet thermocycler. The primers used were ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1998). For the PCR reaction the thermal cycles were initial denaturation: 3 min at 95°C, annealing: 35 cycles of 1 min at 95°C, extension: 40 s at 54°C, denaturation: 40 s at 72°C, and final extension: 10 min at 72°C. After PCR amplification, the products were purified with QIAquick columns (Quiagen Inc.) according to manufacturer's instructions. 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

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(Tamura *et al.*, 2007), and then were subjected to blast analysis in National Center for Biotechnology Information (NCBI). Phylogenetic bootstrap tests were performed using Neighbor joining algorithm in Mega 4.1 software (Tamura *et al.*, 2007).

#### RESULTS

#### Collection of nematode samples.

The parasitism percentage on vermiform nematodes from greenhouse samples was 21.2%, and the percentages from Limestone, Henry, and Baldwin counties were 3%, 23.2%, and 5.6%, respectively. In the greenhouse samples, frequency of fungi associated with visually colonized vermiform life stages were *Arthrobotrys dactyloides* (49%), *Paecilomyces lilacinus* (12.5%), *Penicillium waksmanii* (9.7%), *Aspergillus glaucus* Group (6.5%), and *Penicillium herbarum* (3.2%). No fungal growth was observed in 19.4% of the visually colonized, cultured, reniform nematodes. Fungal frequency for the egg stage was *Paecilomyces lilacinus* (50%), *Phoma exigua* (12.5%), *Cladosporium cladosporioides* (6.3%), *Fusarium oxysporum* (6.3%), *Torula herbarum* (6.3%), and an unidentified basidiomycete (6.3%). No fungal cultures were found in 12.5% of the discolored eggs (Figure 2.1).

From the Limestone field location, only *Phoma exigua* (25%) was associated with vermiform stages of *R. reniformis*; however, 75 % of the colonized vermiform nematodes did not produce any fungal growth. The Henry field was colonized with *Arthrobotrys dactyloides* (95.8%), and *Dactylaria brochophaga* (4.2%). In the Baldwin location, *Arthrobotrys dactyloides* (20%) was also isolated along with *Aspergillus fumigatus* (20%). As seen previously, 60% of the nematodes colonized did not grow on culture medium (Figure 2.2.).

From all the samples taken, the most frequently isolated fungi from *R. reniformis* were *A. dactyloides* (49%) and *P. lilacinus* (15%). *Penicillium waksmanii* and *P. exigua* were isolated from 3.8% of the nematodes, followed by *Aspergillus glaucus* group (2.5%), and *C. cladiosporoides*, *C. herbarum*, *F. oxysporum*, *T. herbarum*, *D. brachophaga*, *A. fumigatus*, and unidentified basidiomycete were less frequent (1.25%). A high percentage (17.5%) of colonized nematodes did not produce a fungal culture on the media (Figure 2.3.). Four out of the eleven species of fungi found colonizing *R. reniformis* have been previously reported attacking other species of nematodes. The fungi were *Arthrobotrys dactyloides*, *Dactylaria brochopaga*, *Paecilomyces lilacinus*, and *Fusarium oxysporum*.

#### Morphological characteristics

Arthrobotrys dactyloides (Fig. 2.4A) is a nematode-trapping fungus that produces rings as organs of nematodes capture. The rings were 20 - 32  $\mu$ m diameter and composed of 3 individual arcuate cells (Fig. 5A,B). Conidia are ellipsoidal in shape, 40 to 48  $\mu$ m long, 8 to 9  $\mu$ m wide, and divided in two equal cells by 1 septum. Chlamydospores have been reported on this fungus (Haard, 1968), but they were not observed in any of our strains. Culture surface and reverse were white on PDA. *Dactylaria brochophaga* (Fig. 2.4B) is also a nematode-trapping fungus producing rings of 20 to 35 $\mu$  in diameter, composed by three arcuate cells. Conidia are ellipsoidal yet slightly curved, 30 to 40  $\mu$ m long, 5 to 8  $\mu$ m wide, and divided in 4 cells by three septa. *Dactylaria brachophaga* shows a general parallelism with *Arthrobotrys dactyloides*. Culture surface and reverse were also white in appearance on PDA. However, the more abundant septation of its conidia is the feature most decisively distinguishing it as a species (Drechsler, 1937; Haard, 1968; Barron, 1977). *Paecilomyces lilacinus* (Fig. 2.4C) produced erect conidiophores 400-600  $\mu$ m with flask shaped phialides 10-12  $\mu$ m long. Phialides were swollen at the base tapering into a long and slender neck. Conidia were oval and smooth shaped 3.0 x 2.0  $\mu$ m. Hyphae were 2.0 – 3.0  $\mu$ m wide. The colony appearance on PDA is vinaceous in color with an uncolored reverse (Domsch *et al.*, 1980). *Fusarium oxysporum* (Fig. 2.4D) produces macro and micro conidia on branched or unbranched monophialides. The monophialides bearing the macroconidia were short, sub-cylindric, 8-14  $\mu$ m long and 2.5-3  $\mu$ m wide. Single-celled microconidia are oval shaped and abundant, 4.0 x 8.0  $\mu$ m. Macroconidia are sickle shaped, with an apical cell attenuated, basal cell is foot shaped, usually 3-septate, measuring 35 x 4.2  $\mu$ m. Chlamydospores were present, with measurements 10 x 13  $\mu$ m, from a hyphae 4.0-5.0  $\mu$ m wide. Coloration of the colony at the top is white with a medium to dark purple undersurface (Nelson *et al.*, 1983; Gerlach and Nirenberg, 1982).

#### Molecular identification of fungi.

The morphological identification of the fungi reported above as nematophagous fungi was confirmed by PCR amplification and blast analysis on GenBank. The three strains of *Arthrobotrys dactyloides* (GH-*A. dactyloides*, HN-*A. dactyloides*, BW-*A. dactyloides*) providing 100% coverage of the sequence indicated there was a 99% identity with *Arthrobotrys anchonia* and 97% with *Drechslerella dactyloides*. *Paecilomyces lilacinus* and *Fusarium oxysporum* isolates presented a 100% coverage of the sequences and a 100% identity when blast in the GenBank (Table 2.1)

Arthrobotrys dactyloides is closely related to Arthrobotrys anchonia, as both are ringtrapping fungi, but they differ in the shape and size of the conidia. Arthrobotrys anchonia produce elongated ovoid conidia with unequal cells within each of the conidia, while the *Arthrobotrys dactyloides* conidia are ellipsoid with equal cell lengths (Haard, 1968).

All of the isolates of *Arthrobotrys dactyloides* group were in a clade in the phylogram, and group close to *Arthrobotrys anchonia*, and *Drechslerella dactyloides*. *HN-Dactylaria brochopaga* grouped with *Drechslerella brochopaga*. The isolates of *P. lilacinus* grouped together in a monophyletic clade. Also, GH-*Fusarium oxysporum* grouped together with a biocontrol strain. *Rhizoctonia solani* was used in this graph as an outgroup (Figure 2.6.).

#### DISCUSSION

Continuous cultures of *R. reniformis* maintained in cotton plants in the greenhouse increased the population of antagonist organisms to this nematode. Parasitism on vermiform stage nematodes was higher on plants in the greenhouse than in the field. Wang *et al.* (2004), obtained fungal isolates from building up populations of antagonists under greenhouse conditions using cotton field soil. Of the isolated fungi, only *Arthrobotrys dactyloides*, *D. brachophaga*, *P. lilacinus*, and *F. oxysporum* have been reported as pathogens on other nematode species (Kiewnick and Sikora, 2006; Stirling and Smith, 1998; Freitas *et al.*, 1995). *Arthrobotrys dactyloides* and *D. brachophaga* have been reported to reduce populations of *Meloidogyne graminicola* on rice (Singh et al., 2007). Additionally, Stirling and Smith (1998a) reported reductions in populations of *Meloidogyne javanica* on tomato roots using isolates of *A. dactyloides* have been attempted (Stirling et al., 1998b) but need to be improved.

In the samples from Alabama, the most predominant nematode-trapping fungus was *A*. *dactyloides*, which was isolated from the greenhouse and two counties (Henry and Baldwin). *Dactylaria brachophaga* is closely related to *A*. *dactyloides* and was only identified in Henry County.

*Paecilomyces lilacinus* is typically a soilborne fungus and is common in the rhizosphere of a number of plants (Domsch et al., 1980). This fungus has been reported on *R. reniformis* on tomato plants, where it reduced the populations of the nematode in greenhouse and microplot trials (Walters and Barker, 1994). This fungus was also very predominant on the cotton plants used for culturing *R. reniformis* in the greenhouse.

*Fusarium oxysporum* has been previously reported to be destructive on eggs of the soybean cyst nematode in Alabama soybean fields (Morgan-Jones & Rodriguez-Kabana, 1981). There are some biotypes capable of penetrating eggs and causing disorders on the embryonic development through enzymatic and/or toxic effects (Morgan-Jones & Rodriguez- Kabana, 1988). This fungus has never been reported on *R. reniformis* so it is necessary to evaluate the biocontrol potential for this nematode.

Morphological and molecular fungal identification are in agreement for HN-*D*. *brochopaga*, GH-*P. lilacinus*, and GH-*Fusarium oxysporum*. Only the three strains of *Arthrobotrys dactyloides* showed differences between the two types of identification. Molecular identification showed 99% of identity with *A. anchonia* and 97% identity with *A. dactyloides*. While, referring back to morphological characters, we observed that the conidia from the three isolates are ellipsoid and have equal cell size. This allowed us to confirm that the fungi we isolated are *A. dactyloides*. For future studies it will be named *Drechslerella dactyloides*, based

on the classification proposed by Scholler et al. 1999, which also renames *A. brochopaga* as *Drechslerella brochopaga*.

Overall, our results suggest that *Drechslerella dactyloides*, *Dr. brochopaga*, *Fusarium oxysporum*, and *Paecilomyces lilacinus* are pathogens of *R. reniformis*. Hence, in the future evaluations of these fungi under *in vitro* and greenhouse conditions must be done to evaluate their biocontrol potential. Also, is important to develop and evaluate different media and techniques on isolation and identification of the fungi that did not growth on the artificial media. There is a possibility that this are endoparasitic fungi (complete the entire life cycle inside the host) or bacteria pathogenic to nematodes. Knowing most of the natural enemies of *R. reniformis* in different stages of the life cycle will give the base to develop a biocontrol program that could be added to an integrated pest management.

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Figure 2.1. The percent occurrence of fungi isolated from symptomatic Rotylenchulus reniformis

greenhouse samples.



**Figure 2.2.** Percent occurrence of fungi isolated from symptomatic *Rotylenchulus reniformis* vermiform life stages and eggs from the field locations.



**Figure 2.3.** Percent frequency of occurrence of the total fungi isolated from symptomatic *Rotylenchulus reniformis* from all samples.

**Table 2.1.** Relationship of fungal isolates colonizing *Rotylenchulus reniformis* with other fungi

 from the blast analysis in GenBank.

	FRAGMENT SIZE	HIGHEST MATCH IN THE
ISOLATE	(bp)	GENBANK
BW- Arthrobotrys dactyloides	552	99% Arthrobotrys anchonia
		91% Drechsterenti auctytotaes
GH- Arthrobotrys dactyloides	549	97% Drechslerella dactyloides
		99% Arthrobotrys anchonia
HN- Arthrobotrys dactyloides	547	97% Drechslerella dactyloides
HN- Dactylaria brochopaga	552	100% Drechslerella brochopaga
GH-Fusarium oxysporum	688	100% Fusarium oxysporum
GH-Paecilomyces lilacinus	543	100% Paecilomyces lilacinus
Paecilomyces lilacinus Strain251	543	100% Paecilomyces lilacinus



**Figure 2.4.** Conidia of *Arthrobotrys dactyloides* (A), *Dactylaria brachophaga* (B), *Paecilomyces lilacinus* (C), and *Fusarium oxysporum* (D). Magnification: 100X



**Figure 2.5.** Trapping rings produced by *Arthrobotrys dactyloides* and *Dactylaria brachophaga*. Open ring (A), Swollen ring (B) Magnification 100X



**Figure 2.6.** Phylogram from the fungi isolated from *Rotylenchulus reniformis* nematodes based on ITS and 5.8s rDNA (Neighbor-joining algorithm).

# III. EVALUATION OF *DRECHSLERELLA DACTYLOIDES*, *DRECHSLERELLA BROCHOPAGA*, *FUSARIUM OXYSPORUM*, AND *PAECILOMYCES LILACINUS* FOR BIOCONTROL OF *ROTYLENCHULUS RENIFORMIS* UNDER *IN VITRO*, GREENHOUSE, AND MICROPLOT CONDITIONS.

# ABSTRACT

The objective of this work was to evaluate the biological control potential of *Drechslerella dactyloides*, *Drechslerella brochopaga*, and *Paecilomyces lilacinus* under *in vitro*, greenhouse, and microplot conditions. *In vitro* pathogenicity tests with *Drechslerella dactyloides* and *Dr. brochopaga* revealed the start of conidial germination after 14 hours, and 72 hours later the nematodes were trapped. *Paecilomyces lilacinus* conidia start germination 12 hours after culturing. Nematode eggs were parasitized by 24 hours after the initial exposure. Under greenhouse conditions pathogenicity of these fungi was tested using two soil types and three fungal carriers applied at seeding. In autoclaved soil, *Dr. dactyloides*, *Dr. brochopaga*, and *P. lilacinus* reduced ( $P \le 0.05$ ) the number of vermiform *R. reniformis* nematodes in soil 60 days after planting. *Paecilomyces lilacinus* and *F. oxysporum* reduced ( $P \le 0.05$ ) number of juveniles and eggs in the roots. In natural soil, *Dr. dactyloides* and *F. oxysporum* reduced *R. reniformis* vermiforms but none of the fungal isolates affected the number of *R. reniformis* eggs in roots. The fungal isolates exhibited no phytoxicity and did not reduce plant shoot or root mass or reduce plant height. Microplot tests none of the isolates showed reduction of *R. reniformis* in

cotton field soil. Results obtained demonstrate that *Dr. dactyloides*, *Dr. brochopaga*, and *P. lilacinus* are parasitizing *R. reniformis* under *in vitro* and greenhouse conditions, using autoclaved soil. Under microplot conditions and using non-autoclaved soil in the greenhouse the fungi evaluated did not reduce number of *R. reniformis*. These suggest that even if the fungi are parasites of *R. reniformis* they require to have advantage to compete with other microorganisms in the soil when they are released.

#### INTRODUCTION

Rotylenchulus reniformis is a constant threat to cotton production in the United States, especially in Mississippi, Alabama, and Louisiana, where 9% (142,000 bales), 8.5% (41,463 bales), and 4% (32,558 bales) of the total cotton production are lost due to this nematode (Blasingame et al., 2008). Management options are limited to the use of crop rotation (Davis et al., 2003; Gazaway et al., 2000) and nematicides, such as aldicarb, metam sodium, oxamyl, 1,3 dichloropropene, avermectin, and thiodicarb (Koenning et al., 2007; Kinloch and Rich, 2001; Lawrence et al., 1990). Nematicide degradation such as aldicarb is accelerated by soil microflora, and even increasing the doses does not increase the efficacy of aldicarb (McLean and Lawrence, 2003). Also, seed treatments with abamectin and thiodicarb have shown increases in cotton yields (Lawrence and Lawrence, 2007). Although seed treatments with abamectin, suppressed penetration and infection by R. reniformis on cotton taproots at a length of 5 cm, but suppression decreased when the taproot length increased (Faske and Starr, 2007). Nematicides are proven to maintain yields reducing nematode damage in the roots (Koenning et al., 2007; Kinloch and Rich, 2001; Lawrence et al., 1990), but there is a concern of the toxicological and environmental effects of this practice (Koenning et al., 2004; Jatala, 1986). There is an economic

need to reduce the impact of *R. reniformis* on cotton crops and search for new control strategies such as biocontrol that can be incorporated in integrated pest management.

Biological control of nematodes in cotton is not practiced in the U.S. (Robinson, 2007). There is a misconception that nematodes are impossible to control using biocontrol (Sikora, 1992). Numerous fungal and bacterial antagonists have been reported on *R. reniformis*. The fungi *Pochonia chlamydosporia, Paecilomyces lilacinus*, and an unidentified fungus named Arkansas Fungus (ARF) have been reported to parasitize eggs of *R. reniformis* (Wang *et al.*, 2005; Wang *et al.*, 2004; Walters and Barker, 1994), and *Arthrographis* sp., *Pseudorobillarda* sp., and *Fusarium equiseti* have been reported to reduce vermiform stages (McLean *et al.*, 2000). Strains of bacterium *Pseudomonas fluorescens* were isolated from the rhizosphere of cotton plants in India and reduced the population of *R. reniformis* 70.4% in soil and 44.8 % in roots. Recently, isolates of *Pasteuria* spp. were isolated from *R. reniformis* from Alabama, Florida, and Mississippi cotton fields (Hewlett *et al.*, 2009). Soils from Lower Rio Grande Valley of Texas have been reported to be suppressive to *R. reniformis* by up to 95%, but the organisms involved in this suppression have not been identified (Robinson *et al.*, 2008).

In Alabama cotton crops, *Drechslerella dactyloides* (formerly *Arthrobotrys dactyloides*), *Drechslerella brochopaga* (formerly *Dactylaria brochopaga*), *Paecilomyces lilacinus*, and *Fusarium oxysporum* have been isolated from *R. reniformis* nematodes (Castillo *et al.*, 2008). *Drechslerella dactyloides* and *Dr. brochopaga* were previously reported reducing populations of *Meloidogyne graminicola* in rice (Singh *et al.*, 2007; Kumar and Singh, 2006), and were also found parasitizing juveniles of *R. reniformis* nematodes. These two fungi produce constricting rings that trap the nematode. *Paecilomyces lilacinus* reduces populations of eggs of *R. reniformis* in tomato plants by up to 36% at harvest season (Walters and Barker, 1994). *Paecilomyces*  *lilacinus* penetrates the egg shell by production of penetrating hyphae and appressoria (Lopez-Llorca *et al.*, 2008). Toxins produced by *Fusarium oxysporum* have been reported to destroy eggs of *Heterodera glycines*, and *Meloidogyne arenaria* (Morgan-Jones & Rodriguez-Kabana, 1985; Morgan-Jones & Rodriguez-Kabana, 1981).

Our hypothesis is that the fungal strains *Dr. dactyloides*, *Dr. brochopaga*, and *P. lilacinus* isolated from *R. reniformis* are pathogens that will reduce *R. reniformis* under *in vitro*, greenhouse, and microplot conditions. Our objectives are to evaluate *in vitro*, greenhouse, and microplot conditions the biocontrol potential of *Drechslerella dactyloides*, *Drechslerella brachopaga*, and *Paecilomyces lilacinus* against *R. reniformis*.

#### MATERIALS AND METHODS.

### 1. In vitro pathogenicity observations and Scanning Electron Microscopy (SEM).

Conidia were collected from seven-day old pure cultures of *Drechslerella dactyloides*, *Dr. brachopaga*, and *Paecilomyces lilacinus* on corn meal agar (CMA) (Sigma Chemical Co.). Plates were flooded with 10 ml of sterile water, and conidia were dislodged aseptically with a spatula and transferred to a sterile beaker. Conidial solutions were enumerated, quantified using a hemacytometer, and standardized to 6,250 conidia/ml each for *Dr. dactyloides*, *Dr. brochopaga*, and 7,500 conidia/ml of *P. lilacinus*.

The *R. reniformis* life stages were extracted from stock cotton plants in the Plant Science Research Center of the Alabama Agricultural Experiment Station on the campus of Auburn University. Vermiform stages were extracted from the soil by gravity screening followed by sucrose centrifugation-flotation, and eggs were washed from cotton root systems by shaking the roots in 6.0% NaOCl for 4 minutes at 120 rpm speed on the shaker (Hussey and Baker, 1973). Extracted vermiform life stages and eggs were disinfected by immersion in streptomycin sulfate (200 mg/L) (Sigma Chemical Co.) for 30 seconds followed by a second 30 second wash in vancomycin (10 mg/L) (Sigma Chemical Co.) and a final rinse in sterilized distilled water.

In the *in vitro* colonization test treatments were: 1) *Dr. dactyloides* + 20 *R. reniformis* vermiforms, 2) *Dr. brochopaga* + 20 *R. reniformis* vermiforms, 3) *P. lilacinus* + 20 eggs, 4) 20 vermiforms *R. reniformis*, and 5) 20 eggs of *R. reniformis*. Each treatment contains four replicates, and treatments 4 and 5 that contains eggs or vermiforms were used as controls to verify that there was no fungal or bacterial contamination in the cultures. Conidia were pipetted onto glass slides with 3.5 cm circular depression containing 1% CMA adjusted to 6.6 pH. To observe colonization of *Dr. dactyloides* and *Dr. brochopaga*, twenty juveniles of *R. reniformis* were added to the slides of *P. lilacinus*. All the cultures were incubated at 22°C without light. Observations of conidia germination parasitism of the nematode eggs and vermiform life stages by the fungi were recorded every 6 hours. A linear correlation of the percentage of nematodes infected vs. time were analyzed on SAS Software SAS 9.1.3 (SAS Institute Inc.) using the REG procedure for the linear regression of each of the fungi, and GLM procedure to compare the linear regressions of *Dr. dactyloides* and *Dr. brochopaga*.

The vermiform life stages of *R. reniformis* nematodes trapped by rings of *Dr. dactyloides* and *Dr. brachopaga*, eggs parasitized by conidia of *P. lilacinus*, and females naturally attacking the cotton root were observed using SEM. Vermiform life stages and eggs parasitized by fungi were removed from the culture, and females with the root fragment were placed on 12mm diameter aluminum stubs. Fixation of the tissues included vapor exposure to a 2% aqueous solution of osmium tetroxide (OsO4 2%) in the dark for 2 hours. Samples were air dried for 2

more hours, and afterward the stubs were transferred to the sputter coater (EMS 550x) for gold layering. SEM observations were made with a Carl Zeiss EVO 50 microscope.

# 1. Greenhouse trial.

The six fungal isolates were increased on three different fungal carriers (oat seed, wheat seed, or cornmeal) and placed in two types of soil (natural or autoclaved) in the presence or absence of the nematode. In addition one control contained the fungal carrier without any fungi and the second or absolute control added no fungal carrier at all. The experiment was designed as a factorial arrangement in a randomized complete block design (RCBD) with five replications. The entire experiment was repeated twice, for a total of 960 experimental units. Data were imported, linearized, and tabulated in SAS 9.1 (SAS Institute Inc.) using PROC GLIMMIX to check the residuals, and means were compared by a Dunnett test at the ( $P \le 0.05$ ) level of significance.

### Inoculum preparation.

Three strains of *Drechslerella dactyloides* (*BW-Dr. dactyloides*, *GH-Dr. dactyloides*, *HN-Dr. dactyloides*), *Drechslerella brochopaga*, *Paecilomyces lilacinus*, and *Fusarium oxysporum* were isolated from *R. reniformis* nematodes found across cotton crops in Alabama. The fungal isolates were cultured for 7 days on water agar (WA) and then transferred to a flask carrier culture. In a 250 ml conical flask, 150 cm<sup>3</sup> of the carrier (oat seed, wheat seed, or cornmeal) were added and moistened with 100 ml of tap water. Imbibed seed were autoclaved twice at 121°C and 103.4 kPa for 30 minutes on two consecutive days. Two 5 mm diameter fungal disks from the periphery of 7 day-old WA cultures were aseptically transferred to each flask. Fungal cultures were increased in a growth chamber at 27°C during 30 days and shaken daily to distribute the fungi evenly.

## Inoculation and extraction.

Three cotton ST 5599 BGRR plants were planted in 500 cm<sup>3</sup> pots containing a sandy loam soil (sand, silt, clay of 67.5-20-12.5; 1.4% OM), and 1% v/v of fungal inoculum was added to each pot. The fungal inoculum was mixed into the soil to evenly distribute the fungus. Additionally, 3000 *R. reniformis* vermiform life stages and eggs in 3 ml of water were added to each pot by pipeting at planting. After 60 days under greenhouse conditions, plants were harvested, and variables of plant height, fresh and dry shoot weight, and fresh and dry root weight were recorded. *Rotylenchulus reniformis* vermiform life stages were extracted from the soil and eggs were extracted from the cotton roots as previously described. Vermiform life stages and eggs were counted using an inverted TS100 Nikon microscope.

# 2. Microplot trial.

Treatments for the microplot trails included : 1) untreated natural soil control, 2) Aldicarb (Temik 15G applied at 5.7 Kg/Ha), 3) *Drechslerella dactyloides*, 4) *Drechslerella brochopaga* 5) *Paecilomyces lilacinus*, and 6) *Dr. dactyloides* plus *Dr. brochopaga* and *Paecilomyces lilacinus*. The experimental design was a RCBD with five repetitions and the entire experiment was repeated twice. Data analysis was the same as described above in the greenhouse trial.

# Inoculation and extraction.

Microplots consisted of 4,400 cm<sup>3</sup> black plastic pots placed 30 cm deep in 20 cm of pine bark shavings under a 40% shade cloth house. Microplots were filled with a Decatur silt loam soil (sand, silt, clay of 24-49-29; 1.0% OM, pH 5.6) from a cotton field collected on the Tennessee Valley Research and Extension Center near Belle Mina, AL. At planting, each microplot was inoculated with the appropriate 1.1% v/v of fungal inoculium which was mixed into the top 15.2 cm of soil in each microplot. Six cotton ST 5599 BGRR seed were planted in a single row in each pot. After which 10,000 juveniles and eggs of *R. reniformis* were added by pipetting 2 ml of inoculum solution into three depressions in the pot and covering the inoculum with soil to eliminate desiccation. The cotton in the moicroplots was allowed to grow for 120 days under natural conditions with supplemental watering as needed. Soil samples were taken at mid and late season approximately 60 and 120 days after planting, and nematodes and eggs were extracted as described above.

#### RESULTS

#### 1. In vitro observations and SEM.

In the presence of *R. reniformis*, *Drechslerella dactyloides* conidia began germinating 8-12 hours after culturing on the CMA slides at 27 °C (Figure 3.1A). Hyphal strands spread across the plate within 14 hours (Figure 3.1B), and nematode trapping rings were formed by 32 hours (Figure 3.1C, 3.1D). Trapping rings ensnared 5% of the nematodes at 72 hours (Figure 3.1E), 16% at 78 hours, 24% at 84 hours, 35% at 90 hours, and 59% after 96 hours. Nematode entrapment followed a linear pattern over time. The linear regression model obtained for *Dr. dactyloides* is the percent of nematodes captured equals -103.6 + 1.6 \* time (hours) with an  $r^2=0.84$ . Based on the regression, conidia are able to germinate, form rings, and start trapping nematodes within 64, and can kill the nematodes within 127 hours (Figure 3.2). *Drechslerella brochopaga* growth was similar to that of *Dr. dactyloides* but differences in timing were observed. The conidia started to germinate at 10 hours after culturing, which was two hours slower than *Dr. dactyloides*. Hyphae extended across all the plates at 14 hours, which was similar to the hyphal extension observed with *Dr. dactyloides*. The ring formation was initiated at 24 hours with complete ring formation by 32 hours after culturing. First nematodes were trapped after 66 hours, which was 6 hours

before any nematodes were trapped by *Dr. dactyloides*. At 72 hours 5% of the nematodes were trapped, at 78 hours 15%, at 84 hours 30%, at 90 hours 47%, and after 96 hours 69% (Figure 3.2). The linear regression model obtained for *Dr. brochopaga:* is the percent of nematodes captured = -125.5 + 1.9 \* time (hours) with an r<sup>2</sup>=0.81. *Drechelerella brochopaga* should require 118 hours to kill the nematodes (Figure 3.2). *Drechslerella dactyloides* and *Dr. brochopaga* have the same three celled ring mechanism of attacking *R. reniformis*, and the time they require from the conidia stage to trapped nematodes is not significantly different (*P*<0.0994).

*Paecilomyces lilacinus* is an egg pathogen and the infection process required less time than the nematode-trapping ring fungi. Germination of conidia was observed at 12 hours after culturing (Figure 3.3A). The first eggs were surrounded by hyphae at18 hours and parasitized by 24 hours after the initial exposure (Figure 3.3 A, B,C). *Paecilomyces lilacinus*-colonized eggs were observed with conidiophores with conidia rupturing through the egg shell after 72 hours (Figure 3.3D). This fungus began parasitizing eggs at 18 hours (2% of the total eggs) and increased mortality in a linear fashion killing 82% of the eggs present after 42 hours. Linear regression of percentage of eggs parasitized equals -28.1 + 2.4 \* time (hours) with an r<sup>2</sup>=0.89; then eggs could be parasitized after 53 hours (Figure 3.4).

SEM observations illustrate the differences between the conidia of *Dr. dactyloides* and *Dr. brochopaga* (Figure 3.5A, 3.5B). Nematode trapping rings and the assimilative hyphae were observed inside the body of the parasitized *R. reniformis* nematodes (Figure 3.5C, 3.6D). *Pecilomyces lilacinus* surrounded the nematode eggs with hyphae and lemon shaped conidia (Figure 3.6A, B). Other observations of *R. reniformis* females naturally parasitizing the cotton roots illustrate a protuberance blisters that erupt from the body in the dorsal and vulva region (Figure 3.7) compared to healthy females (Figure 3.8). These symptoms reveal the presence of a

new organism invading females because those symptoms do not coincide with any of the fungi evaluated.

# 2. Greenhouse trial.

Phytopathogenicity to the cotton plants was not observed for any of the fungal isolates. Plant shoot mass was not affected by the fungal isolates, soil types (autoclaved or natural), presence or absence of *R. reniformis*, or the fungal carrier. No interactions were observed between the fungal isolates, carrier, and presence or absence of the nematode (P<0.998), and no significant differences were found among the carriers within the soil treatments (P<0.074) (Table 3.1). However, in autoclaved soil, shoot mass was greater than in natural soil (P<0.001). In treatments with corn meal carrier, plant shoot mass was lower than plants with oat (P<0.015) and wheat carriers (P<0.039) (Figure 3.9). Also, in treatments without nematodes the shoot mass was higher (P<0.019).

Root mass was affected by soil types, fungal inoculums carrier, and the presence or absence of the nematode (P<0.006) (Table 3.2). Cotton root mass was 60% higher in natural soils than in autoclaved soils (P<0.001) (Table 3.10). Plants with the nematode present had 41% lower root mass than the plants without the nematode (P<0.001). Plants with cornmeal carrier in natural soil presented higher root mass than plants with oat (P<0.001) and wheat carrier (P<0.001). When the nematode was present, root mass of the plants exposed to the oat carrier was higher than the wheat and cornmeal carriers in the autoclaved or natural soils (P<0.001) (Figure 3.10). For plant height, there was no carrier effect with soil combinations (P<0.0126) (Table 3.3). Plants grown in natural soil were taller than the ones from autoclaved soil and also taller when nematode was not present (P<0.001) (Figure 3.11).

The total number of *R. reniformis* nematodes in the soil was lower in all six fungal isolate treatments compared to the two controls (P<0.001) in the autoclaved soil. In natural soil, the fungal isolates BW-*Dr. dactyloides* (P<0.003), and *F. oxysporum* (P<0.014) reduced 64% and 44% populations of *R. reniformis* compared to the two controls (Table 3.4). The total number of *R. reniformis* eggs extracted from the roots was 48% lower averaged over all six fungal isolates treatments compared to the no carrier control in the autoclaved soil. However, the isolates of *F. oxysporum* (P<0.003) and *P. lilacinus* (P<0.007) reduced *R. reniformis* populations 46% and 60% when compared to the two controls, respectively. There was no reduction of nematode population in natural soil (Table 3.5). The numbers of eggs and vermiform life stages of *R. reniformis* per gram of root was significantly reduced over all fungal isolates by 62% compared to the no carrier control (P<0.001), but no difference was observed compared to the carrier control (P<0.06), and no nematode reduction was observed in natural soil (Table 3.6).

#### 3. <u>Microplot trial.</u>

Phytopathogenicity to the cotton plants was not observed for any of the fungal treatments in the microplots. The total number of *R. reniformis* in the mid and final season samples was not reduced by any of the fungal isolates as compared to the untreated control. The combination treatment of the three nemaphagous fungi did not reduce *R. reniformis* at either nematode sampling. In comparison, the nematicide aldicarb did not reduce the number of nematodes as compared to the untreated control or the fungal isolate treatments. (P<0.1551) (Table 3.7).

### DISCUSSION

Drechslerella dactyloides, Dr. brochopaga, and Paecilomyces lilacinus provided control of *R. reniformis* in autoclaved soil under greenhouse conditions but not in natural soil in the greenhouse or microplot conditions. The lack of efficacy in natural soils suggests that when fungi are applied at seeding time, they need to establish in the soil and be able to compete with the soil microflora. Under in vitro conditions, Dr. dactyloides and Dr. brochopaga require at least 72 hours (3 days) to produce trapping rings and ensnare R. reniformis nematodes, and Paecilomyces lilacinus start parasitizing eggs after 24 hours. This coincides with reports by Kumar and Singh (2006), where Dr. dactyloides strains captured 95% of the population in the same amount of time. The differences in the percentage of capture found with the Kumar and Singh (2006) study is probably due to the released of the nematodes (*Meloidogyne incognita*) in 8-day-old fungal cultures of Dr. dactyloides while in our study the conidia and R. reniformis vermiforms were released at the same time. Trapping times of Dr. brochopaga coincides with findings of Sing et al. (2007) where the fungus trapped 60% of Meloidogyne graminicola in 96 hours (4 days). Also, ring trapping formation time is also similar to the studies mentioned above. The conidia of these two fungi have a lag phase to adapt to changing environments and begin forming trapping rings to ensnare R. reniformis vermiforms. The infection times of these fungi suggests that releasing these fungi before planting could improve the biocontrol in natural soils.

Only, the isolate BW-*Dr. dactyloides* showed the ability to control *R. reniformis* in sandy loam soils in the greenhouse trials, but not in the silt loam field soil used under microplot conditions. To have successful control with these fungi it may be is necessary: to improve the formulation of the fungal carrier to provide an advantage to the fungus allowing it to compete in the natural soil environment. Wheat, cornmeal, and oat seed are good food sources to many

microorganisms in the soil. Stirling *et al.* (1998) reduced more than 90% the number of *Meloidogyne javanica* and in 57-98% the galling in tomato plants releasing *Dr. dactyloides* in granules formulations with kaoline and vermiculite as carriers and arabic gum as a binder.

On the other hand, *P. lilacinus* has a fast growth rate, parasitizing eggs in 48 hours (2 days) under *in vitro* conditions. *Rotylenchulus reniformis* second stage juveniles develop and hatch in 6-7 days (Sivakumar and Seshadri, 1971), which make them sensitive to parasitism by *P. lilacinus* before colonizing the roots. Under greenhouse conditions *P. lilacinus* reduced the number of eggs of *R. reniformis* in autoclaved soil when the fungus was applied at the planting time. This coincides with Reddy and Khan (1988) where *R. reniformis* was controlled by this fungus reared in rice seeds after 60 days. Also, Cabanillas and Barker (1989) increased tomato yields and reduced 36% of tomato roots galling with the application of 20 g fungus-infested wheat 10 days after transplanting and at transplanting under microplot conditions. There was no *R. reniformis* reduction on eggs under natural soil and microplot conditions, which suggest that in future studies the dose of the fungus has to be increased, must be applied before planting, and placed close to the site where the seed is going to be, so, it can establish contact with the cotton roots. *Paecilomyces lilacinus* has been proven to grow around and in the epidermis of the roots (Cabanillas et al., 1988) and produce the antibiotics leucinostatin and lilacin (Samson, 1974).

*Fusarium oxysporum* reduced the number of eggs in autoclaved soils. Morgan-Jones and Rodriguez-Kabana (1988) reported that some biotypes of this fungus appear to be capable of penetrating eggs in cyst nematode, affecting the embryonic development through enzymatic and/or toxic effects. Studies on the effect of secondary metabolites produced by the *F*. *oxysporum* strain isolated from *R. reniformis* should be conducted. For future studies it may be necessary to use a carrier that give some advantage in competiveness with the natural soil microflora, increase the dose of these fungi, and evaluate the application of them before planting. Also, there is a need to continue isolating different antagonists of *R. reniformis*, because the SEM observations of the female naturally attacking roots shows symptoms different from the nematophagous fungi evaluated in this work. The eruptions on the cuticle of the nematode suggest that the organism associated can be cyst forming endoparasitic fungi as described by Barron, 1977, or endospores formed by *Pasteuria* spp.

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**Figure 3.1.** *Drechslerella dactyloides* and *Drechslerella brochopaga* trapping process. (A) Initiation of conidia germination 8-12 hours (400 x), (B) hyphae growth at 14 hours (400 x), (C) initial of ring formation at 16 hours (400 x), (D) Complete formation of rings at 24-36 hours (400x), (E) Nematode trapped by a ring at 72 hours (400 x).



**Figure 3.2.** Percentage of capture of *Rotylenchulus reniformis* vermiforms in time, by *Drechslerella dactyloides* and *Dr. brochopaga* under *in vitro* conditions. Linear regression model for *Dr. dactyloides*: Percent of nematodes captured = -103.6 + 1.6 \* Time (hours) r<sup>2</sup>=0.84. Linear regression model for *Dr. brochopaga*: Percent of nematodes captured = -125.5 + 1.9 \* Time (hours) r<sup>2</sup>=0.81.



**Figure 3.3.** *Paecilomyces lilacinus* attacking reniform egg. (A) Reniform nematode egg and non germinated conidia 12 hours (400 x), (B) Germinated conidia surrounding reniform egg at 18 hours (400 x), (C) Egg parasitized by *P. lilacinus* 24-40 hours (400 x) (D) Sporulation of *P. lilacinus* within the egg 72 hours (400 x).



**Figure 3.4.** Percentage of *Rotylenchulus reniformis* eggs parasitized by *Paecilomyces lilacinus* under *in vitro* conditions. Linear regression model: Percentage of eggs parasitized= -28.1 + 2.4 \* Time (hours)  $r^2=0.89$ .



**Figure 3.5.** A) Conidia of *Dr. dactyloides* with one septum (2300x), B) Conidia of *Dr. brochopaga* with three septa (3000x), C) *Rotylenchulus reniformis* vermiform trapped by constricting rings (2500x), D) Assimilative hyphae inside *R. renifromis* vermiform (1000x).



**Figure 3.6.** A) *Rotylenchulus reniformis* egg parasitized by *P. lilacinus* (1400x), B) Conidiophore with lemon shaped conidia of *P. lilacinus* (2250x)



**Figure 3.7.** A) *Rotylenchulus reniformis* female with unkown symptoms feeding from a cotton root (1000x), B) Bumb erupting from the dorsal region of the female (7400x), C) Bumps and hyphae on the cuticle of the female (2900x), D) Bumps erupting from the vulva region of the female (2300x).


**Figure 3.8.** A) Healthy young *R. reniformis* female feeding on a cotton root (1400x), B) Healthy mature *R. reniformis* female feeding on a cotton root (1400x).

Effect	DF	FValue	ProbF
Soil	1	22.4875	0.0001
Carrier	2	4.6051	0.0102
Soil* Carrier	2	2.6054	0.0744
Nematodes	1	5.4945	0.0193
Soil*Nematodes	1	0.0541	0.8162
Carrier*Nematodes	2	0.3480	0.7062
Soil*Carrier*Nematodes	2	0.8011	0.4491
Fungus	7	0.6307	0.7308
Soil*Fungus	7	0.5634	0.7859
Fungus*Carrier	14	0.3713	0.9825
Soil*Fungus*Carrier	14	0.5466	0.9057
Fungus*Nematodes	7	0.1911	0.9873
Soil*Fungus*Nematodes	7	0.3044	0.9520
Fungus*Carrier*Nematodes	14	0.2342	0.9984

**Table 3.1.** ANOVA of cotton shoot mass (g) using corn meal, oat, and wheat as fungal carriers with autoclaved and natural soils (P<0.05).

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**Figure 3.9.** Cotton shoot mass (g) using corn meal, oat, and wheat as fungal carriers with autoclaved and natural soils (P<0.05) (SE= 1.02; df=869, alpha=0.05).

Effect	DF	FValue	ProbF
Soil	1	248.50	0.0001
Carrier	2	17.56	0.0001
Soil* Carrier	2	12.65	0.0001
Nematodes	1	317.93	0.0001
Soil*Nematodes	1	35.62	0.0001
Carrier*Nematodes	2	3.50	0.0305
Soil*Carrier*Nematodes	2	5.14	0.0060
Fungus	7	1.50	0.1683
Soil*Fungus	7	0.97	0.4545
Fungus*Carrier	14	1.27	0.2212
Soil*Fungus*Carrier	14	1.02	0.4342
Fungus*Nematodes	7	1.73	0.0975
Soil*Fungus*Nematodes	7	1.52	0.1581
Fungus*Carrier*Nematodes	14	0.95	0.5055

**Table 3.2.** ANOVA of cotton root mass (g) using corn meal, oat, and wheat as fungal carriers with autoclaved and natural soils (P < 0.05).



**Figure 3.10.** Cotton root mass (g) using corn meal, oat, and wheat as fungal carriers in autoclaved and natural soils and with the presence and absence of the nematode (P<0.05) (SE=0.083; df=869; alpha=0.05) (Yes=*R. reniformis* present. No= *R. reniformis* absent)

Effect	DF	FValue	ProbF
Soil	1	3785	0.001
Carrier	2	0	0.944
Soil* Carrier	2	0	0.975
Nematodes	1	0	0.897
Soil*Nematodes	1	123	0.0001
Carrier*Nematodes	2	4	0.0120
Soil*Carrier*Nematodes	2	4	0.0126
Fungus	7	9	0.0001
Soil*Fungus	7	5	0.0001
Fungus*Carrier	14	2	0.0225
Soil*Fungus*Carrier	14	3	0.0001
Fungus*Nematodes	7	9	0.0001
Soil*Fungus*Nematodes	7	8	0.0001
Fungus*Carrier*Nematodes	14	1	0.1579

**Table 3.3.** ANOVA of cotton height (cm) using corn meal, oat, and wheat as fungal carriers with autoclaved and natural soils (P<0.05).



**Figure 3.11.** Cotton height (cm) using corn meal, oat, and wheat as fungal carriers, in autoclaved and natural soils, and with the presence and absence of the nematode (SE=0.013; df=865; alpha=0.05). (Yes=*R. reniformis* present. No= *R. reniformis* absent).

**Table 3.4.** Rotylencuhlus reniformis populations per  $150 \text{ cm}^3$  of soil as influenced by *Drechslerella dactyloides*, *Dr. brochopaga*, *Fusarium oxysporum*, and *Paecilomyces lilacinus* in natural and autoclaved soil (P<0.05).

Fungi	Autoclaved			Natural		
	<b>R. reniformis</b> /	Dunnett	P vs.	<b>R. reniformis</b> /	Dunnett P vs.	
	150 cm <sup>3</sup>	No carrier	Carrier	150 cm <sup>3</sup>	No carrier	Carrier
No carrier	4851			2138		
Carrier	5539			2107		
BW-Dr. dactyloides	1726	0.0001	0.0001	1138	0.0039	0.0051
GH-Dr. dactyloides	2495	0.0022	0.0002	1492	0.2001	0.2350
HN-Dr. dactyloides	2209	0.0002	0.0001	1590	0.3823	0.4357
HN-Dr. brochopaga	2471	0.0018	0.0001	1890	0.9695	0.9845
GH-F. oxysporum	2702	0.0085	0.0008	1228	0.0140	0.0177
GH-P. lilacinus	2036	0.0001	0.0001	1373	0.0721	0.0876

**Table 3.5.** Number of *Rotylenchulus reniformis* eggs and juveniles in cotton roots as influenced by *Drechslerella dactyloides*, *Dr. brochopaga*, *Fusarium oxysporum*, and *Paecilomyces lilacinus* in natural and autoclaved soil (*P*<0.05).

Fungi	Autoclaved			Natural		
	Eggs and	Dunnett	P vs.	Eggs and	Dunnett P vs.	
	juveniles/ Root	No Carrier	Carrier	juveniles/ Root	No Carrier	Carrier
No carrier	5028			1659		
Carrier	3797			1772		
BW-Dr. dactyloides	2498	0.002	0.131	1388	0.874	0.637
GH-Dr. dactyloides	2779	0.013	0.384	1436	0.950	0.766
HN-Dr. dactyloides	2683	0.007	0.278	1881	0.975	1.000
HN-Dr. brochopaga	2534	0.003	0.153	1192	0.327	0.168
GH-F. oxysporum	1923	0.001	0.003	1455	0.969	0.814
GH-P. lilacinus	2019	0.001	0.007	1411	0.916	0.701

**Table 3.6.** *Rotylenchulus reniformis* eggs and juveniles per gram of cotton root as affected by *Drechslerella dactyloides*, *Dr. brochopaga*, *Fusarium oxysporum*, and *Paecilomyces lilacinus* in autoclaved and natural soils (*P*<0.05).

Fungi	Autoclaved			Natural			
	Eggs and	Dunnett	P vs.	Eggs and	Dunnett P vs.		
	juveniles/ gr of root	No Carrier	Carrier	juveniles/ gr of root	No Carrier	Carrier	
No carrier	474.9			117.3			
Carrier	304.2			123.4			
BW-Dr. dactyloides	178.4	0.0001	0.0526	101.6	0.9672	0.8698	
GH-Dr. dactyloides	222.8	0.0023	0.4697	91.9	0.7097	0.5258	
HN-Dr. dactyloides	213.7	0.0012	0.3400	139.3	0.9224	0.9867	
HN-Dr. brochopaga	182.4	0.0001	0.0685	84.0	0.3956	0.2571	
GH-F. oxysporum	186.8	0.0001	0.0900	92.3	0.7240	0.5400	
GH-P. lilacinus	179.8	0.0001	0.0576	95.3	0.8316	0.6585	

**Table 3.7.** Midseason and final season population of *R. reniformis* in soil under microplot conditions as influenced by the nematicde aldicarb, *Drechslerella dactyloides*, *Dr. brochopaga*, or *Paecilomyces lilacinus* alone or incombination (*P*<0.05).

Control / Fungus	<b>Midseason Population</b>			<b>Final Population</b>		
	<b>R. reniformis</b> /	Dunnett P vs.		<b>R.</b> reniformis/	Dunnett P vs.	
	150 cm <sup>3</sup>	Control	Aldicarb	150 cm <sup>3</sup>	Control	Aldicarb
Control	2981			4555		
Aldicarb	2168			2500		
BW-Dr. dactyloides	2962	0.9815	0.3323	3524	0.1519	0.1551
HN-Dr. brochopaga	2684	0.7156	0.5276	3623	0.1948	0.1191
P. lilacinus	3842	0.2933	0.045	3640	0.2025	0.1144
A.d. + D.b. + P.l.	3019	0.9630	0.2923	3345	0.0943	0.2387

## APPENDIX

**Appendix I.** Differences in cotton shoot mass (g) using corn meal, oat, and wheat as fungal carriers with autoclaved and natural soils (*P*<0.05).

Effect	Estimate	StdErr	df	ProbF
Soil (Autoclaved vs. Natural)	1.11	1.02	869	0.001
Carrier (Cornmeal vs. Oat)	0.93	1.03	869	0.015
Carrier (Cornmeal vs.Wheat)	0.93	1.03	869	0.039
Carrier (Oat vs. Wheat)	1.01	1.03	869	0.939
Nematodes (Present or				
Absent)	1.05	1.02	869	0.019

Appendix II. Differences in cotton root mass (g) in the three way interaction of soil, carrier, and
presence or absence of nematodes (Standard Error=1.12; df=869; alpha=0.05).

Effect Level	S	oil		Estimate	Adjp
Cornmeal w/o nematodes	Autoclaved	vs.	Natural	0.2	0.001
Cornmeal with nematodes	Autoclaved	vs.	Natural	0.53	0.001
Oat w/o nematodes	Autoclaved	vs.	Natural	0.42	0.001
Oat with nematodes	Autoclaved	vs.	Natural	0.74	0.013
Wheat w/o nematodes	Autoclaved	vs.	Natural	0.48	0.001
Wheat with nematodes	Autoclaved	vs.	Natural	0.6	0.001
Effect Level	Nema	atode	s	Estimate	
Autoclaved soil w/ cornmeal	Absent	vs.	Present	1.51	0.001
Autoclaved soil w/ oat	Absent	vs.	Present	1.53	0.001
Autoclaved soil w/ wheat	Absent	vs.	Present	2.51	0.001
Natural soil w/ cornmeal	Absent	vs.	Present	4.02	0.001
Natural soil w/ oat	Absent	vs.	Present	2.7	0.001
Natural soil w/ wheat	Absent	vs.	Present	3.1	0.001
Effect Level	Car	rier		Estimate	
Autoclaved soil w/o nematodes	Cornmeal	vs.	Oat	1.06	0.636
Autoclaved soil w/o nematodes	Cornmeal	vs.	Wheat	0.79	0.052
Autoclaved soil w/o nematodes	Oat	vs.	Wheat	0.75	0.016
Autoclaved soil w/ nematodes	Cornmeal	vs.	Oat	1.07	0.551
Autoclaved soil w/ nematodes	Cornmeal	vs.	Wheat	1.32	0.022
Autoclaved soil w/ nematodes	Oat	vs.	Wheat	1.23	0.089
Natural soil w/o nematodes	Cornmeal	vs.	Oat	2.24	0.001
Natural soil w/o nematodes	Cornmeal	vs.	Wheat	1.93	0.001
Natural soil w/o nematodes	Oat	vs.	Wheat	0.86	0.219
Natural soil w/ nematodes	Cornmeal	vs.	Oat	1.50	0.001
Natural soil w/ nematodes	Cornmeal	vs.	Wheat	1.49	0.001
Natural soil w/ nematodes	Oat	vs.	Wheat	0.99	0.925